Stem Cell Biology and Regenerative Medicine

Peiman Hematti
Armand Keating *Editors*

Mesenchymal Stromal Cells

Biology and Clinical Applications

Humana Press

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ISBN 978-1-4614-5710-7 ISBN 978-1-4614-5711-4 (eBook)
DOI 10.1007/978-1-4614-5711-4
Springer New York Heidelberg Dordrecht London

Library of Congress Control Number: 2012953270

Printed on acid-free paper

Humana Press is a brand of Springer
Springer is part of Springer Science+Business Media (www.springer.com)

To my wife Shirin, and my daughters Neeloufar and Faranak, for their love and support.

Peiman Hematti

To all my students and collaborators who have labored in this challenging and exciting area of research.

Armand Keating

Foreword

Every tissue has some degree of mesenchymal cell contribution whether it be rare as with perivascular cells in the brain or abundant as in bone. In mesenchymal tissues like bone, there is a great deal known about lineage relationships and function, but the same cannot be said to be true for most organs where the epithelial components have largely been the focus of research. The ubiquity of mesenchymal cells in all organs may be one reason why they have generally been understudied as they are regarded as simply a part of supportive stroma.

However, the role of mesenchymal cells in organ formation is well recognized as central to development, providing an interplay with other cell types in guiding patterning and morphology. In adult tissues, they have been most evident as functional participants in wounding but are highly likely to play important roles in the maintenance of the tissue as well. The setting where this is increasingly appreciated is the bone marrow. A number of studies have now indicated a primary role for mesenchymal cells in regulating the hematopoietic stem and progenitor cells. These studies have further indicated that mesenchymal cells are highly diverse even within a given tissue. For example, in the bone marrow, it is evident that cells expressing different marker genes like nestin, leptin receptor, or osterix are not entirely overlapping and express different levels of other proteins implicated in stem cell function. Further, at least some of the cells appear to turn over rapidly and be replenished by a resident stem cell pool. And the function of the mesenchymal cells seems to be important in the integrity of the tissue. Several models of altered mesenchymal cell function have resulted in changes in hematologic function indicating the clear dependence on intact interplay between mesenchymal and parenchymal cell types for the tissue to function normally. Collectively, these kinds of studies are raising awareness of the complexity, dynamism, and centrality of endogenous mesenchymal cells for tissue health. They have to some extent trailed the many studies of mesenchymal cells cultured ex vivo and used therapeutically, and yet, they support and deepen the rationale for such studies.

The behavior of endogenous mesenchymal cells in tissues includes a highly unusual ability to fundamentally change cell state. In development, certain mesenchymal cells such as those of the developing kidney are known to transition to

acquire epithelial features. Mesenchymal to epithelial transition is well accepted in the normal course of tissue formation, particularly in forming the nephron. Similarly, mesenchymal cell characteristics are known to be acquired by some epithelial cells in development such as in gastrulation but are best characterized in pathologic conditions, specifically in cancer. The epithelial to mesenchymal transition (EMT) is regarded as a signature pathologic process of malignancy that is now being associated with cells acquiring more stem cell-like features and to be driven by particular genes like Twist, Snail, and Slug. This process is thought to reflect a potentially more metastasis-prone cell phenotype. It is striking that cells can acquire mesenchymal features with very little evidence that alternative cell states can be achieved. Transition to other somatic cell types is simply not seen. This does raise the issue of whether the mesenchymal cell state is not rigidly constrained and that cells can achieve it perhaps with less epigenetic precision than required for other cell types.

The ability of cells to acquire mesenchymal-like features may be part of the reason why virtually every organ appears to have so-called mesenchymal stem cells. These cells are generally defined by their functions in vitro, the ability to form three lineages, bone, cartilage, and adipose cell types. The cells from different tissues are likely to be distinctive, yet once cultured they have a highly similar phenotype. It is not clear if this is reflective of a uniform phenotype in vivo or even of a uniform gene expression signature after in vitro culture. However, whether such distinctions exist may be of interest but may not have significant functional consequences. The cultured cell populations may very well share critical features selected for by the process of cell culture.

The cultured mesenchymal cells with a shared tri-lineage differentiation potential also appear to have unexpected immunologic features. They are capable of altering the immunologic reactivity of the host upon adoptive transfer and may have immune-modulating properties in residence. While there is skepticism regarding this issue, it should be remembered that tissue resident immunologic functions of dendritic cells and endothelial cells were also regarded skeptically when first proposed and it may well be that mesenchymal cells represent the next generation of such cell populations discovered to play central roles. The putative immunologic effects of transferred mesenchymal cells are not a global immune suppression. There is no evidence of a predisposition to infection as occurs with most immunosuppressive drugs. The immunologic alteration may be more subtle and perhaps more of a reset of a hyperreactive immunologic state as is present in most of the conditions where the cells have been tested. The mechanistic basis by which the cells can induce an effect that apparently lasts longer than the persistence of the infused mesenchymal cells has many hypotheses. Most of these are related to elaboration of proteins exerting a paracrine effect. Some of these effects may also alter activities like hematopoietic stem cell engraftment.

The volume expertly edited here by Drs. Peiman Hematti and Armand Keating represents an assembly of reviews and perspectives from leading investigators in the field. It addresses controversies of the field head-on and covers topics from underlying biology to clinical testing in multiple settings to the all important regulatory considerations in preparing such cells for use in patients. It is an extremely impor-

tant, timely, and thoughtful addition to a rapidly changing field and will be a resource for those entering or active in the study of these intriguing cells. This cell type is likely to become a mainstay in the medical armamentarium. We are still in the early days of defining where and how they can be optimally active. A guiding resource such as this volume will help move the process forward.

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Preface

Alexander Friedenstein is rightfully credited with discovery of what we now know as mesenchymal stromal cells (MSCs) more than four decades ago. Friedenstein's seminal work showed that bone marrow, in addition to hematopoietic stem cells, contained a population of cells capable of generating hematopoietic supportive stroma, bone, fat, and cartilage. In the last decade, work in the field of MSCs has exploded, and now these cells have become the most commonly used in regenerative medicine only after hematopoietic stem/progenitor cells. The rapidly increasing number of clinical trials in a broad range of applications attests to considerable clinical potential. Applications span from their use as supportive cells for the ex vivo expansion of cord blood cells to treatment of localized defects, wounds and pathologies, and for systemic diseases with different pathophysiology. MSCs have been investigated in small- and large-scale clinical trials for acute and chronic graft-versus-host disease, myocardial infarction, diabetes, chronic obstructive pulmonary disease, systemic lupus erythematosus, systemic sclerosis, lower limb ischemia, stroke, multiple sclerosis, amyotrophic lateral sclerosis, kidney transplantation, nonhealing cutaneous wounds, bone and cartilage defects, and inborn errors of metabolism. Despite the rapid advances into the clinic, an assessment of the therapeutic efficacy of MSCs remains a challenge. It is therefore incumbent on us to carefully evaluate and reappraise the field. This book attempts to address these issues and consists of 38 chapters that comprise a range of topics from basic biology of MSCs to their clinical application. It also addresses the controversies surrounding their mechanisms of action.

The first part of this book discusses the latest advances in our understanding of the biological properties of MSCs in vitro and in vivo. This part covers the basic science involved in immunophenotypic and functional characterization of MSCs, technological innovations of their in vivo investigation, and a new understanding of potential mechanisms of action. Also, views and controversies are discussed in the context of a more recent understanding of cellular and molecular mechanisms of action of MSCs. The second part of the book is a comprehensive resource to readers interested in the translational and regulatory aspects of MSCs as cell therapy. Our goal here is to fill the gaps between "bench" and "bedside" and help connect our

understanding of MSC biology with the requirements of clinical investigation. These chapters provide a guide on how to bring MSCs from basic research laboratories to the clinic. The third part of the book offers an extensive coverage of the clinical use of MSCs involving almost all human tissues and organs that have served as targets for this treatment. These chapters explore the rationale, design, safety, and efficacy of published and ongoing clinical trials.

The authors represent an international group of basic, translational, and clinical investigators from almost all sub-subspecialties of medicine as well as experts in the regulatory aspects of cellular therapy. Moreover, all the contributors have been directly involved in the various aspects of MSC research. We hope that this book will provide a benchmark for the most exciting developments in the investigation and clinical use of mesenchymal stromal cells.

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List of Commonly Used Abbreviations

AT	Adipose tissue
ALS	Amyotrophic lateral sclerosis
ANG1	Angiopoietin 1
ASCs	Adipose-derived stromal/stem cells
BAL	Bronchoalveolar lavage
BDNF	Brain-derived neurotropic factor
BLI	Bioluminescence imaging
BM	Bone marrow
CD	Cluster of differentiation
CFU	Colony-forming units
cGMP	Current good manufacturing practice
CMC	Chemistry, manufacturing, and controls
CNS	Central nervous system
CXCR4	C-X-C chemokine receptor type 4
CXCL12	Chemokine, CXC motif, ligand 12
DC	Dendritic cells
EGF	Epidermal growth factor
FBS	Fetal bovine serum
FCS	Fetal calf serum
FGF	Fibroblast growth factor
G-CSF	Granulocyte colony-stimulating factor
GDNF	Glial cell-derived neurotropic factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GMP	Good manufacturing practice
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
HSCs	Hematopoietic stem cells
IA	Intraarterial
IC	Intracerebral
IFN-γ	Interferon-gamma
IGF	Insulin-like growth factor

IL	Interleukin
IND	Investigational new drug
IP	Intraperitoneal
IT	Intrathecal
IV	Intravenous
LPS	Lipopolysaccharides
M-CSF	Macrophage colony-stimulating factor
MCP1	Macrophage chemotactic protein 1
MLR	Mixed lymphocyte reactions
MCP1	Monocyte chemotactic protein 1
MI	Myocardial infarction
MIF	Macrophage inhibitory factor
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
MSCs	Mesenchymal stromal/stem cells
NGF	Nerve growth factor
NK cells	Natural killer cells
OI	Osteogenesis imperfecta
PDGF	Platelet-derived growth factor
PGE2	Prostaglandin E2
PHA	Phytohemagglutinin
PI3K	Phosphatidylinositol 3-kinase
SAGE	Serial analysis of gene expression
SMA	Smooth muscle actin
SOD1	Superoxide dismutase 1
SVF	Stromal-vascular fraction
TGF-β	Transforming Growth Factor-β
Th1	Type 1 T helper cells
Th2	Type 2 helper cells
TLR	Toll-like receptors
TNFα	Tumor necrosis factor-alpha
T-regs	Regulatory T cells
UCB	Umbilical cord blood
VEGF	Vascular endothelial growth factor

Part I
Basic Biology

Chapter 1
Mesenchymal Stromal Cells in Regenerative Medicine: A Perspective

Peiman Hematti and Armand Keating

Abstract Multipotent mesenchymal stromal cells (MSCs) of bone marrow origin not only provide a supportive cellular niche for hematopoiesis inside the bone marrow but also differentiate into mesodermal cells such as bone, fat, and cartilage. Clinical uses of culture-expanded MSCs were originally investigated for their presumed hematopoietic-supportive activities. Their use in the clinic was later expanded to the treatment of steroid-resistant acute graft-versus-host disease based on unique immunomodulatory properties shown in a variety of in vitro experiments and in vivo models. Systemically administered MSCs participate in tissue regeneration through diverse biological activities, including paracrine effects that are not necessarily dependent on cell engraftment. Although there is an impressive record of safety in clinical trials, most outcomes have been assessed in the short term, and their efficacy has yet to be shown conclusively in randomized controlled trials. Forty years after their original description and 20 years after their use in humans, culture-expanded MSCs, and particularly their in vivo counterparts, remain poorly understood. However, unless or until better therapeutic options for debilitating disorders are found, the notion that MSCs could be potentially useful warrants further investigation to establish long-term safety and efficacy in well-designed clinical trials.

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_1,

Introduction

Friedenstein was the first to isolate fibroblast-looking cells from bone marrow (BM) and to show that they were capable of regenerating rudiments of bone and supporting hematopoiesis in vivo [1]. These cells were later shown to be capable of differentiating into fat and cartilage and thus were given the name mesenchymal stem cells (MSCs) [2]. However, to be more technically accurate and to better reflect their true biological properties, it has been suggested that the name multipotent mesenchymal "stromal" cells (with the same acronym) be used for the heterogenous population of cells that is cultured *ex vivo*, while the term mesenchymal stem cell be used only for the cells capable of both self-renewal and multi-lineage differentiation [3]. Although the correlation between *ex vivo*-generated MSCs and their in vivo counterparts still remains poorly understood, culture-expanded MSCs derived from BM or other tissues are currently being investigated for an ever-expanding number of clinical indications based on their tissue-regenerative, immunomodulatory, anti-inflammatory, and trophic paracrine effects. In this chapter, we provide an overview of the role of these cells in the nascent but exciting field of regenerative medicine.

Four decades after their original description, there is still much debate about the exact anatomical location of MSCs inside different tissues (including BM) and their true physiological role. MSCs comprise a very small population (<0.1%) of adult BM cells. It is believed that these cells, or their progeny such as osteoblasts, constitute the supportive cellular niche for hematopoietic stem cells (HSCs) inside the BM [4–7]. Derivation of cells with similar phenotypic characteristics from non-BM tissues has just added to the uncertainty surrounding the true identity and physiological roles of these cells. For example, similar populations of cells have been isolated from almost all other adult and neonatal tissues including fat [8], skeletal muscle [9], synovium [10], dental pulp [11], placenta [12], amniotic fluid [13], umbilical cord blood [14], and fetal lung, liver, and blood [15]. Importantly, MSCs isolated from these non-BM tissues share cell-surface markers similar to BM-derived MSCs and have similar differentiation potential into bone, fat, and cartilage. However, since no physiological role can be imagined for cells with bone- and cartilage-forming potential in organs such as heart and adipose tissue, it can be argued that these in vitro observations are artifacts of our experimental assays with no correlation to the true homeostatic role of MSCs in different tissues. Surprisingly, MSCs isolated from these non-BM sources possess similar immunomodulatory properties [16–18].

It should be noted that there is a vast difference between what cells do in their normal in vivo environment under physiological conditions and what they can potentially do if they are tested out of their physiological context. For example, functional properties and capabilities of BM-derived MSCs that are culture expanded may be very different from their in vivo counterparts. Furthermore, these cells are usually transplanted in very large numbers to a new location for repair of damage in a tissue different from their tissue of origin. Consequently, MSC transplantation

bears significant differences with HSC transplantation in which cells are usually transplanted with minimal manipulation. However, for clinical cell therapists, what matters most is that the transplanted cells result in some beneficial effects and do not cause harm, whatever the mechanism. This view of regenerative medicine is different from that of investigators whose primary focus is to understand the basic biology of cells and their mechanism of actions. Nevertheless, the maximum potential value of MSCs in regenerative medicine involves a swinging back and forth between bench and bedside and is in the best interest of laboratory researchers and clinicians alike.

Mechanism of Action of MSCs

Mesenchymal stromal cells have generated huge interest in both public and scientific communities because of their potential to regenerate a wide variety of tissues. The place of these cells in clinical medicine was originally thought to be due to their presumed hematopoietic-supportive activities or bone- and cartilage-forming potential. However, our view of the potential mechanisms of action of MSCs and thus of the potential indications in regenerative medicine has evolved considerably over the years. A major reason for the initial enthusiasm for MSCs in the non-bone marrow transplant field was a multitude of studies suggesting MSCs not only differentiate into other types of cells of mesodermal lineage but also into cells of endodermal and ectodermal lineages, including cardiomyocytes [19], endothelial cells [20], lung epithelial cells [21], hepatocytes [22], neurons [23], and pancreatic islets [24]. However, the degree of contribution to different tissues through trans-differentiation is now considered very unlikely given that many later studies using more sensitive and appropriate techniques could not duplicate the results of original reports [25–27]. Thus, it has now become more accepted that, despite the fact that under certain experimental conditions, these cells might assume some characteristics shared by other cells, this process, if it occurs at all, is probably a rare event in vivo and is certainly insufficient to explain the positive results observed in animal models and human studies and thus is of no clinical significance.

While under normal circumstances, we expect that MSCs will preferentially home to BM after intravenous infusion [28, 29], experimental models show that *ex vivo* culture-expanded MSCs infused intravenously can be detected at low levels in many tissues [30, 31]. Indeed, these cells preferentially home to damaged tissues, probably via the SDF1/CXCR4 axis [32, 33]. The prevailing view is that MSCs home to sites of tissue injury/inflammation, secrete trophic factors to promote recovery of injured cells, and recruit and expand resident progenitors to replace damaged cells. Likewise, they participate in tissue regeneration through matrix remodeling and exert desirable immunomodulatory and anti-inflammatory properties, making them ideal candidates for use in disorders affecting many different organs [34–36].

Originally, robust structural integration of the MSCs into patient tissue was considered a requirement for achieving the desired end points. For example, it was thought that MSCs should ideally be able to substitute affected tissues. However, the assumption that persistence of the transplanted cells in the recipient is necessary to yield a therapeutic effect is being replaced by other mechanistic paradigms that involve mainly anti-inflammatory and paracrine effects. For example, recent studies in animal transplant models have shown that infused MSCs are trapped to a significant degree in the lungs and nevertheless can exert significant beneficial systemic effects (in this case, in the heart) via paracrine effects [37]. These mechanistic insights could influence design of clinical trials, for example, choosing between intravenous delivery of MSCs and their direct intracardiac injection for repair of heart damage.

In addition to the new mechanisms of action proposed for MSCs, our view of the pathophysiology of disease processes has evolved over the years too, including many for which we contemplate using the cells. For example, we now understand that in many disease processes, inflammatory and immunological disturbances play a much bigger role than was appreciated only a few years ago. Consequently, it is no surprise that MSCs, found about a decade ago to have immunomodulatory properties, could potentially be beneficial in conditions that we now know involve immune disturbance and inflammation. Observing beneficial effects in conditions with very poorly documented engraftment of the cells is consistent with these observations. These effects could be due to transient immunomodulation, or paracrine action, including the secretion of cytokines and other trophic factors. Paracrine effects may mediate repair by protecting tissue cells from apoptosis, promoting angiogenesis, or recruiting and activating tissue progenitor cells. Alternatively, MSCs could also change the repertoire of immune and inflammatory cells present in damaged tissue to avoid further immunological damage or promote the generation of tissue-regenerating macrophages. Thus, to exert a beneficial effect, prolonged levels of engraftment might not be needed and, indeed, may be irrelevant. Nonetheless, repeated doses may be required to obtain therapeutic effects.

Clinical Experience with MSCs

Hematologists have been at the forefront of cellular therapies, as in the case of bone marrow transplantation (BMT) decades ago. Hematologists have also been the first to use MSCs clinically, given that the cells are derived from BM and support hematopoiesis in experimental models in vitro and in vivo. Thus, in the field of BMT MSCs were investigated originally to improve hematopoietic engraftment. Lazarus and his colleagues showed not only feasibility of collection and *ex vivo* culture expansion of MSCs from small BM aspirates of patients with different

malignancies but also safety of infusion of autologous MSCs alone [38] or combined with autologous peripheral blood $CD34^+$ cells [39]. They also showed that administration of culture-expanded allogeneic MSCs with their corresponding HSCs in patients undergoing myeloablative HSC transplantation for hematological malignancies was safe and not associated with an increased incidence or severity of graft-versus-host disease (GVHD) [40]. Compared with historical controls, hematopoietic engraftment was not faster, but these studies provided evidence that *ex vivo* culture expansion of MSCs was feasible and intravenous infusion did not cause toxicity. There are hints that MSCs may promote HSC engraftment based on small non-randomized clinical series [41–45]. Improvement of HSC engraftment in these settings is likely not due to a direct HSC niche effect but perhaps is more likely to be related to an immunomodulatory paracrine effect in ameliorating tissue inflammation, a major barrier to HSC engraftment. Indeed, while donor MSCs may exert an effect after BMT, many but not all studies consider them host derived in transplant recipients [46–48].

Almost a decade ago, it was suggested MSCs, including from unmatched third-party donors, may be useful in ameliorating GVHD after allogeneic HSC transplantation [49–51]. Le Blanc et al. were the first to report the treatment of GVHD with MSCs in a 9-year-old boy who received a HSC transplant from an unrelated matched donor [52]. The patient had severe refractory acute GVHD of gut and liver unresponsive to all types of immunosuppressive medications. Infusion of one dose of haploidentical MSCs resulted in an impressive response with resolution of all clinical and laboratory manifestations of GVHD. The infusion of a second dose of MSCs was also effective in treating the GVHD that soon recurred. This landmark case report was followed by another promising small case series of eight patients [53] and then by a phase II trial of 55 pediatric and adult patients, with steroid-refractory acute GVHD [54]. The latter study confirmed that the clinical responses were independent of the source of MSCs; that is, MSCs from human leukocyte antigen (HLA) identical sibling, haploidentical, and third-party HLA-mismatched donors gave similar responses. GVHD is also the only indication in which a phase III randomized double-blind controlled study has been conducted to completion [55]. In this study of refractory GVHD, subsets of patients with liver or gastrointestinal GVHD had an improved response to MSCs. However, the primary end point of the study could not be achieved. Nonetheless, pediatric patients showed a higher rate of response [56] .

Use of third-party MSCs in the context of HSC transplantation without regard to their HLA typing opened the gate to use of unmatched allogeneic MSCs for many other indications. Also, the multitude of paracrine, immunomodulatory, and anti-inflammatory properties of MSCs has been the rationale for initiating numerous phase I–III clinical trials for a wide range of human disorders. Such studies include metachromatic leukodystrophy and Hurler's disease [57], osteogenesis imperfecta [58], myocardial infarction [59], chronic obstructive pulmonary disease [60], amyotrophic lateral sclerosis [61], stroke [62], refractory wounds [63],

diabetes mellitus [64], systemic sclerosis [65], systemic lupus erythematosus [66], Crohn's disease [67], and multiple sclerosis [68]. Although unequivocal efficacy in any of these indications has yet to be shown, what we have learned is that infusion of MSCs, not only intravenously but also intra-arterially [69] and even intrathecally [68], is safe. The use of MSCs for these indications is covered in detail in many other chapters of this book.

Standardization of Culture Methodologies

Considering that more than a few hundreds MSC-related clinical research protocols are listed in www.clinicaltrials.gov and that MSCs have been given to several thousand patients worldwide, there is an urgent need to assess MSC production methodology on clinical outcomes [70]. Currently, there is no standardized culture protocol, and considerable heterogeneity exists in methods for producing MSCs [71–73]. In addition, many of the clinical trials have enrolled small number of patients for whom MSCs were generated in local hematopoietic cell processing laboratories, while some larger studies involved pharmaceutical companies in which MSCs were made under current good manufacturing practice (cGMP) standards and provided limited information on production methodology due to proprietary concerns. Thus, heterogeneity of patient-related characteristics and culture methodology in many MSC studies may prevent definitive conclusions from being drawn. Consequently, definitive studies are needed to show the efficacy of MSCs, preferably in multicenter trials with MSCs produced by a central manufacturing facility or generated according to the same protocol.

MSCs are present in the mononuclear cell (MNC) fraction of BM, and minor changes in processing, including the use of Ficoll density gradient centrifugation, can affect cell characteristics [74]. Clinical results should therefore be interpreted cautiously as the MSCs used may differ based on donor (autologous versus allogeneic, young versus old, male versus female), starting material (fresh versus frozen BM), isolation technique (Ficoll versus no Ficoll), plating density, coating material, culture medium, passage number, and cell expansion protocol specifications. Furthermore, we know that *ex vivo* culture-expanded MSCs comprise a heterogeneous population with potentially different biological characteristics. Thus, it is possible that different culture conditions may favor the growth of certain MSCs with undetermined characteristics.

It is a major challenge to determine if any changes in production methodology have an impact on the final properties in vivo. For example, one major variation is the culture medium used such as fetal bovine serum (FBS) versus synthetic serum-free medium, autologous serum, fresh frozen plasma, or human platelet lysate [75–77]. In one clinical trial, FBS was replaced by human platelet lysate to produce MSCs [78]; however, it is not known whether the generation of MSCs in platelet lysate played a role in the lower response rate observed in this small study ($n = 13$) for the treatment for steroid-refractory GVHD.

There is also no consensus on the release criteria for MSCs. However, when MSCs are used for such diverse conditions as GVHD after allogeneic HSC transplantation, bone repair, and myocardial infarction, a single potency assay is not likely to be feasible but needs to reflect the specific indication. Further work is necessary to address this important issue and will probably be managed on a case by case basis.

Unresolved Issues

Although several thousand patients have received MSCs for a wide variety of indications using different routes of administration, outcomes of most treatments have not been reported in the medical literature. Of further concern is the lack of long-term follow-up to monitor adverse events. Moreover, rare long-term adverse events are likely to be identified only from a database of a large number of treatment recipients. While analyses of blood and marrow transplant database registries have been very helpful in determining outcomes and adverse effects of specific categories of transplant recipients, a similar strategy for persons receiving cell products such as MSCs is significantly more challenging, not the least because many different and separate specialties of clinical medicine are involved that do not have a history of close interaction. Nonetheless, some issues may be possible to address with existing BMT registries, such as assessing the potential for increased relapse or opportunistic infections in allogeneic transplant recipients receiving MSCs for prevention of GVHD. Indeed, in a small open-labeled randomized trial of MSC infusion for prevention of GVHD, an increased risk of early relapse led to early termination of the study [79]. Although such risk has not been seen in similar studies, long-term outcome data collection is needed and could conveniently be collected by transplant outcomes database registries such as the Center for International Blood and Marrow Transplantation [80].

Preclinical animal models are useful in evaluating the safety and efficacy of cellular therapeutics. However, finding a relevant animal model can be challenging because of large biological differences between humans and, especially, inbreed laboratory animals. Even the evaluation of human MSCs in immune-deficient xenogeneic rodent models presents a challenge in simulating an appropriate microenvironment, in addition to accounting for the absence of an intact immune system. Conclusions from murine models have major implications in the design of human clinical trials. For example, a beneficial effect of MSCs in the NZBxNZW F1 model of SLE was not obtained [81]. However, another group, based on their promising results in an MRL/*lpr* murine model of SLE [82], showed, that a single infusion of allogeneic bone marrow-derived MSCs in four patients with lupus nephritis resulted in improvement of serologic markers and kidney function [83]. The same group later reported positive outcomes in 16 SLE cases treated with umbilical cord-derived MSCs [84]. More recently, they reported a positive outcome in 15 patients with active SLE, 14 of whom had nephritis and were refractory to conventional treatments (including the previously published four cases) [85].

All patients showed improvement in autoantibody levels, proteinuria, and non-renal manifestations of SLE after infusion of a small dose of allogeneic bone marrow-derived MSCs (1×10^6/kg by intravenous injection) with no significant acute toxicity. In contrast, in another study the injection of autologous MSCs in two patients had no effect on disease activity despite inhibition of lymphocyte proliferation in vitro [86]. The latter negative result may be due to the small number of cases treated or the possibility that MSCs derived from ill persons may not be as immunosuppressive as allogeneic MSCs from healthy individuals. This raises the possibility that the choice of autologous versus allogeneic MSCs may depend not only on the urgency of the need but also on the specific clinical indication.

The infusion of *ex vivo*-expanded MSCs without regard to HLA status has been repeatedly shown to be safe and was originally based on the assumption that the cells are non-immunogenic. However, total lack of immunogenicity is called into question given the number of studies showing minimal engraftment of these cells. Furthermore, the notion that MSCs always suppress proliferative responses of allogeneic lymphocytes is also debatable, as it has been now shown that MSCs can function as antigen-presenting cells or even activate immune responses under certain conditions [87, 88]. Also, preclinical data on the ability of MSCs to suppress these responses in vivo have been conflicting [89–91]. These discrepancies in basic research literature could be due to many factors, including the strain of mice used to derive MSCs, the culture methodology, the number of cells infused, the passage or cell doubling number, and the timing of MSC infusion. For example, in one murine study, MSCs infused on the day of BMT were ineffective in GVHD prevention, but infusion of cells on day 2 significantly reduced mortality [92]. Furthermore, this study also showed that MSCs contaminated with >3% CD45+ cells and MSCs from late passage (more than 6) did not show a significant effect on GVHD-related mortality. Results may also reflect the dose of cells used. For example, both murine [93] and human studies [94] have shown that MSCs inhibit proliferation of B-lymphocytes stimulated by various means. However, based on the Corcione et al. study [94], the inhibition was dose dependent, as more MSCs led to less inhibition. This contrasts with inhibition of T-cell proliferation, where more MSCs usually lead to greater inhibition of T-cell proliferation. Thus, it is possible that in some clinical scenarios, such as SLE in which B cells play a major pathophysiological role, a lower dose of MSCs may be more effective.

One of the inherent characteristics of cells is that, unlike pharmaceuticals, they are complex and variable. Their in vivo behavior depends on many factors, including the route of administration, autologous versus allogeneic sources, the immune system status of the patient, concomitant medications, and the microenvironment of the tissue to be augmented. Moreover, such factors can be disease specific. The potential for the accumulation of genetic mutations after long-term culture [95–98] theoretically exists and mandates vigilance, especially if cells of multiple doublings are used. However, more important than the theoretical possibility of malignant transformation of MSCs is the possibility of the promotion of the growth of existing tumors or an enhancement of their metastatic potential, as previously documented in some murine models [99, 100]. Nevertheless, it is reassuring that no tumor formation has been found to date in human recipients of MSCs [101].

Conclusion

MSCs were originally isolated from bone marrow and provided a critical step in the in vitro and in vivo study of hematopoiesis. The cells were later found to possess intriguing immunomodulatory and trophic properties both in vitro and in preclinical models, in addition to supporting hematopoiesis. Numerous clinical studies followed investigating the role of MSCs for a wide range of clinical conditions. Currently, MSCs are at the forefront of regenerative medicine and offer the potential to ameliorate serious or debilitating diseases with limited or no other therapeutic options. Many issues remain to be addressed, including mechanisms of action, the best methods for cell production, the optimal dose, frequency and route of administration, and, in particular, appropriate indications for use. The collaborative efforts of scientists and clinical researchers are essential to advance our understanding of the biology and clinical applicability of these intriguing cells.

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Chapter 2
MSCs: Changing Hypotheses, Paradigms, and Controversies on Mechanisms of Action in Repairing Tissues

Darwin J. Prockop and Roxanne L. Reger

Abstract Research on mesenchymal stromal cells (MSCs) has moved at a rapid pace that has been driven by unexpected discoveries about the biology of the cells and their beneficial effects in multiple models for human diseases. There are currently at least three paradigms as to how the MSCs can repair tissues: (I) by engrafting and serving as a niche for stem/progenitor cells; (II) by engrafting and differentiating to repair damaged tissues; and (III) by temporarily engrafting in injured tissues, engaging in extensive "cross-talk" triggered by signals from the injured tissues, and producing factors that both limit injury to the tissues by multiple effects such as modulating excessive inflammatory and immune responses and enhance repair by providing a niche that stimulates the propagation and differentiation of tissue-endogenous stem/progenitor cells. In the background of research to support each of the paradigms is a series of controversies that have not been resolved in spite of the efforts of the thousands of dedicated scientists who have made major contributions to the field. We will review here just a few of these controversies with conclusions that reflect some of our own biases.

Introduction: The Three Paradigms of MSCs

The cells that are the topic of this chapter have generated a tangled history of changing hypotheses and paradigms. The cells were first identified over 50 years ago in the early experiments on bone marrow: They were spindle-shaped cells that adhered

Where there is good science, there is always trouble. (Anonymous)

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_2,

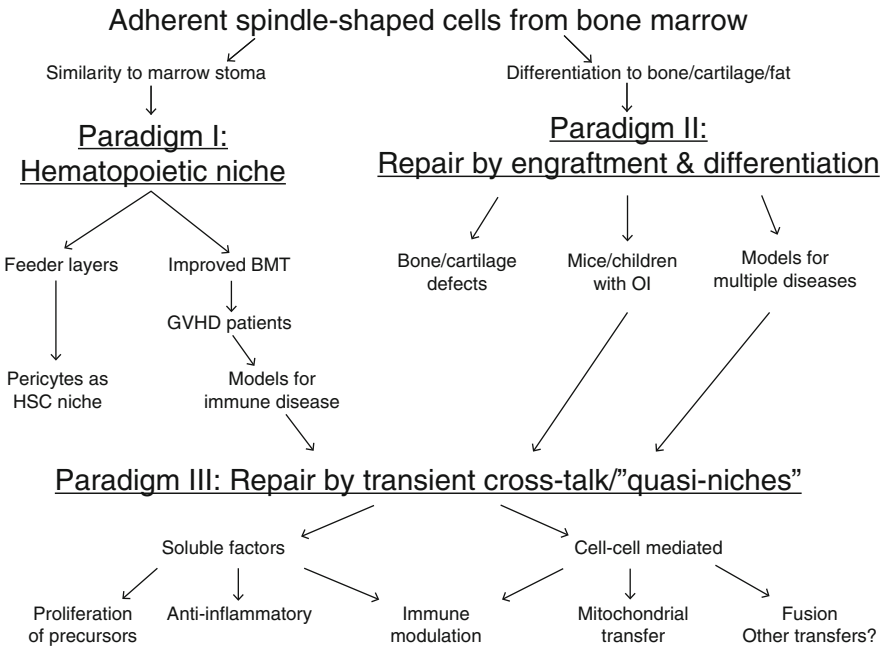

Fig. 2.1 Schematic summarizing three evolving paradigms for the repair of tissues by MSCs. The morphology of a small number of adherent cells from bone marrow suggested the paradigm that the cells served as a niche for hematopoietic cells (Paradigm I). The ready differentiation of the cells in culture suggested that the cells could repair tissues by engrafting and differentiating (Paradigm II). Clinical trials using the cells to improve bone marrow transplants unexpectedly demonstrated that they improved graft-versus-host diseases in a few patients and thereby drew attention to their immunomodulatory properties. Functional improvement without significant engraftment in animal models and a few patients suggested that MSCs enhanced repair by transiently forming microenvironments or "quasi-niches" (Paradigm III) (Reproduced with permission from Prockop et al. [4])

to tissue culture surfaces and were clearly not destined to be hematopoietic. From the outset, the cells were investigated on the basis of two hypotheses or paradigms, in the sense of scientific paradigms as originally defined by the philosopher Thomas Kuhn [1].

Some observers were impressed with the similarity of the cells to cells that formed the stroma of the marrow. This impression prompted the important use of the cells as feeder layers for the culture of hematopoietic cells [2, 3]. In effect, these observers developed the paradigm that the cells provided a niche for hematopoietic stem cells (Paradigm I in Fig. 2.1). Other observers discovered that the cells were readily differentiated into osteoblast-like mineralizing cells, into chondrocytes, and into adipocytes both in culture and in capsules implanted in vivo [5, 6]. They therefore pursued the paradigm that cells were similar to embryonic stem cells and might be used therapeutically to replace many injured tissues by engrafting and differentiating

(Paradigm II in Fig. 2.1). Research based on both paradigms advanced in an irregular, stop-and-go manner as the experimental methods improved. The research also took an irregular course because the cells were at an intersection of the rapidly developing fields of stem cells and of tissue regeneration and repair. As the concepts in these fields evolved, there were major changes in the underlying hypotheses for research on MSCs.

Paradigm I: MSCs as Niche for Hematopoietic Stem Cells

The paradigm that the confluent cultures of MSCs could serve as effective feeder layers or niches for the culture of hematopoietic stem cells has proven to be a major breakthrough in the study of bone marrow and the field of bone marrow transplantation [2, 3]. Direct demonstration of a niche function of MSCs was provided by the observation that islands of hematopoiesis were formed within human MSC-seeded ceramic cubes that were implanted under the skin of immunodeficient mice [7]. Also, the niche function of MSCs was indirectly supported by clinical trials in which the cells were shown to hasten the recovery of the hematopoietic system after bone marrow transplants [8]. The paradigm was further supported by recent studies in mice that identified MSCs as nestin^{+} cells that were part of the neuroendocrine system for mobilization of hematopoietic system [9]. Some of the most direct support for a niche function came from the observation that human MSCs implanted into the hippocampus of mice stimulated the proliferation on endogenous neural stem cells and also their migration and differentiation [10]. The niche function of MSCs may well explain many of the therapeutic benefits that have been reported in multiple animal models for human diseases and a few of the patients in whom therapeutic benefits have been observed in the over 140 of clinical trials with MSCs and related cells that have been registered (clinicaltrials.gov).

Paradigm II: MSCs Repair by Engrafting and Differentiating

The paradigm that MSCs might repair multiple tissues by engrafting and differentiating resonated widely among physicians and scientists interested in new therapies for human diseases [11, 12]. The paradigm reawakened an idea attributed to the classical pathologist Cohnheim who as early as 1867 [13] made observations suggesting that some of the cells involved in tissue repair came from the general circulation and, in the light of further information, from the bone marrow. The paradigm was supported by observations that systemically infused MSCs appeared to be recovered in multiple tissues [14] and that the cells, under some circumstances, differentiated to cells originating from all three germ layers [15–19]. Many of the early experiments were handicapped by inadequate techniques for isolating and characterizing the cells and the lack of markers for the cells that were not readily lost during

differentiation or transferred to other cells. Also, all the potential assay artifacts had not yet been recognized such as artifacts from overlapping cells in microscopic sections labeled with antibodies. As these problems were resolved, reports from multiple laboratories established that MSCs could engraft and differentiate in multiple tissues but the process was robust only in limited circumstances such as in rapidly developing tissues of embryonic mice [16] or chick embryos [20], and with local injection into damaged tissue such as fractured bones [21] or injured cartilage [22, 23]. At the same time, there were convincing reports that MSCs or some related cells from bone marrow engrafted in severely injured tissues in patients such as those undergoing organ rejection following transplants of lung [24]. Also, MSCs or some related cells from bone marrow can generate some of the cells found in epithelial cancers [25]. Therefore, the paradigm appears to have limited applicability, but it is not fully excluded as a therapeutic strategy.

Paradigm III: Repair by Transient Cross-Talk and Niche Functions

As MSCs were explored in many laboratories around the world, several unexpected observations emerged: (a) They frequently repaired tissues even though they were detected in the tissues only transiently, and (b) the cells engaged in extensive communication or "cross-talk" with other cells and tissues [26] that dramatically altered the genes they expressed, including those for secreted factors [27, 28]. The observations have provided the new paradigm that is a partial synthesis of the first two paradigms: The cells temporarily engraft in injured tissues, they engage in extensive "cross-talk" triggered by signals from the injured tissues, and, as a result, they are activated to express genes that (a) limit injury to the tissues by modulating excessive inflammatory and immune responses and (b) enhance repair by providing a niche that stimulates the propagation and differentiation of tissue-endogenous stem/progenitor cells.

The three paradigms are summarized in Fig. 2.1.

In the background of research to support each of these paradigms is a series of controversies that have not been resolved in spite of the efforts of the thousands of dedicated scientists who have made major contributions to the field. We will review some of these controversies and provide conclusions that reflect some of our own biases.

Controversy I: What Are the Criteria for Identifying MSCs?

The commonly employed criteria for defining MSCs are that the cells are (a) highly clonogenic; (b) readily differentiate in culture to osteoblasts, adipocytes, and chondrocytes; and (c) lack epitopes for hematopoietic cells and express several epitopes

that are shared with other non-hematopoietic cells such as CD73, CD90, and CD105 [29]. Unfortunately, each of the criteria is difficult to apply quantitatively. In addition, many reports have not employed them with rigor.

The criterion of clonogenicity lacks rigor as commonly applied. As originally emphasized by Friedenstein [6, 30], the clonogenicity of MSCs is striking. In the case of human MSCs, if the nucleated cells from bone marrow are first plated at high density, incubated for 7–9 days, and the adherent cells then replated at low density, 10% or more of the cells will give rise to single-cell-derived colonies (defined as colony-forming units or CFUs) in about 2 weeks. With some preparations, from 80 to 90% of the cells are CFUs [31, 32]. However, the CFUs decrease dramatically if the same preparations are plated at a high density or are allowed to expand to confluence [32]. Moreover, there are large differences in the clonogenicity seen with cultures prepared from bone marrow aspirates from different normal donors and even between aspirates drawn from the right and left iliac crests of the same normal donor in the same session [33]. The criterion is further confused by observations with MSCs from rodent bone marrow. Cultures of mouse MSCs are particularly confounding. Nucleated cells from mouse bone marrow that adhere to tissue culture plastic are heavily contaminated by hematopoietic cells. The hematopoietic cells can be removed by immunoselection [34] or by repeated passage as adherent cells [35]. However, mouse MSCs are similar to mouse fibroblasts [36] in that they initially grow slowly until a few cells emerge from a "crisis" in the cultures, become transformed, and then are potentially tumorigenic [37]. Moreover, there are differences among MSCs from different strains of mice and some difference in the media required for optimal yields [35]. With rat bone marrow, plating of nucleated cells from bone marrow of some but not all strains gives rise to MSCs that are relatively free of hematopoietic cells [38]. The MSCs from young rats of strains that provide good yields of MSCs grow rapidly when first plated and are highly clonogenic. However, CFU assays on rat MSCs need to be a carried out carefully by plating single cells in separate wells in microtiter plates because of the tendency of the cells from one colony to detach and generate new colonies [38].

The criterion of differentiation has also been applied loosely. In the case of MSCs from human bone marrow, preparations isolated with the same protocol vary in the extent of differentiation into osteoblast-like mineralizing cells, adipocytes, and chondrocytes [33, 39]. In fact, one surprising observation is that if cells from single-cell-derived colonies are replated at clonal densities, they give rise to new single-cell-derived colonies that vary in their potential to differentiate into mineralizing cells and adipocytes (Fig. 2.2). The colonies also vary in size and morphology (Fig. 2.3).

Usually, differentiation is assayed by incubating cultures in a medium conducive to mineralization and stained with Alizarin red S or incubating in medium conducive to adipogenesis and stained with Oil red O. The extent of differentiation is then evaluated qualitatively by microscopy, and the evaluation of the same samples by different observers can vary. Far more reliable data for mineralization and adipogenesis can be obtained with extraction of the dyes for quantitative colorimetric assays [41].

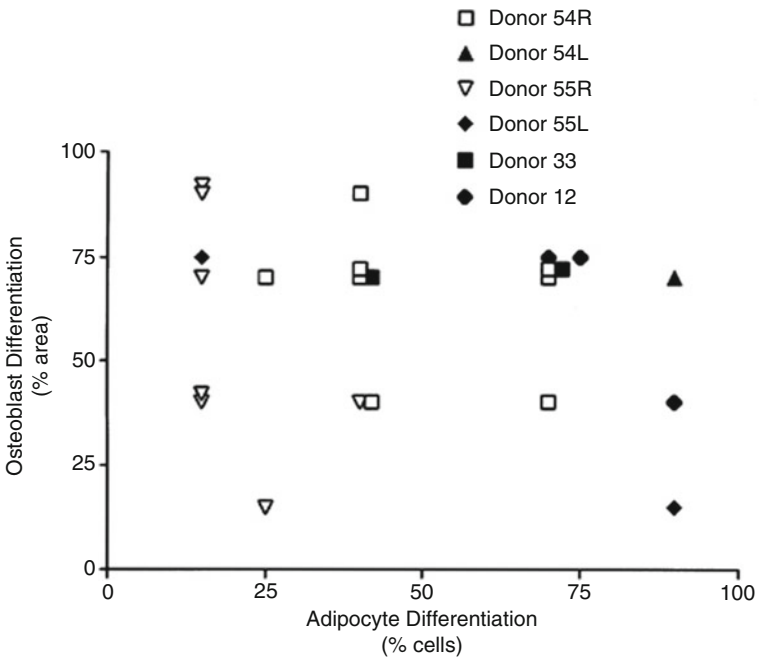

Fig. 2.2 Variation within and between human donors in osteoblast and adipocyte differentiation capacity of MSC colonies from CFU assays at passage 2. *R and L* indicate samples from right and left iliac crests of same donor taken at the same time. One to eight large colonies from the same plate were assayed (Reproduced with permission and modified from Digirolamo et al. [39])

Unfortunately, quantitative assays have rarely been employed. Alternatively, quantitative RT-PCR assays for gene expression are very helpful. Chondrogenic differentiation requires culturing the cells as a micropellet and incubating for 2–3 weeks in a serum-free chondrogenic medium. The pellets are then fixed, sectioned, and stained with either toluidine blue to detect the proteoglycans or Safranin O to detect glycosaminoglycans. Again, the evaluation is qualitative and varies with different preparations and with expansion of the same preparations. Also, the recombinant cytokines used with the serum-free medium (TGF-β3 and BMP-2) are expensive, and the assay is not frequently repeated. The quantitative RT-PCR assays for cartilage-specific mRNAs are very helpful.

The criterion of epitopes is also problematic. There is consensus that human MSCs from bone marrow should be negative for epitopes found on hematopoietic cells. The expression of epitopes found on non-hematopoietic cells is more useful, but again, some of these can be variable among different preparations, different laboratories, different species, and different passage numbers of the same preparations [42].

How will this controversy be resolved? One approach is for reviewers to encourage authors to apply the existing criteria more rigorously. Another is to make available reference banks of MSCs that investigators can use as standards for comparison.

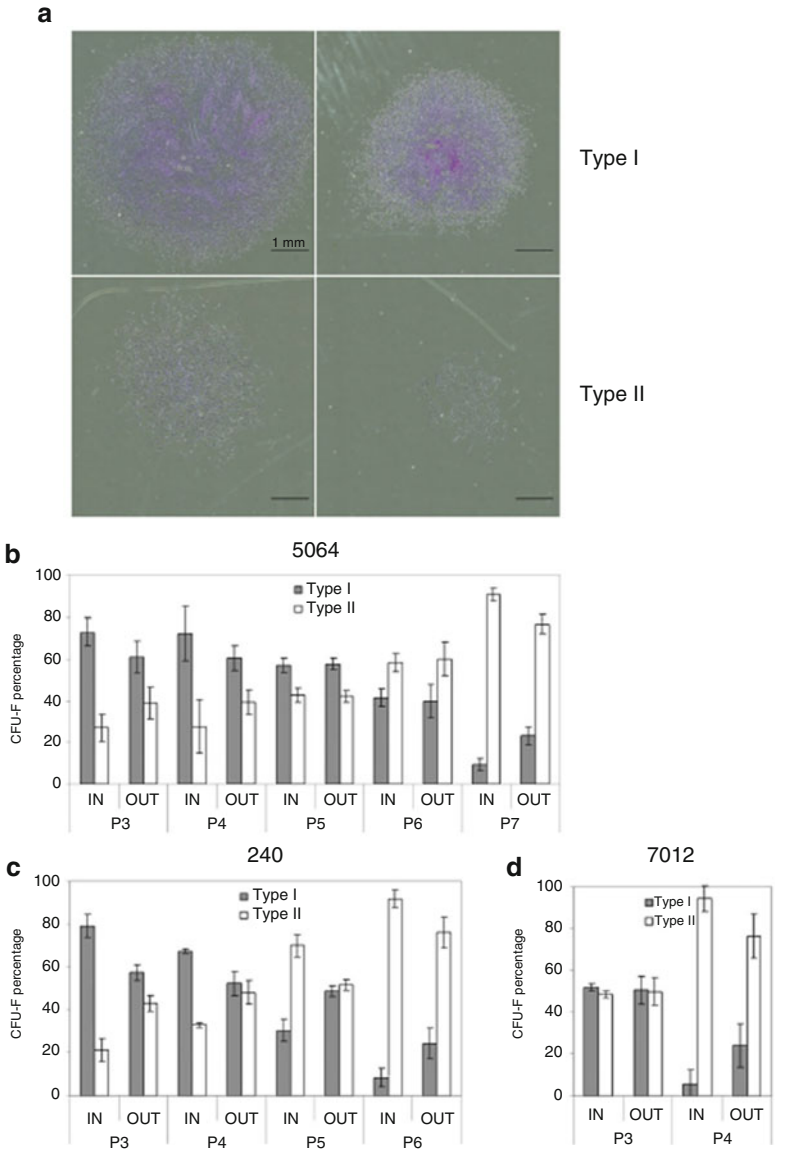

Fig. 2.3 Changes in clonogenicity, colony size, and morphology within and among donors upon subcloning. Passage 1 MSCs were plated at 2 cells/cm^2 and grown for 10–12 days. Cells (passage 2) were then isolated from either the inner dense region (IN) or outer region (OUT) of the colonies and replated and cultured as above. This passaging continued until the cells no longer generated colonies with distinct IN and OUT regions. Representative colonies of donor 240 (P3) stained with crystal violet, measured, and classified as either type I or type II. (**a**) Type I colonies were at least 4 mm in size with a dense IN (upper panel). Type II colonies were either less than 4 mm with a dense IN or larger in size but loose (lower panel). Distribution of type I and type II colonies derived from cells isolated from the IN and OUT regions during subcloning for donor (**b**) 5,064; (**c**) 240; and (**d**) 7,012. The fraction of type II colonies increased with passage number for all 3 donors. However, the passage number at which type II colonies predominated varied among the donors. Error bars: standard deviations; $n=3$. Scale bar: 1 mm. *Abbreviations*: *CFU-F* colony-forming units fibroblast, *IN* inner region of colony, *OUT* outer region of colony, *P* passage (Reproduced with permission and modified from Ylostalo et al. [40])

In support of this suggestion, we obtained an NIH grant to prepare and distribute standardized preparations of MSCs to other investigators (http://medicine.tamhsc.edu/irm/msc-distribution.html). We have provided the cells to over 350 laboratories. A more permanent solution will probably be to develop rapid and more reproducible in vivo assays for either the differentiation of MSCs or therapeutic benefits in one or more disease models.

Controversy II: Can MSCs Differentiate into Non-mesenchymal Cells

Early observations with MSCs generated this controversy because they suggested that MSCs were capable of differentiation into neural cells and fibroblasts (see Fig. 2.4 and Pereira et al. [43], Azizi et al. [44], Kopen et al. [16]).

The observations generated controversy in part because the accepted dogma at the time was that cells could not differentiate across germ lines. Also, the accepted dogma at the time was that stem cells differentiated in a defined sequence of progenitor cells referred to as hierarchical differentiation [45]. The controversy became heated at several different levels, particularly at the political level when it was suggested that research on human embryonic stem cells was unnecessary because of the differentiation potential of "adult stem cells" such as MSCs. The controversy also became heated because the early experiments on differentiation of MSCs were limited by available techniques and the generation of artifacts that had not been previously recognized: Cell labeling reagents such as dyes, and even genetic markers, were unexpectedly transferred across membranes from one cell to another; many antibodies were used without fully defined specificities; overlapping cells in immunocytochemistry of tissues at a time when 3D resolution of microscopic imaging was not available; and artifacts introduced by rare cell fusion events. The controversy has become less heated as the assays, and, therefore, the data generated have improved. For example, in our own laboratory, we were able to use time-lapse microscopy and a series of RNA and protein assays to demonstrate that MSCs cocultured with heat-shocked primary epithelial cells differentiated into epithelial cells both with and without cell fusion, at least under those experimental conditions (Fig. 2.5) [18].

Also, the controversy has become less heated with parallel developments that challenged several dogmas in the field. One of these developments was the discovery of induced pluripotent stem cells that demonstrated the ease with which the genome of cells can be reprogrammed by the introduction of four genes expressed in embryonic cells [46]. The generation of the induced pluripotent cells emphasized the plasticity of the genome, a conclusion demonstrated earlier by experiments in which nuclei of somatic cells were transferred to enucleated embryonic cells [47]. Another development was the recognition that the concept of "hierarchical differentiation" of hematopoietic stem cells had overlooked the important contribution of "niches" in directing differentiation of stem cells [45]. The critical importance of

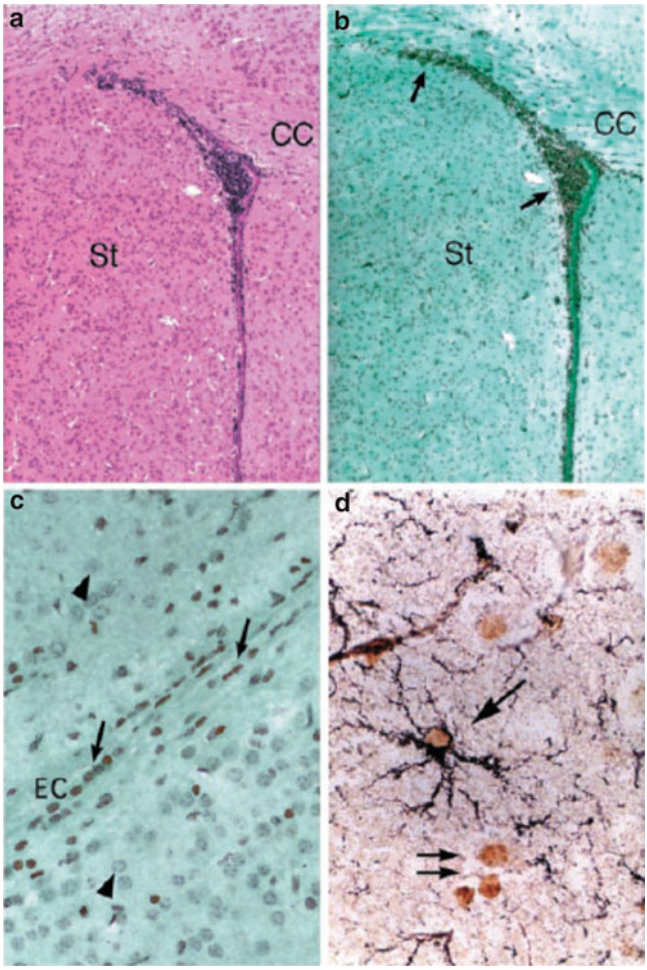

Fig. 2.4 Immunohistochemical localization of BrdUrd-labeled mouse MSCs in transplanted into mouse forebrain. Hematoxylin/eosin (**a**)- or anti-BrdUrd (**b**)-stained serial sections of striatum and lateral ventricle, ipsilateral to the injection site at bregma. (**c**) High-power magnification of BrdUrd-labeled cells in the external capsule. Photomicrograph is from same section as (**b**) but shows a more lateral field. (**d**) MSC-derived astrocyte in the molecular layer of the hippocampus double labeled with anti-BrdUrd and anti-GFAP (*black*). *Arrows*, BrdUrd-labeled nuclei; *arrow-heads*, nuclei negative for BrdUrd labeling (**a** and **b** – ×40; **c** – ×400; **d** – ×1,000) St: Striatum; CC: corpus callosum; EC: external capsule (Reproduced with permission from Kopen et al. [16])

niches in the hematopoietic system was a rediscovery of the role of niches in simpler systems such as ovogenesis in drosophila [48]. Still another development was the extensive observations on epithelial-mesenchymal transition in cancer and other conditions. Among the most remarkable recent publications is the report by Olsen and colleagues [49] that two lines of cultured human endothelial cells can be

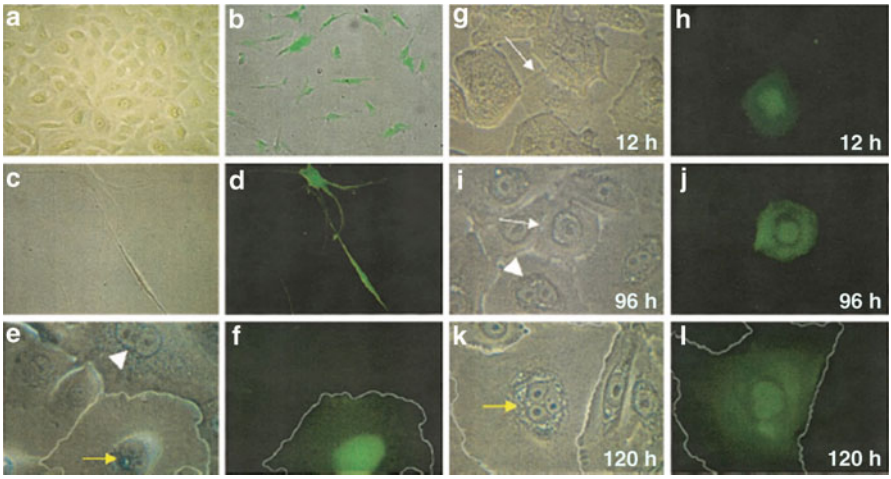

Fig. 2.5 Phase-contrast and fluorescence microscopy of small airway epithelial cells (SAECs), bronchial epithelial cells (BEC), and lentiviral GFP⁺ hMSCs in culture and coculture. Epithelial cells were heat shocked (47 °C, 30 min), and 1–2 h later, GFP⁺ hMSCs were added to the cultures. (**a**) Monolayer of SAECs in SAEC serum-free medium. (**b**) GFP⁺ hMSCs in 20% FBS MSC medium (FITC overlay on phase). (**c**, **d**) GFP⁺ hMSCs cultured in serum-free SAEC medium. (**e**, **f**) Coculture with heat-shocked BEC at 2 week. The differentiated GFP⁺ cell has an epithelial morphology and has repaired the monolayer formed by the epithelium. The cell is binucleated (*yellow arrow*), as is a GFP-negative BEC above it (*arrowhead*). (**g–l**) Time-lapse images of cocultures of GFP⁺ hMSCs and heat-shocked SAECs after incubation for 12–120 h. (**g**, **h**) GFP⁺ cell between SAECs undergoing morphological changes (*arrow*). (**i**, **j**) Differentiated GFP⁺ cell has an epithelial morphology, has repaired the monolayer formed by the SAECs, and has a single nucleus (*arrow*). Adjacent SAEC is binucleated (*arrowhead*). (**k**, **l**) Differentiated GFP⁺ cell has three nuclei (*yellow arrow*). (**e**, **f**, **k**, and **l**) The outermost cytoplasmic edges of the GFP⁺ cells are artificially enhanced (Magnification: (**a–d**) ×10; (**e**, **f**) ×40; (**g–j**) ×20; (**k**, **l**) ×40) (Reproduced with permission and modified from Spees [18])

efficiently transformed in culture to cells with characteristics similar to MSCs by the simple addition of BMP4 or TGFβ2 to the medium.

It is probably too early to conclude that there is a consensus on the differentiation potential of MSCs. However, there are currently 543 entries in PubMed under "mesenchymal stem cells neural differentiation." A quick scan of the entries indicates that a few challenge the possibility of neural differentiation but several offer extensive data on the functional characteristic of the differentiated cells (see Zeng et al. [50], Delcroix et al. [51], Zhang et al. [52]). These publications continue to appear at a time when both the investigators and reviewers have had an adequate opportunity to recognize the artifacts and criticisms encountered previously. Therefore, they deserve serious attention.

Where does this leave the field? Certainly, there is a consensus that the experimental conditions for differentiation of MSCs are not highly reproducible. Also, the molecular events that drive differentiation such as Wnt and Notch signaling have been touched on by some recent reports [53, 54] but not explored in detail. At the end of the day, it seems clear that carefully prepared MSCs have a potential to

differentiate that lies somewhere between mature somatic cells such as skin fibroblasts and ESCs and induced pluripotent cells. The differences from ESCs and iPS cells are probably quantitative ones that hinge on the rigor of the experimental conditions that are required to reprogram the genome.

Controversy III: Are MSCs Pericytes?

Several groups have claimed that a rare population of perivascular $CD45^{-}/CD146^{+}$ pericytes are the progenitors of MSCs capable of generating the hematopoietic microenvironment [7, 55, 56]. The results demonstrated striking similarities between pericytes and bone marrow-derived MSCs. There are, however, some differences that have not been explained to date. One difference is that most pericytes expand slowly in culture. Another is that most pericytes are contractile cells. Still another is that pericytes from different vessels show considerable heterogeneity. In addition, more recently, Mendez-Ferrer and colleagues identified a rare subset of perivascular nestin-positive cells that had essentially all the properties of MSCs, that spatially associated with the hematopoietic stem cells (HSCs), and that were an essential component of the HSC niche [9]. Although not directly compared, the nestin-positive MSCs did not appear to be pericytes.

How do we resolve these apparently contradictory observations? Clearly, the observations themselves were carefully made and cannot be challenged. The resolution is perhaps to accept that pericytes and MSCs could be members of a large family of cells with many properties in common. Most importantly, they have a remarkable plasticity that approaches transmogrification: changing from one phenotype to another when presented with different niches or microenvironments in vivo and in vitro. We have learned to accept the multiple phenotypes of T lymphocytes and the M1/M2 phenotypes of macrophages. The concept seems more difficult to accept for MSCs.

Controversy IV: Can MSCs Rescue Injured Cells by Transfer of Mitochondria?

Several years ago, we made the unexpected observation that MSCs can rescue cells with nonfunctional mitochondria by the transfer of mitochondria [57]. The observation had broad implications for the therapeutic potentials of MSCs because failure of mitochondria is a common event in many diseases, particularly with ischemia and reperfusion of tissues. The mitochondria are damaged by the ischemia and then fail to provide adequate electrons to reduce oxygen when the tissue is reperfused. The result is an increase in reactive oxygen species (ROS) that rapidly damage cells. Paradoxically, low levels of ROS trigger inflammation and high levels cause apoptosis.

The transfer of mitochondria we observed therefore could provide a rationale for the use of MSCs as therapy for stroke, myocardial infarction, and other diseases. All the observations we made, however, were in tissue culture, and we were unable to devise an adequate experiment to prove transfer in vivo. This problem has recently been addressed with an ingenious series of observations on relatively benign genital tumors of dogs that were transmitted as allografts over many generations during coition [58]. Sequencing of two informative regions in mitochondria in 37 samples of the tumors in dogs from four continents indicated extensive capture of host mitochondrial DNA in most of the samples. The results do not conclusively establish that functional mitochondria were transferred, but they do establish the transfer of mitochondrial DNA.

A recent study by Islam et al. [59] is the first demonstration of in vivo mitochondrial transfer from MSCs to LPS-injured mouse lung alveolar epithelial cells leading to promotion of tissue repair and increased survival of the mice. Intravital fluorescence microscopy of ex vivo perfused LPS-damaged lungs was employed to directly observe the interaction of MSCs expressing a fluorescent tag with the lung epithelial cells. The MSCs were administered intratracheally into the lungs and imaged up to 24 h. The MSCs attached to the alveolar cells, and the MSC mitochondria were observed to be transferred in microvesicles and nanotubes to the damaged cells and accompanied by an increase in ATP in the alveolar cells. What are the consequences of transfer of mitochondria by MSCs? One is that some of the beneficial effects of MSCs observed in animal models of human diseases may be explainable by transfer of mitochondria. Another possibility is that if the mitochondria or mitochondrial DNA are transferred in vesicles, the vesicles may also contain microRNAs or even mRNAs that could explain the differentiation of MSCs observed in some cocultures with other cells [18].

Controversy V: Do Intravenously Infused MSCs Escape Entrapment in the Lung?

Early observations with MSCs demonstrated marked improvements in injuries to organ such as the heart, brain, and kidney after intravenous administration of the cells. MSCs were detected in the tissues with assays for markers such as cell-labeling dyes, and marker genes such as GFP. However, subsequent observations demonstrated that many of the experiments were subject to previously unsuspected artifacts. Also, some of the experiments, particularly those with mouse MSCs, were probably confounded by the presence of hematopoietic cells and the tendency of the cells to spontaneously transform. In addition, most of the data were based on manual counting of labeled cells in sections without resort to the quantitative deconvolution and 3D microscopic algorithms now available. To address these problems, we elected to resort to an unconventional approach: infusion of well-characterized human MSCs into either wild-type or immunodeficient mice [10, 27, 28]. The strategy made it possible to use endogenous markers in the MSCs that could be

assayed quantitatively for human DNA by real-time PCR assays and for genes expressed by the human cells by RT-PCR assays. The assays themselves required careful attention because of two variables: the efficiency of extraction of DNA and RNA from different tissues and the efficiency of polymerization of nucleic acid by PCR because of variations in the contaminants in extracts of DNAs and RNAs from different tissues. To overcome these problems, Lee et al. [28] developed separate standard curves for each tissue by adding varying numbers of human MSCs to naïve tissues before extraction of the nucleic acids. They also normalized the value of each assay to the total DNA or RNA in the sample by quantitative PCRs with species-specific primers for both mouse and human GAPDH. The results confirmed previous reports based on qualitative imaging techniques that most intravenously infused MSCs are immediately trapped in the lung, probably during the first pass [28, 60, 61]. The results obtained with the assays indicated that after IV infusion of human MSCs, 99% (±1.07 SD) of the cells were cleared from the circulation within 5 min and that most of the cells were trapped in the lung. The cells disappeared from the lungs with a half-life of about 24 h but did not appear in any significant numbers in other tissues that were assayed: A total of 0.04% of the infused Alu sequences (equivalent to about 4,000 cells) were recovered in six tissues after 48 h and 0.01% after 96 h.

The conclusions from these experiments and the prior observations [28, 60, 61] have not been universally accepted in subsequent publications. One criticism was that the human MSCs employed are larger and perhaps more adherent than mouse MSCs and therefore more likely to be trapped in the lungs. However, one of the earlier experiments that provided qualitative data on extensive trapping in lungs used rat MSCs [60], and one used mouse MSCs [61]. Another criticism was that the immune system of the immunodeficient mice (NOD/*scid*) used in the experiments [28] may have destroyed the human MSCs in the lung before they had a chance to recirculate to other tissues. As discussed below, the use of human MSCs raises concerns but offers distinct advantages, and the concerns can probably be addressed by validating the observations with additional carefully controlled experiments.

At the same time, it seems apparent that the data obtained with quantitative PCR and RT-PCR established that about 90% of human MSCs infused intravenously into mice are trapped in the lungs and very few reach more distal organs, even after selective injury to the organs. Mice appear to have unusually small capillaries, and human MSCs are larger than those from mice. Therefore, larger numbers may escape entrapment in the lung if autologous MSCs are infused in other animals. Also, the number of human MSCs trapped in the lung decreased by 25% when human MSCs were cultured as hanging drops to form spheroids and then are dissociated before being infused intravenously into mice apparently because the MSCs become compacted in spheroids and are about one-quarter the volume of MSCs cultured as monolayers [62]. However, we will not know the extent to which intravenously infused MSCs escape entrapment in the lungs in species other than mice until more quantitative experiments are performed.

At the same time, the impression that most MSCs administered IV are trapped in the lung has prompted great interest in soluble factors released by MSCs that might

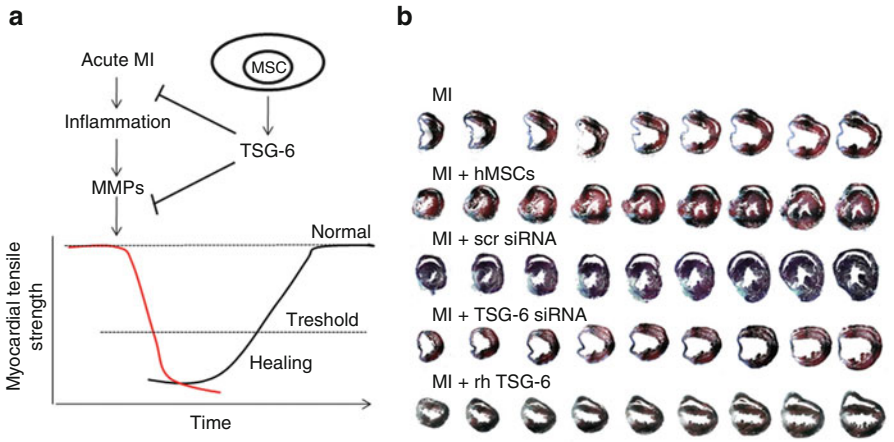

Fig. 2.6 Effects of human MSCs and recombinant TSG-6 in NOD/scid mice with myocardial infarcts (MI). (**a**) Schematic illustrating the progressive damage to the myocardium following MI. The ischemia triggers invasion by inflammatory cells. The inflammatory cells and the matrix metalloproteinases they release accentuate damage to the myocardium. TSG-6 synthesized by MSCs or recombinant TSG-6 limits the injury and thereby enhances repair (Reproduced with permission and modified from Prockop et al. [4]). (**b**) Protective/reparative properties of MSCs and TSG-6 in MI. Three weeks after permanent ligation of the anterior descending coronary artery in mice, each heart was cut from the apex through the base into sequential 5-um sections and stained with Masson trichrome. Every 20th section is shown. Cells (2×10^6) were delivered intravenously (IV) 1 h after MI. *Symbols*: MI only; MI + hMSCs, hMSCs; MI + scr siRNA, hMSCs transduced with scrambled siRNA; MI + TSG-6 siRNA, hMSCs transduced with TSG-6 siRNA; MI + rhTSG-6, 30 ug recombinant TSG-6 protein infused IV 1 h and again 24 h after MI (Reproduced with permission and modified from Lee et al. [28])

explain their beneficial effects. For example, in different models of inflammation, the beneficial effects of MSCs were traced to the cells being activated to express the anti-inflammatory protein TSG-6 (Fig. 2.6) [28], prostaglandin E2 [63], the interleukin 1 receptor antagonist [64], or soluble TNF receptor 1 [65]. Still, other factors produced by MSCs including nitric oxide, indoleamine 2,3-dioxygenase, and CCL2 were reported to explain the immunomodulatory effects of MSCs (below).

Controversy VI: Is It Valid to Experiment with Human MSCs in Rodent Models?

The use of xenogeneic cells in animals has long been an anathema in biology. However, the difficulties inherent in isolating and expanding mouse MSCs tempted us and others to test human cells in rodents. We were encouraged by the evidence that MSCs are at least partially immune privileged and the apparently successful use of unmatched MSCs from universal donors in patients [66]. Further, we were

encouraged by our observations that human MSCs disappeared with about the same half-life whether injected into the hippocampi of wild-type or immunodeficient mice [27]. A number of reports have now established therapeutic benefits from systemic administration of human MSCs in immunodeficient mice with streptozotocin-induced diabetes and with myocardial infarction. Therapeutic benefits were also observed with administration of human MSCs to wild-type rodents that were models for transient global ischemia [27], retinal degeneration [67], peritonitis [68], and meniscal injury (Masafumi Horie, Hosoon Choi, Ryang-Hwa Lee, R.L.R., Joni Ylostalo, Takeshi Muneta, Ichiro Sekiya, and D.J.P., Osteoarthritis and Cartilage, in press). We also note that a number of other investigators have recently used human MSCs from bone marrow in rodent models [69–72]. The use of human MSCs in animal models offers the tremendous advantage of using species-specific markers to follow the cells, including the ability to examine the cross-talk between the human MSCs and endogenous cells and the dramatic changes which result in both cell populations.

Nonetheless, many investigators are still concerned about crossing species barriers in such experiments. The best resolution of this controversy is to define clearly the goals of such experiments. The human MSCs are used in rodents or other species to first establish a therapeutic benefit and then define the mechanisms by which the human cells exert their effects. The strategy provides a powerful tool for following the cross-talk between the MSCs and injured tissues. Autologous or syngeneic MSCs might be more effective in many of the experiments, but the tools for analyzing their effects are much more limited. The happiest resolution of the controversy is probably the one that occurred in observing the effects of human MSCs in a mouse model for myocardial infarction: The beneficial effects were traced to TSG-6, and then the recombinant protein was shown to reproduce most of the beneficial effects of the human MSCs (Fig. 2.5) [28]. Also, most of the beneficial effects of administration of MSCs were lost when the TSG-6 gene was knocked down with an siRNA.

Controversy VII: Can "Universal Donors" of MSCs Be Used in Patients?

The initial efforts to develop clinical therapies generated a dilemma: Would autologous MSCs be required? The question had immediate financial consequences. The autologous MSCs were about twice as expensive (well over $10,000/preparation by our estimates). Quality control was difficult because if freshly prepared MSCs were used, the tests for efficacy of the cells and microbial agents possibly acquired during preparation of the cells could not be completed before the cells were administered. If the samples were frozen before use, the extensive tests required for each autologous preparation were expensive. Also, fresh autologous MSCs were impractical for therapy of acute diseases such as myocardial infarction and stroke. So the most common "business model" for the several biotech companies was to prepare large lots of extensively expanded MSCs from a single donor. We and others persisted with the development of protocols to use autologous MSCs in patients. We pursued this course because we were concerned about (a) administering cells that might

elicit immune reactions, and (b) the extensive expansion or highly confluent cultures necessary to produce a large bank of MSCs would eliminate the early progenitor cells that were enriched in early passage low-density cultures. We developed a protocol to prepare autologous MSCs for therapy of patients with spinal cord injuries on the basis of data from experiments in rats indicating that there was a window for therapy of about 1 week following an acute injury to the spine [73, 74]. Sekiya and colleagues in Tokyo [75] developed a similar protocol preparing autologous MSCs generated from a biopsy of synovial tissue to treat acute injuries to knee cartilage. They launched a phase I/II clinical trial now nearing completion.

In the interim, newly launched biotech companies and academic centers have carried out extensive clinical trials with large banks of MSCs from universal donors. In some of the trials, patients have received repeated infusions of large numbers of MSCs from large banks. To date, no major adverse reactions have been reported. Therefore, the use of universal donors for clinical applications is now supported by a considerable body of data, and our commitment to autologous MSCs is a minority position. However, we are convinced that autologous MSCs may be superior for some applications such as the repair of knee cartilage by local application of the cells as is being done by Koga et al. [75].

Controversy VIII: How Do MSCs Modulate Immune Responses?

Important immunomodulatory effects of MSCs were first discovered in clinical trials to improve bone marrow transplants with MSCs: In a proportion of patients, the MSCs improved the manifestations of graft-versus-host disease (GVHD) [66]. The observations were supported by reports that MSCs inhibited the mixed lymphocyte reaction. These observations in turn prompted experiments that demonstrated IV infusions of MSCs reduced neurological deficits in the experimental autoimmune encephalitis (EAE) model for multiple sclerosis [76]. Extensive efforts have been made to explain the immunomodulatory effects of MSCs, but the field remains controversial, and several different scenarios have been advanced by leaders in the field (Fig. 2.7) (For more complete reviews, see Uccelli et al. [79], Bernardo et al. [80]).

Shi and associates [81] observed that murine MSCs were activated by IFNγ together with any one of three other proinflammatory cytokines (TNFα, IL-1α, or IL-1β) to become immunomodulatory. The activated MSCs expressed several cytokines and inducible nitric oxide synthase (iNOS). The chemokines attracted T cells to the MSCs, and the NO suppressed the T cells. The same authors [81] subsequently found that human and monkey MSCs did not synthesize NO, but the MSCs suppressed T cells by secreting indoleamine 2,3-dioxygenase (IDO) that depleted tryptophan in the medium or generated toxic concentrations of kynurenine and other suppressive metabolites [81].

Galipeau and associates [77] found that the MSCs inhibited activation of the T cells by secreting both CCL2 (monocyte chemotactic protein-1 or MCP-1) and MMP-9

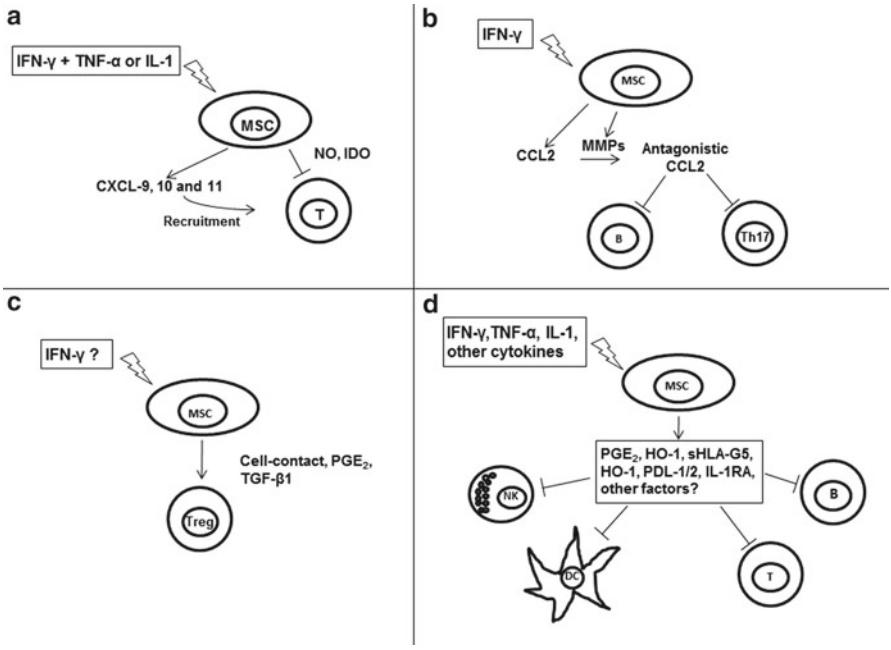

Fig. 2.7 Four putative scenarios by which MSCs modulate immune reactions. (**a**) Stimulation of MSCs by interferon (*IFNγ*) and other proinflammatory cytokines causes MSCs to produce either nitrous oxide (*NO*) or indoleamine 2,3-dioxygenase (*IDO*) and thus suppress T cells. (**b**) MSCs inhibit activation of $CD4^+$ T cells by secretion of CCL2 that is cleaved into an antagonistic fragment by matrix metalloproteinase-9 (*MMP-9*), also secreted by MSCs [77]. (**c**) MSCs enhance T regulatory (*Treg*) cells. Direct contact with $CD4^+$ T cells induces MSCs to secrete transforming growth factor (TGF)-β1 and prostaglandin E2 which in turn induce expression of the Treg markers FoxP3 and CD25 by the $CD4^+$ T cells [78]. (**d**) MSCs act on natural killer (*NK*) cells, dendritic cells (*D*), T cells, T helper cells, and B cells to modulate immune responses [79]

in the murine EAE model for multiple sclerosis. MMP-9 then cleaved the CCL2 into an immune-suppressive derivative. In support of their proposal, they found that conditioned medium from MSCs inhibited activation of $CD4^+$ T cells from mice in which EAE was induced but not T cells from $CCL2^{-/-}$ EAE mice. François et al. also observed that MSCs can produce opposite effects: They can stimulate immune and inflammatory responses. They observed that MSCs cross-presented exogenous antigen and induced an effective $CD8^+$ T cell immune response [77].

Mahon and associates [78] observed that MSCs enhanced the generation of T regulatory cells. They reported that allogeneic MSC induced expression in $CD4^+$ T cells of two markers of T regulatory cells: Forkhead box P3 (FoxP3) and CD25. They proposed a sequential process in which a first step required direct contact between MSCs and $CD4^+$ T cells followed by secretion of TGF-β1 and prostaglandin E2 by the MSCs to drive differentiation of T cells to T regulatory cells.

Uccelli et al. [79] suggested more pleiotropic effects of MSCs. They suggested that MSCs (a) decreased proliferation, cytotoxicity, and cytokine production by NK cells;

(b) impaired maturation and antigen presentation by dendritic cells; (c) decreased proliferation of T cells and impaired T helper cells; and (d) decreased proliferation and antibody production by B cells.

Which of these proposals best accounts for the immunomodulatory effects of MSCs? There is no consensus at the moment and a number of questions remain unanswered. How many of the observations are confounded by the differences between the murine and human immune systems? How many of the differences result from the difficulty of obtaining well-characterized and genetically stable mouse MSCs? How many of these carefully executed and interpreted experiments reflect immune mechanisms that are dramatic in culture but may of secondary importance in vivo? Or are MSCs in fact pleiotropic and does their response depend on the microenvironment of in vivo injury which cannot at this time be completely mimicked in culture? We all await definitive answers to these and many related questions.

Controversy IX: Do Human MSCs Cause Tumors?

The efforts to use human MSCs in clinical trials hit a major roadblock with the appearance of three reports that human MSCs escaped from senescence and generated malignant cells as the MSCs were expanded in culture [82–84]. The reports were surprising since MSCs were regularly observed to become senescent after 35 or so population doublings in culture, and emergence from senescence was not observed in numerous laboratories that had studied the cells for over a decade (see Bernardo et al. [85], Pittenger et al. [86], Digirolamo et al. [39]). In fact, data from two laboratories indicated that human MSCs emerged from senescence at a frequency of much less than 10^{-9} [87, 88]. The discrepancies were resolved by subsequent reports by two of three laboratories [89, 90] that the transformation of human MSCs they initially observed was explained by contamination of their cultures by a small number of malignant cells. Therefore, scientists working with MSCs had rediscovered the danger of cross-contamination of cell cultures by malignant cells, a danger recognized many decades ago but one that still plagues cancer research [91].

It is clear that the danger of generating tumors in patients with any cell therapy must be weighed carefully. DNA replication is not a perfect process, and every cell division poses some risk of activating an oncogene or inactivating a suppressor gene. What years of experience have taught us is that cells vary widely in the stability of their genomes as they are expanded. Mouse fibroblasts and mouse MSCs are at one end of the spectrum in that they regularly pass through crisis and escape senescence in culture after a few passages [36]. As emphasized by a recent report [92], immortal cells such as embryonic stem cells and induced pluripotent cells consistently demonstrate genomic instability and develop numerous mutations as they are expanded. Also they are consistently tumorigenic when administered to mice. Human MSCs occupy the other end of the spectrum in terms of the probability of becoming tumorigenic with expansion. They have been consistently

observed to pass into senescence as they are expanded in culture. The test of senescence in cultures remains the best indication that a culture does not contain any cells that have undergone genomic changes that make them immortal in culture and therefore prone to be tumorigenic and carcinogenic in vivo. In contrast, the test of tumorigenicity in mice, labeled the "most ridiculous assay on the planet," [93] is of limited value because many human cancers will not form tumors in mice. Also, the steadily improving technologies of genomic sequencing and analysis are likely to remain of limited value. There is no strategy that even in theory can detect the presence of few carcinogenic cells in large preparations unless every cell in the preparation is sampled. However, the danger of tumor formation in cell therapies with MSCs must be considered simply as a low probability, not an absolute guarantee, and the low probability must be weighed carefully in the risk/benefit evaluation for treating any patient.

Conclusions

Controversy I: *What are the criteria for identifying MSCs*? The existing criteria of clonogenicity and differentiation potential in culture should be applied more rigorously than they have in many publications in the past. Comparisons with standardized preparations prepared by other laboratories are probably useful. It seems unlikely that a single epitope marker for MSCs will be found given the extensive efforts that have already been made to define such a marker and the remarkable ability of the cells to change in culture and in different microenvironments in vivo. However, we are all awaiting an assay that will accurately define the therapeutic potentials of the cells in vivo and that will reflect the remarkable ability of the cells to respond to different microenvironments.

Controversy II: *Can MSCs differentiate into non-mesenchymal cells*? This controversy has largely been put to bed with the recent observations that reemphasize previous observations that demonstrated the plasticity of the mammalian genome. The differences from ESCs and iPS cells are probably quantitative ones that hinge on the rigor of the experimental conditions that are required to reprogram the genome.

Controversy III: *Are MSCs pericytes*? MSCs may not cleanly fit the classical criteria of pericytes that are defined primarily as contractile cells whose properties differ in different vascular settings. However, MSCs have striking similarities to cells closely associated with blood vessels and therefore probably belong in the same family of cells.

Controversy IV: *Can MSCs rescue injured cells by transfer of mitochondria*? Recent observations on transfer of mitochondria from MSCs to damaged alveolar epithelial cells strongly support the earlier suggestion that MSCs can transfer mitochondria to rescue ischemic cells. Also, recent observations indicate they can transfer microRNAs that change the properties of target cells.

Controversy V: *Do intravenously infused MSCs escape entrapment in the lung*? Our own assays by quantitative PCR and RT-PCR established that about 90% of human MSCs infused intravenously into mice are trapped in the lung and very few reach

more distal organs, even after selective injury to the organs. Entrapment of autologous MSCs is probably somewhat less in other animals with larger capillaries, but we will not know the extent to which intravenously infused MSCs escape entrapment in the lung until more quantitative experiments are performed in species other than mice.

Controversy VI: *Is it valid to experiment with human MSCs in rodent models*? Administration of human MSCs to mouse models for a number of diseases has produced therapeutic benefits that are similar to those obtained by administration of isogeneic MSCs apparently because the cells are at least partially immune privileged. However, both isogeneic and xenogeneic MSCs are degraded, and only a few recovered from most tissues after a week or so. The use of human MSCs provides a wealth of specific assays to follow cross-talk between the MSCs and the host cells, but it is obviously important to extensively verify the observations.

Controversy VII: *Can* "*universal donors*" *of MSCs be used in patients*? No adverse reactions attributable to MSCs have been reported in clinical trials in which cells with large preparations from universal donors were infused into large numbers of patients. Some of the patients received repeated infusions of the cells. However, the results have not excluded the possibility that autologous MSCs may be more efficacious and safer under some circumstances such as local injections of the cells to repair tissues by engraftment and differentiation.

Controversy VIII: *How do MSCs modulate immune responses*? This controversy cannot be resolved without further evidence, primarily from experiments in vivo, for the four explanations currently proposed (Fig. 2.7) and others that are likely to be generated in the near future.

Controversy IX: *Do human MSCs cause tumors*? The probability of human MSCs causing tumors is not zero, but it is extremely low. However, there is convincing evidence that MSCs can enhance tumor growth under some circumstances.

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Chapter 3
MSCs: The Need to Rethink

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Abstract It has long been known that within bone marrow, a non-hematopoietic stem cell, capable of reforming a complete skeletal segment exists. Originally termed a bone marrow stromal stem cell and later renamed a "mesenchymal" stem cell ("MSC"), this cell has the capability to form cartilage, bone, hematopoiesis-supportive stroma, and marrow adipocytes. Furthermore, this cell also organizes marrow vasculature and is a component of the HSC niche, properties that make this cell inherently unique. Based on its cell surface properties (that solely represent connective tissue cells) and the use of less than stringent in vitro differentiation assays, "MSCs" have been reported to be found in virtually any connective tissue, with extensive differentiation capacities. Yet by rigorous criteria, MSCs from different tissues are not the same, are not ubiquitous, and they are not pluripotent. Considering the current interest in the use of MSCs from all types of tissues for not only tissue engineering but also in regenerative medicine (using the cells as a drug), there is an urgent need to get specific about the biology of MSCs from different tissues: what they are, where they came from, and what they can really do.

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_3,

Introduction

As measured by the number of papers published on the topic in the last decade, it is evident that "mesenchymal stem cells" are one of the most intensively investigated types of "stem cells." There are a number of reasons that can explain this turn of events: (1) the current explosion of interest in stem cells at large as a challenging intellectual scenario and a promising source of innovation in medicine; (2) the realization of the potential applicative (and commercial) implications of the concept(s) behind the notion of an "MSC"; (3) the relative ease with which cells regarded as MSCs are cultured; and (4) last but not least, the unique character of the archetypical MSC, a non-hematopoietic stem cell, located in bone marrow stroma, able to give rise to all types of cells required for formation of bone and its marrow. Unbeknownst to most workers in the field, the notion of a non-hematopoietic stem/progenitor cell goes back many years and precedes the current explosion of interest by at least two decades. History aside, the present time is a time of change. Reports during the past decade implying transdifferentiation of MSCs are on the wane. New trends are emerging, suggesting different approaches for the use of MSCs in cell therapy but still in need of a clear mechanistic definition. Translational paradigms are changing worldwide, well beyond the boundaries of this specific area. Overall, a measure of confusion pervades the field as a result of less than stringent definitions and defining assays. Stepping forward out of the confusion may involve taking one step back, first.

From a Revolutionary Concept to the Present-Day Misconceptions

MSC biology has its roots in seminal experiments performed in the 1960s with the aim of understanding whether hematopoiesis could be heterotopically transplanted or would, vice versa, necessarily require its natural physiological frame provided by bone. Initially, in experiments performed by Tavassoli and Crosby, it was quite unexpectedly demonstrated that pieces of bone marrow, lacking any bone at all, would actually rapidly be depleted of hematopoietic tissue and reform bone when transplanted in ectopic sites (e.g., under the kidney capsule), prior to the reappearance of hematopoietic tissue in the newly formed bone [1]. The unknown entity bearing the osteogenic potential revealed by these experiments was later identified as part of the non-hematopoietic, stromal fraction of bone marrow [2] and to coincide with cells capable of initiating clonal growth [3], as well as of multiple differentiation potential toward all cell types normally comprised within a skeletal segment-cartilage, bone, adipocytes, and fibroblasts [4] (Fig. 3.1). These observations led Friedenstein and Owen to postulate that a multipotent progenitor of skeletal tissues exists within the bone marrow stroma – a putative stem cell, which they named "osteogenic" or "stromal" [5]. It was not until quite recently, however, that

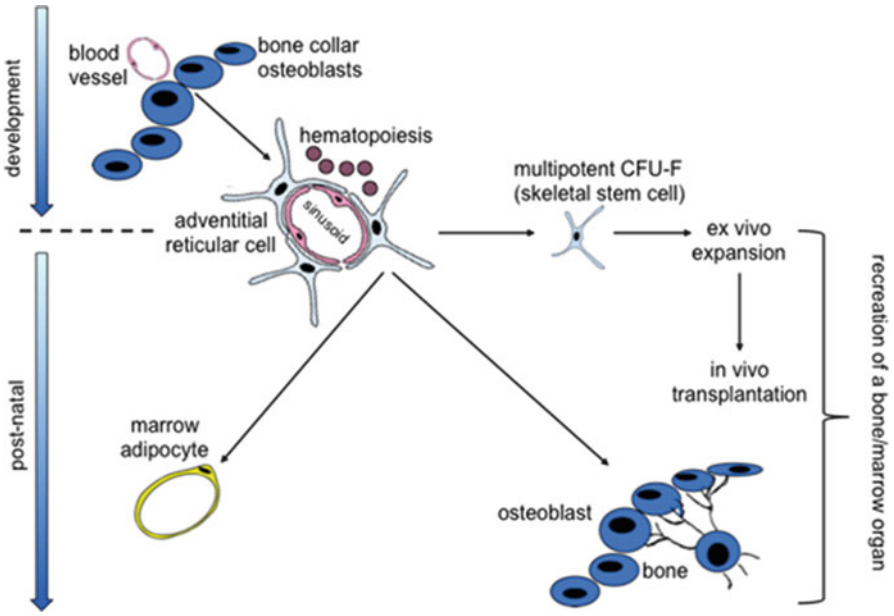

Fig. 3.1 During embryonic development, blood vessels invade developing bone, and in doing so, they associate with committed osteogenic precursors, which in turn form adventitial reticular cells that stabilize and control the caliber of the blood vessel. It is now known that adventitial reticular cells are in fact the colony-forming unit fibroblasts, a subset of which is composed of multipotent and self-renewing skeletal stem cells. During postnatal growth and development, these cells are capable of forming bone (cartilage under certain circumstances), hematopoiesis-supportive stroma, and marrow adipocytes. The clonal progeny of a single skeletal stem cell (MSC) can be expanded in vitro, and upon in vivo transplantation can form bone and adipocytes, and at the same time reconstitute a population of adventitial reticular cells that can be secondarily passaged as CFU-Fs, which represents evidence of self-renewal (Sacchetti et al. [6])

the second feature that defines a bona fide stem cell more stringently than multipotency; that is, the ability to self-renew, received direct support from experimental evidence. At the same time, the in situ identity of bone marrow-derived MSCs was recognized [6, 7].

Stroma, Mesenchyme, and the System of Skeletal Tissues

"Stroma" (from the Greek, στρομα, meaning a mattress, what one rests or lays upon), with regard to bone marrow, denotes the non-hematopoietic (cellular, extracellular matrix-poor) tissue that structurally and functionally supports hematopoiesis. In other organs, it means the interstitial connective tissue that surrounds the vasculature and supports the organ-specific functional tissue (the parenchyma). Hence, the term "stroma" should not be used indifferently for bone marrow and non-bone marrow

tissues, as it alludes to radically divergent functions: hematopoiesis does not take place in the placenta, subcutaneous fat, or muscle – not physiologically and not after experimental or clinical reconstitution via bone marrow transplantation. Hematopoiesis does take place in bone marrow because the bone marrow has a unique, organ-specific, stroma. Therefore, the terms "stroma" and "stromal" should not be used indiscriminately to denote bone marrow or non-bone marrow-derived cells in culture. Unfortunately, the term, "mesenchymal stromal cells," is now widely used to denote cells derived in culture from virtually every tissue. While intended to convey caution as to the actual nature of *cultured* bone marrow "MSCs" as bona fide stem cells [8], the widespread use of this term has contributed to the blurring of the fundamental functional distinction between bone marrow stroma and the stroma of non-hematopoietic tissue (that is, the support of hematopoiesis) and to support the incorrect notion that MSCs with identical functional properties are found in virtually every tissue [9].

Additionally, mesenchyme is the primitive interstitial connective tissue of the embryo. In development, past the time of somite patterning and specification, there are no cells with properties of a common progenitor of all mesoderm derivatives. Tissues commonly seen as part of a varied progeny of a postnatal "mesenchymal" stem cell, such as bone and muscle, do not emanate from a common progenitor past that developmental time point, so that, paradoxically, there is no prenatal "mesenchymal" stem cell. The very term "mesenchymal" stem cell is probably a misnomer, as reviewed elsewhere [10].

The fact that bone is an organ, and that as an organ it includes two functionally interacting systems (hematopoiesis and skeletal tissues), is the simple foundation of hematopoiesis and of transplantability of hematopoiesis. The discovery that each of these two interacting systems emanates from two distinct stem cells was intriguing not only because it highlighted a second type of stem cell in bone marrow but also because it established a link between tissues previously seen as not connected to one another. Bone and marrow adipocytes and cartilage were not conceived as being interrelated prior to Friedenstein's work. Of note, the nature of the interrelation still remains largely unexplored. The notion of an assayable common progenitor for such tissues (a bone marrow-derived MSC) was indeed revolutionary but per se may not exhaust the range of functional relationships that remain to be understood. All cartoons of MSCs and their progeny portray a system of segregated lineages, modeled on the template of the hematopoietic system, by far the best understood, postnatal stem cell-dependent system. However, the relationships of the different stromal "lineages;" (e.g., bone and adipocytes) are far more complex than that of segregated, parallel differentiation cascades. For example, bone and fat (and cartilage) do not develop at the same time. While bone turns over continuously (albeit slowly), little is known about actual turnover of marrow adipocytes in vivo. In humans, marrow adipocytes do not appear until after birth, and cartilage cells are not formed in the postpubertal skeleton in the absence of trauma. Still, we regard MSCs from any tissue as capable of generating, at any time, all these three tissues.

A further distinction between the skeleton and blood or epithelia, the best-known systems emanating from postnatal stem cells is that the turnover rate of the skeleton

is orders of magnitude lower. Yet, bone marrow-derived MSCs are subliminally assumed to be endowed with comparable replicative capacity, even though we know that, for example, they do not express telomerase, in contrast to hematopoietic stem cells (HSCs) [11]. In addition, bone and cartilage, like all connective tissues, are made of cells living within an extracellular matrix that modulates their growth, differentiation, stability, and remodeling, and still, we regard "stem cells" of matrix-free tissues such as blood and epithelium, and MSCs, as part of a common family. Finally, several lines of evidence point to unique features of flexibility, not of bone marrow-derived MSCs per se, but of differentiated stromal phenotypes such as adipocytes, chondrocytes, and osteoblasts [12]. Elucidating the actual in vivo kinetics of tissue turnover within the skeleton, and beyond remodeling of bone proper, seems crucial for understanding the physiology of the system at hand.

Perivascular Bone Marrow Stromal Cells

One of the most important limitations to understanding the biology of bone marrow-derived MSCs has been the difficulty in relating properties of cells explanted from the bone marrow and probed functionally ex vivo (clonogenicity assays) and in vivo (differentiation capacity) to a defined in situ counterpart. While embryonic stem cells (ESCs) are explanted from a defined embryonic structure (the inner cell mass), we have not recognized until recently what cell is explanted and from where exactly in the bone marrow the MSCs emanate. We now know that clonogenic, multipotent, self-renewing stromal cells come from the microvascular compartment of the bone marrow, where they can be identified in situ as cells residing in the vessel wall next to the endothelium of sinusoids [6, 7]. Long known as adventitial reticular cells in classical histology [13–15], these cells had been long surmised, solely based on obvious consideration of the actual bone marrow structure and composition, to represent the prime candidate for the in situ counterpart of MSCs [15–19]. The term adventitial alludes to their position (adventitial to the microvascular wall), while the term reticular alludes to their morphology (Fig. 3.1). These cells are not "undifferentiated" cells, inasmuch as they constitutively express multiple markers characteristic of early osteogenic cells. At the same time, they express multiple markers that are characteristic of pericytes/mural cells in other tissues, which together with osteogenic markers contribute to define a unique signature of bone marrow stromal progenitors, that is, bone marrow-derived MSCs [6]. In addition, they respond to known regulators of pericyte physiology, such as Fibroblast Growth Factor-2 (FGF-2), Transforming Growth Factor-β (TGF-β), and Platelet-Derived Growth Factor (PDGF)-BB, in a manner consistent with the known behavior of pericytes when exposed to the same cues: FGF-2 acts as a mitogen and down-modulates the "pericyte" phenotype, while TGF-β acts as an anti-mitogen and enhances expression of a pericyte phenotype [6]. These responses are consistent with the known in vivo effects and physiological roles of the same factors in the context of microvascular physiology, growth, and functional responses. The mural cell nature of MSCs is also reflected

in the expression of a pivotal regulator of microvascular development, angiopoietin-1, (Ang-1) [6]. Ang-1 is expressed in cells that are recruited to microvascular walls during vascular development and acts specifically in the remodeling of primary microvascular lattices into more definitive networks [20]. Recruitment of mural cells to vascular walls in general, and Ang-1 expression in particular, results in anti-apoptotic effects; in concert with activation of latent TGF-β at sites of contact between endothelial and mural cells, Ang-1 also contributes to the establishment of mitotic quiescence. Of note, Ang-1 exerts similar effects on hematopoietic cells, contributing to a "niche" effect [21].

MSCs and Pericytes

Several earlier lines of evidence had implicated microvascular pericytes with a progenitor function [12, 22, 23]. Noting the evidence, we previously postulated that bone marrow-derived MSCs, which at the time were surmised to possibly coincide with adventitial reticular cells, would represent a tissue-specific variant of pericytes found in other systems [18, 19], a hypothesis that was to be substantiated by direct experimental evidence leading to the identification of self-renewing MSCs in the human bone marrow [6]. During development, bone marrow stroma originates from the vascular invasion of developing bone marrow cavities, which brings osteogenic progenitors from the primitive periosteum-perichondrium into the newly excavated marrow space, in a perivascular position [12] (Fig. 3.1). While this was suggested by classical studies of human embryology [24] and then again from subsequent studies relying on expression of osteogenic markers and BrdU incorporation [25], the same notion has been recently validated by genetic studies in the mouse [26]. Overall, anatomy and ontogeny of the bone marrow clearly converge with recent data in establishing not only the perivascular nature of bone marrow-derived MSCs but also the developmental pathway whereby this association is established [10]. Importantly, these data emphasize that assayable MSCs in the bone marrow originate from preexisting, committed osteogenic cells.

While adventitial reticular cells are unique to the bone marrow, pericytes are found everywhere in connective tissues and parenchymal organs. In skeletal muscle, cells explanted in culture based on markers expressed in pericytes appear to include myogenic cells [27], which however are not osteogenic unless exposed to the reprogramming effects of bone morphogenetic protein (BMP)-2. BMPs induce a non-physiological osteogenic conversion of cells of distinct lineage, potency, and function, such as myoblasts and endothelial cells, which do not share lineage or origin with skeletal cells or bone marrow stromal cells. Nonetheless, the claim was subsequently made that cells isolated from multiple non-bone marrow tissues using the identical approach that we used to isolate MSCs from bone marrow represent a ubiquitous and uniform population of MSCs [9, 28]. This claim, which converges with other claims that cells capable of in vitro "differentiation" behavior similar to that described for bone marrow-derived MSCs, that MSCs can be isolated from

multiple tissues [29], led to the widespread assumption that MSCs are found everywhere, not just in the bone marrow, and coincide with pericytes found in every tissue, with identical potency [9].

Pericytes are defined by anatomy (abluminal cells sharing a basement membrane with endothelial cells) and express a host of markers, which, while characteristic, are not specific. Consistent with the notion that pericytes do not represent a distinct lineage but during embryonic development arise from mesenchymal cells surrounding developing vessels, prevailing evidence indicates that pericytes are highly heterogeneous [23, 30]. The claim that all pericytes outside of bone marrow are MSCs, or vice versa, is not at this time substantiated by convincing evidence, and both the origin and the actual potency of cells isolated using pericyte markers from different tissues remain open to direct and proper experimentation.

Indeed, one of the most significant sources of confusion in MSC biology has been the reliance on in vitro assays of differentiation for defining the potency of test cell strains. This approach has major limitations: first, it employs highly artificial conditions of chemical cuing, which per se, marks a major departure of any observed effect on the natural differentiation ability. Of note, when differentiation of MSCs is classically assessed in vivo, no exogenous cues are applied or involved; the generation of histology-proven bone in such assays is consistent with the nature, ontogeny, and gene expression profile of the transplanted cells. Second, in vitro differentiation assays are usually not conducted on clonally derived cells, which detracts from any claim of "multipotency" in most studies.

The Need for Stringent Criteria

More in general, one important source of confusion has been the widespread adoption of less than stringent criteria for defining what MSCs are. Such less than stringent criteria, when carefully scrutinized, appear to represent some unwanted evolution of fundamental tenets in the field – deformed, however, in the daily practice of many labs around the world. For example, the fact that MSCs are found within the fraction of bone marrow cell suspensions that adhere to plastic was used at the time of Friedenstein's pioneering work to distinguish the stromal fraction from the non-adherent hematopoietic fraction (at a time when no cell sorting was available). If extrapolated to non-bone marrow tissue, this property loses every meaning, as all connective tissue cells are adherent to plastic, without this denoting stemness. Nonetheless, the belief is widespread that MSCs can be defined among other things, by adherence to plastic. Likewise, the pursuit of markers suited to prospective isolation of stem cells was the engine that moved the hematopoiesis field toward identification of HSCs; in the MSC field, the same approach has also produced major advances, including the identification of STRO-1 [31], the identification of the in situ counterpart of MSCs, and the ultimate proof of their ability to self-renew [6, 7]. However, the reliance of markers in vitro is quite different from the pursuit of markers in uncultured cells. All markers used to "characterize

MSCs" in vitro are dramatically modulated in culture (and likely even in vivo), and the limited number of markers considered to identify cultured cells as MSCs are indeed expressed in virtually every culture of every kind of connective tissue cell. Finally, differentiation assays must be clonal, and in vivo, to warrant any conclusion about (multi)potency. The adoption of more stringent criteria, mostly based on the use of rigorous in vivo assays, clonal assays, and prospective isolation, will significantly help to reduce current confusion.

Stem Cells in Culture?

The isolation in culture of human ESCs [32] had a profound impact on stem cell biology at large and influenced the MSC field significantly.

1. On a conceptual level, it was ESC biology that introduced the idea that stem cells can be amenable to continuous culture. This is true for ESCs, but obviously it is not the case for the best understood systems of postnatal stem cells such as HSCs. In other areas of postnatal stem cell biology, such as epithelial stem cells, cell culture is a tenet, but caution has always been retained in noting that cultures include different kinds of progenitors and clearly include their progeny as well. The impact on MSC biology was huge: the idea pervaded the field that like ESCs, MSCs can be maintained in continuous culture and expanded in a non-differentiated state. This was oblivious to the fact that ESCs can, in many ways, be seen as continuous cell lines rather than as primary cultures from tissue explants (pluripotent cells of the inner cell mass of the blastocyst are a fleeting intermediate and do not persist after gastrulation). In noncontinuous (non-transformed) cell lines, growth kinetics is inherently asymmetric, meaning that, by definition, the population internally diversifies as it grows, due to concurrent differentiation and senescence events in progeny of the culture initiating cells.
2. On a methodological level, the impact was even greater. The notion became popular that, as for ESCs, differentiation can be probed in vitro and actually cued by differentiation cocktails that remain for the most part empirical. Slowly, this shifted the very concept of differentiation from an in vivo dimension to an in vitro dimension. As applied to MSCs, the effects of differentiation cocktails were subliminally (as much as wrongly) assumed to have physiological meaning. The use of in vitro readouts (not comparable to the in vivo generation of structurally and functionally sound differentiated tissues) was gradually considered to be proof of physiological differentiation. This led at times to gross misunderstandings, the best example being the use of cAMP agonists to induce "neural" differentiation, while solely inducing, in fact, disruption of actin filaments and reversible cell shape changes mimicking a "neuronal" morphology; or the use of nestin as a positive marker of neural differentiation, while in fact nestin is a widely expressed molecule in perivascular cells, and MSCs are perivascular cells.
3. The third impact was the most misleading of them all – the idea that "stem cells" are the same, embryonic or postnatal, and thus share similar properties. A worldwide

hunt for properties of MSCs that would define them as pluripotent cells (i.e., as capable of transgermal potency as embryonic stem cells) began, and invariably, data supporting the notion that MSCs, or subsets thereof, could be pluripotent were reported and had a large impact in the field [33].

It was specifically on these bases, that a number of studies were initiated, aiming to harness whatever measure of hypothetical "pluripotency" could be attributed to cultured MSCs. These included attempts to regenerate the myocardium, the brain, and virtually every other organ [34–36]. The fact that MSCs are not pluripotent cells is now very well accepted. However, we also now know that they can be reprogrammed to pluripotency through defined factors, just like other kinds of somatic cells that are not stem cells. This can be stated in another way as well: unless reprogrammed through defined factors into an induced pluripotent state, MSCs are not pluripotent. In light of this fact, data reporting beneficial effects of MSC grafting for regeneration of nonskeletal tissues need to be reevaluated. Part of this reevaluation is embodied in the current trend that ascribes to MSCs a therapeutic effect distinct from their nature as local, tissue-specific stem/progenitor cells.

Non-progenitor Functions of MSCs

That MSCs exert some functions in vivo not directly related to the generation of differentiated progeny is, indeed, proven. Bone marrow-derived MSCs establish, transfer, and organize a hematopoietic microenvironment. They do so in vivo, and they do so by reconstituting, in an in vivo transplant, cells with the same anatomy and phenotype as those originally explanted (i.e., sinusoidal adventitial cells). Self-renewal of MSCs coincides with establishment of a stromal compartment within heterotopic ossicles [6], and it is this stromal compartment that establishes the hematopoietic microenvironment (Fig. 3.1). The functional support that bone marrow-derived MSCs provide to hematopoietic cells, once they have resumed their original stromal habit and function in vivo (i.e., once they have actually self-renewed), is in fact a function that bone marrow-derived MSCs exert through direct cell-to-cell contact and by release of bioactive cytokines and chemokines. This function can be directly probed in vivo through a specific in vivo assay and is a defining feature of bone marrow-derived MSCs.

The observation that it is the establishment of a sinusoidal network and a perivascular stroma (Fig. 3.1), rather than of bone and osteoblasts, that allows for establishment of heterotopic hematopoiesis in chimeric ossicles redirected attention to self-renewing osteoprogenitors proper, rather than differentiated osteoblasts, the focus on specific cell compartments maintaining a HSC niche in bone. By highlighting a unique role for a non-endothelial preosteoblastic cell of the sinusoidal wall in this major physiological function, this observation also reconciled the frustrating duality of endosteal and sinusoidal "niches" (posited by independent lines of experimentation [37–39]), into a simple, unifying view [40]. A number of studies in murine systems [7, 41–43] have now substantiated this view, adding evidence for

the direct interaction of HSCs with perivascular stromal cells and opening up interesting questions regarding the key regulators of this interaction that acts on perivascular stromal cells. On the other hand, it has become apparent that the sinusoidal niche is shared by two stem cells (bone marrow-derived MSCs and HSCs) interacting with each other [6, 7, 40], rooting the interaction of bone and hematopoiesis as macroscopic tissues at the level of stem cells. The functional implications of a stem cell niche maintained by a different stem cell are just beginning to be recognized and largely remain to be experimentally explored. Here, pursuing a clear definition of the specific properties of the true MSCs (the multipotent and self-renewing, perivascular skeletal progenitors residing in the bone marrow), and an understanding of how these properties relate to hematopoietic regulation is a major avenue for investigation. The implications of such investigation are obvious – both biological (such as the elucidation of microenvironmental regulation of leukemic or cancer cells in the bone marrow) or therapeutic (e.g., with respect to strategies for HSC expansion [44]).

MSCs also act, in vivo, as organizers of nascent blood vessels. Again, this function can be probed in vivo through specific assays. In standard heterotopic transplants intended to probe the osteogenic capacity of bone marrow-derived MSCs, the organization of a sinusoid type (i.e., of a bone marrow-specific type) of microvessels is integral to the establishment of a heterotopic hematopoietic microenvironment/niche [6]. In other assays that bar the local differentiation of mature osteoblasts, the vessel-organizing capacity of MSCs can be probed in isolation [45–47]. This capacity does not involve the differentiation of grafted MSCs into endothelial cells; nor does it involve the differentiation anew of endothelial cells from mesenchyme. Thus, this capacity does not portray events that are easily categorized as either angiogenesis or vasculogenesis and are therefore better referred to by a different terminology – such as, for example, an "angiopoietic" function.

Aside from these two specific scenarios, a number of less precisely defined non-progenitor functions of MSCs are invoked to explain certain observations, such as the beneficial effects of infused MSCs in preclinical and clinical settings in which none of the established progenitor functions of MSCs are operative (e.g., myocardial infarction, stroke, inflammatory bowel disease, kidney disease, etc.). These effects include anti-inflammatory, immune-modulating, or trophic actions of MSCs, cross-talking to injured tissues. For all intents and purposes, these effects are currently seen by many as the prime effects to be harnessed for therapy [48], leaving aside the true stem cell functions of bone marrow-derived MSCs precisely at the time when the nature of bone marrow-derived MSCs as bona fide stem cells (self-renewing) is established at last. Invariably, these effects are the effects of ex vivo culture-expanded cells, which, while defined as "mesenchymal stem cells," are for all intents and purposes cultures of connective tissue cells, as they are defined by criteria that apply to every culture of nondescript connective tissue cells. Direct experimental evidence is needed to single out the properties of MSC populations that are attributable to stem cells and those that are due to cultured connective tissue cells. These non-progenitor effects, intriguingly, are now often linked to the recent recognition by some, long suspected by others, that bone marrow-derived MSCs are

"pericytes." This implies, directly or indirectly, that the non-progenitor functions of any type of MSC would be consistent with a defined set of in vivo functions played out by a defined set of cells in vivo; that is, that "pericytes" would be the in vivo cell type to which immune modulatory, anti-inflammatory, or trophic effects described for cultured cells called MSCs would have to be traced. The conceptual background underpinning these assumptions is, however, uncertain. For example, pericytes can be as much "pro-inflammatory" as "anti-inflammatory," depending not on the paracrine factors they secrete but the paracrine factors to which they are exposed, in vivo (reviewed in [23]). They are the source of myofibroblasts, which in turn are effectors of tissue scarring (reviewed in [23], (tissue scarring is the biological opposite of tissue regeneration)). There is a substantial lack of biological rationale to account for most assumed "paracrine" functions of MSCs. The void is even more significant when clinical translation of the same effects is sought. Here, the most important consideration is that all putative paracrine effects must have a paracrine mediator(s). Hence, one would assume that identification of the mediator would be key to the development of any unexpected biological property into an application. As much as with anything in the history of medicine and pharmacology, beneficial factors contained within plant or animal tissues (from Digitalis to hormones) need to be identified, isolated, or synthesized in order to develop a therapy. Surprisingly, instead, the prevailing approach has remained infusion or injection of cells at the bedside, rather than moving back to the bench. This is even more surprising in view of the mounting evidence that systemically infused MSCs do not engraft; they embolize in the lungs and are cleared in a matter of hours [49]. Hence, not only paracrine effects of infused MSCs are not linked to a progenitor function – they are not linked to engraftment and are only transient. Still, intravenous infusion of MSCs is often described as beneficial for a number of conditions, both acute and chronic, in many of which a rationale for any benefit is hardly seen. As hundreds of clinical trials are initiated worldwide on these assumptions, a word of caution seems at least prudent, and a more clear distinction of scientific and medical from commercial interests is needed.

What Does It Take to Develop an Effective Stem Cell Therapy?

The only two known, undisputable, striking cases of stem cell-based therapies with historical success in medicine are bone marrow transplantation (BMT) and regeneration of surface epithelia. Of note, neither was developed as a commercial product. What was the key piece of knowledge that led to success in these two cases? Curiously, it was not a refined strategy for prospective isolation of stem cells: bone marrow transplantation was developed [50], before the first direct experimental hint was provided [51], as to the actual existence of the long-postulated HSC. The human HSC phenotype has only been recently defined [52], and the phenotype of epidermal stem cells is still missing [53, 54]. In one case (BMT), there was a solid and extensive preclinical evidence behind the choice to experiment in humans [55], but

in the case of epithelial regeneration, this was not entirely the case. Key to success was, in each case, the following: (1) a clear, precise definition of the type of organ damage to be reversed, that is, irreversible loss of the differentiated tissue; (2) a clear idea that one specific differentiated tissue would be regenerated by specific progenitors, even if not directly identified; and (3) a clear, precise idea of how to deliver the replacement – via the bloodstream or as ex-vivo-generated epithelial patches to be locally implanted, respectively. Of note, this was made possible by the characteristic high turnover as well as the physical nature of the target tissues: a shapeless, fluid tissue in one case and a two-dimensional tissue in the other. The very same perception of these fundamental facts was later to allow, in each case, for the development of gene therapy in stem cells as the next frontier – adenosine deaminase deficiency-induced severe combined immunodeficiency (ADA-SCID) [56] and epidermolysis bullosa [57] were successfully corrected in humans by capitalizing on that perception. Of note, gene correction in MSCs is now feasible in specific genetic settings that are even more complex than loss-of-function mutations. Technically, we can efficiently correct dominant, gain-of function, point mutations in ubiquitous, indispensable genes in MSCs, with striking selectivity and in vitro effectiveness [58]. Yet, we cannot correct the human disease caused by those mutations, as we still miss the key pieces of knowledge – how to deliver cells, how to engraft them systemically, and how long would it take to revert the adverse effects of the disease genotype in 3D structures such as bone. Perhaps no better example can be envisioned to highlight the need to understand the specificities of the stromal system as a key to therapy.

Conclusions

Bone marrow-derived MSCs, as defined in this chapter, are bona fide stem cells; they are progenitors of skeletal tissues, which together comprise a system of lineages with a biology inherently distinct from other systems, and unique in many respects. MSCs are neither pluripotent cells nor ubiquitous progenitors of all mesoderm-derived, nonepithelial tissues. They cannot be easily transplanted systemically but engraft locally with high efficiency when transplanted with an appropriate scaffold. They function as progenitors of all skeletal tissues, as vascular organizers, and as niche cells for HSCs. They can be harnessed for therapies in different ways, which include the development of strategies for tissue engineering; the development of stem cell-based models of disease (a field that has become trendy since the development of induced pluripotent stem cells but was actually initiated within the MSC field); the identification of stem cell-specific mechanisms of disease, which can be targeted in different ways including pharmacological ways; the identification of MSC-released bioactive factors that can be acting on tissues in different ways and can be amenable to purification or synthesis as new drugs. Meanwhile, there is an urgent need to develop stringent, high-quality studies that will tackle the fundamental gaps in our knowledge of the system.

Acknowledgments This work was funded by MIUR, Fondations Roxa Fondations Cevei Bowauetti and Telethon-Grant GGP09227 (PB) and by the DIR, NIDCR of the IRP, NIH, DHHS (PGR).

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Chapter 4
Characterization of MSCs: From Early Studies to the Present

Mark F. Pittenger

Abstract Studies on mesenchymal stem cells/mesenchymal stromal cells (MSCs) have increased dramatically in the last 10 years, and many clinical trials are underway to take advantage of their properties. Early studies on MSC-like cells were performed in laboratories studying either bone repair or hematopoiesis, but the overlap in these studies was not broadly appreciated. The relationship between MSCs, osteoblastic progenitor cells, and the bone marrow stromal cells that provide support for hematopoietic stem cells has emerged. A variety of assays, in vitro and in vivo, allowed for a broader understanding of the MSCs and their characteristics. The MSCs from different animal species have properties similar to those from man, and this has allowed for many animal studies that provided preclinical support for human clinical trials with MSCs. While there are many established characteristics, new understanding of the MSC and the interaction of MSCs with other cell types, including HSCs and those of the immune system, will continue to reveal new and useful understanding of MSC properties.

Introduction

The early, underlying research behind mesenchymal stem cells, also commonly referred to as mesenchymal stromal cells, (MSCs) was slow to develop and can be traced to the fields of bone and blood research. However, by 2012 over 14,000 documents are found when one searches "mesenchymal stem cells" on PubMed. Bone marrow is commonly harvested for MSC isolation because it is considered renewable and does not require the sacrifice of healthy, nonrenewable tissue, although a variety of different tissues can be used to isolate MSCs. This is likely related to the

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_4,

existence of microvascular pericytes found along the capillaries in all tissues that have cells with the properties of MSCs. Isolation and propagation procedures for MSCs should be optimized and followed carefully for reproducibility as the cells are sensitive to culture conditions. Procedures used to isolate and propagate stem cells determine many of the properties of the resulting cells, and this is true for MSCs. Like all multipotent progenitor cells, MSCs are poised to respond to environmental conditions, including growth factors and cytokines, basal nutrients, cell-cell contact, as well as two and three dimension formats. The development of a combination of in vitro and in vivo assays greatly aided the characterization of MSCs and progress in this field. Whether for research purposes or clinical therapy, the time in particular culture conditions plays a role in the properties and characterization of MSCs, as well as their differentiation. In many ways, the MSC is an ideal, model adult stem cell; in the proper media, it does not require feeder cell layers, and it expands a millionfold or more and differentiates to desired or specified lineages in a reproducible fashion. Our early studies to characterize human MSCs from bone marrow provided necessary framework for many subsequent studies and clinical trials that continue today.

Early MSC Characterization: Born of Bone and Blood

Although the field of MSC research is expanding exponentially, the early work in this area was slow to develop. There is a modern body of literature that goes back 50 years from both the fields of blood and bone research that has many elements of research on cells we now call mesenchymal stem cells, multipotential stromal cells, or simply MSCs. Hematologists sought blood stem cells and the feeder cells that could support them in vitro in order to develop therapies for hematological malignancies. In the early 1960s, Till and McCulloch published their seminal work on hematopoietic spleen colony-forming units (CFU-Sp) in several papers where they were able to transplant the blood-forming ability of marrow into lethally irradiated mice [1, 2]. Although the term “stem cell” was already in use, this in vivo assay provided an experimental avenue for isolation of the responsible progenitor cells. The field of bone research long sought the progenitor cells for new bone formation to understand osteobiology and to use for repair strategies for bone and cartilage. Drs. Marshall Urist and Frank McLean transplanted decellularized fragments of bone to ectopic sites in 1953 and described new bone formation, but the responding tissue resident cells were unknown [3]. Dr. Urist’s research led many years later to the isolation and cloning of the bone-inducing molecules termed bone morphogenetic proteins (BMPs). In the 1960s, Alexander Friedenstein was researching interactions between bone and hematopoietic tissues at the Gamaleya Institute, USSR Academy of Medical Science in Moscow. He was culturing and characterizing the fibroblastic colony-forming units (CFU-F) from guinea pig bone marrow. The cells were placed in chambers with dialysis membranes to prevent the ingress of host cells, and the chambers were implanted under the skin of same specie host animals.

After several weeks, the chambers were excised, and some chambers provided evidence of new bone and cartilage. In a series of papers beginning from 1966, Friedenstein characterized the CFU-F from guinea pigs and rabbits [4–6]. These studies suggested the CFU-F was quiescent in vivo (in G_0) as it was resistant to radiation and slow to begin dividing. Dr. Maureen Owen and colleagues, working at Oxford University, investigated similar marrow-derived cells using athymic mice as hosts for cell chambers for better reproducibility [7–9]. Dr. Owen was the first to propose that mesenchymal tissues arose from a common progenitor cell similar to the hierarchal diagrams developed at the time for hematopoietic cells. Dr. Arnold Caplan at Case Western University was investigating bone and cartilage formation and repair in animal models in the 1980s. He championed the mesenchymal lineage hierarchy hypothesis and coined the term "mesenchymal stem cell" to focus attention on these powerful cells [10]. In pursuing the logical goal of isolation and therapeutic use of human MSCs, the Caplan lab developed a reliable in vivo bone-forming assay, which has been used to demonstrate their multilineage capabilities and identify fetal bovine serum lots that can maintain the multilineage potential of MSCs during ex vivo expansion [11]. The Caplan group also developed several monoclonal antibodies against surface antigens on the human MSC that proved useful for identification and further cell characterization [12, 13]. These are the SH-2 and SH-3 antibodies, now known to detect the surface molecules CD105 (endoglin) and CD73 (5′exonuleotidase, a salvage pathway enzyme), respectively [14, 15].

To develop the Caplan MSC methods for clinical use, Osiris Therapeutics, Inc. was founded. My cell biology group was tasked with developing in vitro assays that could be utilized to characterize human MSCs and test their differentiation to mesenchymal lineages. The osteogenic differentiation assay was in use in the Caplan lab and further developed for human MSCs by Scott Bruder [16, 17]. We developed an in vitro adipogenic differentiation assay based on the early work of Dr. Howard Green whereby the cells acquire all the attributes of adult adipocytes [18–20]. The chondrogenic differentiation assay was developed by Drs. Brian Johnstone and Jun Yoo at Case Western, and my group at Osiris, largely based on the in vitro study of rat chondrocytes by Drs. Tracy Ballock and Hari Reddi [21–23]. We presented the results from human MSC differentiation to these three lineages at the American Society for Cell Biology annual meeting in 1996 [24]. We added further gene expression studies, chromosome cytology, telomerase assays, and clonal studies and submitted those results in 1998, which were published in 1999, laying the groundwork for many subsequent studies [20]. Over several years, we performed differentiation assays on over 100 unique donors, providing substantiation for the mesenchymal stem cell paradigm.

In the early 1990s, the therapeutic potential of MSCs was already under study at University Hospital in Cleveland as an autologous treatment to support peripheral blood stem cell or bone marrow transplant for hematological malignancies [25, 26]. At this time, immunology studies on MSCs at Osiris Therapeutics demonstrated that human MSCs did not stimulate allogeneic T cell proliferation, and this was reported at international meetings and later published Klyushnenkova et al. [27]. These data correlated well with the lower than expected graft-versus-host disease incidence in

cancer patients undergoing matched unrelated bone marrow or mobilized peripheral blood transplants. That is, patients under treatment for hematological malignancies were found to have poor production of MSCs from their bone marrow, and matched donor MSCs were investigated as an improved therapy [28]. Allo-MSCs were also tested in patients with metachromatic leukodystrophy and Hurler syndrome [29]. (The hMSC immunology studies will be covered in other chapters.) It became apparent that allogeneic MSCs may be just as potent as autologous MSCs in preventing GVHD and stimulating beneficial responses from host cells and tissues and since then many studies have utilized allogeneic bone marrow MSCs. The autologous versus allogeneic MSC debate remains lively, and each may see therapeutic use in the future.

It should be emphasized that clinical use of MSCs has required careful characterization of the identity, purity, viability, potency, and stability of the therapeutic "product," and that the supporting preclinical studies performed in several mammalian species required a similarly rigorous if not quite as thorough characterization of the species' MSCs. In this regard, MSCs from rat [30–34], guinea pig [4], rabbit [35–37], dog [38, 39], goat [40], pig [41–46], and nonhuman primates [47–51] have very similar characteristics to their human counterparts. Therefore, many of these species have been useful for developing the necessary preclinical studies that allowed clinical development of MSC therapies. It is worth remembering that the rat has long been the preferred animal model for understanding aspects of human physiology/biology prior to gene knockout techniques that catapulted the mouse to the head of the line for understanding questions of gene functions and development.

Studies with mouse MSCs are plentiful, and many efforts have gone toward isolating mouse MSCs by similar methods as the above species [52–55]. However, the inherent co-purification/co-proliferation of mouse MSCs and cells derived from the hematopoietic lineages during ex vivo culture, something not seen in the other species listed above, has brought up the question of which cells in the cultures may be responding in experiments. This issue of mouse MSCs containing HSC progeny that continue to coculture throughout the in vitro culture process remains a problem today for the study of mouse MSCs. A method to eliminate HSCs progeny in mouse MSC preparations requires a final immunoselection step to negatively select and eliminate the HSCs before experimenting with the mouse MSCs [56]. This can limit the number of mouse MSCs available for study as they may no longer propagate without the presence of the hematopoietic cells.

"Mesenchymal stem cell" captures the potential of these cells to do more than differentiate to one or two lineages in vitro and in vivo, although to date there are no methods to differentiate these cells to *all* mesenchymal lineages. Other names including mesenchymal progenitor cell, multipotential stromal cell, and multipotential mesenchymal stromal cell are used by different investigators, yet the abbreviations MSC and MSCs, for the plural, are universally common. While some researchers continue to refer to MSCs as mesenchymal stromal cells, the reader should recognize that not all stromal cells are MSCs – actually very few. We suspect that MSCs were probably part of the bone marrow stromal cell preparations used in past years to propagate hematopoietic progenitor cells, but this is not assured

because MSCs are very rare in the bone marrow and their in vitro expansion while retaining multilineage potential is dependent on *optimized* in vitro *culture conditions*. The reliance of hematopoietic stem cell research on irradiated feeder layers led to the isolation of a number of characterized stromal cell lines, both mouse and human, but these generally were not tested for differentiation to any other lineages as such methods were not developed. Early cultured populations of stromal cells may have supported hematopoietic expansion, but they were only partially characterized, and what differentiation potential to other lineages or immunemodulatory capacity these cells may have had was not tested. Therefore, the percentage of early cultured stromal cells with the properties of MSCs cannot be known. Hence, given the rarity of MSCs in adult bone marrow and the need for careful culture methods for their propagation, a general claim of stromal cells as MSCs must be thoughtfully examined.

Source Tissues for MSCs

Isolation of MSCs (BM-MSCs) from human adult bone marrow drawn from the iliac crest is common, and this marrow or the isolated and cultured cells can be ordered from vendors. Adipose tissue has been used as a MSC source (AT-MSCs), the MSCs likely deriving from the adipose vascular pericytes [57–59]. The discarded placenta and umbilical cord *tissues* appear to be good sources of MSCs, although cord blood has very few MSCs [60–65]. Even the pulp of shed teeth has been used as a source of MSCs [66, 67]. From all species, bone marrow is most commonly used and adipose the next likely source as it is easily harvested in small quantities, or in larger amounts through liposuction procedures (for veterinary uses, see www.VetStem.com). Despite the origin of MSCs, they need to be characterized before use for in vitro or in vivo experiments. Clinical use requires highly characterized MSCs and full compliance with current Good Manufacturing Practices (cGMP). Many of the methods presented here have been adapted for Good Laboratory Practice (GLP) and cGMP use.

Fetal Calf Serum Qualification and the "In Vivo Cube Assay"

Following their initial studies with cells from rats and rabbits, Arnold Caplan and colleagues specifically sought to isolate human mesenchymal stem cells that could be expanded in culture and used for clinical studies for hematopoietic support and/or bone and cartilage therapies. Steve Haynesworth working with Dr. Caplan sought to isolate and study human MSCs and developed several monoclonal antibodies that identified rare cells in bone marrow that could be isolated and cultured in vitro. These were the SH-2, SH-3, and SH-4 antibodies now known to bind to cell surface

endoglin, or CD73, (SH-2), and the two that bound epitopes on 5′-exonucleotidase or CD105 (SH-3, SH-4). The other aspect was the development of an in vivo assay to test the ability of the isolated cells to differentiate to bone and cartilage and demonstrate endochondral bone development [11]. This entailed choosing a porous osteoconductive matrix material that was not osteoinductive. Specifically, hydroxyapatite/tricalcium phosphate matrix would allow osteo differentiation of cells attached to it, but would not induce osteoid formation when placed into tissue. The cells in question were allowed to attach for several hours and then implanted under the skin of athymic mouse recipients with up to six cubes per mouse. Usually 3 and 6 weeks later, the cubes were removed and examined histologically for the presence of new bone and cartilage. Culture medium and supplements, particularly fetal bovine serum (FBS), have been analyzed by the cube assay to develop a method to reliably grow human MSCs. When the porous cubes with no cells were implanted, no bone or cartilage was seen, and only some host fibrous tissue may be present. Similarly, when fibroblasts were placed in the cubes, no bone or cartilage was seen when the cubes were sectioned and analyzed. However, when a "good" prospective MSC population was placed in the cubes, abundant bone and cartilage tissue could be found in the matrix pores (see Fig. 4.1), whereas poor MSC preparations showed limited bone and little cartilage. The astute reader will recognize that the cube assay analyzes the results of the in vitro culturing of the cells of interest and their in vivo differentiation. Therefore, the cube assay can be used to develop the in vitro culture conditions as well as examine the resultant cultured cells. Importantly, the cube assay can identify and qualify fetal bovine serum that supports the expansion of multipotential MSCs. This iterative "boot strapping" process of both culture conditions and isolated cells was essential to the development of human MSCs, and no hindsight or prospective MSC isolation procedure suffices to replace the process even today because FBS or its replacement(s) must still be optimized and qualified in some manner. Most vendors of reagents for MSC research use some version of the cube assay or other methods to qualify lots of fetal bovine serum for MSC growth. Fetal serum is a complex solution of growth factors, and cytokines and quantities of each factor vary from one calving season to the next, and from lot-to-lot. Simply using more FBS does not seem to work. Although many efforts to use defined growth factors instead of FBS have been published, each growth factor needs to meet its own release criteria following manufacturing, and most are not produced to clinical standards.

Flow Cytometric Analysis of MSCs

Fluorescence-activated cytometry or simply flow cytometry can analyze the presence of known molecules on the cell surface with the use of antibodies, although internal molecules and some other characteristics can be analyzed as well. For MSCs, the characteristic surface molecules do not identify MSC stemness per se, but some have proven useful to routinely assay the cultured cells for homogeneity,

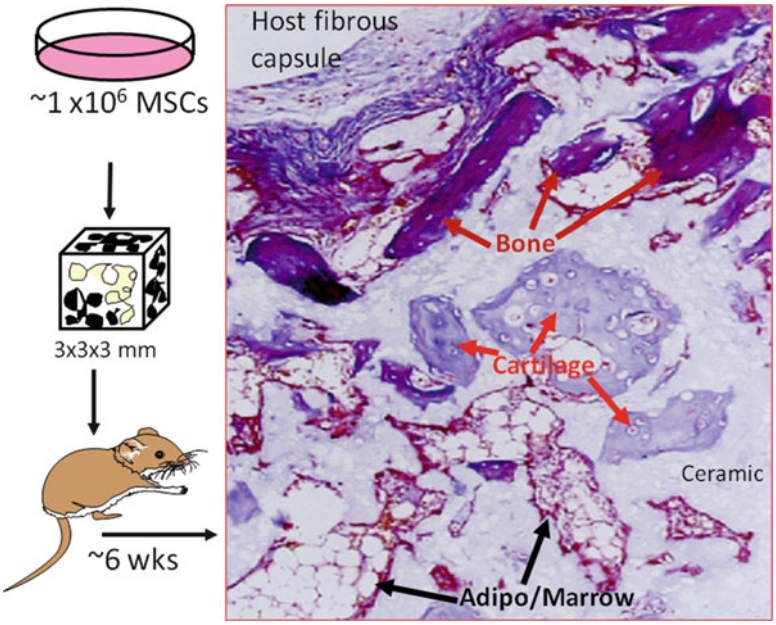

Fig. 4.1 Developing preferred MSC culture conditions. Mononuclear cells are isolated from bone marrow or other tissues sources, propagated in controlled tissue culture conditions which may include different additives such as fetal calf serum, particular growth factors, different basal media, etc. The cultured cells are placed on "inert" carriers and placed under the skin of immune-deficient mice, and the in vivo culture continued for ~6 weeks. The animal is sacrificed; the carriers are recovered and analyzed for the presence of tissues with differentiated cell types such as bone, cartilage, and adipose. This method was used in an iterative fashion to understand and improve the culture conditions for human BM-MSCs

"subpopulations," or any contaminating cells. Isolated MSC populations that performed well in the cube and differentiation assays were found to have consistently high levels of certain surface markers and low or undetectable levels of others. These positive and negative markers can be used as one facet for the characterization of MSCs for research or clinical purposes, and the presence and/or absence of certain surface molecules can help to determine the purity of the sample [68]. The markers are not sufficient to identify stem cells but do indicate the surface molecules available for interaction with other cells, extracellular matrix, etc. Cultured expanded human MSCs are commonly >95% positive for CD29, CD44, CD73, CD105, and CD166 and negative for hematopoietic markers CD11, CD34, and CD45, and a subset of these markers has been used as one aspect to qualify MSCs used in clinical studies. Table 4.1 contains a list of surface markers on human MSCs as analyzed by flow cytometry; this list is not complete, and culture conditions can affect some expression data. Attempts to isolate subpopulations of MSCs based on low and high expression of particular surface molecules has met with limited success, partly due to the limited number of cells isolated, but it is also debatable whether further isolation identifies "new cells" or just reveals temporal variations in expression. It is

Table 4.1 Cell surface molecules on MSCs by flow cytometry

Surface antigen	Pos/Neg
CD11a,b	Neg
CD13	+
CD14	Neg
CD18 integrin β2	Neg
CD29 integin β1	+
CD31 PECAM	Neg
CD34	Neg
CD44	+
CD45	Neg*
CD49b integrin α2	+
CD49d integrin α4	Neg
CD49e integrin α5	+
CD50 ICAM3	Neg
CD51 integrin αV	+
CD54 ICAM1	+
CD56 NCAM	+
CD62E E-selectin	Neg
CD71 transferrin rec	+
CD73 SH-3	+
CD90 thy-1	+
CD105 endoglin, SH-2	+
CD106 VCAM	+
CD117	Neg
CD133	Neg
CD166 ALCAM	+
CD271 p76 LNGFR	+
Trk A, B, C	+
HLA A, B, C	+
HLA-DR	Neg, IFNγ inducible
B2 microglobulin	+
Nestin	+
SSEA-3	+
SSEA-4	+

*Primary MSCs may be poscultured MSCs are ref

important to note that the flow cytometry data are routinely presented on a log scale, and purified populations of cells often have positive expression levels that vary tenfold or more. Another aspect of flow cytometry that has been useful in MSC characterization is the shift in expression levels of some surface molecules when MSCs are treated with certain biologically active molecules. For example, when MSCs are treated with interferon-γ, they now express HLA-DR on their surface. A shift in a single peak is found in the flow cytometry results, further suggesting MSCs are a

single population of cells responding uniformly to a biological stimulus and not a heterogeneous population.

In Vitro Differentiation of MSCs

In vivo assays such as the cube assay involve the added complexity of different host animals and several handlers at different steps and can lead to varying results. We developed a series of in vitro assays to test the differentiation of human MSCs [20, 24, 68] which also perform well for MSCs from other species. The assays were then miniaturized to allow for full testing with a minimum number of cells to compare parental cells and progeny derived from single cell clones. That is, from a single human cell from bone marrow, we expanded the progeny 21–22 population doublings (PD) to yield 500,000–1,000,000 cells that could be analyzed by flow cytometry and in vitro differentiation illustrating that the differentiation of the parental cells to different lineages was due to their multilineage potential rather than the outgrowth and subsequent differentiation of separate subpopulations [20]. If some MSC clones do not differentiate to each lineage, it indicates either that the colony has expanded beyond its capability to differentiate to all lineages or that the original single cell did not have multilineage capacity.

Adipogenic Differentiation of MSCs

The method for adipogenic differentiation of human MSCs is similar to the method developed by Dr. Howard Green for differentiation of 3T3-L1 preadipocytes [19]. With 3T3-L1 cells, the differentiation occurs easily in a few days, but with MSCs, repeating the induction conditions commits more cells to adipocytes, so several treatments were found to be optimal [20]. Briefly, MSCs are cultured as monolayers in dishes with low glucose (1 g/l) DMEM with 10% FBS and allowed to become confluent. The cells are cultured for ~3 days more, and then the medium is changed to adipogenic induction medium (MDI + I medium) containing 0.5-mM methylisobutylxanthine, 1-μM dexamethasone, 100-μM indomethacin, 10-μg/ml insulin, and 10% FBS in low glucose DMEM. The MSCs are then incubated for 48–72 h, and the medium is changed to adipogenic maintenance medium (AM medium) containing 10 μg/ml insulin and 10% FBS in the DMEM for 24 h. Greater commitment to the adipogenic lineage is seen when the cells are retreated with (MDI + I) for a second and third treatment round. The cultures are then maintained in AM medium for about 1 week to allow the lipid vesicles to enlarge and coalesce and then assayed. Nile Red, a fluorescent vital dye, is used to quantify lipid vacuoles using a UV plate reader and counterstaining the cells with DAPI to label DNA content as described

[20]. If desired, the adipogenic MSCs can then be fixed and stained with oil red O for nonquantitative histological presentation [20].

Chondrogenic Differentiation of MSCs

The chondrogenic differentiation of human MSCs utilizes an in vitro culture method described for rat chondrocytes and optimized for human MSCs [21–23]. Although MSCs are usually cultured in low glucose (1 g/l glucose) and 10% FBS, during chondrogenic differentiation in a compact micromass, this leads to cell death so it is important to use high-glucose (4.5 g/l glucose) DMEM but no FBS. In the micromass or "pellet culture," there is little or no cell proliferation, but the abundant amount of extracellular matrix produced leads to enlargement of the pellets. For chondrocytic differentiation of human MSCs, approximately 250,000 cells are placed in a polypropylene conical tube (to prevent easy adhesion) with DMEM, and cells are gently centrifuged to the bottom. Cells will form a cell micromass in 24 h that should be dislodged and free floating. The chondrogenic media consists of high-glucose DMEM supplemented with ITS+ (6.25-μg/ml insulin, 6.25-μg/ml transferrin, 6.25-μg/ml selenous acid, 5.33-μg/ml linoleic acid, 1.25-mg/ml bovine serum albumin), 0.1 μM dexamethasone, 10-ng/ml TGF-β3, 50-μg/ml ascorbate 2-phosphate, 2-mM pyruvate, and antibiotics. This medium is changed every day due to the labile TGF-β. The TGF-β3 is stored at −80 °C in small aliquots. For rat MSCs, BMP-2 is added at 10 ng/ml to improve chondrogenic differentiation. During the first week, little change is observed, but in 2–3 weeks, the extensive extracellular matrix leads to larger hard cell pellets that appear cartilaginous (if not obvious, extend the culturing for another week). The chondrogenic MSCs can undergo further maturation in vitro to become hypertrophic chondrocytes with addition of thyroxine, demonstrating their chondrocyte biology [21, 23]. Gene expression studies, immunofluorescence, and histological examination will reveal extensive differentiation that resembles neo-cartilage during embryonic development, and electron microscopy evaluation will show the glycoproteins are extensive but perhaps less cross-linked than adult cartilage [21].

Osteogenic Differentiation of MSCs

The osteogenic differentiation of MSCs is perhaps the easiest assay and has been used for many years to demonstrate the potential of bone-derived osteoblasts as well as MSCs [16]. We refer to the in vitro differentiated cells as osteoblasts and not osteocytes because the cells first proliferate (blasts) but do not encase themselves in mature bone extracellular matrix as osteocytes. However, the in vivo-differentiated MSCs are found as osteocytes with extensive matrix production around each cell. For in vitro osteogenic differentiation of MSCs, approximately 3×10^4 cells (low density) are seeded onto 35-mm dishes or six well plates in low glucose DMEM

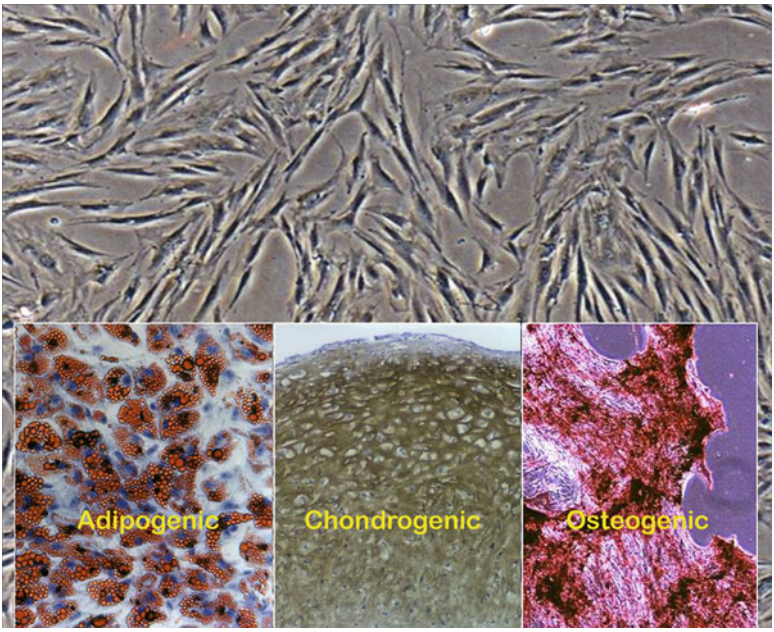

Fig. 4.2 Cultured MSCs can be exponentially propagated in culture and tested for in vitro differentiation. We demonstrated culture conditions that resulted in complete differentiation of human BM-MSCs. Under these protocols, virtually every MSC in the culture progressed to the fully differentiated cell type and exhibited gene expression and properties of the differentiated phenotype of the adult tissue, that is, it was not a mixture of differentiated and undifferentiated cell types. *Left to right*: Adipogenic oil red O stained lipid vesicles; chondrogenic MSCs immunostained for type II collagen shows abundant extracellular matrix in *brown* (DAB staining); osteogenic MSCs stained for alkaline phosphatase (*red*) and calcium deposits by silver staining by the von Kossa method (*black*)

with 10% FBS, glutamine, and antibiotics. In 24 h, the medium is replaced with the same supplemented with 50-μM ascorbate 2-phosphate, 10-mM β-glycerol phosphate, and 100-nM dexamethasone. The medium is changed every ~3 days, and periodically a sample is stained with Alizarin Red and compared to MSCs maintained in their normal culture medium. The differentiation is largely complete in 10 days. The culture wells can otherwise be stained for increased expression of alkaline phosphatase and deposition of mineralization by silver staining by the method of von Kossa [17]. In a separate set of culture wells, mineralization is quantified by measuring calcium deposition using commercially available kits [17] (Fig. 4.2).

Stromal Support Assay

Cultured MSCs produce a large number of cytokines and growth factors that are necessary for support of hematopoietic stem cells or even human embryonic stem cells. MSCs produce macrophage colony-stimulating factor (M-CSF), granulocyte

colony-stimulating factor (G-CSF), and granulo-macrophage colony-stimulating factors (GM-CSF). MSCs also produce interleukins IL-1a, IL-1b, IL-6, IL-7, IL-8, IL-10, IL-11, IL-12, IL-14, and IL-15. MSCs and each of these can be assayed by western blot, ELISA, or Elispot assays [20, 68]. MSCs also express surface molecules including intercellular adhesion molecules and vascular cell adhesion molecules, ICAM and VCAM, respectively, which interact with receptors on HSCs or ES cells. These surface molecules are easily assayed by flow cytometry [20]. Therefore, MSCs can be used to provide stromal support for the expansion of HSCs in culture.

Gene Expression MicroArrays in MSC Characterization

Analyzing gene expression of MSCs by microarray analysis is very promising, and several studies have been completed. The power of microarray analysis is the ability to analyze thousands of transcripts in a single experiment. Phinney and colleagues utilized serial analysis of gene expression (SAGE) to sample 2,300 transcripts from MCSs and found mRNAs from multiple cell lineages [75]. We previously used an array of 8,400 gene tags with highly purified MSCs, and the results demonstrated MSCs-transcribed genes normally associated with many differentiated cell types including astrocytes, neurons, epithelial and endothelial cells, as well as osteocytes, myocytes, tenocytes, adipocytes, chondrocytes, and other mesenchymal lineages (unpublished.) Analyses of the proteomes and transcriptomes of various MSC preparations from lab to lab reveal the transcription and translation of the genes of multiple lineages, but results can be dependent on lab-specific culture conditions. Moreover, microarray analysis is also dependent on initial cell sample isolation and multiple steps isolation of RNA, reverse transcription and amplification of the DNA copy – and performing these steps in an identical manner is essential for reproducibility and validation. From a clinical regulatory perspective, microarray data should only include that which is reliable and necessary, and variations should be within a specified range or be otherwise explicable. In this regard, a downsized custom microarray of ~100 transcripts may be more useful for MSC characterization for clinical purposes. Any “data for information purposes only” can be useful for future cell characterization but should not be confusing or non-reproducible, because they raise concern among regulatory agencies.

MSC Population Properties: Homogeneous or Heterogeneous MSCs?

MSCs constitute a discrete cell population that can be isolated reproducibly from bone marrow and other tissues and become a highly homogeneous population with consistent assayable properties after only a few passages ex vivo. Such properties

are maintained after further expansion through many passages. The expanded cells from different donors are found to have the same flow cytometric profile of positive and negative cell surface molecules; the growth characteristics and morphology are the same; and the results of differentiation assays are remarkably consistent. The flow cytometry scattergrams show a highly reproducible normal distribution with few outliers. Further, the search for the presence of known cells of other lineages is characteristically negative or produces a nominal 0–2% of uncharacterized cells. The contribution of any small population of contaminating cells to assay results is likely to be very small, and any contaminating cells with an uncharacterized phenotype are likely fewer than other clinical therapeutic preparations such as mobilized peripheral blood mononuclear cells. From a clinical perspective, it is necessary to have reproducible methods and a thorough description of the cellular product and its possible contaminants. When procedures are established and consistently followed, laboratories thousands of miles apart using marrow from different donors isolate MSCs that are indistinguishable from one another. Many clinical trials are underway with MSCs characterized similarly to the methods described in this chapter with the understanding that the methods are reproducible. In virtually all respects, cultured MSCs are much more homogeneous than other stem cells such as ESCs, iPSCs, neural stem cells, and others.

Immunoselection of cells from fresh bone marrow with different antibodies can obtain a subpopulation of the cells present in bone marrow. We previously selected cells from human bone marrow using a variety of antibodies and expanded them in culture. The resultant cells had similar properties to MSCs isolated by density centrifugation or direct plating. Other researchers have also utilized antibodies to select bone marrow cells. For example, Covas et al. used anti-CD146 to select cells from bone marrow aspirates, and the cultured cells had the desired phenotype of MSCs [69]. Similarly, McGonagle and coworkers used anti-CD271 immunoselection to select a population of primary cells from bone marrow to produce MSCs [70]. Other authors have utilized antibodies whose antigen is unknown and claimed that they have isolated a superior population of MSCs, but the field has been slow to confirm such claims. The antibody selection of a desired subset of bone marrow cells may be advantageous in focusing attention on the population of interest in an immediate fashion but the selected and culture-expanded cells appear to be virtually identical to the MSCs derived from density centrifugation or direct plating methods from other labs, rather than obtaining unique stem cell populations.

Recent studies of MSC heterogeneity usually choose a point in time and analyze differences in gene expression in isolated subpopulations and assume no or limited interconvertibility. The question is whether these are unique stable phenotypes or a phenotype that is time- and culture condition-dependent. For HSCs, it is known that subpopulations can be interchangeable, and that phenotype can depend on cell cycle, injury proximity, cytokines, and interactions among homologous and heterologous neighboring cells [71]. For MSCs, it seems apparent that their plasticity is at least as complex as HSCs, yet it is quite possible to use the “MSC population properties” to design revealing studies and clinical trials. Nevertheless, MSCs can exhibit microheterogeneity within the isolated cell population, and this may be a common and useful property of all stem cells. MSCs express a variety of surface

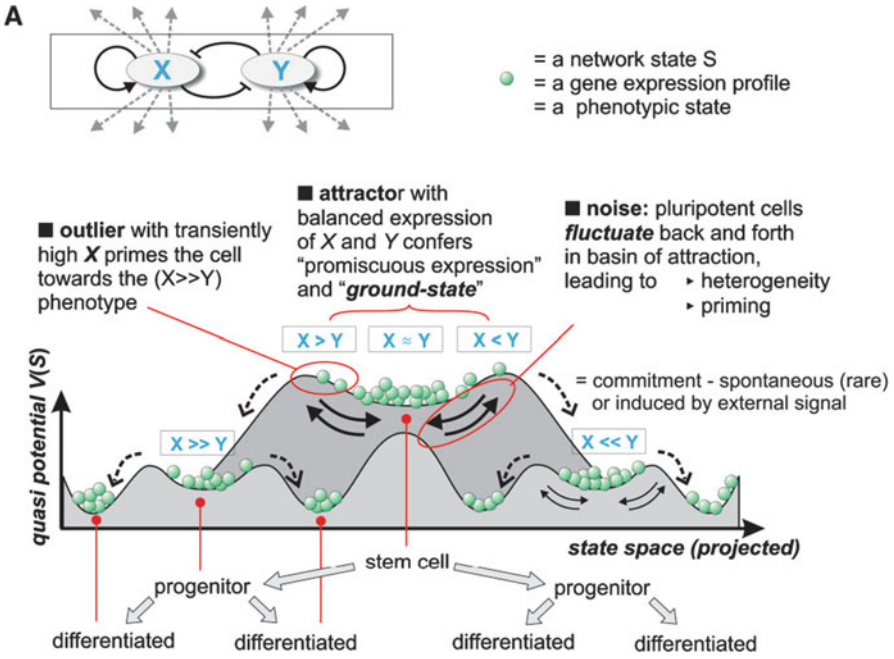

Fig. 4.3 Stem cells can express a range of genes without losing identity or differentiating. A hypothetical epigenetic landscape with a field of multipotential cells. A population of MSCs, even if clonally derived, can express a range of genes, and the levels of gene and protein expression can oscillate, although some states are more common or "preferred." X and Y may represent master genes leading to different pathways such as X = PPARγ (adipocyte pathway) and Y = BMP2 (osteocyte pathway) (Drawing from Figure 3A in Huang [74]. With permission from *Bioessays*)

receptors and internal signaling pathways that allow them to respond to neighboring cells and different external signals. It should be recognized that dispersed single MSCs growing in the culture dish at low confluency have different and measurable properties from MSCs that have contacts with their neighbors, or those that are confluent, or contact non-MSCs such as endothelial cells, lymphocytes, or HSCs. Thus, culture conditions partly determine the properties of any stem cell populations; hence, closely following protocols results in greater reproducibility.

Mesenchymal stem cell microheterogeneity may be an adaptation to the needs of the cells in the embryo, expanding fetal tissues, or repairing adult tissues where the stem cell is constantly modifying its response to environmental input. Several papers in system dynamics have modeled stem cell biology by describing preferred states within a continuum that allows the interchange between states, some more likely than others, based on transcription factors energy levels and other factors that must be overcome to pass from one state to the other [72, 73, 74] (see Fig. 4.3). Thus, describing MSC gene expression with its stochastic fluctuations yet constrained by interacting gene regulatory pathways, the availability of ATP, and input from the environment and neighboring cells, gives a dynamic yet stable phenotype. Such

approaches are powerful and useful alternatives to the stem cell heterogeneity paradigm. Moreover, such a view explains well the notion that multipotential stem and progenitor cell states are not so rigid and can sometimes move back and forth.

Conclusions

Current methods can produce highly reproducible populations of mesenchymal stem cells for research purposes or clinical therapies. The characterization of MSCs will continue to improve until highly successful or preferred methods become more obvious, and agreement among investigators is achieved. New assays will always be needed to further characterize stem cells, including RNA microarrays, glycoprotein arrays, transcription factors, DNA methylation sites, and differentiation assays for new lineages. Some such work has been published, but more is needed. Due to the inherent microheterogeneity of stem cells, it may be necessary to constrain some parameters in the assays to limit the cells' degrees of freedom. This can be accomplished by controlling one or more dominant parameters such as using defined medium containing a single growth factor or a culture surface that signals through a particular cell adhesion molecule. Well-characterized animal models and in vivo assays are also further needed to develop particular clinical therapies with MSCs. It is just as important to define the limitations of MSCs as well as their diverse potential. Overall, it seems it is not the MSCs that are limiting; it is the ingenuity and creativity of the investigators.

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Chapter 5
MSCs as Therapeutics

Arnold I. Caplan

Abstract Marrow has long been recognized as a source of osteoprogenitor cells. Such cells are a member of a heterogeneous group of cells that I have termed mesenchymal stem cells (MSCs) because they can be induced to form a number of differentiated mesenchymal cell types. With the realization that many of these MSCs are perivascular cells, pericytes, also comes the realization that they secrete a large array of bioactive molecules that are immunomodulatory and trophic. In this context, the differentiation capabilities are less important than their medicinal capacity and their regenerative potential in a number of diseases and medical conditions. Thus, we propose the suggestion that they could be called medicinal signaling cells (MSCs).

Introduction

The twenty-first century brought the "Age of the Stem Cell" into sharp focus. Started in the 1950s and 1960s with the experimental demonstration of adult hematopoietic stem cells (HSCs) in human bone marrow, the focus on the turnover and control elements in hematopoiesis reemphasized the spectrum of molecules and factors contributing to embryonic tissue development and how all of life was a genomically controlled temporal pattern of change [1–5]: The temporal pattern of change is initiated when the sperm fertilizes the egg, then to form a primitive tri-layered embryo, then to form all the organs and specialized tissues, to have the newly configured organism grow and mature into an adult, and then the obvious genetic and environmental basis for late onset disease and the new biologic complexity associated with

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_5,

longevity [4, 5]. Clearly, a bone-fracturing fall for a 5-year old has hugely different consequence compared to a fall of an 80-year old: The orthopedic consequence of very rapid bone-fracture repair in the young compared to that of the older adult has both a molecular and cellular basis [6]. It is within this context that I invented the term "mesenchymal stem cell," the MSC, to account for both the embryonic formation of diverse skeletal tissues and the implications for their functioning in adult tissues as the controlling cellular elements of turnover, maintenance, and repair [7, 8]. Indeed, orthopedic surgeons long ago recognized that adding extra amounts of autologous bone marrow had associated osteogenic units that could add value to fracture repair or spinal fusions [9].

How the lessons learned in studying adult bone marrow MSCs ramified into deeper understanding of MSC function is the focus of this chapter with emphasis on orthopedics. It is safe to say that current clinical uses of adult MSCs represent the new and startling insight into their normal in vivo functions. These new clinical uses are the "new medicine" which will change the way healthcare is delivered. Although bone marrow MSCs, because of their relationship to HSCs, are well studied, the fact that adult MSCs can be isolated from almost every tissue and organ in the body implicates these cells in a broader context [10]. This broad context challenges us to understand how MSCs naturally function and, more importantly, how to optimally manage these cells both outside and inside the body for clinical benefit.

Historic Perspective

Marrow has long been recognized as a source for osteogenic cells [9, 11, 12]. The quality of the marrow, fatty yellow marrow compared to red marrow, is likewise associated with bone degradation and loss and bone formation and maintenance, respectively. The bone-forming units, osteoblasts, are present in vivo as monolayer sheets of electrically coupled and coordinately controlled secretory cells that form laminar sheets of organic matrix, osteoid, that eventually become mineralize into bone. The orientors of these sheets of osteoid-secreting cells are the blood vessels, the vasculature. This leads me to infer that vasculature is both the driver and orientor of bone formation [13, 14]. Likewise, in the chick embryo, we established that there was a sequential series of differentiation steps between the osteoprogenitor cells and these secretory osteoblasts and, thus, established the details of an osteogenic lineage progression [15]. The link between the osteoprogenitor cells (i.e., MSCs) and vasculature becomes more obvious in the sections below.

The experimental findings of the orthopedic surgeon and clever scientist Marshall Urist in describing the bone-forming effects of demineralized bone matrix (DBM) when implanted into the muscle of adult rodents also infers that in muscle (or in other sites such as subcutaneous pouches) that DBM attracted osteochondrogenic progenitor cells (i.e., MSCs) from the surrounding adult host tissue [16, 17]. Indeed, by implanting chips of DBM in subcutaneous pouches, these osteochondrogenic progenitors were, likewise, in association with the host vasculature: with the vasculature

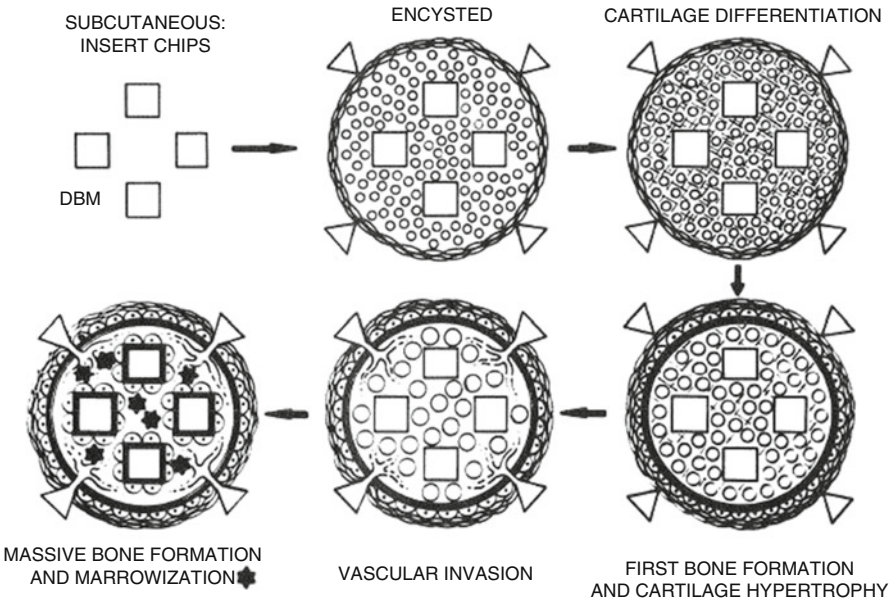

Fig. 5.1 Diagrammatic representation of low-magnification view of the sequence of cellular and tissue events involved with the implantation of DBM (*empty squares*) into a subcutaneous location [18]. The DBM particles are surrounded by mesenchymal cells (presumably MSCs), and this composite field is walled off by a three- or four-cell thick layer of "stacked-cells" to encyst the host cell-implanted particles of DBM. Outside the encysting layer (also probably MSCs) are blood vessels (*empty triangles*). The cells within the cyst differentiate into chondrocytes due to the bioactive factors released from the DBM [19]. From the bottom of the stacked cell layer, vascular-driven osteogenesis produces a layer of mineralized bone (*black circle*). The osteoid bony layer inhibits nutrient entrance which cases the internal chondrocytes to become hypertrophic and then to expire. These expiring chondrocytes secrete VEGF that causes the vasculature to erode through the bony layer bringing in resorptive cells that remove the cartilage ECM and replace it with newly forming blood vessels and new pericytes (MSCs) that form the first bony layer (*bold squares*) on the surface of the DBM particles. Eventually, marrow is established around the newly fabricated bone (With permission from [18])

orienting a 3–4-cell layer that encysted the DBM particles and, subsequently, the internal, expiring hypertrophic chondrocytes secreting chemoattractants to bring the host vasculature with new MSCs to replace the cartilage matrix with bone and eventually newly formed marrow (see Fig. 5.1) [18, 19]. The experiments of Reddi and his colleagues established both the cellular and molecular details as the implanted DBM experienced the sequential temporal phases of acute inflammatory events, the encysting, chondrogenesis, osteogenesis, and finally the formation of marrow [19].

Based on the above, in the late 1980s, I proposed that marrow contained an adult skeletal stem cell that I called a mesenchymal stem cell (MSC) that was capable of entering different expressional lineages to form, separately, bone, cartilage, muscle, the highly differentiated marrow stromal (marrow connective tissue) that houses hematopoiesis, tendon/ligament, fat, and other connective tissues; I summarily

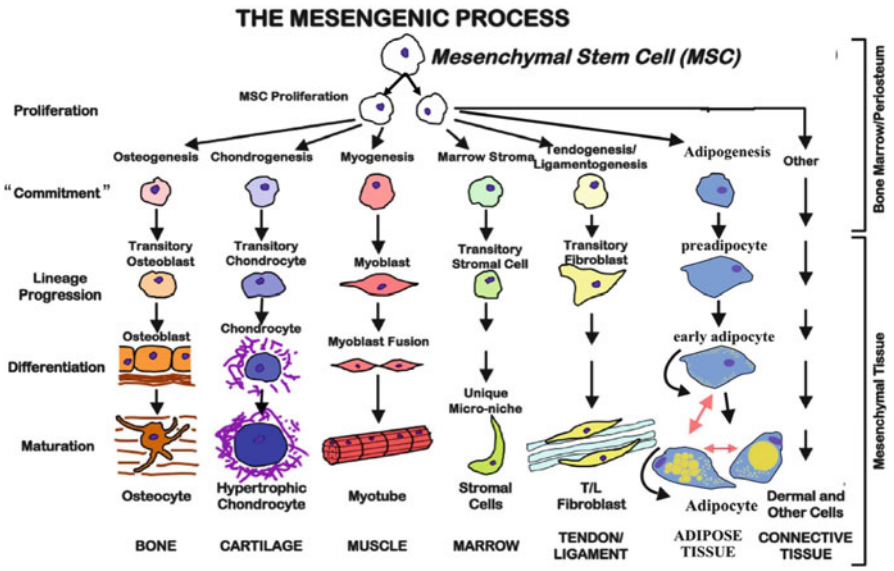

Fig. 5.2 The Mesengenic Process: Originally conceived in the late 1980s, the scheme was hypothesized to represent the existence of a stem cell whose progeny could separately lineage progress into a variety of mesenchymal phenotypes. The basis for the figure was the details known for hematopoiesis and the existence of an osteogenic lineage [7, 8, 20–22]

referred to this pathway as "mesengenesis" (see Fig. 5.2). The proposition of a widely distributed adult stem cell, the MSC, was quite contrary to the dogma of the 1980s that held that HSCs were the only progenitor cell in marrow. Based on this initial mesengenic hypothesis, Stephen Haynesworth and I developed the technology to isolate and culture expand human MSCs in a scale that could allow the clinical uses of MSCs [23].

Although unknown to me at that time, it was Alexander Friedenstein who first documented that osteogenic cells could be isolated and cultured from marrow [11, 12]. However, it was Maureen Owen who first put the lineage progression of MSCs into the same format as was pictured for HSC lineage progression [20]. Indeed, it was Owen who popularized Friedenstein's early work. In this context, Friedenstein is often recognized as the first to isolate a mesenchymal-like progenitor from marrow. By the mid-1990s, we published studies showing that MSCs from marrow (and also periosteum and synovium [24, 25]) could form bone [26, 27], cartilage [28], muscle [29], hematopoietic supportive stroma [30], tendon [31], and fat [21].

Also, the first use of MSCs in humans as a support for bone marrow transplantation in cancer patients documented their safety [32] and, eventually, their efficacy [33]. Based on these reports and on several patent applications, Osiris Therapeutics, Inc. was started in December 1992 as a "BioOrthopedic" company committed to the tissue-engineered restoration of skeletal tissues using MSCs.

Also by the mid-1990s, we published two reports in which, I am embarrassed to say, we overlooked the key biologic implications until many years later. The first was a study of the bioactive molecules secreted into the growth medium in 24 h by

G-CSF	+	+	+++
GM-CSF	O	O	+++
M-CSF	+	+	+++
LIF	-	+	+++
IL-6	-	+	+++
IL-11	-	+	+++
SCF	+	+	+
IL-3	O	O	O
TGFβ2	O	O	O
OSM	O	O	O

Fig. 5.3 The media from 24-h incubation of human MSCs initiated into osteogenic or marrow stromagenic pathways compared to cells in growth were analyzed by ELISA for specific bioactive molecules listed on the *left*. The relative percent change was scored with +, ++, +++ representing fold increase over baseline (Taken from [34])

hMSCs in growth, in osteogenesis, or in stromagenesis [34]. An ELISA assay for each molecule listed in Fig. 5.3 allowed us to vertically identify a comparative molecular signature for these three cell states. What we missed is that the MSCs innately secreted massive quantities of various bioactive molecules (to be discussed below). The second publication documented that some MSCs were localized on or integrated with blood vessels in human skin of young donors [35]. Again, the implications of this observation eluded us until 2007–2008 [10].

From the late 1980s until 2006–2008, the focus of the MSC technology was within a tissue engineering context. Inherent in this approach was the supposition that MSCs were the natural progenitors for skeletal tissue turnover, maintenance, and repair. This is certainly the case for marrow-derived MSCs and bone and to some extent bone marrow itself [36]. However, the presence of the muscle satellite cells compared to muscle-derived MSCs both challenges and confuses the issue of which is the "true" turnover progenitor for muscle [37, 38].

Fracture Repair

Fracture repair in long bones had been the subject of numerous studies [22]. What is agreed upon is that a "blastema" (high-cell-density fracture-spanning tissue) is the key element of the repair process. If the fracture is mechanically stable, newly forming blood vessels can span the break, can span the blastema, and spanning bone will form

as driven by these mechanically stable blood vessels. If the fracture is mechanically unstable, the blastema forms a spanning avascular plug of cartilage in a connective tissue sheath [6, 7]. Outside this plug of cartilage, a vascular-driven outer shell of bone forms (much like the encysted DBM referred to earlier). This outer mineralized bony callus mechanically stabilizes the fracture, and the central cartilage becomes hypertrophic; these hypertrophic cells expire and cause blood vessels and new MSCs to invade that eventually forms bridging endochondral bone [18, 19]. Eventually, the callus and over-repaired bone are remodeled to provide weight-bearing bone spanning the previous fracture site. For emphasis, we now recognize this close relationship between MSCs and newly forming blood vessels, as will be discussed next.

MSCs Are Pericytes?

It was long ago recognized that all blood vessels, large and small, arterial or venous, have cells of mesenchymal origin in ablumenal locations; for simplicity, I refer to these perivascular cells as pericytes. Over the last few years, we now recognize that if one purifies or immunostains cells using pericyte markers, one obtains cells that also have MSC cell surface markers [10, 39, 40]. This observation leads me to hypothesize that "all MSCs are pericytes." The reverse that all pericytes are MSCs is not correct since some pericytes are so highly differentiated that they cannot exhibit the multipotent properties of MSCs. Recently, pericytes (i.e., MSCs) have been isolated from the thick connective tissue surrounding major blood vessels (these cells have distinctive markers compared to pericytes from capillaries) [41]. The fact that *every* blood vessel in the body has MSCs in perivascular locations explains the fact that MSCs can be isolated from every vascularized tissue of the body; the best characterized MSCs are from marrow [42, 43], fat [44, 45], and muscle [37, 38], but also from placenta [46] and from umbilical cord [47]. This latter source is quite interesting in that there are no MSCs in cord blood; a few MSCs can be isolated and expanded from cord blood probably resulting from the needle which is inserted thru the external tissue to enter the lumen of the cord to harvest the blood. I suspect that this needle pushes a pericyte or two into the collection stream. This also accounts for the fact that 30–60 % of cord blood specimens do not yield expandable cultures of MSCs.

The new hypothesis that all MSCs are pericytes now explains the close association of MSCs and blood vessels in fracture repair, DBM-controlled bone formation, endochondral bone formation, and in the embryology of bone formation. Moreover, the issue of "what do MSCs do naturally?" can be addressed relative to their close association with blood vessels.

Clinical Use of MSCs: The New Medicine

We like to say that we scientists take our bench work and translate it to the bedside, to clinical utility. In the case of MSCs, we have, indeed, done that but because of the new clinical information derived from the use of MSCs [48], we must take them

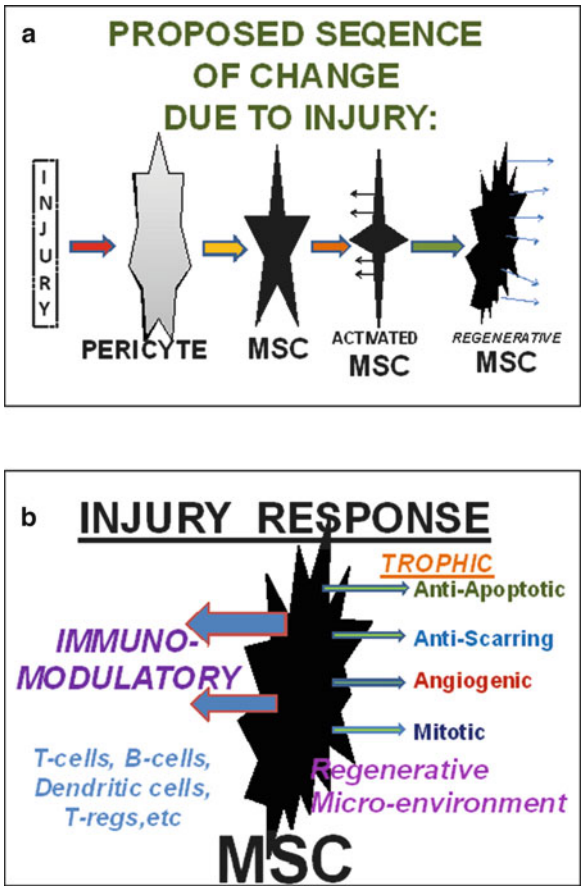

Fig. 5.4 Proposed sequence of cellular events following injury where pericytes are released from their ablumenal positions (**a**). These released pericytes become MSCs that are activated to secrete (**b**) bioactive molecules that are immunomodulatory and trophic [42, 43, 49]

back to the bench to determine how they function clinically since these results have nothing to do with their multipotency nor lineage progression pictured in Fig. 5.2. Without detailing all of the data now available, it is clear that MSCs have both an immunomodulatory and trophic function [49]. The immunomodulation allows allogeneic MSCs to be used clinically and allows the use of human MSCs in mouse models of disease such as asthma [50], MS [51], or inflammatory bowel disease [52]. The immunomodulation can affect a variety of immunocompetent and surveillance cells by shutting them down [53]. My hypothesis is that this is how the MSCs function once an inflamed or injured blood vessel releases the pericyte from its ablumenal surface. In this case, the injury site activates the MSC to interfere with the inflammatory process at its residency site (see Fig. 5.4). This inhibits immunosurveillance of the damaged tissue and, thus, inhibits autoimmune reactions from developing.

The trophic activity is much more complicated and probably involves different secreted molecules at different anatomic or diseased sites [42]. It seems obvious that MSCs function in the brain of a stroke patient differently from the same MSC

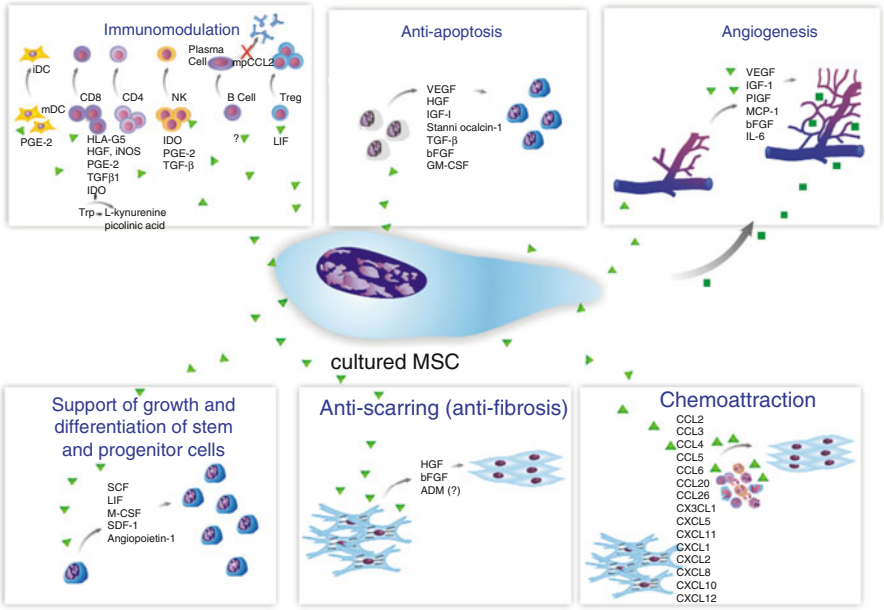

Fig. 5.5 The listing of bioactive molecules that have been identified [42] to contribute to MSC-controlled immunomodulation, anti-apoptosis, angiogenesis, mitosis of tissue-specific progenitors, anti-scarring, and chemotaxis

functioning at the infarct site of a heart attack patient. However, the trophic activity is similar at these different anatomic sites: anti-apoptotic (ischemic cells do not undergo apoptosis because of protective factors secreted by MSCs), anti-scarring (either by inhibiting the entry or functioning of myofibroblasts to form scar tissue), angiogenic (not only do MSCs secrete a huge amount of VEGF that attracts vascular endothelial cells, but MSCs again become pericytes and stabilize newly forming blood vessels), and mitotic for tissue intrinsic progenitors such as cardiac stem cells (see Fig. 5.5) [42, 43, 49].

It is now clear that our previous observations that MSCs intrinsically secrete massive quantities of bioactive molecules [34] account for both the immunomodulation and trophic activities observed clinically. Indeed, it is both of these activities that allows MSCs to structure regenerative microenvironments and provides the generalized mechanism for their clinical utility. Thus, one could foresee that in rural areas of Georgia, bags of MSCs for infusion could be stored at local urgent care or health centers and, thus, be a realistic way to treat heart attack patients; the challenge is to make this therapy at a few pennies per bag. This *new medicine*, delivered intravenously, will provide immune-silent allogeneic MSCs to home to sites of injury or inflammation.

Given the above, we can now more clearly understand the relationship between MSCs and blood vessels in fracture repair: Whether cells will form bone or cartilage will depend on the stability of the fragile, newly forming vessels. Moreover, the

regeneration of muscle, tendon, skin, and other well-vascularized tissues is not only dependent on the local microenvironments but also on the local vascular density and, thus, MSC titers. Regeneration of excised or damaged neonatal or pediatric (less than 3 years of age) skeletal tissues including the joints of fingers has been reported [54]; this regeneration will not occur in older individuals or in wounds that have been sutured. Like amphibians that can regenerate arms or legs, if the natural repair process is interrupted with sutures, regeneration will not occur. I would infer that a blood clot to close the wound followed by the acute inflammatory response followed by a sequence of MSC activity and vascular penetrance is required for regeneration. The interruption of this will result in nonunions, scarring, and lack of wound closure. The role of endogenous MSCs in these processes is the subject of current experimentation.

Conclusions

Based on the over 200 clinical trials now going on using MSCs from marrow, fat, and other tissues, it is clear that the principal use of MSCs clinically is not in the tissue-engineered fabrication of replacement tissues as we originally envisioned. Quite the contrary, the immunomodulatory and trophic properties of MSCs are now being investigated for graft-versus-host-disease, Crohn's disease, multiple sclerosis, diabetes, amyotrophic lateral sclerosis (ALS), tendonitis, osteoarthritis, rheumatoid arthritis, spinal cord injury, stroke, acute myocardial infarction and chronic cardiac insufficiency, asthma, and other indications. Based on these clinical uses, I would assert that MSCs are the "New Medicine" of this era, and cell-based therapies will change the course of healthcare delivery. For example, someday if someone has an infarct or stroke, they can receive a bag or two of allogeneic MSCs as a primary therapy, perhaps without major hospitalization. It is possible that this new use will make the "Miracle Drugs" of the last century seem insignificant by comparison. The new use of MSCs in orthopedics will likewise generate new products and approaches including smarter tissue-engineered tissue fabrication and implantation schemes that not only optimize the lineage translation and differentiated properties of MSCs, but also their medicinal activities (immunomodulation and trophic). With this in mind, I have recently suggested for non-tissue engineering uses that the MSC be renamed as the Medicinal Signaling Cell [55]. Thus, the future holds great promise when considering this new medicine both for the use of MSCs for specific disease states and also for aspects of longevity where vascular density plays a key role.

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Chapter 6
MSC Niche for Hematopoiesis

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Abstract Blood cell production is maintained throughout life by hematopoietic stem cells (HSC), which reside in specific areas of the bone marrow (BM) referred to as niches. These niches regulate the self-renewal, proliferation, and migration of HSC and also integrate signals from the periphery to respond to the hematopoietic demand. In the last decade, several putative cellular components of the HSC niche have been identified. Here, we briefly review current knowledge on different putative niche cells and their regulation.

Introduction

Every day millions of mature blood cells are released into the blood. Blood cell production, hematopoiesis, is a highly regulated hierarchical process in which immature progenitors differentiate into lineage-committed progenitors until fully mature cells arise [1]. Single HSC can reconstitute all hematopoietic lineages in irradiated recipients [2–4]. HSC/progenitors transplantation is of enormous clinical importance as it is the only curative treatment for many malignant and nonmalignant hematopoietic diseases [5].

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_6,

Hematopoiesis is migratory during development [6] but settles in the adult BM. The adult BM is made of two major components: an abundant hematopoietic fraction formed by the HSC progeny in various maturation stages and a stromal fraction formed by non-hematopoietic cells. It is important to distinguish among the concepts of BM stroma, hematopoietic microenvironment, and HSC niche. The stroma generally refers to the non-hematopoietic cell fraction which comprises a heterogeneous cellular mixture of mesenchymal-derived constituents, including osteoblasts, endothelial cells, and other less well-defined stromal cells. By contrast, the hematopoietic microenvironment more broadly refers to the cues that are necessary for hematopoiesis to take place [7]. These cues originate from stromal cells, hematopoietic cells (e.g., macrophages), or extramedullary cells (e.g., hormonal). The HSC niche refers to the regulatory subunit of hematopoietic microenvironment that controls the maintenance, self-renewal, differentiation, and migratory ability of HSC [8–10]. The HSC niche integrates signals from the periphery and coordinates an appropriate response [6, 11]. Understanding how the niche regulates HSCs may allow the development of novel strategies for expanding HSC ex vivo and therefore may increase the efficiency of BM transplantation. Further, several studies suggest that niche deregulation can cause hematopoietic disease [12–15] and that the niche can be occupied by cancer cells that colonize the BM [16, 17]. In the last few years, significant efforts have been devoted to the identification and regulation of various HSC niche components. Here, we will review the current knowledge in this area and discuss more specifically the recent evidence showing that perivascular mesenchymal stem and progenitor cells represent a critical niche constituent. We will also present an overview of the major systems that have been shown to regulate niche activity and the studies that implicate BM niche dysregulation in hematopoietic diseases.

From the Hematopoietic Microenvironment to the Hematopoietic Stem Cell Niche

Although the concept of a hematopoietic stem cell niche was proposed as early as 1978 [18], it took 25 years and major advances in mouse genetics and imaging technology to demonstrate the existence of putative niche cells in murine models [19, 20]. The first evidence of the existence of a hematopoietic microenvironment necessary for hematopoiesis came from experiments in which hematopoietic cells were transplanted into irradiated recipients. Despite the fact that donor cells recirculate throughout the body and interact with multiple organs, only the BM and the spleen are colonized by the donor cells [21]. The unique ability of BM stroma to support hematopoiesis was demonstrated by the development of long-term bone marrow cell cultures (LTBMC or Dexter cultures), where stromal cells can maintain HSC for months without losing their reconstitution potential [22–24], and by the fact that transplantation of BM stromal cells into a host animal can generate heterotopic ossified tissues containing a hematopoietic-supportive stroma and newly formed bone [25].

Early microscopy studies identified reticular cells as the most abundant stromal cells in a hematopoietic BM. These cells were identified by the presence of collagen type III fibers in their cytoplasm (also known as reticulin) and subdivided in two classes: fibroblastic reticular cells distributed along the BM and adventitial reticular cells that formed a layer covering the abluminal side of BM vessels [26]. Direct cellular contact between reticular and hematopoietic cells was demonstrated by electron microscopy [27]. Importantly some reticular cells were shown to be committed to the osteoblastic lineage and to associate with granulocytic progenitors in vivo [28]. The BM is a highly vascularized organ, and thus endothelial cells are also an important component of its microenvironment [29]. Adipocytes are relatively rare in the hematopoietic BM, but their number increases as hematopoietic activity decreases, suggesting that they are negative regulators of hematopoiesis [30]. In addition, analyses of LTBMC cultures revealed that BM macrophages are also a component of the hematopoietic-supportive microenvironment and that they secrete important factors that regulate hematopoiesis [31, 32].

Stromal cells regulate hematopoiesis by direct cell-to-cell contact, release of regulatory factors, and secretion of extracellular matrix proteins with regulatory properties [29, 33]. In LTBMC, HSC and progenitors cells are strongly attached to the adherent stromal cell layer [34, 35]. Direct cell contact is required for their maintenance [36] and depends on several molecules produced by the stromal cells including kit ligand (KL) [37] and vascular cell adhesion molecule-1 (VCAM-1) [38, 39]. Stromal cells also secrete cytokines and growth factors that act on multiple progenitor cells to regulate hematopoiesis [40]. As more knowledge was obtained on how the different stages of blood cell production were regulated by stromal cells, the focus shifted to identifying the specific cells and molecules responsible for regulating and maintaining HSC. In an effort to identify better the factors that specifically act on HSCs, several cell lines were derived from LTBMC [41–43]. The ability to support HSC in vitro varied widely among cell lines, although a few were shown to be able to maintain HSC (i.e., capable to reconstitute an irradiated recipient) for several months in culture [44]. Interestingly, some of these cells exhibited the capacity to differentiate into adipocytes or osteoblasts [45, 46]. These and other experiments which showed that the hematopoietic-supportive stroma was derived from multipotent progenitor cells [47, 48] suggested that multipotent mesenchymal progenitor cells may play an important function in establishing the hematopoietic microenvironment. It is important to note, however, that not only immature mesenchymal cells are able to maintain HSC in culture, both osteoblasts [49, 50] and endothelial cells [51] were shown to support HSC expansion in vitro, suggesting that they may also represent functional components.

The HSC Niche in the Bone Marrow

The study of the hematopoietic niche is particularly challenging by the fact that it is enclosed within the bone and by the lack of markers that allow the visualization of

endogenous HSC and niche cells. However, advances in imaging techniques, the discovery of novel markers that identify HSC and niche cells, as well as genetic models that allow for the deletion of specific cells and molecules have greatly increased our knowledge on the HSC niche. Recent excellent reviews have discussed in depth the nature of the different components of the HSC niche [8–10, 52–54]. Here we will provide a brief overview of the different putative niche cells identified thus far.

Cellular Components of the Hematopoietic Stem Cell Niche

Osteoblasts

Osteoblasts are located in the endosteal surface of the bone and are actively synthesizing new bone. That osteoblasts might serve as niche cells was suggested decades ago when hematopoietic progenitor activity was found to be enriched in areas closer to the bone [55, 56], and after transplantation, progenitors were located near the endosteum [57]. Differentiated osteoblasts were also shown to be able to maintain HSC and progenitor activity in vitro [49, 50]. Osteoblasts are reported to produce factors that can regulate HSC such as CXCL12 [58], angiopoietin-1 [59], KL [60], thrombopoietin [61, 62], and osteopontin [63].

In 2003, two studies supported the existence of hematopoietic niches in vivo. Expression of a constitutionally activated form of the receptor for the parathyroid hormone (caPPR) in osteoblastic cells led to an increase in trabecular bone that was accompanied by an increase in total HSC numbers [19]. Treatment with parathyroid hormone in wild-type mice also led to increased trabecular bone and HSC numbers [19]. Conditional deletion of the bone morphogenetic protein receptor IA (*BMPR1a*) in hematopoietic and non-hematopoietic cells caused an increase in trabecular bone and osteoblasts and HSC numbers [20]. Although these reports demonstrated that osteoblastic cells were components of the HSC niche, the specific role of mature osteoblasts as niche cells remained unclear. Indeed, the models discussed above [19, 20] affect all osteoblastic lineage cells in the BM and not only osteoblasts. Further, changes in mature osteoblast numbers are not always followed by changes in HSC [64, 65]. In addition, the development of novel HSC markers showed that, although some HSC are located near the bone, most were located near the vasculature [66].

Endothelial Cells

The discovery that the SLAM family members CD48 and CD150 could be used to prospectively isolate HSC facilitated detailed histological analyses determining HSC location in the BM [66]. Surprisingly, these studies revealed that HSC were not restricted in the endosteal surface but were rather located in perivascular areas [66]. This suggested

the existence of a vascular HSC niche [9, 66]. This observation, together with the fact that endothelial cells can maintain HSC in vitro [51, 67, 68] and that co-transplantation of HSC and endothelial cells [69] or endothelial progenitors [70] increases the efficiency of HSC engraftment, suggests that endothelial cells may be a niche component. Perhaps the best evidence for a role of endothelial cells in the niche is the fact that deletion of the gp130-signaling subunit in endothelial cells caused progressive BM failure [71]. In addition, expression of a constitutively active form of Akt in endothelial cells is accompanied by HSC expansion in vivo [72]. These reports argue that (at least some) endothelial cells have niche activity in vivo. Different vascular microdomains have been identified in the BM vasculature [73, 74], indicating that further investigations will likely determine which endothelial cell fraction promotes HSC function.

Mesenchymal Stem and Osteoprogenitor Cells

Recent reports have reconciled observations that niche cells may belong to the osteoblastic lineage [19, 20] with the fact that most HSC are in a perivascular location [66]. Immunofluorescence analyses of mice in which the GFP protein is under the control of the CXCL12 promoter revealed that most HSC (94–97 %) are closely associated with a population of GFP^+ perivascular reticular cells (CAR cells for CXCL12-abundant reticular cells) [75]. In humans, $CD45^-CD146^+$ adventitial reticular cells that exhibit osteoprogenitor activity also have the capacity to generate a functional hematopoietic microenvironment in heterotopic ossicles [76]. Similarly, $CD45^-CD105^+Thy1^-$ cells obtained from fetal BM were able to generate bone and a hematopoietic microenvironment when injected under the kidney capsule [77]. These reports clearly demonstrated that immature osteoblastic progenitor cells could create a hematopoietic microenvironment supporting HSC and progenitors, but the nature of the cells comprising the HSC niche remained unclear.

Because the sympathetic nervous system (SNS) regulates HSCs' attraction to their niche and their mobilization in blood [78, 79], the identification of the cell(s) regulated by the SNS was predicted to provide insight into the niche constituents. Recent studies have identified a rare subpopulation of perivascular stromal cells using mice in which the GFP gene is under the control of the nestin promoter [80]. In these mice, $CD45^-$ nestin-GFP^+ cells were found intimately associated with the BM vasculature and with HSC (60 % of $Lin^-CD48^-CD150^+$ HSC are in direct contact with at least one nestin-GFP^+ cell). $Nestin^+$ cells express high levels of molecules known to regulate HSC function like CXCL12, KL, VCAM-1, and angiopoietin-1. Interestingly, these genes are downregulated by stimuli that elicit HSC release from the niches [80, 81]. $Nestin^+$ cells are self-renewing mesenchymal stem cells (MSC) grown as nonadherent spheres (called "mesensphere") with osteogenic, chondrogenic, and adipogenic potential and containing all the colony-forming unit-fibroblast (CFU-F) activity within the BM [80]. Ablation of $nestin^+$ cells in vivo caused a reduction in HSC numbers in the BM. Finally, transplanted $nestin^+$ cells are able to self-renew and support hematopoietic activity in heterotopic

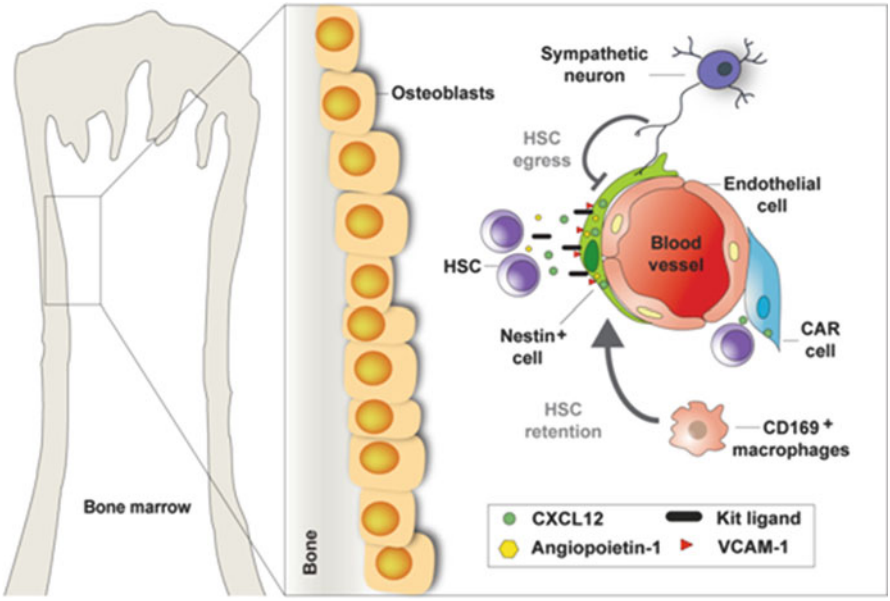

Fig. 6.1 The bone marrow hematopoietic stem cell niche. Within the bone marrow, HSC are mainly located near the blood vessels. Most HSCs are adjacent to populations of perivascular nestin$^+$ mesenchymal stem cells and CXCL12-abundant reticular (CAR) cells that promote HSC maintenance. Nestin$^+$ cells express high levels of molecules known to regulate HSC function and retention like CXCL12, kit ligand, angiopoietin-1, and vascular cell adhesion molecule 1 (VCAM-1). The bone marrow is also heavily innervated by nerve fibers; sympathetic neurons modulate HSC trafficking and mobilization by acting on nestin$^+$ niche cells, this leads to the downregulation of HSC retention factors and HSC release into the blood. The function of the sympathetic nervous system in the niche is antagonized by bone marrow CD169$^+$ macrophages that produce soluble factor(s) that upregulate(s) HSC retention genes in nestin$^+$ cells, contributing to HSC maintenance in the niche

bone ossicle assays [80]. Further studies on the CAR cells, which are more abundant than nestin$^+$ cells, also suggest that they have osteoprogenitor activity [82]. These studies thus suggest that the HSC niche is formed by two types of stem cells, the mesenchymal stem cell and the hematopoietic stem cell that are present in the bone marrow (Fig. 6.1).

Regulation of the Hematopoietic Stem Cell Niche

Along with the advances made in our understanding of the cellular constituents of the niche, recent reports have begun to unravel the network of signals acting on niche cells, which in turn, regulate HSC activity. Several of these signals have been identified and include cytokines, hormones, and the nervous system. Here we will discuss the major cell types thus far known to regulate niche activity.

Sympathetic Neurons

The BM is heavily innervated by a heterogeneous set of nerve fibers, most of which are found along BM vessels [83] but also in the stroma [84] and the endosteal surface [85, 86]. Hematopoietic cells express receptors for several neurotransmitters that can potentially modulate hematopoiesis [87, 88]. Specifically, sympathetic neurons can modulate HSC activity by targeting niche cells in the BM. This observation was made following studies investigating the mechanisms by which fucoidan, a sulfated glycan, elicits HSC release from the BM [89, 90]. In these studies, it was hypothesized that fucoidan might mimic the activity of endogenous BM sulfated glycans. Mice lacking the enzyme UDP-galactose:ceramide galactosyltransferase (Cgt), which is essential for the synthesis of sulfatide and galactocerebrosides [91, 92], failed to mobilize after granulocyte colony-stimulating factor (G-CSF) or fucoidan treatment [78]. In wild-type mice, G-CSF induced osteoblast "suppression," characterized by changes in osteoblast morphology that switched from a cuboidal to a "flattened" morphology against the bone matrix. Interestingly, $Cgt^{-/-}$ osteoblasts constitutively exhibited this suppressed morphology. Since $Cgt^{-/-}$ mice have deficient nerve conduction due to lack of galactocerebrosides [91, 92], the role of the sympathetic nervous system (SNS) in mobilization was investigated. Mice with pharmacological or genetic loss of function of SNS activity showed reduced mobilization after G-CSF treatment, whereas sympathetic agonists increased mobilization efficiency, thus demonstrating a critical role of the SNS in regulating HSC release [78]. The SNS also controls circadian HSC trafficking during steady state [79]. A major target of SNS activity is the β3 adrenergic receptor expressed by $nestin^{+}$ MSC. β-adrenergic activation induces downregulation of CXCL12 [78, 79], KL, angiopoietin-1, and VCAM-1 in $nestin^{+}$ cells and elicits HSC release from the niche [80]. Thus, the SNS regulates HSC trafficking and mobilization by acting on $nestin^{+}$ MSC, leading to the downregulation of HSC retention factors and HSC release into the blood (Fig. 6.1).

Bone Marrow Macrophages

The function of the SNS on the niche is antagonized by BM macrophages. β-adrenergic activation only induces a modest mobilization unless homing receptors are blocked [78, 79]. In addition, expression of the G-CSF receptor (encoded by *Csf3r*) on a hematopoietic cell distinct from the HSC was required for mobilization [93]. These results suggested that hematopoietic cell(s) also regulated HSC attraction to its niche. Recent studies have indeed revealed that BM macrophages act on niche cells to regulate HSC trafficking [81, 94, 95].

In one study, it was found that G-CSF treatment reduced the number of macrophages in the endosteal region [94]. In addition, in vivo deletion of myeloid cells using "Mafia" mice [96] or phagocytes using clodronate-loaded liposomes [97] increased the number of circulating HSC/progenitors [94]. This depletion also

caused a reduction in osteoblast numbers (determined by osteocalcin immunohistochemistry) and reduced niche activity as determined by reduced *CXCL12*, *KL* and, *angiopoietin-1* levels in total BM extracts [94]. This strongly suggested that BM phagocytes can signal to the niche to maintain HSC in the BM.

A separate report on mice showed that *Csf3r* gene expression in $CD68^+$ cells, which identifies BM monocytes/macrophages, rescued the G-CSF-induced mobilization defect of $Csf3r^{-/-}$ mice [95]. This demonstrates that *Csf3r* expression in monocytes/macrophages may be sufficient for mobilization [95]. In vitro studies also showed that BM macrophages can increase osteoblast growth [95, 98] and promote the upregulation of CXCL12 and osteocalcin in osteoblasts by releasing an unidentified soluble factor [95].

Independent studies defined BM macrophages as $CD115^+Gr1^-F4/80^+CD169^+$ cells, whereas monocytes are either $CD115^+Gr1^+CD169^-$ or $CD115^+Gr1^-CD169^-$ cells, wherein CD169 expression is restricted to BM macrophages [81]. In vivo depletion of BM phagocytes with clodronate-loaded liposomes or depletion of mononuclear phagocytes in "Mafia" or CD11b-DTR mice, described by Cailhier et al. [99], led to increased number of circulating HSC/progenitors and *CXCL12* downregulation [81]. BM monocytes/macrophages do not produce CXCL12 but secrete a soluble unidentified factor(s) that promotes *CXCL12* synthesis by stromal cells [81]. BM monocyte/macrophage depletion led to significant reductions in CXCL12, angiopoietin-1, KL, and VCAM-1 levels in $nestin^+$ niche cells but not in osteoblasts [81]. Selective in vivo depletion of BM macrophages using CD169-DTR mice [100] was sufficient to induce HSC mobilization and downregulation of HSC retention genes by $nestin^+$ niche cells [81]. These findings demonstrate that BM $CD169^+$ macrophages produce soluble factor(s) that upregulate(s) HSC retention genes in $nestin^+$ cells, maintaining HSC in the niche. Thus, $CD169^+$ macrophages antagonize the SNS activity, where adrenergic stimulation causes the downregulation of HSC retention genes in $nestin^+$ cells and promotes HSC release from the BM (Fig. 6.1).

Osteoclasts

Although osteoclasts have been implicated in regulation of the HSC niche, their specific role is unclear. Osteoclasts are multinucleated hematopoietic cells that are responsible for bone resorption. Bleeding or lipopolysaccharide (LPS) injections elicit HSC mobilization and an increase in the number of osteoclasts [101]. Similarly, receptor activator of nuclear factor kappa-B ligand (RANKL) treatment increased osteoclast numbers and induced mobilization, whereas mice with reduced osteoclast function showed reduced G-CSF mobilization efficiency [101]. These data thus support a role for osteoclasts in promoting HSC release from the BM niche. However, although RANKL-activated osteoclasts release cathepsin K and this enzyme has the potential to cleave CXCL12 in vitro [101], G-CSF treatment does not increase cathepsin K synthesis [94]. To complicate matters further, mice treated

with different bisphosphonates (drugs that inhibit osteoclast function) have yielded contradictory results. Mice acutely treated with zoledronate show efficient G-CSF mobilization, suggesting that osteoclasts do not regulate HSC niches [94]. On the other hand, mice chronically treated with alendronate showed a mild reduction (~20 %) in BM HSC numbers thus suggesting a role for osteoclasts in regulating HSC niches [102]. Given the aforementioned function of macrophages, an effect of bisphosphonates on macrophage function remains possible [103]. Although further research is needed to determine their precise contribution, a role of osteoclasts in HSC and niche maintenance is likely. It is possible that osteoclasts regulate indirectly the niche by increasing bone resorption and in turn activating nestin$^+$ cells and osteoblastic progenitors to differentiate into mature osteoblasts thus changing the composition of the niche.

Bone Marrow Adipocytes

In young humans, hematopoiesis takes place in the marrow of the long bones, but after puberty, hematopoiesis is progressively lost from the epiphyseal portion of long bones and persists in the metaphyses, sternum, and short ribs [104]. During this process, epiphyseal BM is progressively occupied by adipocytes. In mice, hematopoiesis is maintained through life in the long bones, although some bones, such as the tail vertebrae, tend to be occupied by adipocytes. Surgical removal of the adipocyte-rich marrow in rabbits induced transient regeneration of a hematopoietic-supportive stroma [105]. Adipocytes in LTBMC do not support hematopoiesis [106]; instead the presence of adipocytes seemed to correlate with reduced hematopoietic activity [106]. Indeed, adiponectin produced by BM adipocytes reduces the proliferation of hematopoietic progenitors [107]. Importantly, recent analyses have revealed an inverse correlation between the number of adipocytes in the BM and HSC frequency [30]. Adipocytes secrete factors that reduce HSC expansion in vitro, probably by increasing HSC quiescence. Intriguingly, pharmacological inhibition of adipogenesis increased survival and BM regeneration in transplanted mice, and this was associated to increased osteogenesis [30]. This suggests that adipocytes might be negative regulators of the niche and that removing them might increase mesenchymal stem cell activity.

Bone Marrow Microenvironment: Implications in Hematopoietic Diseases

Since the interactions between niche cells and HSC regulate hematopoiesis, it is reasonable to hypothesize that an altered niche may also contribute to the pathophysiology of hematologic diseases. Increasing evidence points toward critical roles of

microenvironmental factors in the development and/or progression of various hematological malignancies. This idea has been supported by the observation that allogeneic donor cells can become leukemic in transplanted recipients [108]. This is the result of oncogenic transformation of apparently normal donor hematopoietic cells in a diseased recipient [109]. Interestingly, in long-term follow-up studies of patients and their donors, all of the donors remained healthy, suggesting that the microenvironment of the recipient contributed to the disease [110, 111]. Further studies support the view that pathological hematopoiesis can be induced by a defective BM microenvironment. Microenvironment-induced myeloproliferative-like disease (MPD) was shown to occur in mice deficient for retinoic acid receptor gamma (RARγ) [14] or after conditional deletion of the retinoblastoma protein (RB) [13]. In both mouse models, a reduction in trabecular osteoblasts correlated with the disease progression, and this was accompanied by loss of HSC in the BM and increased mobilization to extramedullary tissues [13, 14]. In agreement with these studies, mice lacking the E3-ubiquitin ligase Mind Bomb 1 (Mib1), a notch ligand regulator, also develop MPD [15]. This is not due to an independent effect of Mib1 loss in hematopoietic cells, but rather to a defective BM, as the transplantation of BM cells from *Mib1*$^{-/-}$ mice into wild-type recipients resulted in a normal phenotype [15]. Interestingly, this Mib1 deficiency did not result in faulty Notch activation in hematopoietic cells, but rather in defective Notch signaling in the microenvironment [15]. Recent studies also revealed that deletion of the RNase III enzyme Dicer1 specifically in osteoprogenitor cells not only impaired their differentiation but also resulted in disrupted blood formation and secondary leukemia [12]. Furthermore, the conditional deletion of the glycoprotein 130 (gp130) in endothelial cells, the main cellular component of the vascular BM niche, resulted in hematopoietic disease and premature mice death in the first year of life [71]. Importantly, these studies revealed that disruption of niche signaling in mice can model human hematopoietic diseases.

Primary myelofibrosis is the classical example of a chronic MPD where perturbations in the microenvironment play an important role in its pathogenesis. This is characterized by severe hematopoietic alterations with prominent mobilization of hematopoietic progenitors from the BM to alternative niches (spleen and liver) [112]. These alterations are associated with profound modifications of the BM stroma as demonstrated by the progressive development of collagen and reticulin fibers in the BM, neoangiogenesis, and osteoblast proliferation [113]. Interestingly, microenvironmental signals can also affect lineage determination of premalignant cells, which may progress into acute myeloid, lymphoid, or mixed-lineage leukemias depending on the presence of distinct cytokines or the actual recipient mouse strain [114].

The dynamic BM microenvironment in constant remodeling with a high concentration of growth factors and cytokines renders it a permissive organ for cancer cell homing and survival [115]. Leukemic cells can in fact use the migratory pathways of normal cells to usurp the niche, possibly via the same molecules that healthy cells use to interact with their niche [116]. In a mouse model of Nalm-6 pre-B acute lymphoblastic leukemia, malignant cells were able to metastasize to specific CXCL12-

positive vascular niches in the BM that overlap with perivascular HSC niches [73]. In addition, leukemic cell growth was also shown to be able to disrupt normal hematopoietic BM niches, creating abnormal tumor microenvironments [117].

Conclusions

The characterization of precise cellular elements comprising the HSC niches in both normal and diseased states may shed new light on the molecular circuitry contributing to microenvironment-induced malignancies. This may allow the development of therapeutic modalities targeting both the malignant clone and the altered stromal signals that perpetuate the disease.

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Chapter 7
Immunomodulatory Properties of MSCs

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Abstract Mesenchymal stromal cells (MSCs) are multipotent cells that can be isolated from several human tissues and expanded *ex vivo* for clinical use. They comprise a heterogeneous population of cells, which, through production of growth factors, cell-to-cell interactions and secretion of matrix proteins, play a key role in the regulation of haematopoiesis. In recent years, several experimental studies have shown that MSCs are endowed with potent immunomodulatory properties directed in vitro at all cells involved in immune responses. Due to their immunomodulatory and engraftment-promoting properties, MSCs have been tested in the clinical setting both to facilitate haematopoietic engraftment and to treat steroid-resistant acute graft-versus-host disease (GvHD). More recently, experimental findings and clinical trials have focused on the ability of MSCs to home to injured tissues and to produce paracrine factors with anti-inflammatory properties, resulting in functional recovery of damaged tissues. The mechanisms through which MSCs exert this pleomorphic

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_7,

therapeutic potential rely on some key properties of these cells: the capacity to home to sites of injury, the ability to blunt exaggerated immune responses and the ability to secrete soluble factors capable of stimulating both the survival and recovery of injured cells. This chapter focuses on recent advances in MSC biology and summarises the clinical studies on their immunomodulatory and anti-inflammatory properties, particularly in the setting of allo- and autoimmune disorders.

Introduction

In addition to haematopoietic stem cells (HSCs), the bone marrow (BM) also contains mesenchymal stromal cells (MSCs). These cells were first recognised more than 40 years ago by Friedenstein et al. who described a population of adherent cells from the BM which were non-phagocytic, exhibited a fibroblast-like appearance and could differentiate in vitro into bone, cartilage, adipose tissue, tendon and muscle [1]. Moreover, after transplantation under the kidney capsule, these cells gave rise to the different connective tissue lineages [2]. Human MSCs were first identified in postnatal BM and later in a variety of other human tissues, including periosteum, muscle connective tissue, perichondrium, adipose tissue (AT), umbilical cord blood (UCB) and fetal tissues, amniotic fluid and placenta [1, 3–8]. One of the hallmarks of MSCs is their multipotency, defined as the ability to differentiate into several mesenchymal lineages [9]; usually trilineage differentiation into bone, adipose tissue and cartilage is taken as a criterion for multipotentiality. Recently, the existence of pluripotent cells has been reported that have the ability to differentiate into cells of the mesodermal lineage but also into endodermal and neuroectodermal cell types, including neurons, hepatocyte and endothelium [10–12]. MSCs have been also demonstrated to display chemotactic ability, to migrate to sites of inflammation and injury [13], as well as to secrete paracrine mediators able to reverse acute organ failure [14]. MSCs have been successfully used in repairing tissue injury, occurring after allogeneic haematopoietic stem cell transplantation (HSCT) [15]. In view of their immunosuppressive properties, as well as of their role in sustaining tissue repair and trophism, MSCs represent a promising tool in immunoregulatory and regenerative cell therapies [16, 17].

MSC *Ex Vivo* Expansion

Due to the low frequency of mesenchymal progenitors in human tissues, in vivo use of MSCs requires that the cells be extensively *ex vivo* manipulated to achieve the numbers that are necessary for their clinical application [18–20]. Standard conditions for *ex vivo* expansion of MSCs are based on the presence of 10% fetal bovine serum (FBS), and serum batches are routinely prescreened in order to guarantee both the optimal growth of MSCs and the biosafety of the cellular product [18–20].

However, the use of FBS raises concerns when utilised in clinical grade preparations, because it might theoretically be responsible for the transmission of zoonoses as well as cause immune reactions in the host, especially if repeated infusions are needed. This may lead to the risk of rejection of the transplanted cells [21, 22]. In view of these considerations, serum-free media, appropriate for extensive expansion and devoid of the risks connected with the use of animal products, are being developed.

Both autologous and allogeneic human serums have been tested for in vitro expansion of MSCs [23]; several serum-free media, based on the use of cytokines and growth factors, such as basic fibroblast growth factor (b-FGF) and transforming growth factor β (TGF-β), have been proposed in experimental conditions [24, 25]. Platelet lysate (PL) has been demonstrated to be a powerful substitute for FBS in MSC expansion, especially in terms of cell growth due to its high concentration of *natural* growth factors [26–28]. Doucet et al. first demonstrated that the growth factors contained in PL are able to promote MSC expansion in a dose-dependent manner [26]. This was further substantiated by data published by other groups, showing that culture medium with 5% PL added is superior to 10% FBS in terms of clonogenic efficiency and proliferative capacity of MSCs, while preserving MSC immunomodulatory functions [27, 28]. It has, however, to be emphasised that clinical data on the safety and efficacy of MSCs have been obtained, so far, mainly with cells expanded in the presence of FBS, whereas relatively little in vivo experience is available with MSCs cultured in alternative medium supplements. Therefore, cells expanded in the presence of alternative expansion media require extensive experimental and clinical testing before being safely and effectively employed to substitute cells generated in the presence of FBS-based media.

MSC Surface Markers and Prospective Isolation

Little is known about the characteristics of the primary mesenchymal precursors in vivo; this has been mainly due to the inability to prospectively isolate the most primitive mesenchymal cells from bulk cultures because of their low frequency and the lack of specific markers. To date, MSC isolation/identification has relied mainly on morphology and adherence to plastic; immunophenotyping by flow cytometry has been applied to identify *ex vivo*-expanded MSCs and to define purity. No specific marker has been shown to identify true MSCs, and *ex vivo*-expanded cells are characterised by a combination of both positive (CD105, CD73, CD90, HLA class I) and negative (CD34, CD45, CD14, CD31) markers [9, 29], at least in case of BM-derived cells (indeed, a proportion of AT-derived MSCs express CD34) [5, 9, 29].

Recently, the identification and prospective isolation of mesenchymal progenitors, both in murine and human adult BM, have been reported, based on the expression of specific markers [30–40]. Anjos-zfonso et al. have reported the identification, isolation and characterisation of a population of multipotent mesenchymal cells in

murine BM, based on the expression of the stage-specific embryonic antigen-1 (SSEA-1) [30]. In human cells, with the aim to prospectively isolate MSCs, surface markers such as SSEA-4, STRO-1, the low affinity nerve growth factor receptor (CD271) and MCAM/CD146 (melanoma cell adhesion molecule) [31–38] have been employed. Battula et al. have recently isolated by flow cytometry MSCs from human BM, using antibodies directed against the surface antigens CD271, mesenchymal stem cell antigen-1 (MSCA-1), CD56 and SSEA-3, and identified novel MSC subsets with distinct phenotypic and functional properties [38, 39]. In particular, CD271, which has been employed for prospective isolation of MSCs from BM, has been reported to define a subset of MSCs with immunosuppressive and lympho-haematopoietic engraftment-promoting properties in vivo [35]. Moreover, it has been shown that only $CD271^{bright}$, but not $CD271^{dim}$, cells give rise to clonogenic MSCs and these populations differ considerably in their morphological appearance [34, 35, 39]. Similarly, a 100-fold enrichment in fibroblast colony-forming cells (CFU-F) was found in the STRO-1^{+} population in the bone marrow [33]. MCAM/CD146 molecule, which has been shown to allow for CFU-F enrichment, was expressed on both MSCs and pericytes [37]. A STRO-4 monoclonal antibody has been demonstrated to be specific for mesenchymal precursors cells from human and ovine tissues, being capable of providing enrichment in CFU-F when employed for MSC isolation from BM [40].

Despite the identification of these new MSC markers [30–40], none presently available has demonstrated to be, by itself, capable of identifying the true mesenchymal stem cell. Whether culture-expanded MSCs differ from their progenitors in vivo is uncertain, as proliferation on plastic surfaces and culture conditions may induce both phenotypic and functional changes. Future research should focus on the identification of MSC-specific markers which will hopefully allow to dissect the developmental hierarchy of MSCs and facilitate the generation of homogenous cellular products.

MSC Tissue Sources for Clinical Use

As previously mentioned, MSCs, after their first identification in BM [1], have been isolated from a variety of other human tissues. Although similar MSCs can be cultured from different fetal and adult tissues [3–8], clinical experience has been mainly gained with *ex vivo*-expanded BM-derived cells; only few studies have employed different sources, such as AT [41]. The frequency of mesenchymal progenitors, their proliferative capacities and differentiation potential, as well as their immunophenotype and immunomodulatory properties have been shown to vary in different sources [42, 43]. Intrinsic diversities of MSCs residing in a tissue, as well as their physiological role in that tissue, might influence the properties of a specific source, as compared to other MSC sources. The frequency of cells with lineage-specific differentiation capacity may differ between tissue sources, and therefore, MSCs with the ability to differentiate into bone-forming cells might be present with higher

frequency in BM rather than in fetal lung or placenta. Also the culture conditions employed for MSC *ex vivo* expansion might influence their biological properties, leading to the commitment of MSCs towards a specific function or cell lineage.

These differences should be taken into account when considering the clinical application of MSCs in the various clinical settings, together with the method of collection from a specific tissue (invasive procedure for BM and AT vs. noninvasive collection of UCB) and their isolation efficiency (100% success rate when isolating MSCs from BM and AT vs. 20–63% success rate when culturing MSCs from UCB) [44–47]. Whether one specific source might be more useful in a defined clinical setting depending on its biological and functional properties needs to be further investigated.

Safety Data on Malignant Transformation

It has been suggested that *ex vivo* manipulation of both human and murine MSCs may alter the functional and biological properties of the cells, leading to the accumulation of genetic alterations [48–52]. A high susceptibility to malignant transformation was also reported in murine BM-derived MSCs by different groups [50, 52]. Other researchers, using human MSC, did not confirm a propensity to develop morphological and genetic changes [27, 53–55]. In particular, both BM- and UCB-derived human MSCs, expanded in the presence of FBS or PL, could be safely cultured for long term without losing their phenotypical and functional characteristics and without showing the presence of chromosomal abnormalities [27, 53, 54]. French researchers have reported the presence of aneuploidy in a number of MSC preparations for clinical use; by further characterising these genetic abnormalities, they found that these alterations were not related to cell transformation, but rather to senescence of the cells [55]. While earlier reports indicated that human AT- and BM-derived MSCs are prone to undergo malignant transformation after long-term *ex vivo* expansion [48, 51], recently, it was shown that the tumour cells that they had described were unrelated to the original MSCs and were derived from contaminating tumour cell lines in these laboratories [56, 57]. Human bone marrow-derived MSCs have been long term expanded until senescence or until independent clones emerged. These cultures represented 8–15 passages and 33–55 population doublings, and no independent clones emerged. The likelihood of malignant transformations was estimated to be $<10^{-9}$. Altogether these data indicate that under the commonly used culture conditions, tumorigenesis is likely to be an extremely uncommon event [58]. This incident highlights the risk of cross-contamination and emphasises on the importance of cell line verification with DNA fingerprinting.

In light of these observations, phenotypic, functional and genetic assays, although known to have limited sensitivity, should be routinely performed on MSCs before in vivo use to verify whether the clinical application of *ex vivo*-expanded MSCs is safe.

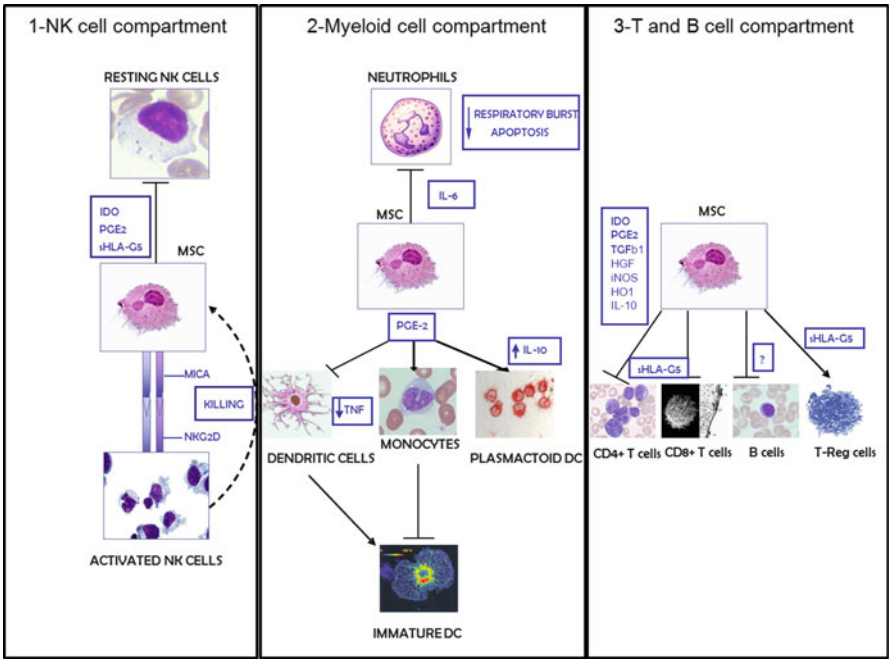

Fig. 7.1 Possible mechanisms of interaction between MSCs and cells of the immune system. (1) Mesenchymal stromal cells (*MSCs*) can inhibit the proliferation and cytotoxicity of resting natural killer (*NK*) cells; these effects are mediated by indolamine 2,3-dioxygenase (*IDO*), prostaglandin E2 (*PGE2*) and soluble HLA-G5 (*sHLA-G5*) released by *MSCs*. Killing of MSCs by cytokine-activated NK cells involves the engagement of activating receptors expressed by NK cells and of their ligands expressed by MSCs. (2) MSCs inhibit the differentiation of monocytes into immature myeloid dendritic cells (*DCs*), skew mature DCs to an immature DC state, inhibit tumour necrosis factor (*TNF*) production by DCs and increase *IL-10* production by plasmacytoid DCs. MSC-derived PGE2 is involved in all these effects. MSCs dampen the respiratory burst and delay the spontaneous apoptosis of neutrophils by constitutively releasing *IL-6*. (3) Direct inhibition of $CD4^+$ T-cell function depends on the release by MSCs of soluble molecules (IDO, PGE2, transforming growth factor-b1 (*TGFb1*), hepatocyte growth factor (*HGF*), inducible nitric oxide synthase (*iNOS*), heme oxygenase-1 (*HO-1*) and IL-10). MSCs inhibit $CD8^+$ T-cell cytotoxicity and stimulate the differentiation of regulatory T cells a.o. through the production of sHLA-G5. MSC-mediated inhibition of B-cell function involves both cell-to-cell contact and soluble mediators. → indicates a stimulatory effect; ⊥ indicates an inhibitory effect (Adapted from Uccelli et al. [62])

Immunomodulatory Properties of MSCs In Vitro

MSCs display broad and potent immunomodulatory properties that have been first demonstrated in vitro and, subsequently, in vivo both in animal models and in humans. Initially, most studies focused on the effects of MSCs on T lymphocytes; however, it is now evident that these cells display their effects on other cells involved in immune response, including B lymphocytes, dendritic cells (DCs) and natural killer (NK) cells [59–61]. See Fig. 7.1.

MSCs and T Cells

MSCs were first demonstrated to suppress in vitro T lymphocyte proliferation induced by alloantigens [63], mitogens [64], CD3 and CD28 agonist antibodies [65, 66]. MSCs have been reported to inhibit the effects of cytotoxic T cells (CTLs), probably due to suppression of CTL proliferation [67]. The inhibition of T-cell proliferation and cytotoxicity mediated by MSCs is not HLA restricted; in fact, MSCs are able to induce a similar degree of inhibition in the presence of both autologous and allogeneic responder cells [63, 66]. This observation supports the concept that MSCs can be considered *universal suppressors*. Since the separation of MSCs and PBMCs by transwell experiments does not completely abrogate the suppressive effect, most human MSC-mediated immunosuppression on activated T lymphocyte has been attributed to the secretion of antiproliferative soluble factors, such as TGF-β, hepatocyte growth factor (HGF), prostaglandin E2 (PGE2), indoleamine 2,3-dioxygenase (IDO, an enzyme causing depletion of tryptophan, an essential factor for lymphocyte proliferation), nitric oxide (NO), heme oxygenase-1 (HO-1) and interleukin (IL)-10 [59–61, 68]. In particular, more recently, the stress-inducible enzyme HO-1 which is able to exert potent anti-inflammatory, anti-oxidative and anti-apoptotic activities has been found to be expressed in rat and human MSCs and to be involved in MSC-mediated immunosuppression [69]. However, published data do not exclude that a part of the immunosuppressive effect exerted by human MSCs on alloantigen-induced T-cell activation be dependent on cell-to-cell contact mechanisms. Of interest, the calcineurin inhibitors, cyclosporine-A and tacrolimus, employed to both prevent and treat graft-versus-host disease (GVHD), enhance the immunosuppressive effect of human MSCs, in particular the in vitro activation of alloantigen-specific and the T-cell-mediated cytotoxicity [70]. Some authors have shown that the unresponsiveness of T cells in the presence of MSCs is transient and that T-cell proliferation can be reinitiated after MSC removal [59, 64, 66]. Inhibition of lymphocyte proliferation by MSCs has not been associated with the induction of apoptosis, but is rather interpreted as due to inhibition of cell division, thus preventing T lymphocyte capacity to respond to antigenic triggers while maintaining these cells in a quiescent state [64, 66, 71]. Indeed, T cells in the presence of MSCs remain in the G0/G1 phase of the cell cycle, and this, at a molecular level, translates into the inhibition of cyclin D2 expression [71].

MSCs are also capable of inducing in vitro regulatory T cells (Treg), as demonstrated by the increase in the population of $CD4^+CD25^+FoxP3^+$ cells in mixed lymphocyte reactions (MLRs) in the presence of MSCs [72, 73]. Very recently, the cytoprotective enzyme HO-1, produced by MSCs, has been shown to promote differentiation of IL-10^+ Tr1 and TGF-β^+ Th3 Treg subsets in a MLR system, as well as to produce IL-10, a suppressive cytokine produced by regulatory cells [74]. It is conceivable that suppression of T-cell proliferation and induction of Tregs are related events.

MSCs and DCs

MSCs have been reported to interfere in vitro with DC differentiation, maturation and function. Differentiation of both monocytes and CD34+ progenitors into $CD1a^+$-DCs is inhibited in the presence of MSCs, and DCs generated in this latter condition are impaired in their function, in particular in their ability to induce activation of T cells [75, 76]. Transwell experiments have demonstrated that the suppressive effect of MSCs on DC differentiation is at least partly mediated by soluble factors, namely, IL-6, macrophage-colony stimulating factor (M-CSF), PGE2 and IL-10 [76].

Incubation of MSCs with mature DCs reduces the latter's expression of HLA class II and co-stimulatory molecules, inhibits TNF production and impairs antigen presentation, therefore favouring the induction of regulatory APCs through which they could indirectly suppress T-cell proliferation [75, 77]. Moreover, MSCs could act as non-professional antigen-presenting cells early in immune responses, in the presence of low levels of interferon γ (IFN-γ). However, the increase in the levels of INF- γ leads MSCs to later switch to their immune suppressive function [77, 78].

MSCs and B Cells

The ability of MSCs to inhibit B cell proliferation was first reported in murine studies [71]. Thereafter, human MSCs have been demonstrated to suppress in vitro the proliferation of B cells activated with anti-Ig antibodies, soluble CD40 ligand and cytokines, as well as to interfere with differentiation, antibody production and chemotactic behaviour of B lymphocytes [79]. Corcione et al. also demonstrated that MSCs do not induce apoptosis, but determine a block of B cells in the G0/G1 phases of the cell cycle, as already shown for T cells [79]. Krampera et al. have reported that MSCs are able to reduce the proliferation of B cells in vitro in the presence of IFN-γ, thanks to its ability to induce IDO activity by MSCs [80]. In contrast with these observations, Traggiai et al. have reported that BM-derived MSCs are able to promote proliferation and differentiation into immunoglobulin-secreting cells of transitional and *naive* B cells isolated from both healthy donors and paediatric patients with systemic lupus erythematosus (SLE) [81].

These conflicting in vitro results on MSC effect on B lymphocyte function/proliferation may partly reflect the differences in experimental conditions employed by the different authors, although, overall, the majority of reports suggest that in vivo B cell proliferation, as well as differentiation and expression of cytokines are inhibited by MSCs [79, 80]. Moreover, as T cells orchestrate B cell function, whatever be the ultimate effects of MSCs on B cell functions are, B cells are likely to be significantly influenced by the MSCs-mediated T-cell inhibition.

MSCs and NK Cells

It has been reported that MSCs are able to suppress NK-cell proliferation after stimulation with IL-2 or IL-15 [67, 82]. Indeed, while MSCs do not inhibit the lysis of freshly isolated NK cells [67], these latter cells when cultured for 4–5 days with IL-2 in the presence of MSCs display a reduced cytotoxic potential against K562 target cells [80]. Transwell experiments have suggested that the suppression of IL-15-driven NK-cell proliferation as well as of their cytokine production by MSCs is mediated by soluble factors [80, 82]. On the contrary, the inhibitory effect displayed by MSCs on NK-cell cytotoxicity required cell-cell contact [82].

Although MSCs were initially considered immunoprivileged and therefore capable of escaping lysis by freshly isolated NK cells [67], recent experiments have demonstrated that IL-2 activated both autologous and allogeneic NK cells are capable of effectively lysing MSCs [83]. Although MSCs express normal levels of MHC class I that should protect against NK-mediated killing, they display ligands that are recognised by activating NK receptors that, in turn, trigger NK alloreactivity [83]. Moreover, it has been recently demonstrated that MSCs can be lysed also by cytotoxic T lymphocytes or antibodies, when infused into MHC-mismatched mice, resulting in their rejection [84].

MSC Mechanisms of Action In Vitro

Several studies have demonstrated that MSCs, in vitro, are capable of modulating the function of different cells active in the immune response, although a clear view of MSC mechanisms of action has yet to be obtained. Cell-cell contact and soluble factors are thought to be required for the induction of MSC-mediated immunosuppression [62]. Primary contact between MSCs and the target cell is initiated by adhesion molecules [85]. Most studies demonstrate that soluble factors are involved, as the separation of MSCs and peripheral blood mononuclear cells (PBMCs) by a transwell permeable membrane does not prevent the inhibition of proliferation [64]. It has been demonstrated that MSCs release several soluble molecules either constitutively or following crosstalk with other cells [62]; these include TGF-β, PGE2, IDO, IL-10, NO and HO-1 [59–61, 68, 69]. Release of IFN-γ by target cells induces the release of IDO by MSCs, which, in turn, depletes tryptophan, an essential amino acid for lymphocyte proliferation [80, 86]. IDO is necessary to inhibit proliferation of Th1 cells and, together with PGE2, inhibits NK-cell function [68, 80]. IFN-γ can, in a murine model where pro-inflammatory cytokines are added, stimulate chemokine production by MSCs, resulting in T-cell attraction and increased inducible NO synthase (iNOS) [87]. T cells are inhibited by the subsequent production of NO [88]. Moreover, cytokines produced by target cells can increase the release of some of these MSC-derived soluble factors [62]. Soluble HLA-G5 (sHLA-G5) released by MSCs suppresses T-cell proliferation, as well as $CD8^+$ T cell and NK-cell cytotoxicity [88]. Conversely, MSCs through the release of sHLA-G5 initiate the up-regulation

of $CD4^+CD25^+FoxP3^+$ cells [88, 89], although their depletion has no effect on the inhibition of T-cell proliferation by MSCs [90].

The complexity and mechanisms whereby MSCs interact with cells of both the adaptive and innate immune system are schematically represented in Fig. 7.1. Whether these effects are displayed through real suppression of immune responses or through a nonspecific antiproliferative effect is still unclear. The mechanisms by which MSCs display their immunosuppressive effect are largely restricted to in vitro studies. The in vivo biological relevance of the in vitro observations needs to be addressed in appropriate in vivo models.

The Importance of Host Factors: Pro-inflammatory Environment

The clinical potential of MSCs might be also influenced by host factors; it has been suggested that MSCs need to be activated in the host environment in order to mediate their immunomodulatory effect [91]. In this sense, MSCs are not constitutively inhibitory, but they acquire their immunosuppressive functions after being exposed to an inflammatory environment [91]. This became clear after the observation that anti-IFN-γ receptor antibodies can block the suppressive effect of MSCs. The various techniques employed to activate an immune response in vitro may involve the release of IFN-γ which, in turn, activates the immunosuppressive activity of MSCs [80, 92]. Moreover, the level of IFN-γ and the contemporary presence of other inflammatory cytokines, such as TNF-α and IL-1ß, can influence the immunosuppressive effect of MSCs, as well as induce changes in their immunophenotype [93]. Indeed, IFN-γ, TNF-α or IL-1ß are able to induce the up-regulation of HLA class I. How about HLA-II? ICAM-1 and VCAM-1 on MSC surface, while IFN-γ alone can induce the activity of IDO [93]. Different cytokine combinations, and consequently the heterogeneity in the host environment, can produce different effects on MSC function; this may explain the variability of response that is observed in patients enrolled in clinical trials and treated with MSCs.

MSCs also express a large number of toll-like receptors (TLRs), and their stimulation has been shown to affect MSC immunomodulatory properties [94]. TLRs are non-catalytic receptors that recognise molecules derived from microbes and mediate the activation of immune responses of both innate and adaptive immunity [95]. In analogy with the functional status of monocytes/macrophages, Waterman et al., by using a short-term TLR-priming protocol, identified two functionally different MSC populations: the TLR4-primed MSC population which exhibits a pro-inflammatory profile (MSC1) and the TLR3-primed MSC population which delivers immunosuppressive signals (MSC2). In accordance with this theory, T-cell inhibition is achieved only in case of co-culture with MSC2, whereas T-lymphocyte activation takes place following MSC1 co-culture [96].

MSCs may exert direct antiproliferative effects on T cells, NK cells and B cells and in this way direct suppress effector immune mechanisms. At the same time, they may exert indirect modulatory activities by inducing tolerogenic immune

responses though the induction of regulatory T cell and tolerogenic dendritic cells. A pro-inflammatory environment may lead to the activation of MSC and may be critical for the induction of suppressive mediators.

Based on these findings which underline the importance of host factors, it has been proposed to mimic the in vivo pro-inflammatory environment by activating MSCs in vitro with the addition of cytokines and to use these activated cells for the treatment of allo- and autoimmune disorders, as well as in the repair of tissue damage.

Immunomodulatory Properties of MSCs In Vivo in Animal Models

The immunomodulatory and reparative/anti-inflammatory properties of MSCs have been tested in a variety of animal models (see Table 7.1).

Animal Models of HSC Engraftment

MSCs have been reported to secrete cytokines important for haematopoiesis and to promote engraftment of haematopoietic stem cells (HSCs) in experimental animal models, especially when the dose of transplanted HSCs was low [97, 98]. Systemic infusion of allogeneic BM-derived MSCs from baboons has been demonstrated to suppress lymphocyte proliferation and prolong the survival of allogeneic skin grafts, as compared to animals not receiving MSCs [122]. Almeida-Porada et al. observed that co-transplantation of human MSCs into pre-immune fetal sheep resulted in enhanced long-term engraftment of human cells in the BM and in higher levels of donor cells in the circulation [97]. Another study performed in NOD/SCID mice demonstrated that co-infusion of fetal lung-derived MSCs and cord blood-derived $CD34^+$ cells is associated with enhancement of engraftment of human HSCs in the BM of the animals, the effect being particularly evident when relatively low doses of HSCs were transplanted [98]. In NOD/SCID mice, co-transplantation of placenta-derived MSCs resulted in both enhanced engraftment of double umbilical cord blood transplantation (UCBT) and reduced single cord predominance [99]. In non-human primates, co-transplantation of MSCs improved HSC engraftment after autologous intra-BM transplantation, and this was associated with increased chimerism in the peripheral blood [100]. Kuci et al. demonstrated that CD271-positive MSCs were capable of promoting significantly greater lymphoid engraftment, as compared to an unselected population of plastic-adherent MSCs, when co-transplanted with CD133+ HSCs in NOD/SCID mice [35].

It has been demonstrated that allogeneic MSCs are not intrinsically immunoprivileged, since, under appropriate conditions, they can induce an immune response, resulting in their rejection when infused into MHC-mismatched mice [84]. In contrast, infusion of syngeneic host-derived MSCs resulted, in the same model, in enhanced engraftment of allogeneic haematopoietic cells [84]. These observations

Table 7.1 Immunomodulatory properties of MSCs in vivo in animal models

Animal model	Outcome	Ref. no.
Part A: HSC engraftment		
Pre-immune fetal sheep	Enhancement of human HSC engraftment	[97]
NOD-SCID mouse	Enhancement of human HSC engraftment	[98]
NOD-SCID mouse	Enhanced engraftment of double UCBT, reduced single-donor predominance	[99]
Non-human primate	Enhancement of autologous HSC engraftment	[100]
NOD-SCID mouse	Promoted lymphoid engraftment	[35]
MHC mismatched mouse	Promotion of graft rejection	[84]
MHC mismatched mouse	Promotion of engraftment	[84]
Part B: GvHD		
NOD-SCID mouse (MSC at weekly intervals)	Prevention of GvHD; mice increased survival	[101]
NOD-SCID mouse (1 MSC dose, d + 0 after Tx)	No effect on GvHD prevention	[102]
NOD-SCID mouse (MSC at weekly intervals)	Prevention of GvHD	[103]
NOD-SCID mouse (1 MSC dose, d + 2 or d + 20)	Prevention of GvHD; mice increased survival	[104]
Part C: AID and regenerative medicine		
Mouse, EAE	Prevention of EAE development	[105, 106]
Mouse, SLE	Ameliorated signs and symptoms of SLE	[107]
Mouse, STZ diabetes	Ameliorated diabetes and kidney disease	[108–110]
Rat, glomerulonephritis	Stimulated glomerular healing	[111]
Mouse, AKI	Ameliorated renal function and tubular cell injury	[112, 113]
Rat, experimental colitis	Stimulated intestinal mucosa healing	[114–116]
Rat, acute hepatic failure	Protected against hepatic injury	[14, 117]
Mouse, rat, pig, myocardial infarction	Improved cardiac function	[118–120]
Mouse, CIA	No beneficial effect; accentuation of Th1 response	[121]

HSC haematopoietic stem cells, *MSC* mesenchymal stromal cells, *NOD-SCID* nonobese diabetic severe combined immunodeficient mice, *UCBT* umbilical cord blood transplantation, *GvHD* graft-versus-host disease, *Tx* transplantation, *AID* autoimmune diseases, *EAE* experimental autoimmune encephalomyelitis, *SLE* systemic lupus erythematosus, *STZ* streptozotocin, *AKI* acute kidney injury, *CIA* collagen-induced arthritis, *Ref. n.* reference number

suggest that MSCs may promote engraftment, provided that they survive in vivo and are not rejected as the result of an alloimmune response. See also Table 7.1, part A.

Animal Models of Graft-Versus-Host Disease (GVHD)

Several animal studies have addressed the issue of the suppressive effect of MSCs in the context of GVHD prevention/treatment; however, conflicting results have been published, in particular on the role of MSCs in GVHD prevention.

In one study, AT-derived MSCs have been infused systemically in mice early after transplantation of haploidentical HSCs and were able to rescue the animals from lethal GvHD [101]. Sudres et al. have reported that a single dose of BM-derived MSCs at time of allogeneic BM transplantation did not affect the incidence and severity of GVHD in mice [102], whereas UCB-derived MSCs administered at weekly intervals were able to prevent GVHD development after allogeneic transplantation of human PBMCs in NOD/SCID mice [103]. The same cells were not effective when administered prophylactically right after PBMC infusion, as well as when infused late in the course of GVHD development [103]. Polchert et al. tested the ability of MSCs to prevent GVHD by administering a single dose of the cells at different time points: only when MSCs were infused at day +2 or +20 after the allograft they were able to significantly increase the survival of the recipient mice. At these time points, the levels of IFN-γ were found to be particularly high in the animals, this corroborating the observation that MSCs need to be activated by inflammatory cytokines present in the host microenvironment to deliver their immunosuppressive effect [104]. See also Table 7.2, part B. The studies suggest that MSCs may prevent GVHD following allogeneic stem cell transplantation, but are not effective in the treatment of acute GVHD. These results are in contrast with preliminary results of clinical studies, where MSCs have been used to treat acute GVHD (see section "Clinical Trials of MSC Infusion to Treat GvHD").

Animal Models of Autoimmune Diseases and Regenerative Medicine

Due to their ability to home to inflamed sites and to repair injured tissues, together with their immunomodulatory and anti-inflammatory properties, MSCs have been also tested in animal models of tissue injury and autoimmune disorders (see Table 7.1, part C) [14, 105–121].

Murine MSCs have been demonstrated to ameliorate experimental autoimmune encephalomyelitis (EAE), a model of post-vaccinal encephalitis with many aspects resembling those of human multiple sclerosis (MS), through the induction of peripheral T-cell tolerance against the central nervous system (CNS)-restricted antigens [105, 106]. In a murine model of SLE, MSCs were able to inhibit autoreactive T and B cells, thus ameliorating the signs and symptoms of the disease [107]. MSCs have been also employed for the experimental treatment of diabetes in a mouse model, and their infusion was associated with an increase in the number of pancreatic islets and insulin-producing β cells, as well as with the repair of renal glomeruli [108]. Moreover, the administration of congeneic MSCs in a murine model of type 1 diabetes was shown to suppress both diabetogenic T-cell proliferation and generation of myeloid/inflammatory DCs, resulting in long-term reversal of hyperglycemia [109, 110]. The infusion of rat MSCs in an experimental model of glomerulonephritis was able to stimulate glomerular healing, resulting in the repair of the damaged renal tissue [111]. Intravenous infusion of MSCs in immunodeficient mice with

Table 7.2 Clinical applications of MSCs in phase I–II studies

Clinical context	Outcome	Ref. no.
Part A: HSC engraftment		
Breast cancer; autologous HSCT	No tox. Rapid haematopoietic recovery	[123]
Haematological malignancy; allogeneic HSCT	No tox. Prompt haematopoietic recovery	[124]
Haematological disorders; haploidentical T-cell-depleted HSCT	No tox. Graft rejection prevention. Accelerated leukocyte recovery	[18]
Haematological disorder (1 pt); double UCBT	Alleviated single-donor predominance	[125]
Haematological disorders; UCBT	No tox. Prompt haematopoietic recovery	[126]
Haematological disorders; UCBT	No tox. No effect on engraftment and haematopoietic recovery. GvHD prevention	[19]
Haematological disorders; UCBT + 3rd PD HSC	No tox. No effect on kinetics of engraftment and GvHD	[127]
Part B: aGvHD		
Grade IV aGvHD after allogeneic HSCT (1 pt)	Complete resolution of grade IV acute GVHD	[128]
Grade II–IV aGvHD after allogeneic HSCT/DLI (55 pt)	Overall response rate: 69%; improved OS in responders	[20]
Grade III–IV aGvHD after allogeneic HSCT/DLI (37 peds)	CR 59%; improved OS if early treatment	a
Part C: AID and regenerative medicine		
MS and ALS	Demonstration of safety + increase in Treg	[129, 130]
Fistulizing refractory CD (intrafistular injection)	Demonstration of safety + fistula healing + increase in Treg	[41, 131]
Refractory CD (intravenous injection)	Safety + some clinical response + increase in Treg	[132]
Cirrhosis	Safety + improved clinical conditions	[133]
Myocardial infarction (intravenous infusion)	Improvement in overall health + increase of LVEF	[134]
Myocardial infarction (intracoronary infusion)	Improvement of LVEF not maintained over time	[135]

HSC haematopoietic stem cells, *HSCT* haematopoietic cell transplantation, *tox*. toxicity, *pt* patient, *UCBT* umbilical cord blood transplantation, *PD* party donor, *aGvHD* acute graft-versus-host disease, *DLI* donor lymphocyte infusion, *peds* paediatric patients, *CR* complete response, *OS* overall survival, *MS* multiple sclerosis, *ALS* amyotrophic lateral sclerosis, *Treg*, regulatory T cells, *CD* Crohn's disease, *LVEF* increase of the left ventricular ejection fraction

[a]Accepted as oral presentation at the Annual Meeting of the European Bone Marrow Transplantation Group, EBMT 2011

cisplatin-induced acute kidney injury ameliorated both renal function and tubular cell injury and prolonged survival due to the inhibition of oxidative damage [112, 113]. Topical implantation of BM-derived MSCs has been shown to be beneficial in promoting the healing process of experimental colitis in rats, confirming the ability of these cells to induce tissue repair [114]. In similar models of experimental colitis,

MSCs of different tissue origin alleviated the signs and symptoms of the disease by displaying immunomodulatory functions and ameliorating inflammation-related tissue destruction [115, 116]. In a rat model, MSC-derived conditioned medium proved to be effective in reversing fulminant hepatic failure, this suggesting that MSC-derived molecules are able to promote regeneration of hepatocytes [14, 117]. Recent preclinical studies have suggested that MSCs could be employed to mediate cardiac repair after myocardial infarction, as well as after chronic progressive cardiac failure. In particular, human MSCs have been shown to differentiate into cells with a cardiomyocyte phenotype in the adult murine heart [118] and to improve cardiac function after transplantation in porcine and rat models [119, 120].

While the vast majority of reports indicate a favourable role of MSCs in the promotion of tissue repair, infusion of MSCs had no beneficial effects on collagen-induced arthritis (CIA), a murine model of rheumatoid arthritis. In particular, in this context, MSC treatment was associated with an enhanced Th1 response, although MSCs could not be detected in the articular environment [121].

Potential MSC Mechanisms of Action In Vivo

The mechanisms through which MSCs exert their therapeutic potential, although not fully established, might rely on some key properties of the cells: (i) the ability to secrete soluble factors capable of stimulating both survival and functional recovery of injured cells; (ii) the ability to home to sites of damage; and (iii) the ability to modulate immune responses. In most of the reported studies, the therapeutic effect of MSCs was not associated with their differentiation into the resident cell types, but, rather appeared to be mostly related to antiproliferative and anti-inflammatory effects, as well as to the capacity to stimulate survival and functional recovery in injured organs, likely through paracrine mechanisms [14, 111–113, 117]. It is conceivable that the therapeutic benefit is due to the release of soluble factors (such as HGF, insulin-like growth factor, PGE2, NO, IDO) produced by the cells and/or by the local microenvironment and that MSC survival is not strictly necessary for the clinical effect [14, 117]. Also the engraftment-promoting effect might be obtained through the secretion of paracrine factors produced by MSCs, which might promote the creation of a favourable microenvironment for the survival, proliferation and engraftment of HSCs.

Experimental and clinical data obtained so far indicate that sustained engraftment of MSCs does not occur or it is limited to a small number of cells. In this regard, studies in baboons using a green fluorescent retroviral construct suggest engraftment of MSCs in the gastrointestinal tract and in various tissues in the range of 0.1–2.7%, with comparable results for both autologous and allogeneic cells [136]. Although little is known about MSC homing to target tissues after infusion, it might be largely regulated by chemokines and growth factors released during systemic and/or local inflammatory conditions and be mediated by the interaction with integrins and selectins expressed on the surface of MSCs. In this respect, Wynn et al.

showed that homing of MSCs to BM depends on stromal-derived factor-1 (SDF-1) which interacts with CXCR4 on the MSC surface, thus promoting their migration [137]. Similar mechanisms have been shown to regulate migration of MSCs to pancreatic islets [138] and ischemic tissues [139]. In view of these experimental data, a possible strategy to facilitate homing of MSCs involves the modification of surface structures that play a role in migration to specific tissues, as suggested by Sackstein et al. [140]. These authors converted the native CD44 glycoform expressed on MSCs into E-selectin/L-selectin ligand (HCELL) (expressed on HSCs) using fucosyltransferase. Intravital microscopy in NOD/SCID mice showed BM infiltration by HCELL(+) MSCs within several hours after intravenous infusion [140].

The inhibition of inflammatory and immune responses by MSCs might also be due to the generation of regulatory T cells, as shown in an experimental murine model of Crohn's disease, in which MSC infusion was efficacious in both preventing and curing colitis, probably through the induction of FoxP3^{+} regulatory T cells [116].

Clinical Applications of MSCs

Clinical Trials of MSC Infusion to Promote Engraftment

The first clinical trial on the use of MSCs for accelerating haematological recovery was performed in 28 patients with breast cancer given autologous transplantation of peripheral blood HSCs and co-infused with $1–2 \times 10^{6}$ MSCs/kg body weight. No MSC-related toxicity was recorded, and rapid haematopoietic recovery was noted [123]. After this study, a multicenter phase I/II trial aimed at evaluating the safety of MSC infusion was conducted in 46 patients affected by haematological malignancies and receiving allogeneic HSCT from an HLA-identical sibling [124]. MSC co-infusion was not associated with adverse events, and haematopoietic recovery was prompt for most patients; moderate to severe acute GVHD was observed in 28% of the patients. In a phase I/II, multicenter clinical trial, infusion of MSCs proved to be safe in children given a T-cell-depleted allograft from an HLA-disparate relative [18]. All patients given MSCs showed sustained haematopoietic engraftment without any adverse reaction, this finding comparing favourably with 20% graft failure rate observed in the historical controls. Leukocyte recovery was faster in children given MSCs, as compared to the historical controls. In the setting of UCBT, MSCs were first employed in a single patient transplanted with UCB-derived cells with the aim of improving the outcome of double-unit UCBT [125]. In this patient, MSCs were administered without clinical adverse effects, and the single unit predominance described after multiple UCBT was not observed. In a paediatric, phase I–II clinical trial, including eight children given co-transplantation of unrelated donor UCB cells and *ex vivo*-expanded third-party MSCs, infusion of MSCs proved to be safe and patients had neutrophil recovery a median time of 19 days after the allograft [126]. In another paediatric, phase I/II clinical study,

the safety of co-transplantation of parental MSCs was confirmed in 13 paediatric patients given UCB-derived HSCs [19]. In contrast with preclinical results [98] and the experience reported in the haploidentical transplants [18], no difference was found in engraftment rate and speed of haematological recovery between study patients and controls receiving UCBT alone, although much less study patients were given granulocyte-colony stimulating factor (G-CSF) as compared to controls. Interestingly, MSC co-infusion significantly prevented the incidence of life-threatening acute GVHD and GVHD-associated transplantation-related mortality (TRM), as compared to controls [19]. In adult patients receiving UCBT with co-infusion of third-party donor mobilised HSCs, MSC administration at time of transplantation had no effect on the kinetics of UCB cell engraftment, as well as on GVHD prevention [127]. See Table 7.2, part A.

Altogether these data indicate that co-transplantation of HSCs and MSCs is safe, whereas the efficacy of MSCs on promoting engraftment of donor cells and accelerating the speed of haematological recovery remains to be demonstrated. In some contexts (such as T-cell-depleted allograft from an HLA-disparate relative), MSCs may modulate host alloreactivity and/or promote a better engraftment of donor haematopoiesis, reducing the risk of graft failure. The difference between the haploidentical and UCBT settings may be related to the mechanisms underlying graft failure in UCBT, which might be inherent to the low numbers of HSCs infused in UCBT and/or to altered homing mechanisms.

Despite reports indicating engraftment of MSCs after systemic infusion in animal models [97, 141], the transplantability and sustained engraftment of MSCs in humans has not been demonstrated. A number of studies have documented that marrow stroma remains of host origin after allogeneic HSCT in the majority of patients [142–144], whereas others have shown limited engraftment capacity of MSCs following HSCT in both adult and paediatric patients [18, 19, 22, 145, 146].

Clinical Trials of MSC Infusion to Treat GVHD

The most impressive clinical effect of MSCs in vivo has been observed in the treatment of acute GvHD (aGvHD) developing after allogeneic HSCT or donor lymphocyte infusion (DLI). The first striking report of this effect was reported by Le Blanc et al. who described a paediatric patient experiencing grade IV aGvHD of the liver and gut after allogeneic HSCT from an unrelated volunteer, resistant to multiple lines of immunosuppressive therapy. The child was rescued by the infusion of BM-derived MSCs isolated from the mother [128]. More recently, the benefit deriving from the infusion of MSCs in patients with steroid-resistant aGvHD has been confirmed in a study reporting 55 adult and paediatric patients, treated in six different institutions. Infusion of MSCs appeared to be safe, and no major toxicities were observed. Treatment with MSCs resulted in a response in the majority of patients, this resulting into a significant difference in survival between complete responders and partially responding/nonresponding patients [20]. When compared to adults,

children seemed to have a better response rate and a greater probability of overall survival [20].

The outcome of 37 children receiving MSCs for grade III–IV acute GVHD refractory to steroids have been recently reported [147]. A median of two infusions were administered, with a median cell dose of 2×10^6/kg; MSCs were from third party HLA-mismatched donors in the majority of the patients. Complete response (CR) was observed in 22 children (59% of the overall population), transplantation-related mortality (TRM) being 14%. Fifteen children had either no ($n=6$) or partial ($n=9$) response to MSCs, TRM in this group being 60% ($p=0.005$). With a median follow-up of 2.3 years, overall survival (OS) was 62%, the values for patients who did or did not achieve CR after MSCs being 87 and 27%, respectively ($p<0.001$). Children treated after 2009 had received less second-line treatment and had received MSCs earlier after onset of steroid treatment (mean day 8 vs. day 24 for children treated before 2009). This translated into a significantly better OS for children enrolled in the study after 2009 (93% vs. 65% for those treated before 2009; $p<0.05$). These data indicate that MSCs are a safe and valuable therapy for children with severe, refractory aGVHD, better results being obtained when treatment is employed early in the disease course. See Table 7.2, part B.

The real efficacy of MSC infusion in the management of patients with GVHD will be tested in a randomised controlled trial being conducted in Europe.

Clinical Trials of MSC Infusion in Autoimmune Disorders and Tissue Repair

Following the numerous reports showing a beneficial effect of MSC treatment in experimental models of autoimmunity and acute tissue injury [14, 105–120], clinical data on the use of MSCs in regenerative medicine have become available (see Table 7.2, part C) [129–135, 148–150].

A phase I clinical study including ten patients with multiple sclerosis and treated with autologous MSC infusion has shown the feasibility and the safety of the approach [130]. In 15 patients with MS and 19 patients with amyotrophic lateral sclerosis treated with intrathecal and/or intravenous MSC infusion, the procedure was found to be safe and was associated with an increased proportion of $CD4^+CD25^+$ regulatory T cells in the peripheral blood of the patients [131], suggesting that the possible effects of MSCs involve the induction of regulatory T cells.

In a phase I clinical trial, autologous, AT-derived MSCs have been successfully employed to treat complex perianal fistulas of cryptoglandular origin or associated with Crohn's disease with promising results [41]. Sustained closure of fistula tracks, together with a parallel reduction of Crohn's disease and perianal disease activity indexes, has been obtained in patients with refractory fistulizing Crohn's disease through local injections of autologous BM-derived MSCs [132]. In another phase I/II study, intravenous infusion of autologous MSCs proved to be feasible and safe in nine patients with Crohn's disease refractory to conventional treatments, three

of them showing clinical response [133]. In these latter two studies, an increase in regulatory $CD4^+CD25^+FoxP3^+$ T cells in mucosal biopsies was found after MSC treatment, as compared with what observed before treatment initiation [132, 133].

MSCs have been also employed to treat liver cirrhosis in a limited number of patients. Kharaziha et al. reported a phase I–II clinical trial in which eight patients with end-stage liver cirrhosis were treated with autologous injection of MSCs via either a peripheral vein or the portal vein; preliminary results confirm the safety of the approach and suggest some improvement in the clinical conditions of the patients [148]. Similar findings were obtained by Mohamadnejad et al. who also showed that MSCs are superior to HSCs in treating liver cirrhosis [134, 149].

A randomised placebo-controlled clinical trial has been conducted in patients within 10 days following acute myocardial infarction [135]. As compared to patients treated with placebo, patients receiving MSC intravenous infusion of MSCs experienced an improvement in overall health, coupled with an increase of left ventricular ejection fraction (LVEF) 1 year after treatment. However, patients treated with intracoronary administration of MSCs after MI did not maintain a significant improvement in LVEF over controls at the 18 months follow-up evaluation [150]. The heterogeneity in the route of administration, timing of MSC infusion after myocardial infarction and number of cells administered render definitive conclusions difficult to draw on the efficacy of this approach.

Additional studies have been initiated in other autoimmune and inflammatory disorders, such as type 1 diabetes mellitus, systemic sclerosis and SLE, acute kidney injury, gastrointestinal (autoimmune enteropathy) and pulmonary (chronic obstructive pulmonary disease) disorders [151–155]. The results of these studies will become available in the near future.

Conclusions

In addition to their regenerative properties, MSCs have been shown to exert immunomodulatory effects. Extensive *ex vivo* studies have indicated that they affect a broad range of immune functions, including those of T cells, B cells, DCs and NK cells, mainly through the secretion of soluble mediators. These mediators may act directly on immune cells, to inhibit their proliferation or to inhibit apoptosis. They may also act indirectly through intermediate cells, including monocytes, to induce regulatory responses that result in the induction of regulatory T cells or tolerogenic DCs.

Further investigations aimed at better defining the role played in vivo by human MSCs in developing peripheral immune tolerance are desirable. Relatively little is known about the functional differences between MSCs derived from different tissue sources, i.e. BM-derived versus AT-derived MSCs. It is reasonable to hypothesise that different MSC subsets may be responsible for specific functional activities in vivo. Few surface markers are nowadays available for the prospective identification of MSC subsets, and it is still uncertain to what extent functional

properties are preserved/modified following *ex vivo* expansion. The importance of the host tissue microenvironment has recently become apparent as being a possible determinant of in vivo function of MSCs. It is conceivable that host factors play a crucial role in activating or priming MSCs to exert their immunomodulatory properties. Identification of such factors may lead to novel strategies to functionally activate MSCs prior to infusion in order to enhance/optimise their therapeutic effects.

Acknowledgements This work has been partly supported by grants from Istituto Superiore di Sanità (National Program on Stem Cells), MIUR (Ministero dell'Istruzione, dell'Università e della Ricerca, Progetti di Rilevante Interesse Nazionale, PRIN), from Associazione Italiana per la Ricerca sul Cancro (AIRC) special grant "5 x 1000" to FL and from AIRC IG9062 to MEB and by grants 11.600.1016/17 from ZON-MW (Zorg Onderzoek Nederland, Medische Wetenschappen).

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Chapter 8
MSCs and Innate Immune Responses: A Balancing Act

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Abstract The immunoregulatory functions of bone marrow-derived stromal cells (BMSCs), also called mesenchymal stromal/stem cells (MSCs), have been studied extensively in recent years. Although there is still some confusion in the literature about the nomenclature, for the sake of simplicity, we will use the abbreviation MSC below, referring to cells isolated by their adherence to plastic, which might be derived from a variety of tissues (and might not have identical features). Most investigators who examined the mechanisms responsible for the immunomodulatory actions of MSCs focused on the interactions between the latter and cells that comprise the adaptive immune system (T and B cells). Recently, however, an increasing number of publications have described interactions between MSCs and neutrophil granulocytes and have provided data suggesting that effects on monocytes and macrophages may play a major role in MSC-induced immunomodulation. For example, MSCs were shown to enhance the antibacterial activities of neutrophil granulocytes preventing organ injury caused by the uncontrolled activation of these cells. Furthermore, MSCs were reported to modulate the functions of the monocyte/macrophage lineage by inducing these cells to acquire an anti-inflammatory phenotype. This phenotypic switch seems to be critical not only in the prevention of sepsis-induced multiorgan failure but also in the protection by MSCs seen in autoimmune settings. In fact, data suggest that MSC-macrophage interactions may even be a key intermediate step in the MSC-mediated protection from T cell-driven autoimmunity. In this chapter, we summarize the most important findings that led to these conclusions.

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_8,

Introduction

In 2004, Katarina Le Blanc reported the first successful treatment of steroid-resistant, acute graft-versus-host disease (GVHD) using bone marrow-derived mesenchymal stromal cells (MSCs) in a patient [1]. This case report opened up a new chapter in the history of MSC biology and stimulated stem cell biologists and immunologists to work on MSC-driven immunomodulation. After Le Blanc's paper appeared in the literature, hundreds of publications from several research groups improved our understanding of the unique immunomodulatory properties of MSCs [2–6]. The overwhelming majority of investigators in the field focused on interactions between MSCs and lymphocytes [7–10]. Soon research groups studying dendritic cells and dendritic cell-T cell interactions joined the field and showed how MSCs influence the process of antigen presentation and MHC-dependent T cell activation [11–14]. Other groups examined how B lymphocytes [15] and NK cells [16] behave in the presence of MSCs. Some time elapsed, however, before the first study calling attention to the interactions between MSCs and two other major players of the innate immune system – macrophages and neutrophil granulocytes [17] – was published. Numerous other studies followed, each adding some important new details to the field [4, 18, 19]. In this chapter, we summarize the information currently available regarding interactions between MSCs and monocytes and macrophages and neutrophil granulocytes. We will discuss the importance of these interactions in antibacterial defense and their possible role in the modulation of a variety of immune responses.

MSC Interactions with Immune Cells

Therapeutic use of MSCs was originally tested in GVHD patients because the pathological processes in GVHD are primarily mediated by T lymphocytes [20], and MSCs were reported to suppress lymphocyte proliferation in vitro [8, 21, 22]. Due to the initial success, a variety of groups started to test the cells in autoimmune disease models, hoping that suppressing T cell clones that attack the body might ameliorate such disorders. Zappia et al. demonstrated that MSCs had a beneficial effect in the experimental autoimmune encephalitis (EAE) model [23] of multiple sclerosis. In this system, immunosuppression was attributed to MSC-induced T cell anergy. In a similar study, Rafei et al. identified CD4+ Th17 cells as the most important target of MSC-induced immunomodulation [24]. Other autoimmune diseases were also targeted; MSCs were shown to have a beneficial effect in diabetic NOD/SCID mice, in which glucose and insulin levels normalized after treatment [25]. When MSCs were cocultured with human peripheral blood mononuclear cells (PBMCs) obtained from patients with type 1 diabetes, they abrogated proinflammatory T helper type 1 response to an islet antigenic stimulus in type 1 diabetes seen in vitro. The MSCs induced interleukin (IL)-4-production, suggesting a possible switch to an anti-inflammatory T helper type 2 signaling of T cells [26]. On the basis of encouraging animal studies, clinical trials have been

initiated to determine whether MSCs could be useful in treating multiple sclerosis, primary Sjogren's syndrome, systemic lupus erythematosus, systemic sclerosis, and Crohn's disease [5].

Antimicrobial Role of Bone Marrow MSCs

The first paper suggesting a connection between MSCs and macrophages emerged in 2007 [27]. Based on in vitro coculture experiments, the authors suggested that MSCs decrease LPS- and silica-induced TNF-α (tumor necrosis factor alpha) production by macrophages in a dose-dependent manner. Later, three independent studies reported beneficial effects of intravenously delivered bone marrow MSCs or intraperitoneally administered adipose tissue stem cells in a standardized assay of sepsis in mice, called the cecal ligation and puncture (CLP) model [17, 28, 29]. In each of these studies, MSC-injected animals showed a markedly increased survival compared to the control animals. It is well established that exaggerated neutrophil and macrophage responses [30] followed by immune paralysis play a critical role in the development of septic shock. It is also accepted that other classes of immune cells (e.g., dendritic cells and CD4+ T cells) affect survival of the animals subjected to CLP. To pinpoint the host cells targeted by injected MSCs in the CLP model, Nemeth et al. repeated the studies in mice that lack B and T cells (Rag−/− mouse) and in mice without NK cells. Interestingly, the beneficial effect of MSCs was still present in these animals, suggesting that they worked independently of lymphocytes. However, when liposomal clodronate was used to eliminate monocytes/macrophages, the MSCs were no longer effective [17]. The improved survival of MSC-treated mice correlated with reduced bacterial titers in the blood and decreased organ damage [17]. MSCs were also found to protect organs when LPS was used to induce systemic inflammation in mice [27, 31, 32].

Much of the organ damage seen in systemic infections results from oxidative damage caused by tissue-invading neutrophil granulocytes. A common finding following MSC injections into septic or LPS-treated mice is a marked reduction in the number of organ-infiltrating neutrophil granulocytes (measured by myeloid peroxidase activity) as well as a substantial increase in the phagocytic, antibacterial activity of the cells that remain in the bloodstream.

We wondered how MSCs induce these changes. To answer this question, we first wanted to know where MSCs that are injected intravenously go in the body and whether they kill pathogens themselves. Even if they are able to destroy pathogens, MSCs must also need to collaborate with the host immune system, since as Nemeth et al. demonstrated, MSC can no longer protect the septic mice from dying in animals lacking macrophages. Thus, it was important to try to understand the nature of this collaboration.

Regarding the fate of intravenously administered MSCs, it is generally accepted that in mice following tail vein injection, the majority are trapped in the lung and eventually disappear [33, 34]. Fischer et al. studied the entrapment and concluded

that in addition to their large size, specific adhesion factors (CD49d or VCAM1) play a significant role in mediating this phenomenon [34]. In their study of septic animals, Nemeth et al. found that most injected MSCs were indeed found in the lung. The MSCs were surrounded by many macrophages, and most of the MSCs disappeared after 24 h. Based on these results, it seems that the MSCs encounter the macrophages in the tissues (following IV injections in the lung) and have an intimate connection with them. While they are connected, MSCs appear to reprogram the macrophages and induce them to abandon the diseased (proinflammatory) state and return to a more normal one. Indeed, many investigators have suggested that in pathological environments, MSCs produce cytokines and chemokines that are needed to restore health. In order to affect this "balancing act," MSCs must sense changes in their environment. They are equipped to do this; they express several toll-like receptors [19], one of which is TLR4, the receptor for lipopolysaccharide (LPS). When they detect LPS, MSCs increase their IL-8 production [36] and attract neutrophils. They also increase their IL-6 secretion. IL-6 is a potent antiapoptotic factor [37] and may protect the freshly recruited neutrophils from dying 35. In addition to this direct action on neutrophil survival, MSCs affect neutrophils indirectly as well via their effects on neighboring monocytes/macrophages. Macrophages that are reprogrammed by MSCs decrease TNF-α production [27, 28] and increase IL-10 output [17, 38]. The IL-10 seems to prevent neutrophils from migrating into tissues [39–42], thus reducing oxidative damage and mitigating multiorgan injury. Since they stay in circulation longer and do not migrate into the tissues, neutrophils build up in the bloodstream and exert a stronger antibacterial activity.

In addition to secreting factors that affect macrophages and neutrophils, MSCs also attack pathogens with antibacterial factors. Recently, human MSCs were also reported to produce cathelicidin, an antimicrobial peptide that effectively reduced the growth of gram-negative bacteria [43]. MSCs may have other antimicrobial molecules in their arsenal.

MSC-Macrophage Interactions: Changing the Balance

Macrophages can be classified as either classically activated M1 or alternatively activated M2 species [44]. M1 macrophages are produced upon exposure to acute-phase proteins (such as TNF-α), Th1 cytokines (such as IFN-γ), or microbial products (such as LPS). M1 macrophages produce high levels of proinflammatory TNF-α, IL-1β, IL-6, IL-12, IL-23, and CXCL10 and release significant amounts of reactive oxygen and nitrogen species (NO, peroxynitrite, hydrogen peroxide, superoxide, etc.). M2 macrophages, on the other hand, arise in a Th2-dominant environment (in response to IL-4, IL-13, or IL-10) and during phagocytosis of cell debris (including apoptotic neutrophil granulocytes). M2 macrophages possess a regulatory phenotype characterized by increased production and release of the anti-inflammatory cytokines, IL-10 and TGF-β, and by the synthesis of IL-1Ra,

decoy IL-1RII, scavenger, mannose, and galactose receptors, and the chemokine CCL22 [45, 46]. M1 and M2 macrophages have markedly different functions. M1 macrophages are activated by invading pathogens and play a critical role in combating infection during the initial phase of inflammation. Although their proinflammatory actions are beneficial to the host, the persistence of unbridled inflammatory processes may result in tissue damage. Gradual appearance of M2 macrophages ensures control over the first inflammatory phase and sets the stage for the resolution phase, during which normal tissue homeostasis returns. There are several pathophysiological scenarios where lack of an effective resolution phase can lead to chronic inflammation and destruction of the involved organs (e.g., the joints in rheumatoid arthritis or the colon and small intestine in Crohn's disease) [47]. Therapeutic interventions that move an overly activated M1 state toward an anti-inflammatory M2 state are desirable because they prevent tissue destruction and promote regeneration, but drugs with this effect may be hard to regulate. Such agents may target too many cells, producing unwanted changes in some of them (Fig. 8.1).

Surprisingly, when MSCs are introduced into an inflammatory environment, they seem to make appropriate adjustments. In short, they are smarter than single molecular species. Interestingly, MSCs themselves have been recently described to be able to shift between pro-inlammatory (MSC1) and Anti-inflammatory phenotype [48]. Furthermore, MSCs have been shown to produce TSG-6, an anti-inflammatory agent that significantly contributes to their beneficial actions [49]. Several studies using human and rodent MSCs collected from a wide variety of sources (bone marrow, adipose tissue, gingiva, parotid gland, etc.) have revealed that the alteration in macrophage TNFα/IL-10 release described earlier corresponds to an M1/M2 phenotypic switch yielding macrophages with a largely anti-inflammatory phenotype [38, 50]. A good example of such an in vivo effect was demonstrated in a recently published wound healing study. Zhang et al. injected MSCs into wounded mice and showed that the accelerated healing in the MSC–treated group was associated with a gradual appearance of regulatory, CD206 positive M2 macrophages in the wounded area [51]. Macrophages play a key role in wound healing by terminating the initial inflammatory phase of injury [52]. Phagocytosis of apoptotic polymorphonuclear neutrophils and other cell debris leads to the next (proliferative) phase of wound healing, thus providing the basis for angiogenesis, granulation tissue formation, and finally, re-epithelization of the injured area.

Although didactically the innate and the adaptive immune systems are usually treated as separate entities, in reality they always work together and continuously interact with each other. A recent study by Parekkadan et al. provided an important link between MSC-monocyte/macrophage interactions and in vivo suppression of T cell-mediated disease processes [53]. This study demonstrated MSC-dependent recruitment of regulatory T cells and showed that subsequent amelioration of T cell-dependent autoimmunity depends on the presence of monocytes/macrophages (CD11b+cells).

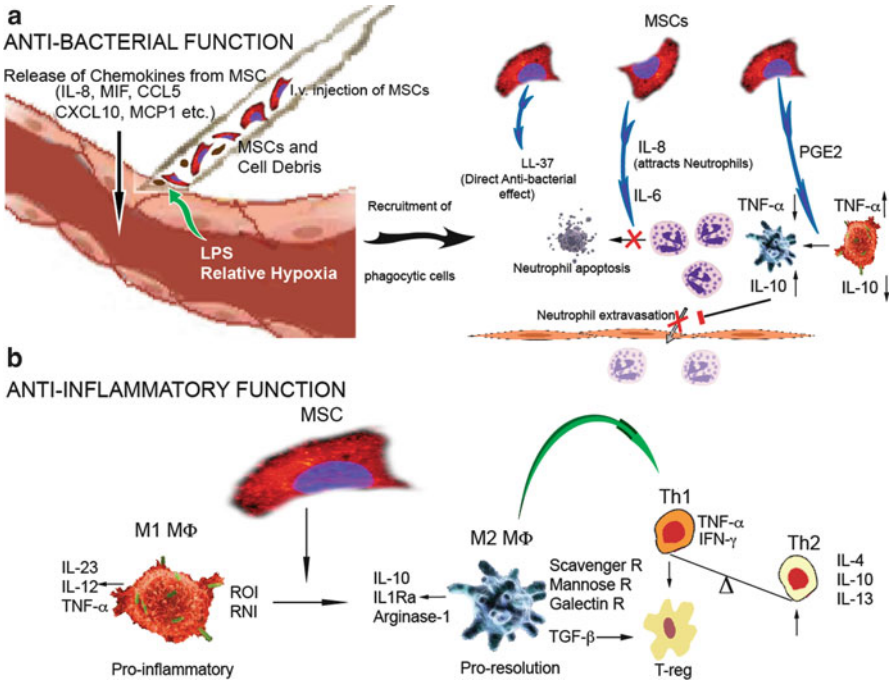

Fig. 8.1 Schematic drawing to demonstrate the (**a**) antibacterial and (**b**) anti-inflammatory functions of MSCs. When MSCs are injected IV, the cultured cells sense a relative hypoxia (compared to the culture conditions) and circulating LPS and release a variety of cytokines and chemokines. These together with the arriving cell debris will attract phagocytic cells, such as monocytes/macrophages and neutrophils. The MSC will release direct antibacterial agents (LL-37) IL-6 that reduces neutrophil apoptosis and PGE2 which will reeducate the proinflammatory (M1, *red*) to anti-inflammatory (M2, *blue*) macrophages. These macrophages will then make and release IL-10, which will help to keep the attracted neutrophils in the circulation, thus increasing bacterial clearance there while decreasing the oxidative damage in the organs. In its anti-inflammatory role (**b**) the MSC will balance the composition of cytokines and chemokines in the tissue in a way that the originally M1/Th1-dominant environment will slowly move back into "neutral" – the seesaw will be flat again

Conclusions

We focused this chapter on the interactions between MSCs and neutrophil granulocytes or monocytes/macrophages. These interactions appear to play a significant role in determining the final outcome of MSC treatment of diseases. MSCs are plastic and powerful: they can sense the environment they are placed in and are able to adjust their responses to counter imbalances detected. They employ a wide variety of cytokines and chemokines (even bactericidal agents) that affect the pathogens directly or recruit and "advise" other members of the immune system. It seems that no matter what the cause of the pathology, MSCs may have the ability to rebalance the immune system and put things in physiological order again. There is still much to learn about how they achieve this amazing regulatory balancing act.

Acknowledgment The work was supported by the DIR, NIDCR of the Intramural Research Program, NIH, and DHHS.

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Chapter 9
MSCs: Paracrine Effects

Siddiraju V. Boregowda and Donald G. Phinney

Abstract Historically, mesenchymal stromal/stem cells (MSCs) have been characterized by their capacity to support hematopoiesis and differentiate into various connective tissue cell types. However, in the past decade, the field of MSC research has witnessed tremendous growth, spurred principally by studies showing that the cells are efficacious in treating a broad array of diseases. Renewed interest in MSC biology has also yielded new insights into their developmental origin, contribution to the hematopoietic stem cell niche, and mechanism of action in promoting tissue repair and regeneration. In the latter case, MSCs have been shown to secrete a bevy of proteins and other molecules that exhibit trophic, angiogenic, immunomodulatory, neuro-regulatory, anti-inflammatory, and anti-apoptotic activity and that function to restore homeostasis at sites of tissue injury and in response to disease. Herein, we provide an overview of the paracrine functions of MSCs by describing the different classes of proteins secreted by cells, the influence of the local microenvironment on their expression, and their therapeutic effects in various experimental animal models of disease.

Introduction

Friedenstein and coworkers were the first to identify a cell population in bone marrow, distinct from hematopoietic stem cells (HSCs), with the ability to generate in vivo a heterotopic osseous tissue capable of supporting hematopoiesis (Chap. 1).

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_9,

This population, now referred to as mesenchymal stromal cells (MSCs), was subsequently exploited to establish long-term bone marrow cultures in vitro, which provided a unique opportunity to dissect the cell-type specific interactions and soluble factors that regulate various aspects of hematopoiesis. These studies revealed much about the phenotype and function of MSCs and as such revealed for the first time insight into their unique paracrine functions [1]. Therefore, we will begin by examining the role of MSCs in hematopoiesis, which continues to evolve.

Role of MSCs in Hematopoiesis

A number of reviews have been published describing in detail the important role played by MSCs in regulating hematopoiesis [2, 3]. Therefore, the topic is discussed only briefly here. MSCs secrete several classes of proteins including cytokines, chemokines, growth factors, neuropeptides, and extracellular matrix proteins that modulate hematopoiesis. For example, the matrix proteins fibronectin, laminin, vitronectin, thrombospondin, haemonectin, thrombopoietin, tenascin, and collagens function as organ and lineage-specific binding proteins for hematopoietic cells [4–12]. These proteins also directly bind cytokines and growth factors and present them in biologically active forms to hematopoietic cells, which then stimulate growth and maturation [13–15]. Many growth factors including stem cell factor, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor, kit ligand, leukemia inhibitory factor, interleukin 1 beta (IL-1B), interleukin 3 (IL-3), interleukin 6 (IL-6), interleukin 7 (IL-7), interleukin 8 (IL-8), interleukin 11 (IL-11), insulin-like growth factor 1 (IGF-1), and transforming growth factor beta 1 (TGFβ1) are also secreted by MSCs [16, 17]. In addition, their expression may be altered in response to external stimuli, thereby providing a mechanism to modulate hematopoiesis in response to stress, infection, and injury. For example, treatment of MSCs with IL-1β, IL-6, and lipopolysaccharides (LPS) stimulates, whereas treatment with interferon α suppresses expression of GM-CSF and G-CSF [18]. Activin A, a potent stimulator of erythroid differentiation and negative regulator of B cell lymphopoiesis, is also strongly upregulated in MSCs by inflammatory cytokines and suppressed by glucocorticoids [19]. Moreover, platelet-derived growth factor (PDGF) enhances the ability of MSCs to support growth of colony forming unit-granulocytes macrophages (CFU-GM) by inducing secretion of GM-CSF, IL-3, and IL-6 [20]. Other factors secreted by MSCs that have been shown to play a role in hematopoiesis include Flt-3 ligand [21], hepatocyte growth factor (HGF) [22], jagged1 [23], substance P [24], and calcitonin gene-related protein [25]. Additionally, secreted frizzled-related protein-1 (sFRP1) inhibits osteoclast formation [26] as well as maintains homeostasis of HSCs in marrow via extrinsic regulation of beta-catenin [27].

More recent studies have identified MSC subpopulations in marrow, discriminated based on secretion of specific cytokines, that bind to functionally distinct T and B cell lineages [28]. For example, MSCs expressing CXCL12 (chemokine,

CXC motif, ligand 12) have been shown to interact specifically with pre-pro-B cells and memory plasma cells, while MSCs that express IL-7 but lack expression of CXCL12 interact specifically with memory $CD4^+$ T cells. These MSC subpopulations also express vascular cell adhesion molecule 1 but lack expression of endothelial cell adhesion molecule 1. These studies indicate that the bone marrow reticular system may be inordinately complex and contain distinct stromal subtypes that specifically interact with different hematopoietic lineages to sustain hematopoiesis [1]. Various groups have also reported that MSCs and or MSC-derived osteoprogenitors physically interact with HSCs in bone marrow and as such contribute to the HSC niche (see Chap. 3).

It is important to note that human CD34+HSCs as well as CFU-GM, CFU-megakaryocytes, and blast-forming unit erythrocytes also secrete various growth factors, cytokines, and chemokines that function via autocrine and paracrine mechanisms to regulate hematopoiesis [29, 30]. For example, human CD34+ HSCs stimulate secretion of G-CSF and IL-6 from MSCs [31]. Therefore, hematopoiesis is orchestrated by the concerted action of many secreted proteins, expression of which is controlled both by changes in the external environment and cross-talk between stromal and hematopoietic cell lineages within marrow.

Regeneration Versus Replacement: Shifting Paradigms of MSC Function

Early studies demonstrating the multi-potency of MSCs led to the assumption that the cells function in tissue homeostasis by serving as a reservoir of connective tissue progenitors and as such were first employed clinically to treat osteogenesis imperfecta [32]. The established role of MSCs in supporting hematopoiesis was also exploited in clinical trials to speed hematopoietic recovery following bone marrow transplantation [33]. Although results from these trials were encouraging, reports published in the early 2000s indicating that MSCs possessed unexpected plasticity as evidenced by their transdifferentiation into cardiomyocytes [34], astrocytes [35], hepatocytes [36], lung epithelium [37, 38], and other lineages sparked renewed interest in the therapeutic potential of the cells. While further research revealed that "transdifferentiation" occurred at a low frequency in vivo [39], MSCs continued to demonstrate measurable therapeutic effects in experimental animal models of disease. Consequently, this prompted a major paradigm shift with respect to the anticipated function of MSCs. Rather than contribute directly to tissue replacement via directed differentiation, the ability of MSCs to alter the tissue microenvironment via paracrine signaling rapidly established itself as the principle mechanism by which the cells affected tissue repair and regeneration. This new paradigm has rapidly gained acceptance following the identification of various proteins secreted by MSCs that have demonstrated angiogenic, anti-apoptotic, anti-inflammatory, and trophic effects in various disease models.

The MSC Transcriptome

The diverse array of paracrine-acting factors secreted by MSCs was predicted in large part by analysis of their transcriptome using genomics-based approaches. For example, our laboratory was the first to analyze the transcriptome of human [40, 41] and primary rodent MSCs [42] via serial analysis of gene expression (SAGE). Interrogation of these databases revealed expressed transcripts encoding proteins that regulate a variety of functions necessary to maintain homeostasis of bone and bone marrow including angiogenesis, hematopoiesis, cell migration and communication, neural activities, immunity, and defense. These findings were confirmed by other groups [43–45]. We also validated the biological activity in vitro and in vivo of a subset of secreted proteins identified by SAGE. For example, we demonstrated that primary mouse MSCs ameliorated bleomycin-induced lung injury in mice and that secretion by cells of interleukin receptor 1 antagonist (IL-1RN), a protein with potent anti-inflammatory activity, contributed significantly to this effect [46]. We also showed that neural regulatory factors secreted by MSCs promoted survival and neurite outgrowth from neuroblastoma cells and spinal nerves from the dorsal root [47]. Finally, we identified a large number of angiogenic factors secreted by MSCs that induce growth and branching morphogenesis of human vascular endothelial cells [48]. Results from genomics-based studies have been codified by proteomics-based analyses showing that MSCs secrete chemokines [49], chemoattractants [50], and angiogenins [51] that play roles in tissue injury and repair.

SAGE analysis also revealed that a single cell-derived colony of human MSCs simultaneously expressed a diverse array of lineage-specific mRNAs characteristic of skeletal and muscle tissue [40]. These data suggested that MSCs exist in a ground state with respect to mRNA expression, similar to that proposed for HSCs [52], and as such are poised for rapid differentiation in response to external stimuli. A similar ground state may exist for MSCs with respect to their paracrine function (Fig. 9.1). For example, MSCs constitutively secrete a number of mitogens including fibroblasts growth factor 2 (FGF-2), brain-derived neurotrophic factor (BDNF), IGF1, and HGF, angiogenins including vascular endothelial growth factor-A (VEGF-A), angiopoietin 1 (ANG1), and CRY61, as well as various cytokines and chemokines, such as GM-CSF, G-CSF, IL-6, CXCL12, and TGFβ1 that are important for bone homeostasis and hematopoiesis. However, a growing number of studies have shown that secreted levels of these proteins are altered and/or induced in MSCs following exposure to external stimuli, such as infection, inflammation, and changes in oxygen concentrations. Importantly, these conditions typify the microenvironments encountered by MSCs when transplanted ectopically to sites of tissue injury or disease. For example, MSCs express several toll-like receptors (TLRs) that allow cells to sense and respond to infectious agents [53]. TLR activation leads to secretion by MSCs of pro-inflammatory cytokines and chemokines [54], growth factors [55], and soluble mediators that regulate immune cell function [56]. MSCs also express receptors for tumor necrosis factor-alpha (TNF-α) and IL-1, which upon ligand binding induce expression of secondary mediators of inflammation,

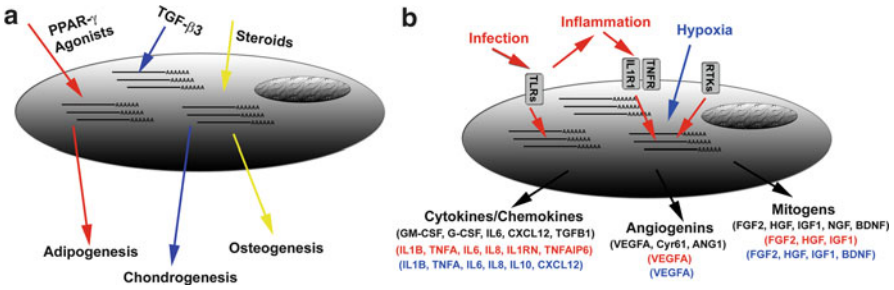

Fig. 9.1 MSCs are poised for rapid lineage specification and activation of paracrine signaling in response to external stimuli. (**a**) Genomics-based studies have shown that a single cell-derived colony of human MSCs expresses mRNAs characteristic of skeletal and muscle tissue [40], indicating that the cells are poised for rapid lineage specification in response to specific external stimuli as illustrated by the red (adipogenic), blue (chondrogenic) and yellow (osteogenic) arrows. (**b**) MSCs have also been shown to constitutively secrete a variety of cytokines/chemokines, angiogenins, and mitogens that play important roles in tissue homeostasis. Moreover, secreted levels of these proteins can be significantly altered in response to a variety of external stimuli, such as infection, inflammation, and hypoxia. Importantly, these conditions typify the microenvironments encountered by MSCs when transplanted ectopically to sites of tissue injury or disease. Therefore, these data suggest that the paracrine functions of MSCs also exist in a ground state and are poised for rapid activation in response to tissue injury and disease. Protein expression in MSCs is constitutive (*black arrows*), induced by infectious and inflammatory agents (*red arrows*) or by hypoxic conditions (*blue arrows*)

such as IL-6, and other proteins including HGF [57], monocyte chemotactic protein-1 (MCP1), cathepsin L, and several matrix metalloproteases [58]. Importantly, engagement of these receptors also leads to secretion of several potent anti-inflammatory proteins including IL-1RN [46] and TNF-α-induced protein 6 (TNF-αIP6) [59], which promote healing by limiting the extent of tissue inflammation. MSCs also induce mature dendritic cells to secrete interleukin 10 (IL-10), which has anti-inflammatory properties, and T helper 2 cells to secrete interleukin 4 (IL-4), which induces IL-10 secretion from macrophages [60]. Exposure of MSCs to interferon-gamma (IFN-γ) has also been shown to augment secretion of HGF, IL-10, TGFβ1, and indoleamine 2,3-dioxygenase, thereby enhancing their immunomodulatory effects [61] (Chap. 6).

Similarly, hypoxia has been shown to elicit a pro-angiogenic program in human MSCs by stimulating expression of VEGF, IL-6, and IL-8 and suppressing expression of TNF-α, interleukin 12 (IL-12), and tissue inhibitor of metallo-protease 1 [62]. Others have confirmed and extended these studies by demonstrating that hypoxia also induces expression of FGF2, HGF, IGF1 [55] and IL-1β, TNFα, and IL-10 [63]. Consistent with these studies, MSCs derived from heme oxygenase-1 (HO1) knockout mice secreted lower levels of CXCL12, VEGF-A, and HGF and exhibited a lower angiogenic potential in vitro [64]. Therefore, HO1 also contributes to the paracrine response of MSCs following exposure to environmental stressors.

The ability of MSCs to respond to and modulate the inflammatory response has broad implications with respect to their use in clinical medicine. For example, although inflammation plays a central role in the elimination of infectious agents and reparation of tissues following injury, unremitting inflammation also is characteristic of many disease states, such as nonhealing wounds, interstitial lung disease, arthritis, psoriasis, inflammatory bowel disease, and others. Owing to their potent anti-inflammatory effects, MSCs are being evaluated in phase I/II clinical trials for the treatment of Crohn's disease, osteoarthritis, muscle and skeletal trauma, and diabetes (www.clinicaltrials.gov). Moreover, inflammation also contributes prominently to other common maladies such as obesity and drug addiction and plays a role in allograft rejection. In the later case, MSCs may modulate regulatory T cell-dependent allograft acceptance by limiting the extent of tissue inflammation. Therefore, similar to their capacity to undergo multi-lineage differentiation, MSCs may also be primed to respond to a broad array of aberrant tissue microenvironments and restore homeostasis to these microenvironments via the secretion of paracrine-acting factors (Fig. 9.1). This capacity is exemplified by the broad therapeutic effect of MSCs demonstrated in the following disease models.

Paracrine Effects in Ischemic Diseases

Myocardial Infarction

From their onset, clinical studies have demonstrated the safety of intracoronary infusion of MSCs for the treatment of myocardial infarction and shown this yields a measurable improvement in overall left ventricular function [65] (Chap. 6). Factors implicated in contributing to the therapeutic effect of MSCs in myocardial infarction include IGF, HGF, VEGF, and FGF, and in some cases, MSCs have been genetically engineered to overexpress these factors to augment their therapeutic effect [66–69]. Importantly, the mechanism of MSC action in myocardial infarction is complex as the cells exhibit beneficial effects at various stages of disease progression. For example, MSCs exhibit anti-apoptotic activity and protect cardiomyocytes from hypoxia-induced death by downregulating expression of the pro-apoptotic protein Bax and augmenting expression of FGF, VEGF, and CXCL12 in heart tissue [70]. In addition, MSCs engineered to overexpress the murine thymoma viral oncogene homolog 1 (AKT1) were found to be superior to wild-type MSCs for cell therapy of acute myocardial infarction in a rat model [71]. Herein, genetic modification altered the repertoire of secreted paracrine factors in MSCs based on the finding that conditioned media from AKT1-modified but not wild-type MSCs exerted cardioprotective effects in vivo. A subsequent study identified soluble frizzled-related protein 2 as a key AKT1-regulated paracrine factor secreted by MSCs responsible for reparative effects and myocardial survival [72].

Similarly, overexpression in MSCs of GATA-binding protein 4 (GATA-4) has also been shown to augment secretion of IGF-1 and VEGF and enhance the cells' cardioprotective effects in vivo [73].

MSCs also promote neovascularization in infracted myocardium, which is necessary to prevent cell death, promote tissue remodeling and improve overall cardiac function [74]. For example, autologous MSC administration in a rat model of myocardial ischemia significantly increased capillary density within the ischemic heart tissue [70]. Enhanced vasculogenesis appears to be a common outcome seen following MSC administration in other ischemic diseases. For example, in animal models of limb ischemia, local delivery of MSCs augments collateral perfusion. This effect is mediated, in part, via paracrine mechanisms since antibodies against VEGF and FGF-2 partially inhibit the capacity of MSC-conditioned media to promote proliferation of endothelial and smooth muscle cells [75]. Other factors secreted by MSCs, such as HGF and IGF-1, augment aortic endothelial cell growth and survival, a response not observed with fibroblast conditioned media [76]. MSCs modified to overexpress GATA-4 or preconditioned by exposure to hypoxic conditions in vitro also exhibit enhanced anti-apoptotic and angiogenic effects on endothelial cells [73, 77]. Hypoxic preconditioning enhances expression of VEGF, IL-6, MCP1 and CXCL12 in MSCs as well as other unidentified factor(s) that activate the phosphatidylinositol 3-kinase (PI3K)-AKT pathway in endothelial cells. The latter is consistent with the fact that PI3K signaling mediates angiogenesis in vascular endothelial cells [78].

The paracrine action of MSCs in myocardial infarction is exemplified by the observation that the cells exhibit cardioprotective effects when administered not only locally (transcardial and/or intraventricular) but also intravenously. In the latter case, most MSCs accumulate rapidly in lung tissue in the first few hours after administration and then are slowly released over a few days into the circulation in low numbers. Lee et al. [59] reported that MSCs trapped in emboli within lung tissue secrete high levels of TNF-αIP6, which antagonizes the function of TNF-α and ameliorates tissue inflammation that contributes to the pathogenesis of myocardial ischemia. These data are consistent with other studies showing that MSC administration attenuates increases in CD68-postiive inflammatory cells and expression levels of MCP-1in heart tissue in a rat model of acute myocarditis [79]. Nguyen et al. [80] further showed that intracoronary injection of MSC-conditioned media reduced cardiac troponin-T levels and improved cardiac output, stroke volume, and wall motion score index in a swine model of acute myocardial infarction.

Ventricular remodeling in response to ischemic injury is typically characterized by hypertrophy and apoptosis of cardiomyocytes and tissue fibrosis. Depending upon the size of the infarction, aberrant remodeling can lead to decreased cardiac output and increased susceptibility to a second heart attack. Paracrine factors produced by MSCs limit the extent of aberrant remodeling by supporting regeneration of cardiomyocytes [81]. However, the identity of factors responsible for this effect is indeterminate. MSCs may promote regeneration of myocardium by stimulating growth and survival of cardiac progenitor cells (CPC). For example, human growth hormone and

IGF-1 are part of an autocrine loop that maintains muscle tissue integrity, but their expression declines rapidly with aging. Similarly, HGF is necessary for CPCs to migrate to areas of tissue damage and promote repair [82]. Therefore, IGF-1 and HGF secreted by MSC may promote myocardial regeneration by stimulating proliferation of CPCs resident in heart tissue.

Whether MSCs also prevent tissue fibrosis remains unclear based on the available data. We previously showed that MSCs prevent fibrosis in a mouse model of acute lung injury, but this effect was a consequence of their potent anti-inflammatory activity [46]. MSC administration has been shown to reduce fibrous tissue deposition in heart in rat models of ischemia/reperfusion injury [83] and dilated cardiac myopathy [84]. However, whether this outcome is secondary to the anti-apoptotic and angiogenic activity of the cells remains uncertain. It is anticipated that HGF secreted from MSCs has anti-fibrotic activity based on its ability to suppress expression of TGFβ-1 [85, 86]. Moreover, intramyocardial injection of IGF-1/HGF affinity bound alginate biomaterial has been shown to reduce fibrosis, attenuate infract expansion, and increase vessel formation at the site of infarct in a rat model of acute myocardial infarction [87]. Therefore, MSCs may limit the extent of tissue fibrosis via secretion of factors that antagonize TGFβ-1 activity. However, it is important to note that MSCs express an array of collagens, matrix proteins, and metalloproteases and under certain conditions can adopt a myofibrocyte phenotype [88]. Therefore, understanding how expression of these proteins is altered when MSCs encounter an ischemic or fibrotic milieu is necessary to better clarify their anti-fibrotic potential. Contradictory reports exist regarding the anti-fibrotic effects of MSCs in models of liver injury [89, 90], which may be related to differences in the timing of cell administration and extent of liver damage. MSCs have been reported to prevent renal fibrosis although this outcome may also be secondary to the anti-inflammatory effects of the cells in this model [91, 92].

In summary, evidence supporting a paracrine hypothesis of MSC action in myocardial infarction includes poor engraftment and retention of MSCs in heart tissue following transplantation, minimal capacity to transdifferentiate into cardiomyocytes in vivo, ectopic overexpression of genes that augment expression levels of secreted proteins enhances the therapeutic efficacy of MSCs in vivo, MSC-conditioned media has cardioprotective effects in vitro and in vivo, and neutralizing antibodies against secreted proteins diminishes the therapeutic effect of MSCs. Nevertheless, it is likely that the list of cardioprotective factors secreted by MSCs is incomplete. Other cardioprotective factors produced by MSCs may include insulin-like growth factor binding protein 7 (IGFBP7) [93], which stimulates prostacyclin production in cultured bovine endothelial cells [94]. Prostacyclin is both antithrombotic and a vasodilator. Therefore, IGFBP7 may play a beneficial role in myocardial infarction and peripheral vascular disease by inhibiting thrombosis and vasoconstriction. Mining of genomic databases is likely to facilitate discovery of additional paracrine-acting factors secreted by MSCs that contribute to their cardioprotective effects in vivo.

Stroke

Similar to effects in myocardial infarction, MSCs have also been shown to positively impact various stages of disease progression in stroke. Although studies defining the role of individual proteins are lacking, the prevailing data indicate that paracrine factors secreted by MSCs reduce ischemic damage [95] and apoptosis [96, 97], induce neurogenesis [98], angiogenesis, synaptogenesis [99], neurite outgrowth [100, 101], enhance neuroplasticity [102], and restore cognitive functions [103]. Proteins secreted by MSCs that have therapeutic effects in myocardial ischemia are also implicated in providing a therapeutic benefit in stroke. For example, the ability of MSCs to promote neuronal cell survival and ameliorate neurological deficits in a rat model of middle cerebral artery occlusion (MCAO) was partially attenuated when cells were transduced prior to injection with a VEGF-RNAi lentivirus [104]. Other studies have reported that IGF1 expression is upregulated in MSCs engrafted within the infarct border in the brains of rats subjected to MCAO, and endogenous levels of VEGF, epidermal growth factor (EGF), and FGF-2 are also upregulated in brain [105]. Lastly, exposure of human MSCs to extracts from ischemic brain tissue augments expression of BDNF, nerve growth factor (NGF), VEGF, and HGF [106]. Therefore, the ischemic brain microenvironment is capable of altering the paracrine activity of MSCs, similar to that seen by exposing cells to hypoxic conditions in vitro. Additionally, trophic factors produced by MSCs engrafted within the ischemic brain are anticipated to modulate the production and expression levels of autocrine and paracrine factors produced by the brain parenchyma [97, 107–110]. This feed forward affect may account for the potent therapeutic effects of MSCs in vivo.

Genetic modification of MSCs has also been used to enhance their therapeutic effects in experimental stroke models. For example, MCAO rats administered human MSCs engineered to overexpress ANG1 and VEGF showed enhanced structural and functional recovery as compared to untreated rats and those administered wild-type MSCs [111]. Placental growth factor gene-modified MSCs also elicited greater angiogenesis and a larger reduction in lesion volume compared to native MSCs [112]. Moreover, BDNF and glial cell line-derived neurotrophic factor (GDNF) but not ciliary neurotrophic factor (CNTF) or neurotrophin 3 (NT-3) gene-modified MSCs are reported to exhibit enhanced capacities to promote functional recovery and reduce infarct size in MCAO rats [95, 113, 114]. In addition to trophic factors, MSCs also secrete extracellular matrix proteins that support the growth of neurons, astrocytes, and oligodendrocytes in vitro by increasing their metabolic rate and protecting cells from nutrient and growth factor deprivation [115]. However, whether these proteins are secreted from cells engrafted within brain tissue has yet to be examined.

MSCs may also improve functional recovery after stroke by modulating cytokine expression in the brain. For example, MSC administration increases brain expression levels of IL10, which has anti-inflammatory and neuro-protective activity [97, 116] and decreases levels of the pro-inflammatory cytokine TNFα [116]. MSCs

also increase bone morphogenetic protein 2/4 expression in ischemic astrocytes, which enhances subventricular progenitor cell gliogenesis by activating relevant signaling pathways [107]. The cells also increase tissue plasminogen activator activity and downregulate plasminogen activator inhibitor 1 activity within the ischemic boundary of MCAO mice and in astrocytes cultured in vitro. These changes resulted in enhanced neurite outgrowth form cortical neurons [101]. Importantly, behavioral recovery and neurogenesis in a rat stroke model was more pronounced when animals were administered early versus late passage MSCs. Moreover, endogenous levels of trophic factors, such as GDNF, NGF, VEGF, and HGF, were higher in early passage MSC-treated brains [117]. These findings illustrate that culture expansion and/or methods of isolation may significantly impact the paracrine functions of MSCs.

Paracrine Effects in Lung Disease

Pulmonary Fibrosis

Our lab was the first to show that MSC administration ameliorated inflammation and fibrosis in a mouse model of bleomycin-induced lung injury [37]. A critical result from these studies was the demonstration that MSC administration at the time of bleomycin exposure but not 1 week later significantly reduced the extent of neutrophil infiltration into lung tissue and upregulation of pro-inflammatory cytokine expression. Based on this result, it was apparent that the principal effect of MSCs was anti-inflammatory in nature and this was subsequently linked to secretion of high levels of IL-1RN [46]. Other studies have shown that MSC administration suppresses bleomycin-induced increases in TGFβ1, IGF-1, and PDGF in lung tissue and laminin and hyaluronan expression in bronco-alveolar lavage (BAL) in rats [118]. Similarly, MSCs derived from umbilical cord blood attenuate expression of TGFβ1, IL-10, IFN-γ, and macrophage migration inhibitory factor in mice exposed to bleomycin [119]. In related studies, coculture of polarized human alveolar epithelial type II cells after inflammatory insult with MSCs was shown to preserve their protein permeability. ANG1 secretion was responsible for this beneficial effect in part by preventing actin stress fiber formation and claudin 18 disorganization via suppression of nuclear factor kappa-B activity [120].

In lung as in heart, it remains unclear whether MSCs actually exhibit anti-fibrotic effects or if their capacity to block fibrosis is merely a consequence of their potent anti-inflammatory properties. Salazar et al. [121] recently showed that MSCs from mouse bone marrow and human umbilical cord blood secrete high levels of PDGF-AA and TGFβ1 and stimulated growth and collagen production of lung fibroblasts. Interestingly, antagonism of TGFβ1 reduced collagen expression in lung fibroblasts, but their growth was inhibited by the Wnt antagonist sFRP1. These data suggest that MSCs also secrete Wnt ligands that stimulate

fibroblast proliferation. Lee et al. [88] also recently reported that treatment of MSCs with connective tissue growth factor upregulates collagen type 1 and tenacin-C expression as well as collagen type III, fibronectin, and matrix metalloprotease type I. Further exposure to TGFβ1 induced the cells to adopt a myofibroblast phenotype and undergo fibrogenesis instead of ectopic mineralization in vivo. These studies suggest that MSCs may be pro-fibrotic under specific conditions.

Endotoxin-Induced Lung Injury

MSCs from mouse bone marrow can also significantly reduce lung inflammation, edema and decrease levels of IFN-γ, IL-1B, IL-6, macrophage inflammatory protein 1-alpha, and IL-8 in peripheral blood in a mouse model of endotoxin-induced acute lung injury [122]. Human MSCs produce a similar effect that is paracrine in nature based on their capacity to downregulate expression of TNF-α by macrophages stimulated with LPS in vitro [123]. In related studies, Lee et al. [124] showed that human MSCs also restored alveolar epithelial fluid transport and lung fluid balance in an *ex vivo* perfused human lung preparation injured by E. coli endotoxin. Importantly, conditioned media from MSCs yielded a similar outcome and knockdown studies demonstrated that keratinocyte growth factor (KGF) secreted from MSCs contributed to their therapeutic effect in this model.

Asthma

In a ragweed-induced mouse asthma model, Nemeth et al. [125] demonstrated that MSCs administered i.v. at the time of antigen challenge significantly reduced the extent of eosinophil infiltration and mucus production in the lung; decreased expressed levels of IL-4, IL-5, and interleukin 13 (IL-13) in BAL; and also lowered serum levels of IgG1 and IgE. In this study, allogeneic and autologous MSCs exhibited similar therapeutic effects, while skin fibroblasts reduced the total number of cells in BAL but not the number of eosinophils compared to untreated mice. Skin fibroblasts also significantly reduced circulating levels of IL-13 but not IL-4. Treatment of mice with MSCs from TGFβ1 knockout mice failed to have a therapeutic effect, consistent with in vitro studies showing that IL4 within BAL from ragweed-challenged mice induced TGFB1 expression in MSCs. Similar results were obtained by Bonefield et al. [126] in an ovalbumin model of asthma. In the latter study, MSCs also reduced systemic levels of IL-1β, suppressed inducible nitric oxide synthase expression from lung infiltrating monocytes, and enhanced IFN-γ levels in BAL fluid.

Paracrine Effects in Wound Healing and Diabetes

Wound Healing

MSCs constitutively express a variety of growth factors important for wound healing including PDGF, EGF, TGFβ1, VEGF, KGF, FGF2, and HGF [127]. Moreover, expression of PDGF, EGF, KGF, and HGF is significantly elevated when MSCs are exposed to LPS or IL-1B. Similarly, treatment of MSCs with superfusates from wounded abdominal tissue upregulated expression of TGFB1, EGF, and VEGF. Human MSCs and adipose-derived cells also secrete appreciable levels of IGF1, VEGF, and HGF, the latter two of which were upregulated by exposure to TNF-α [128]. Therefore, exposure to the wound microenvironment appears to induce in MSCs expression of various angiogenins and mitogens that normally participate in the wound healing process. MSCs have also been reported to secrete significantly higher levels of ANG1, KGF, IGF-1, PDGF, and erythropoietin as compared to dermal fibroblasts [129, 130], which may explain their superior healing capacity [130]. They also secrete high levels of cysteine-rich angiogenic inducer 61 (CYR61), and depletion of this protein from MSC-conditioned media abolished their angiogenic activity [51]. This is consistent with studies showing that MSC-conditioned media also stimulates wound healing [129]. Therefore, paracrine signaling via release of angiogenins (VEGF, EPO, CYR61), growth factors (EGF, FGF2, IGF-1, KGF, PDGF, TGFβ1), and other soluble factors promote angiogenesis, keratinocyte proliferation, and migration and may also modulate the activity of inflammatory cells. MSCs also secrete a large array of extracellular matrix molecules including collagens, fibronectin, and various matrix metalloproteinases and as such may directly contribute to tissue repair by functioning akin to fibroblasts.

Diabetes

MSCs have been shown to normalize blood glucose levels when administered to streptozotocin-induced hyperglycemic mice [131, 132]. In these studies, MSCs were shown to increase the number of pancreatic islets and beta cells producing mouse insulin and also prevent renal damage. Consistent with these results, other studies have shown that islets from MSC-treated animals expressed high levels of pancreas/duodenum homeobox protein 1 and insulin and that peripheral T cells from these animals exhibited a shift toward IL10/IL13 production [133]. Coculture of human pancreatic islets with MSCs also improves their adenosine-5′-triphosphate/adenosine-5′-diphosphate ratio and insulin secretory function in vitro. MSC-conditioned media was shown to contain high levels of IL-6, VEGF-A, HGF, and TGFβ1, factors known to improve the survival, function, and angiogenesis/revascularization of islets [134]. Consistent with this result, Xu et al. [135] reported that exposure of MSCs to rat pancreatic extracts significantly upregulated secretion of

IGF-1, VEGF, and FGF2 and conditioned media from pancreatic extract-treated MSCs was able to lower blood glucose levels when administered to diabetic rats. Finally, MSCs also reportedly restore normoglycemia in diabetic rats by promoting vascularization and enhancing survival of islet grafts via secretion of VEGF [136].

Paracrine Effects in Neurodegenerative Diseases

The anti-inflammatory and immunomodulatory affects of MSCs may be advantageous in the treatment of various neurodegenerative diseases since inflammation is thought to contribute significantly to their pathogenesis. For example, elevated levels of pro-inflammatory cytokines in brain tissue are detected in mouse models of lysosomal storage diseases, and the degree of inflammation has been shown to coincide with the onset of clinical symptoms in these models [137–139]. In most cases, microglia activation occurs in response to aberrant neural cell function or as part of a wider stress response in the brain and typically precedes neuronal cell loss. Inflammation is also a prominent feature in other neurodegenerative disorders such as Alzheimer's and Parkinson's disease [140].

With respect to animal models of storage disease, injection of unmodified MSCs into the cerebellum markedly reduces the extent of microglial and astrocyte activation and reduces levels of macrophage colony-stimulating factor, a microglial activator, in a mouse model of Niemann-Pick type C disease [141]. Similar results were also obtained with adipose tissue-derived stem cells [142]. In the latter studies, cell transplantation directly to the cerebellum resulted in rescue of Purkinje neurons as evidenced by their enhanced electrical activity and suppression of neuroinflammation based on decreased glial cell activation and decreased expression levels of IL-1B, IL-6, and TNF-α protein in the cerebellum.

MSCs administration also delays disease progression, improves motor performance, and decreases microglial activation and astrogliosis in the spinal cords of mice carrying a glycine 93 to alanine (G93A) mutation in the superoxide dismutase 1 gene (SOD1), a model of amyotrophic lateral sclerosis (ALS) [143, 144]. The therapeutic effect of MSCs in this model is also likely paracrine in nature based on studies showing that exposure of MSCs to extracts from the brains or spinal cords of SOD1 mutant transgenic rats significantly upregulated expression of VEGF-A, HGF, and NGF and suppressed expression of FGF2 and IGF1. Moreover, spinal cord but not brain extracts induced expression of BDNF and GDNF [145]. Other studies have shown that exposure to G5 supplement, FGF2, and CNTF induces mRNA and protein expression of the glutamate transporter 1 (GLT-1) in MSCs, which results in an enhanced ability of the cells to uptake aspartate. This result suggests that MSCs may be neuro-protective by restoring glutamate homeostasis in response to disease [146]. Importantly, MSCs from SOD1(G93A) mutant rats showed reduced aspartate uptake despite expressing higher level of GLT1 mRNA and protein. These results indicated that MSCs from mutant rats expressed a non-functional GLT1 receptor and therefore were unable to protect neurons from

glutamate toxicity. This finding questions the suitability of autologous stem cell grafts for treatment of familial forms of ALS. Similarly, Cho et al. [147] reported that secreted levels of several trophic factors including FGF2, HGF, IGF-1, CXCL12, and VEGF-A are decreased in MSCs derived from the bone marrow of human ALS patients. Nevertheless, direct administration of MSCs from human ALS patients into the cistern magna of mice engineered to overexpress the human mutant SOD1(G93A) gene resulted in a dose-dependent increase in life span and survival of motor neurons in the ventral horn of the spinal cord [148]. Therefore, patient-specific MSCs may still provide some degree of therapeutic benefit when administered in vivo, despite their reduced paracrine activity.

Direct administration of MSCs into the cerebellum of newborn Lurcher mutant mice, a model characterized by the selective early postnatal death of Purkinje cells, resulted in significant improvement in motor function as evidenced by improvement in the rotarod test. At 2-month posttransplant, histological analysis demonstrated a significant increase in Purkinje cell numbers in treated versus control mice and revealed that many of the surviving MSCs in brain were juxtaposed to the Purkinje cell layer in the cerebellum. This outcome was attributed to secretion by MSCs of BDNF, NT-3, and GDNF, neurotrophins important for Purkinje cell survival [149]. MSCs also decreased the extent of glial activation, oxidative stress, and apoptosis within the hippocampus and improved memory function and learning in a mouse model of acute Aβ-induced Alzheimer's disease [150].

Collectively, these studies indicate that paracrine mechanisms also contribute significantly to the therapeutic effect of MSCs in a variety of neurodegenerative diseases. This is consistent with genomics-based studies showing that MSCs secrete various neurotrophins as well as other factors that promote neural cell survival and neurite outgrowth under stressful conditions and following injury in vivo [47, 151, 152]. This capacity of MSCs is likely related to the fact that bone and marrow are innervated by nervous tissue, providing a means by which sympathetic efferent input can modulate hematopoiesis. Despite the benefits afforded by MSCs in the aforementioned disease models, it should be noted that one study has shown that MSC-conditioned medium promotes glial cell activation and upregulates expression of TNF-α and IL-6 in organotypic cultures of the hippocampus, leading to neuronal cell death [153]. These results should be carefully assessed as they suggest that MSC-based therapies may yield adverse or unforeseen outcomes that may exacerbate the disease state. The latter necessitates incorporating dose response studies into translational and clinical trials and metrics to measure potential adverse side effects.

Conclusions

Only a decade ago, MSCs were thought to function specifically as a reservoir of progenitor cells to maintain homeostasis and facilitate repair of connective tissues following injury. While exploring their potential use in tissue engineering remains an avid area of research, the field in general has witnessed a major paradigm

shift with respect to the function and potential clinical applications of MSCs. Specifically, it is now believed that the principal mechanism by which MSCs affect tissue repair is by paracrine signaling. This paradigm shift has gained support from genomics and proteomics-based studies, which revealed that MSCs secrete an array of proteins that exhibit angiogenic, anti-inflammatory, immunomodulatory, and trophic activity, and studies demonstrating that MSCs or MSC-conditioned media ameliorate disease and promote tissue repair in a broad array of experimental animal models of disease and human clinical trials. Moreover, evidence is mounting that MSCs express a large repertoire of surface receptors that coordinately regulate paracrine function in response to changing environmental conditions, such as hypoxia, inflammation, and infection. Therefore, it appears the pendulum has swung full circle. In the 1970s, cytokine secretion by MSCs was exploited to establish long-term bone marrow cultures, which led to the identification of the HSC. Most MSC-based therapies currently being pursued today also exploit the paracrine activity of MSCs. Despite these advances, aspects of MSC biology remain indeterminate including their origin during development, the molecular mechanisms that regulate self-renewal and lineage specification, and how paracrine functions are specified within populations. Efforts aimed at addressing these questions will likely lead to the procurement of even more potent cell-based vectors for clinical therapies. Therefore, MSC research likely will remain an exciting and rewarding enterprise for some time to come.

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Chapter 10
Cross-Talk Between MSCs and Their Environments

Thomas P. Lozito and Rocky S. Tuan

Abstract The mesenchymal stromal/stem cell (MSC) has garnered attention as a promising candidate cell type for cell-based therapeutics, partly, by virtue of its ability to differentiate into a variety of cell types. However, the true therapeutic potential of MSCs may lie in the regulatory influences they exert on their environments. Indeed, as a result of their natural homing response to wound sites, MSCs come into contact with a variety of environments and cell types as they leave their perivascular niches. This chapter describes the interactions between MSCs and four such environmental signals, specifically the vasculature, the extracellular matrix, the immune system, and cancer. In vivo and in vitro studies detailing the effects of MSCs on each are presented, with special attention paid to cases of cross-talk in which MSCs alter the very environmental signals acting upon them. Finally, MSC performance in clinical trials is discussed and compared to expectations based on basic science findings. This chapter also identifies gaps in knowledge and current understandings where future research will prove most effective.

Introduction

Adult mesenchymal stromal/stem cells (MSCs) were first discovered in bone marrow and described as mononuclear cells that culture ex vivo as adherent colony-forming unit fibroblasts (CFU-F) [1–3]. In the decades since, MSCs have been

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_10,

identified from mesoderm-, endoderm-, and ectoderm-derived tissues, including mesodermal (trabecular bone [4], synovium [5, 6], cartilage [7], fat [8, 9], muscle [10, 11], blood vessels, and tonsil [12]), endodermal (e.g., thymus [13]), ectodermal (e.g., skin [14], hair follicle [15], dura mater [16], and dental pulp [17]), and prenatal and perinatal tissues (umbilical cord [18], umbilical cord blood [19], and placenta [20]). Because they have been isolated from a wide range of tissues, MSCs are known by many different names in addition to the original "mesenchymal stem cells" coined by Arnold Caplan [21], including mesenchymal stromal cells [22], bone marrow stromal cells [23], marrow-isolated adult multipotent inducible cells [24], and multipotent adult progenitor cells [25]. MSCs have traditionally been thought of in terms of their multi-lineage differentiation potential, including osteogenesis, chondrogenesis, and adipogenesis [26]. Since their initial description, however, the inherent cell biology of MSCs has come into focus due to their emerging roles in a variety of physiological and pathological processes, and these will be the focus of this chapter.

Interactions Between MSCs and the Vasculature

There is strong evidence to suggest that MSCs occupy a perivascular niche in a variety of vascularized tissues, affording them a prime location for regulating vascular events such as angiogenesis [27–31]. Furthermore, numerous similarities have been described between MSCs and pericytes, a microvascular cell type analogous to the smooth muscle cells (SMCs) of macrovessels [31, 32]. For example, MSCs express pericyte markers and vice versa; cultured bovine pericytes are positive for STRO-1, an MSC marker [27], and MSCs from the bone marrow express the pericyte markers CD106 (vascular cell adhesion molecule-1(VCAM-1)), CD146 (melanoma cell adhesion molecule), and smooth muscle α-actin [27, 28]. Dental pulp MSCs express the pericyte marker 3G5 [27], and murine MSCs are positive for two perivascular markers, SAB-1 and SAB-2 [27]. Also, like pericytes, MSCs enhance vessel formation and stabilization through paracrine interactions [29, 30], and both cell types display similar differentiation capabilities [31–34].

However, interactions between MSCs and endothelial cells (ECs), the primary cell type of the vasculature, have implications beyond basic biology. Due to their natural abilities to home to wound sites, suppress inflammation, and support local cells and tissue healing, MSCs show a great promise for inclusion in cell-based therapies. Since the majority of these therapies involve intravenous (IV) or intra-arterial (IA) injection of MSCs into patients, the need to understand the interactions between MSCs and the vasculature becomes apparent. Indeed, current studies suggest that, while the therapeutic potential of MSCs to positively benefit the wound environment is strong, difficulties arise in physically delivering IV- or IA-delivered MSCs to the sites of injury. These difficulties are due to the fact that the vast majority of IV-injected MSCs embolize in the capillaries of the lungs [35, 36]. This passive arrestment of MSCs appears to be due to the large size of MSCs relative to the

small diameter of microvessels [35, 36]. Many of the trapped MSCs die, while a small number first spread out on the lumenal sides of microvessels before extravasating to the perivascular niche [35]. However, substantial evidence exists that not all exogenous MSCs embolize at the precapillary level and that some actively home to sites of injury. For example, MSC homing to organs other than the lungs increased significantly in a mouse injury model, suggesting that MSCs exhibit higher engraftment efficiencies within sites of inflammation or injury [37]. These results also suggest that MSC engraftment to damaged tissues is an active process, while the presence of MSCs in the lungs is due to passive entrapment. These in vivo findings are linked to in vitro studies in which MSCs demonstrate increased adhesiveness for damaged ECs treated with proinflammatory cytokines and proapoptotic agents [38]. However, the most convincing evidence that MSCs actively home to injured tissues comes from studies employing receptor blocking/knockout methodologies. For example, MSC interactions with ECs under shear flow were shown to be dependent upon EC-expressed P-selectin and VCAM-1 and MSC very late antigen-4 (VLA-4) [39, 40]. Prestimulating either MSCs or ECs with proinflammatory cytokines enhanced these interactions. On the other hand, blocking integrin β1 specifically was shown to interfere with MSC myocardial engraftment [41]. Such studies provide information on the identity and mechanisms of action of the receptors involved in MSC homing to various organs and tissues. A summary of these interactions is provided in Table 10.1.

Interactions Between MSCs and the Extracellular Matrix

Tightly wrapped around the vessels, pericytic MSCs also interact with another critical regulator of the vascular environment, the vascular basement membrane (VBM). The VBM is a specialized extracellular matrix (ECM) that surrounds the blood vessels of the body and is regulated through a control system involving proteases, which alter and degrade the matrix, and protease inhibitors, which maintain and protect the VBM from disruption [51]. This interplay between proteases and protease inhibitors and its effects on the VBM profoundly influences vessel stability and, hence, many physiological and pathological processes. For example, disruption of the VBM is an early step in angiogenesis [51–57]. During tumor growth and metastasis, cancer cells secrete proteases that degrade the VBM, allowing new blood vessels to sprout and nourish the growing tumor [51, 55, 58]. These extrinsic factors potentially tip the balance between proteases and protease inhibitors toward vascular disruption. As residents of the perivascular niche, MSCs are in a prime location to alter their local environment by affecting this balance.

As stem cells multipotent for lineages of the musculoskeletal system, MSCs are profoundly influenced by signals originating from their local environments, particularly when it comes to differentiation. Effects on MSC differentiation are often tissue dependent [59]. There is evidence to suggest that this tissue-instructive differentiation is actually supported by the tissue-specific composition of the

Table 10.1 Interactions between MSCs and the vasculature

Effects of MSC-produced factors on vasculature			
Vascular cell type	Factor	Effect	References
ECs	Cysteine-rich protein 61 (Cyr61)	Induces EC chord formation on Matrigel in vitro, induces Matrigel plug neovascularization in athymic mice in vivo	[42]
Apoptotic ECs	Unknown	ECs treated with proinflammatory cytokines and proapoptotic agents exhibited increases adhesion for MSCs in vitro	[38]
ECs	Unknown	MSCs enhance and stabilize EC tubes on HFF feeder layers and on Matrigel in vitro	[43]
ECs	MMPs	Enhance tube formation through high-density fibrin gels in vitro	[44]
ECs	P-selectin, VCAM-1/VLA-4	Mediate rolling and adhesion between MSCs and ECs under shear flow. Adhesion increased when ECs were prestimulated with TNF-α	[39]
ECs	VCAM-1 (NOT ICAM-1)	MSCs injected intraventrically adhered to ECs. Pre-activation of MSCs with TNF-α enhanced cardiac homing in a VCAM-dependent process	[40]
Heart	Unknown	MSCs promote wound repair and regeneration in damaged hearts	[45–50]

extracellular matrix [60–68]. Indeed, interactions with various matrix molecules, including those derived from ECs, modulate MSC behavior and differentiation [61–76]. As part of the perivascular niche, MSCs are subjected to various signals originating from the vascular environment and the VBM. For example, proteolytic degradation alters the biological activity of a variety of these matrix molecules by revealing cryptic domains [77–81], releasing bioactive fragments [51, 72–74, 79, 82–92], and liberating stores of matrix-bound and matrix-regulated growth factors [51, 83, 89, 93–102]. Interestingly, MSCs secrete a variety of molecules that regulate matrix remodeling [29, 52, 55, 56, 67, 68, 103–105].

A specific class of extracellular matrix-degrading metalloenzymes, the matrix metalloproteinases (MMPs), and their endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMP), are specifically linked with VBM remodeling [106]. Of the approximately 26 currently recognized MMPs, several are of particular relevance to the perivascular environment [106]. For example, MMP-2 and MMP-9 are unique among MMPs in that they contain type II fibronectin domains, allowing them to bind gelatin, collagens, and laminin [107]. This allows MMP-2 and MMP-9 to bind intact matrix,

where they degrade gelatin as well as laminins and collagen type IV, the main matrix components of the VBM [56]. Furthermore, membrane type 1-MMP (MT1-MMP), working pericellularly, degrades a wide range of matrix molecules, including those of the VBM [107]. The TIMPs are the main MMP inhibitors, binding 1:1 stoichiometrically with the MMP active-site cleft [107]. Four TIMPS (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) have been identified [107], and each TIMP is composed of distinct N- and C-terminal domains [108]. The N-terminal domains take part in the inhibitory actions, while the C-terminal domains mediate non-inhibitory complexes with MMPs. While the TIMPs as a group are largely specific in their inhibition for MMPs over other proteases, each of the four TIMPs exhibit important differences among their binding properties for specific MMPs. For example, the N-terminal domains of TIMP-2, TIMP-3, and TIMP-4, but not TIMP-1, are potent inhibitors of MT1-MMP [108, 109]. Furthermore, the C-terminal domains of TIMP-2 and TIMP-4 bind the hemopexin domain of MMP-2, while TIMP-1 and TIMP-3 do not [54, 108, 109]. All MMPs are initially expressed as inactive zymogens and require proteolytic removal of N-terminal inhibitory pro-peptides for activation [56]. For example, proMMP-2 activation occurs at the cell surface in a process that requires TIMP-2 and active MT1-MMP [110]. The N-terminal domain of TIMP-2 binds to the active cleft of MT1-MMP on the surface of the cell, while the C-terminal domain binds to the proMMP-2 hemopexin domain [54]. ProMMP-2 is then activated though proteolytic processing by other, noncomplexed MT1-MMP. Only TIMP-2 is able to mediate the ternary complex proMMP-2/TIMP-2/MT1-MMP. TIMP-4, which binds the hemopexin domain of proMMP-2 and potently inhibits MT1-MMP but does not support the ternary complex, competes with TIMP-2 for proMMP-2 and MT1-MMP binding.

MSCs secrete high levels of TIMPs that stabilize vessels and protect the VBM from MMP-induced degradation [105]. MSC secretion of TIMPs and the consequent vessel-protective properties of MSCs were sustained even under simulated disease conditions. This last feature was not exhibited by ECs, suggesting that MSCs, acting as robust sources of TIMP-1 and TIMP-2, are an important protective element of the perivascular niche from protease-mediated degradation.

Interactions Between MSCs and Immune System

Perhaps one of the most significant discoveries involving MSCs concerns their abilities to suppress the immune system. The first such findings concerned the ability of MSCs to suppress T cell proliferation [111, 112]. While the exact mechanisms remain only partially known, cell-cell contact and soluble factors are thought to support various levels of MSC suppression of T cells. For example, cell-cell signaling involving programmed death-1 (PD-1) has been found to mediate contact-driven MSC/T cell interactions [113], while other studies have traced MSC immunosuppressive abilities to MSC-secreted factors, including transforming growth factor-β1 (TGF-β1), hepatocyte growth factor (HGF), soluble isoform of histocompatibility antigen, class I, G (HLA-G5), and indoleamine-pyrrole 2,3-dioxygenase (IDO) [112, 114–117]. Still

other studies have focused on the involvement of proteases such as MMP-2 and MMP-9, which cleave interleukin-2 receptors on the surface of T cells, in MSC modulation of T cell biology [118]. Importantly, the effects of MSCs on T cell proliferation do not appear to involve apoptosis, instead, MSCs promote T cell survival in a quiescent state [119]. The effects of MSCs on other types of T cells have also been investigated. For example, MSCs were found to decrease interferon-gamma (IFN-γ) production in type 1 helper T cells (T_H1 cells) and increase interleukin-4 (IL-4) secretion in type 2 helper T cells (T_H2 cells), indicating a shift from a pro- to an anti-inflammatory state [120–122]. MSCs have also been shown to downregulate cell killing of cytotoxic T lymphocytes (CTLs) and to induce expansion of regulatory T cells (T_{Reg} cells), both of which act to suppress immune system activity [114, 117, 123].

Whatever the mechanism, the influence of MSCs on the immune system is not restricted to T cells. Acting as links between the innate and adaptive immune systems, dendritic cells represent an important target of MSC modulation. MSCs have been shown to inhibit myeloid dendritic cell (DC) differentiation and impair the critical antigen-presenting functions of DCs [121, 124–129]. MSCs also increase IL-10 secretion by plasmacytoid dendritic cells (pDCs), which ultimately promotes T_{Reg} cell proliferation and immune system suppression [121, 128].

Interactions between MSCs and natural killer (NK) cells is complicated by the findings that NK cells effectively lyse MSCs [130]. On the other hand, MSCs decrease NK cell cytokine secretion and interfere with the ability of NK cells to kill other cells. The susceptibility of MSCs to NK cell-mediated cytotoxicity is dependent upon the naturally low levels of major histocompatibility complex (MHC) class I expression in MSCs, and treatment with factors, such as IFN-γ, that increase expression of MHC class I work to partially protect MSCs from NK cell-targeted killing. The relationship between MSCs and B cells is also difficult to interpret due to conflicting reports on the effects of their interactions. Most studies have found that MSCs, either through soluble factor or cell-cell contact, inhibit B cell proliferation and antibody production [113, 131, 132], while others have demonstrated MSC support of B cell survival, proliferation, and differentiation. In the end, however, the interactions between MSCs and B cells may be secondary to the primary roles T cells play in the regulation of B cell activity. Indeed, several in vivo studies have detected reduced levels of antibodies and T cell activity, indicating that MSCs may modulate B cell antibody production in vivo via reduced proliferation of T cells [133].

Perhaps one of the most interesting aspects of MSC interactions with the immune system is the high degree of back-and-forth cross-talk between them and other cells; often stimulation by immune cells is involved in activating MSC modulation of the same or different cells of the immune system. For example, IFN-γ released by immune cells triggers MSCs to release nitric oxide (NO) and IDO, which in turn inhibit immune cell activity and proliferation [122, 134, 135]. Similarly, IFN-γ and other cytokines stimulate MSC production of T cell-attracting chemokines and inducible nitric oxide synthase (iNOS), which inhibits T cell activation via NO [120, 122, 134, 136, 137].

These interactions between MSCs and the immune system are summarized in Table 10.2.

Table 10.2 Interactions between MSCs and the immune system

Effects of MSC-produced factors on immune system			
Target cell type	Factor	Effect	References
T cells	Cell-cell contact: PD-1	Suppress T cell proliferation	[113]
T cells	Secreted factors: TGF-β1, HGF, HLA-G5, IDO	Suppress T cell proliferation	[112, 114–117]
T cells	Secreted proteases: MMP-2, MMP-9	Reduced T cell proliferation and expression of CD25 in vitro; reduced hypersensitivity responses to allogenic antigens and prolonged survival of allogenic islet grafts in vivo	[118]
T cells	unknown	Promote T cell survival in a quiescent state, decrease IFN-γ production in vitro and in vivo	[119]
T cells	iNOS and NO	Inhibit T cell activation	[120]
T_H1 cells	IDO?, PGE_2?	Decrease IFN-γ production	[121, 122]
T_H2 cells	PGE_2?	Increase IL-4 secretion	[121]
$CD^4CD25^{high}FOXP3^+$ T_{reg} cells	HLA-G5	Induce expansion	[114]
CTL	HLA-G5?	Downregulate CTL-mediated cytotoxicity	[117, 123]
DCs	IL-6?, M-CSF?, TGF-β1?, Notch?, others?	Inhibit progenitor cell differentiation into DCs, impair antigen-presenting function of DCs	[121, 124–129]
DCs	PGE_2?	Inhibit production of TNF-α, induce production of IL-10	[121]
pDCs		Induce pDC production of IL-10, which goes on to promote T_{Reg} Cell expansion	[121, 128]
NK cells	IDO, PGE_2, HLA-G5, cell-cell contact?	Decrease IFN-γ secretion, inhibit proliferation in resting NK cells, inhibit NK cell cytotoxic activity by downregulating receptors involved in NK cell activation	[121] [114, 130, 135, 138]

(continued)

Table 10.2 (continued)

Effects of MSC-produced factors on immune system			
Target cell type	Factor	Effect	References
Neutrophils	IL-6	Delay apoptosis	[139]
B cells	soluble factors?, cell-cell contact?: PD-1?	Inhibit proliferation in vitro	[113, 131, 132]
B cells	unknown	Support B cell survival, proliferation, and differentiation to antibody-secreting cells	[140, 141]

Effects of immune system-produced factors on MSCs			
Source cell type	Factor	Effect	References
Immune cells/wound environment	TNF-α, LPS, hypoxia	Increased MSC production of VEGF, FGF2, HGF, and IGF	[142]
NK cells	NKp30, NKG2D, DNAM-1	Lyse autologous and allogeneic MSCs	[130]
Various immune Cells	IFN-γ	Low levels of IFN-γ induce MHC class II expression in MSCs, allowing them to act as APCs	[136, 137]
Immune cells	IFN-γ	Trigger release of IDO and NO in MSCs	[122, 134]
Immune cells	IFN-γ, TNF, IL-1α, IL-1β	Increase MSC production of T cell-attracting chemokines and iNOS	[120]
Immune cells	TNF, IFN-γ	Increase MSC production of PGE_2	[121]
T cells	Cell-cell contact + IL-10	Stimulate HLA-G secretion by MSCs	[114]

Table 10.3 Interactions between MSCs and cancer cells

Effects of MSC-produced factors on cancer			
Cancer type	Factor	Effect	References
Breast cancer cells	CCL5 (RANTES)	Increase motility, invasion, and metastasis	[143]
Renca adenocarcinoma or the B16 melanoma cell lines	unknown	Low numbers of MSCs induced tumor rejection; higher numbers enhanced tumor progression	[144]
Kaposi's sarcoma	Cell-cell contact? (E-cadherin/Akt?)	MSCs inhibit tumor growth and AKT activation	[145]
Adenocarcinoma	IL-6	Promote tumor growth	[146]

Effects of cancer-produced factors on MSCs			
Cancer type	Factor	Effect	References
U87 and LN229 glioma cells	PDGF-BB	Mediates MSC tropism for gliomas	[147]
Breast cancer cells	MCP-1	Responsible for MSC homing to tumors	[148]
Ovarian tumors	LL-37	Recruit MSCs to tumors and induce MSC secretion of proangiogenic factors	[149]
Adenocarcinomas	unknown	Convert MSCs to TAFs	[146]

Interactions Between MSCs and Cancer

The topic of MSCs and cancer offers a good review of the various facets of MSC environmental interactions due to the wide range of physiologic and pathologic processes that underlies cancer progression (see Table [10.3] for a summary of these interactions). MSCs naturally home to sites of injury as part of the body's natural wound healing response through their interactions with immune cells and the vasculature [31, 150]. These cellular activities are hijacked by cancer cells, which create local environments that share many similarities with chronic, unresolved wounds. The abilities of MSCs to leave their perivascular niche and migrate toward tumors and sites of injury and metastasis have been well established [148, 150–155]. Even exogenous MSCs injected into the circulation of animals with breast cancer tumors exhibit highly specific migration to the tumor microenvironment [143]. This strong chemotactic response has been attributed to tumor-produced and tumor-induced inflammatory cytokines, such as platelet-derived growth factor (PDGF-BB),

monocyte chemotactic protein-1 (MCP-1), and the N-terminal peptide of human cationic antimicrobial protein 18 (LL-37) [147–150]. Upon integrating with the tumor microenvironment, MSCs modulate tumor growth and metastasis, but the precise mechanisms remain unclear [156]. Most studies conclude that MSCs are overall pro-tumorigenic and promote cancer metastasis, but again the specifics remain unresolved. For example, the MSC-secreted chemokine (C-C motif) ligand 5 (CCL5) is reported to directly increase cancer cell motility, invasion, and metastasis [143], while other studies suggest MSCs play more indirect tumor-supporting roles by suppressing the immune system and promoting angiogenesis [144]. However, like the other areas of study concerning MSC environmental interactions, there exists a large degree of controversy. For example, several studies suggested that MSCs inhibit tumor growth through direct cell-cell contact [145], while others found a biphasic response of MSCs on tumor progression, wherein MSCs either promoted or inhibited tumor development depending on the number of cells involved in the experiment and independent of direct contact between MSCs and tumor cells [144]. In another similarity to the trends seen in the other avenues of MSC interactions, there also appears to be a great deal of back-and-forth cross-talk between MSCs and tumors. For example, exposure to cancer-secreted factors is reported to convert MSCs to tumor-associated fibroblasts (TAFs). These TAFs act to promote tumor progression through secretion of IL-6 [146]. Similarly, tumors produce LL-37, which recruits MSCs and induces their expression of pro-tumor and proangiogenic factors, including interleukin (IL)-6, IL-10, CCL5, vascular endothelial growth factor (VEGF), and MMP-2 [149]

Filling in the Gaps: Areas for Potential Future Work

The vast majority of interactions between MSCs and their microenvironment remain largely unstudied and poorly characterized. Among these areas of future study, several connections have been outlined in separate studies, and future work needs only connect the dots. In the most common examples, a group of studies describe MSC production of a particular factor, while a distinct pool of findings describes the response to the same factor in some other cell type. Connecting these two seemingly unrelated areas of study would surely yield some interesting findings. Table 10.4 summarizes a number of possible considerations. For example, the antiangiogenic properties described for the TIMPs are noteworthy. Several independent research groups have found that TIMP-1, TIMP-2, and TIMP-3 inhibit angiogenesis [157–161]. At least in the cases of TIMP-2 and TIMP-3, these antiangiogenic properties appear to result from inhibition of signaling between receptor tyrosine kinases (RTKs) and growth factors, either by competing with the growth factors for receptor binding [160] or through interactions with third-party cell surface receptors [158, 159]. Angiogenesis is an important step in cancer development, and at least TIMP-1 has been shown to slow tumor development through interfering with angiogenesis [157]. When one considers the fact that MSCs secrete high levels of functionally

Table 10.4 Potential areas of future study

Factors produced by MSCs		
Factor	Effect	Proposed connection
TIMP-1	Inhibits tumor growth and angiogenesis [157]	Does MSC-secreted TIMP-1 affect tumor progression?
TIMP-2	Interacts with integrin α3β1 and inhibits RTK-growth factor signaling, including angiogenic FGF and VEGF signaling in endothelial cells [158, 159]	Is MSC-secreted TIMP-2 an autocrine and/or paracrine inhibitor of growth factor signaling?
TIMP-3	Blocks VEGF binding to KDR and inhibits downstream signaling and angiogenesis [160]. Inhibits VEGF- and FGF-induced chemotaxis and FGF-induced angiogenesis [161]	Does MSC-secreted TIMP-3 inhibit angiogenesis?
MMP-3, MMP-7	Induce cancer cell metastasis by degrading E-cadherin	Do MSC-secreted MMPs promote cancer development?

Factors produced by other cells		
Factor	Effect	Proposed connection
Exosomes	Discharge of β-catenin and suppression of β-catenin-mediated Wnt signaling [162]	Could MSCs receive/lose β-catenin via exosomes?
Exosomes	Transfer mRNAs and microRNAs between cells [163]	Could MSCs receive/send RNA from/to other cells via exosomes?
EC- and cancer cell-derived microparticles	Bind proteases, including plasmin and MMPs, at their surfaces [164–167]	Could microparticles transfer MMPs from cancer cells to MSCs?
Immune cell-derived microparticles	Induced expression of select MMPs and cytokines in synovial fibroblasts [168]	Do microparticles affect MSC MMP/cytokine production?

activeTIMP-1 and TIMP-2 [105], it begs investigating whether MSCs affect angiogenesis and cancer development via TIMPs.

MSCs are also known to secrete proteases with demonstrated regulatory roles in breast cancer tumor progression [51, 56, 152]. For example, proteases facilitate the changes in cell-cell contacts exhibited by breast cancer cells as they transform from normal breast epithelial cells to malignant migratory cells. This epithelial to mesenchymal transition is highly regulated by E-cadherin, a homotypic cell-cell adhesion molecule that facilitates normal epithelial cell contacts and whose continued expression inhibits breast cancer metastasis [169]. As breast cancer cells become malignant, E-cadherin is degraded by proteases, weakening interactions between cancer cells and the surrounding tissue and releasing E-cadherin fragments that signal breast cancer cells to migrate [170]. MMP-7 and MMP-3, proteases secreted by MSCs, are known to degrade E-cadherin [106]. Thus, the effects of MSCs on cancer

metastasis through degradation of E-cadherin contacts remain a potential topic of study.

One of the most interesting, and often overlooked, areas of study involving interactions between cells and their environment centers on microparticles and exosomes. Both microparticles and exosomes are membrane vesicles that are released into the extracellular environment by a variety of cell types [171–177]. Microparticles and exosomes differ in size (50–1,000 nm in diameter for microparticles [171, 178], 50–100 nm for exosomes [179]) and in composition and origin. Exosomes are enriched in tetraspanins, milk fat globule-EGF factor 8 (MFG-E8), and MHC class II molecules [180], while microparticles are associated with their own set of markers, including MMPs [164, 181]). Microparticles, also known as ectosomes, are formed directly by ectocytosis [171, 177, 181], whereas exosomes originate from multivesicular bodies (MVBs) that result when endosomes bud inwardly into their lumens [182–184]. Exosomes are released as MVBs fuse with the plasma membrane and release their intraluminal vesicles. Both are distinct from apoptotic bodies, which are larger (1–4 μm), formed at the end of apoptosis, and are usually immediately taken up by macrophages [185, 186].

Both microparticles and exosomes contain membrane and cytosolic components that can be transferred from one cell to another as the particles are released and fuse with neighboring cells. For example, exosomes released by human mast cell lines are capable of transferring mRNAs and microRNAs to other mast cells. Once inside the recipient cell, this "exosomal shuttle RNA" (esRNA) is functional and affects cell behavior [163]. While the effects of esRNA on MSCs have yet to be considered, given the various cell types that MSCs interact with, the implications of MSCs receiving functional RNA from neighboring cells are very interesting.

Exosomes have also been shown to discard membrane and cytosolic proteins [162, 176]. For example, release of β-catenin from cells via exosomes has been shown to suppress β-catenin-mediated Wnt signaling. While this study did not consider intercellular transfer of β-catenin via exosomes, the notion is intriguing considering the importance of Wnt/β-catenin signaling in MSC biology; activation of canonical Wnt signaling in MSCs, which is mediated via β-catenin, is reported to keep the stem cells in a self-renewing and undifferentiated state and suppress adipogenesis and early osteogenesis and late chondrogenesis [187–190]. However, other reports describe activation of myogenesis and late-stage osteogenesis by canonical Wnt signaling in MSCs [191–193], and effects on chondrogenesis appear to be largely dependent on the specific Wnt ligand and the developmental state when Wnt is engaged [194–198]. Clearly, Wnt signaling is closely regulated in MSCs, and the shuttling of β-catenin via exosomes may represent a previously unexplored avenue by which MSC Wnt/β-catenin signaling is influenced by surrounding cells.

Formed by budding of the plasma membranes, microparticles contain a wide range of membrane-associated proteins. For example, microparticles have been shown to mediate the intracellular transfer of the chemokine receptor CCR5. While no study has focused on the transfer of membrane proteins to MSCs as of yet, the possibility is intriguing. For example, transfer of exogenous receptors to MSCs by microparticles could influence how MSCs respond to both autocrine and paracrine factors.

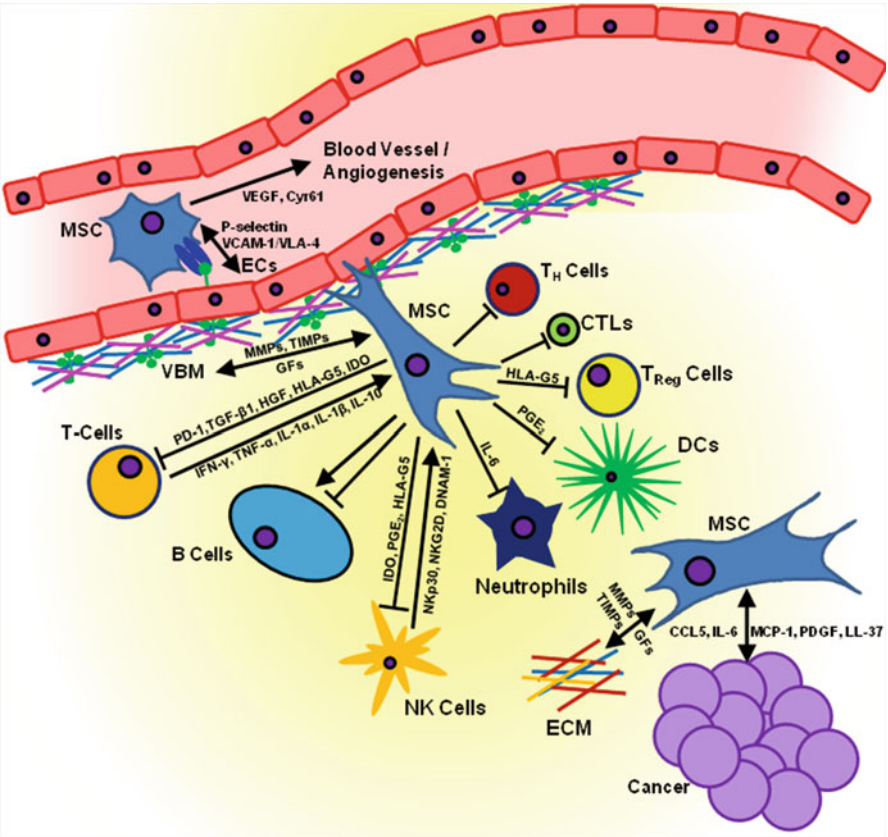

Fig. 10.1 MSC environmental interactions. MSCs influence, and are influenced by, a variety of cells, matrix molecules, and cytokines as they home to wound sites. Within the vasculature, MSCs interact with ECs, particularly those activated by the wound environment. MSCs also secrete factors that affect blood vessel structure and promote angiogenesis by regulating the extracellular matrix of the VBM. At the wound site, MSCs suppress the immune system by regulating the proliferation and activation of various immune cells. If cancer is present at the wound site, cross-talk between cancer cells and MSCs may potentiate tumor growth and metastasis (see text for abbreviations and detailed descriptions)

Furthermore, microparticles derived from cancer cells and ECs contain proteases [164–167], and EC microparticles have been shown to bind MSC-secreted MMPs [164]. Transfer of these proteases to the surfaces of MSCs could have profound effects on MSC migration and tissue invasion. Microparticles have also been shown to signal changes in cell behavior, such as induction of MMPs and cytokine expression in synovial fibroblasts [168]. This brings up the interesting possibility of microparticles and exosomes mediating long-range cell-cell interactions. Both microparticles and exosomes have been shown to display cell adhesion molecules, including E-, N-, and VE-cadherin; P-selectin; and integrins [162, 164, 180, 199]. Signaling through such

molecules are usually restricted to cells in direct physical contact with one another, but perhaps microparticles/exosomes provide a means for MSCs to interact with the surface receptors shed from other cells over longer distances.

Conclusion

The current understanding of the interactions between MSCs and their environment strongly suggests a dynamic relationship in which cells alter their surroundings and vice versa (Fig. 10.1). Studying these interactions has demonstrated unique attributes in MSCs that threaten to overshadow their differentiation capabilities as their most therapeutically important characteristics. Indeed, two of the most exciting properties of MSCs were discovered by considering their interactions with other cell types. These include the abilities of MSCs to home to sites of injury and to suppress the immune system. Several clinical trials involving MSCs that exploit the potential benefits of these properties have already concluded. These studies showed that IV delivery or direct injection of MSCs into patients with hematological pathologies, heart diseases, or cancer/chemotherapy represents a viable form of therapy with reduced chances of toxicity and adverse reactions. Furthermore, many studies observed improved healing in patients, with a variety of disorders, that were treated with MSCs. Taking into consideration that the majority of infused MSCs embolize in the lungs, these results suggest that lung-engrafted MSCs are still able to effect systemic healing in remote tissues. Perhaps the most interesting clinical results involving MSC-based therapies to promote wound repair and tissue regeneration concern cardiovascular diseases of the heart. Given the results of such studies demonstrating the proangiogenic capabilities of MSCs, the exact mechanism by which therapeutic MSCs effect improvements in impaired hearts and other wounded tissues is probably multipronged. Future research will be needed to tease apart the intricacies of these specific interactions and also to address potential side effects of MSC-based therapies, particularly those related to cancer and immunosuppression. The study of MSC and their environmental interactions thus holds the promise of generating therapies virtually impossible by any other means.

Acknowledgment Supported in part by funding from the Commonwealth of Pennsylvania Department of Health.

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Chapter 11
Human MSCs from Bone Marrow, Umbilical Cord Blood, and Adipose Tissue: All the Same?

Patrick Wuchter and Anthony D. Ho

Abstract In addition to bone marrow (BM), umbilical cord blood (UCB) and adipose tissue (AT) represent promising starting materials for the isolation and expansion of mesenchymal stromal/stem cells (MSCs). MSC preparations from these different sources are associated with distinct features and advantages: Whereas MSCs derived from UCB show the best expansion potential, the highest yield of MSC can be recovered from AT. MSCs from these starting materials have been analyzed and characterized in numerous studies. Clinical trials have been activated to define their roles in a variety of disorders. However, no specific cellular markers or marker constellation for MSCs have yet been identified. MSC preparations derived from various protocols are highly heterogeneous and differ widely in their functional properties. It is therefore essential to develop universal criteria for the quality control of starting cell populations as well as for the cell products after expansion. For clinical use, it is also advisable to use well-defined and, preferably, serum-free culture media under current good manufacturing practice conditions.

Introduction

Fibroblast-like cells derived from the bone marrow that demonstrated multilineage differentiation potentials in vitro and in vivo had already been described in the 1960s [1–3]. The term "mesenchymal stem cells" (MSC), however, was coined 30 years later [4] and referred to plastic-adherent cell preparations isolated from bone marrow or other tissues able to differentiate into bone, cartilage, and adipose tissue under specific conditions.

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_11,

As MSCs were originally isolated from the bone marrow, the latter represents the most commonly used source for generating human MSCs. Marrow-derived MSCs are also well characterized. In the past decade, different preparative protocols have been shown to yield MSC-like cell lines from sources as diverse as adipose tissue [5–7], umbilical cord blood [8], (mobilized) peripheral blood [9, 10], and skeletal muscle [11]. There is strong evidence indicating that MSC-like populations reside in many postnatal organs and tissues [12]. In addition, MSCs can also be derived from various fetal tissues, such as lung, liver, and blood [13], and even from human embryonic stem cells [14]. However, the differentiation potentials of MSCs beyond cartilage, fat, and bone tissues have remained controversial [15]. It is commonly accepted that a rare mesenchymal progenitor cell population exists in the bone marrow that can *bona fide* give rise to different cell lineages.

Mounting evidence indicates that most, if not all, MSC populations are heterogeneous and consist of several subpopulations [16]. These might contain different precursor cells that gradually overgrow under specific culture conditions – thereby mimicking the phenomenon of differentiation. Most MSC preparations might not fulfill all criteria for "stem cells" and should therefore be named "multipotent mesenchymal stromal cells" [17–19]. Consequently, the acronym "MSC" stays the same, while the term "mesenchymal stem cells" should only be used for cells that meet specified stem cell criteria.

Gene expression analysis has provided evidence that a significant number of genes are differentially expressed in MSCs isolated from different tissues [20]. Correspondingly, the differentiation potential and functional implications vary significantly among MSC preparations derived from different origins or cultured under different conditions [16, 21, 22]. There is, indeed, a lack of common standards for isolation, preparation, and cultivation of MSCs [15, 23]. Consequently, comparing results from different research laboratories is considerably hampered, underlining the need for the development of universal criteria for quality control of the starting cell populations as well as for the resulting cell products.

Characterization of MSCs from Bone Marrow, Umbilical Cord Blood, and Adipose Tissue

Harvesting bone marrow (BM) is an invasive and painful procedure, requiring local or general anesthesia. The quantity, differentiation potential, and, possibly, the life span of bone marrow-derived MSCs gradually decline with increasing age of the donor, as well as with the number of passages in culture [24–26]. Umbilical cord blood (UCB) and adipose tissue (AT) might be promising alternative sources of MSCs for clinical applications. In this chapter, we will focus on the characteristic features of MSCs isolated from these three sources. A concise summary of recent studies that have defined the differentiation potential, functional properties, as well

as immunophenotype and gene expression profiles of MSCs derived from different sources is shown in Table 11.1.

Isolation and Immunophenotyping of MSCs

Over the past two decades, a variety of protocols for isolation and cultivation of MSCs from bone marrow has been reported. Most protocols make use of the fact that MSCs adhere to plastic and gradually overgrow all remaining hematopoietic cells within 2–3 days, without specific enrichment (e.g., [28–30]). Several protocols have been developed with the aim to initiate the expansion with more homogeneous cell populations by preselecting the marrow cells using specific markers like STRO-1 [31–33], CD271 [34], CD73, and CD105 [35]. Conversely, CD45, Ter119, and glycophorin A (CD235) were used for the negative selection of MSCs [36, 37]. Another panel of surface markers, including platelet-derived growth factor receptor-D (CD140b), HER-2/erbB2 (CD340), and frizzled-9 (CD349), within the CD271-bright population was described by Bühring et al. [38]. However, none of these protocols has emerged as a universally accepted standard. Although some of the markers may lead to an enrichment of MSCs, the resulting cell populations remain heterogeneous. Thus far, a standardized protocol for the isolation and expansion of MSCs has not been established. To address this problem, the International Society for Cellular Therapy (ISCT) proposed, in a position paper in 2006, minimum criteria for defining multipotent mesenchymal stromal cells [17].

Wagner et al. compared the immunophenotype of MSCs and human fibroblast cell lines (HS68 and NHDF) by applying a panel of 22 surface markers without detecting a significant difference between the two cell types [20]. However, osteogenic, adipogenic, and chondrogenic differentiation was exclusively observed in MSC preparations, but not in differentiated fibroblasts [39]. Taken together, surface markers alone are not sufficient to reliably identify MSC populations, and there exists no commonly accepted set of surface markers distinctively describing MSCs.

Culture Media

Several studies have clearly shown the significant impact of different culture media and culture conditions on the functional characteristics of the corresponding MSC populations. These include differences in cell proliferation, morphology, gene expression, and proteome analysis [20, 39]. Most preparative protocols contain bovine serum additives such as fetal bovine serum (FBS) in concentrations between 2 and 10%.

Table 11.1 Comparison of MSCs derived from bone marrow (BM), umbilical cord blood (UCB), or adipose tissue (AT)

	BM-MSC	UCB-MSC	AT-MSC
Morphology[a,b,c,d]	Typical fibroblast-like cell shape.		
Immuno-phenotype[a,b,d]	*Negative* for CD10, CD14, CD24, CD31, CD34, CD36, CD38, CD45, CD49d, CD117, CD133, SSEA4, and HLA-DR		
	Positive for CD13, CD29, CD44, CD73, CD90, CD105, CD166, and HLA-ABC		
Success rate of isolating MSC[a,b,c,d]	100%	28–63%	100%
Colony frequency (no. of CFU-F per well) in colony-forming assay[a,d]	83 ± 61	0.002 ± 0.004	557 ± 673
Maximal no. of cell passages in culture prior to cellular senescence[a]	7	>10	8
Senescence ratio up to passage 2[a]	24%	35%	6%
Differentiation potential[a,c,d]			
Osteogenic	+	+	+
Chondrogenic	+	+	+
Adipogenic	+	(+)/−	+
Supportive function of hematopoiesis in coculture with HSC[e]	+	+	−
Gene expression profiling[b]	25 overlapping and upregulated genes (including, e.g., fibronectin, ECM2, glypican-4, ID1, NF1B, HOXA5, and HOXB6) found in all MSC preparations from AT, UCB, and BM as compared to HS68 fibroblasts.		

[a][21]
[b][20]
[c][8]
[d][27]
[e][22]

The choice of culture medium probably depends upon the source of MSCs and the desired application. In our hands, the medium described by Reyes et al. for generation of bone marrow mesenchymal stromal cells with 2% FBS [37] has resulted in the highest MSC proliferation rate. Verfaillie et al. used this medium in combination with a special isolation method to create their so-called multipotent adult progenitor cells (MAPC). However, we only used the medium for expansion of our standard-isolated BM-MSC, resulting in MSC, not MAPC. The cells appeared morphologically as rather thin spindle shaped and fibroblast like. This medium was also used for AT-MSC. Another expansion medium for BM-MSC we used for a variety of studies is a commercially available product (MSCGM™ Mesenchymal Stem Cell Growth Medium [PT-3001, Lonza]) with 10% FBS. With this medium, no precoating of the plastic dishes is necessary, probably due to the relatively high level of FBS. Intercellular junction formation seemed to be most frequently observed in this medium. However, the manufacturer does not reveal the exact composition of the ingredients. For the expansion of CB-MSC, we have achieved the best results with a commercially available medium (MesenCult® MSC Basal Medium [STEMCELL Technologies]) with additive stimulatory supplements according to the manufacturer's instructions as described previously by L. Hou and colleagues [40].

As more and more investigators attempt to produce MSCs under GMP conditions for clinical trials, there is a growing demand for bovine-free culture media. More recently, alternative culture protocols for the expansion of MSCs based on reagents of human origin (i.e., platelet lysate, plasma or serum, etc.) have been developed [41–47]. As most of these human media supplements result in substantial differences in cell morphology and growth kinetics [48], at present, it is not clear to what extent they potentially alter the composition of the cell preparation. Moreover, for clinical applications, in most countries, it is mandatory to process the cells at all stages under current good manufacturing practice (cGMP) conditions [49].

Cultivation Techniques

Besides the composition of the culture media, many other factors need to be taken into account. For instance, there is evidence that oxygen tension plays a role as MSC differentiation is accelerated under hypoxic conditions [50]. The cell density of in vitro cultures is another important factor. MSCs can lose some of their differentiation potential when grown to confluence [37, 51, 52]. Furthermore, MSCs are usually cryopreserved with dimethyl sulfoxide (DMSO) in liquid nitrogen. Although there is evidence that cryopreserved and non-cryopreserved MSCs possess the same differentiation potential, a detrimental effect of the freezing and thawing procedure on their biological properties cannot be entirely excluded [53, 54]. Also, as plastic adherence is the major criterion for enrichment of MSCs, the molecular structure of the culture dish surface (e.g., roughness, hydrophobicity) may significantly affect the cell populations and their properties [55, 56]. Several authors have used additional

protein coating with fibronectin, bovine serum albumin (BSA), hyaluronic acid, gelatine, or collagen to increase cell adhesion [57–59]. The impact of these factors has not been systematically determined.

We observed a distinct change in the morphology of the MSCs cultured on plates or flasks precoated with fibronectin or gelatin. When the level of FBS in the expansion medium was ≥10%, no precoating of the plastic dishes (e.g., Nunc® flasks with 75 cm^2 by Nalge Nunc, Naperville, Ill., USA) was necessary. For media with less than 10% FBS, precoating with fibronectin (5-μg fibronectin per 500 ml PBS) yielded better results. However, for certain assays, it might be necessary to culture MSCs on glass slides (e.g., for examination with electron microscopy). In this case, precoating the slides with 0.1% BSA enabled us to achieve a better adherence of the cells to the glass surface without morphologic changes. For immunofluorescence staining, it was helpful to culture MSCs in 8-well Lab-Tek® Chamber Slides® on Permanox® (by Nalge Nunc, Naperville, Ill., USA), which allows staining of MSC directly on the slide.

Above all, MSCs cannot be expanded in vitro indefinitely – after a limited number of cell passages, they become senescent. From our experience, the best time point to obtain MSCs for functional assays is between passages 3–6. At later passages (>7), MSCs gradually change their morphology, show lower division kinetics, and ultimately stop to divide. This process of cellular aging is reflected in significant changes of the molecular profile and functional features of the cells [60–65]. Surprisingly, similar alterations in cellular and molecular characteristics between replicative senescence and MSC preparations from different age groups were observed, ranging from UCB to subjects over 60 years [66].

Gene Expression Profiling and Proteomics

Gene expression analysis represents an important means for the molecular characterization of cell preparations. Wagner and colleagues compared the gene expression profiles of MSCs derived from bone marrow, adipose tissue, and cord blood (Fig. 11.1) [20, 23, 39]. While MSCs derived from different donors under identical conditions yielded a consistent gene expression profile, many genes were differentially expressed in MSCs from different tissue sources or under different culture conditions. However, an overlapping upregulation of at least 25 genes was reproducibly found in all MSC preparations irrespective of origin and culture conditions as compared to HS68 fibroblasts. This set of genes included extracellular matrix proteins like fibronectin 1 (FN1), glypican-4 (GPC4), latent-transforming growth factor beta-binding protein 1 (LTBP1), and extracellular matrix protein 2 (ECM2) as well as transcription factors (nuclear factor I/B [NF1B]), homeobox genes (HOXA5 and HOXB6), and inhibitor of differentiation/DNA binding (ID1). Many genes upregulated in MSCs were involved in extracellular matrix, morphogenesis, and development, whereas several inhibitors of the Wnt pathway (DKK1, DKK3, SFRP1) were highly expressed in fibroblasts.

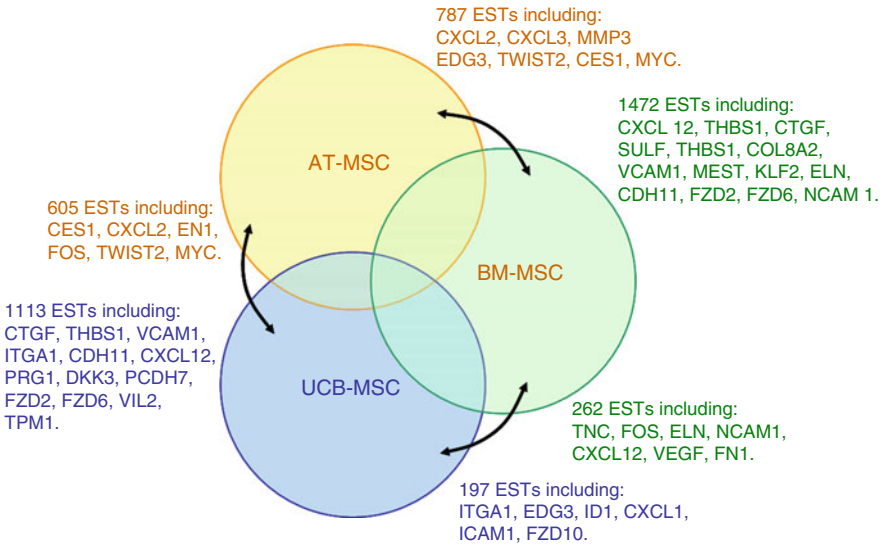

Fig. 11.1 Pairwise comparison of MSC derived from AT, UCB, and BM by gene expression profiling The numbers of expressed sequence tags (*EST*) that revealed highly significant upregulation in the corresponding cell types are shown ($p < 0.001$) (Wagner et al. [20], with permission from Elsevier)

The proteome of different BM-MSC preparations was also analyzed by Wagner et al. [39]. One hundred thirty-six protein spots were unambiguously identified by matrix-assisted laser desorption– time-of-flight mass spectrometry (MALDI-TOF-MS), and most of them play a role in cytoskeleton, protein folding, and metabolism. By combining the datasets of genomics and proteomics, a correlation in differential gene expression and protein expression was found in BM-MSCs cultured with two different culture media. Interchanging culture conditions for 8 days revealed that differential expression was retained in several genes whereas it was altered in others. These data demonstrate that while homogeneous BM-MSC preparations can be isolated in a standardized setting, culture conditions still have a major impact on the transcriptome, proteome, and cellular organization of MSCs. It seems therefore likely that a combination of genomic and proteomic signatures rather than a single genomic or proteomic marker might better define multipotent MSCs.

Cell Junctions of MSC

In a series of studies to define the molecular and cellular interactions between human MSCs and hematopoietic stem cells (HSCs), we initially characterized the interactions among MSCs and among HSCs themselves. Whereas no evident intimate contacts of any significant duration could be identified among HSCs, human bone

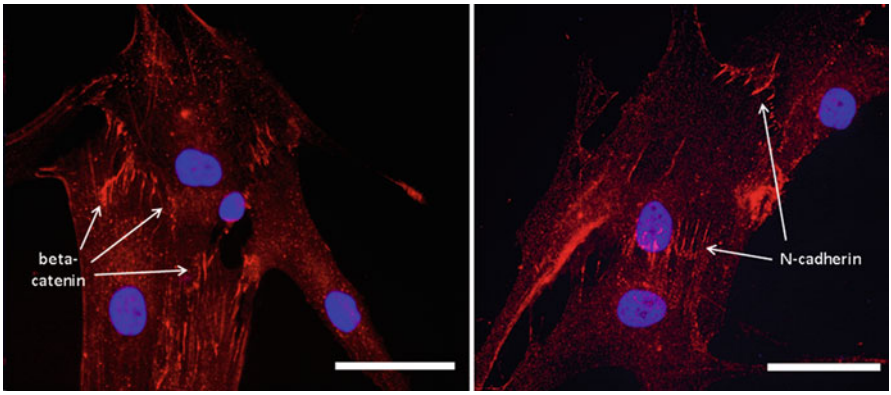

Fig. 11.2 Beta-catenin- and N-cadherin-based junctions between human BM-MSCs Immunofluorescence staining of beta-catenin and N-cadherin (*red*). The cell nuclei are stained in DAPI (*blue*). Scale bar: 50 μm

marrow-derived MSCs are interconnected under in vitro conditions by complex villiform-to-vermiform cytoplasmatic cell protrusions termed *processus adhaerentes* [67]. These processes are inserted tightly into deep plasma membrane invaginations of the neighboring cell, often forming batteries of interdigitating cell-cell connections (Fig. 11.2). Additionally, long tentacle-like cell processes could be observed that cover distances of more than 400 μm and made junctional contacts with up to 8 other MSCs. *Processus adhaerentes* were characterized by a molecular complement comprising N-cadherin and cadherin-11, in combination with the cytoplasmic plaque proteins α- and β-catenin, together with p120ctn, plakoglobin, and afadin. The frequency and morphology of these junctional complexes are greatly affected by culture conditions [16]. A similar type of homotypic cell-cell interaction was described previously by Franke and coworkers in studies of primary mesenchymal cells of the mouse embryo [68], indicating that this special type of cell junction is probably more widespread in embryonal and other tissues and might be relevant for the primitive function of MSCs and heterotypic interaction with other cell types.

Intrinsic Heterogeneity of MSC Preparations

It was demonstrated that an occasional cell among thousands in MSC preparations that could be consistently positive for cardiac type a-actin myofilaments or smooth muscle a-actin. These spontaneously emerging cell culture subtypes are subject to the phenomenon of spontaneous changes of protein synthesis in patterns that suggest random processes [16]. Our conclusion is that MSC preparations that appear homogeneous, in fact, consist of heterogeneous subpopulations. This challenges the hypothesis of the true transdifferentiation potential of MSCs. Predetermined

subpopulations of MSCs may overgrow the predominant cell type and, depending upon the conditions of a given differentiation assay, mimic the phenomenon of differentiation.

In contrast, some features are shared by all MSCs. For example, the intermediate filament protein vimentin was clearly positive in virtually all cells in MSC preparations from all sources. Vimentin is among the most highly expressed proteins in MSCs and has been suggested to serve as a key protein to identify progenitor cells of mesodermal origin [69, 70]. It is the first intermediate filament protein to be expressed during cell differentiation. Although its specific function in MSCs is still unclear, recent data suggest a major role in developmental dynamics. MSCs are highly proliferating cells under in vitro culture conditions. As the phosphorylation of vimentin is significantly enhanced during cell division [71, 72], it is not surprising that a highly active phosphorylation of vimentin was found in MSC cultures.

Another issue currently under debate is the role of nestin+ MSCs as a niche for the regulation of hematopoiesis. Simón Méndez-Ferrer and colleagues described recently that nestin+ MSCs are spatially associated with HSCs and directly connected to adrenergic nerve fibers. They also highly express HSC maintenance genes, thereby forming a unique bone marrow niche [73]. Although their data are convincing, the results have been derived solely from a murine model and require validation in human hematopoiesis. Our preliminary results indicate that 5–40% nestin+ cells can be found in human BM-MSC cultures (manuscript in preparation). It is yet unclear if these cells play a role as the niche for human hematopoiesis, comparable to their murine counterparts.

Specific Properties of Umbilical Cord Blood-Derived MSCs

Umbilical cord blood (UCB) can be harvested readily and without any health hazards. It contains the most primitive available adult stem cells and can be obtained without requiring any invasive measures. Human HSCs as well as MSCs can be recovered from umbilical and placental blood [74]. For more than 20 years, UCB has served as an alternative source of donor cells for hematopoietic stem cell transplantation [75]. Transplantation with UCB is effective in the treatment of children with hematological malignancies and a number of nonmalignant diseases (e.g., marrow failure, hemoglobinopathies, and inherited metabolic diseases; [76]).

In contrast to BM- and AT-MSC, UCB-MSCs could be cultured for a longer period, e.g., for more than 10 passages. They also showed the highest proliferative potential and could be expanded to a much higher quantity of cells [21, 27]. Key issues for the successful isolation of MSC-like cells from UCB were a time span from collection to isolation of less than 15 h, a net UCB volume of >33 ml, and a cell count of more than 1×10^8 mononuclear cells (MNC). Another critical step in the isolation process from UCB is the precoating of the tissue culture flasks with FBS to remove contaminating monocytic and phagocytic cells. FBS may alter the

adherence kinetics of monocytes, enabling the cells to be removed with the medium change after overnight adherence [8].

Several groups have reported that UCB-MSCs, in contrast to BM- and AT-MSCs, showed a lower or no adipogenic differentiation capacity. The adipogenic differentiation capacity of UCB-MSCs has remained controversial [20, 74, 77–79]. There is strong evidence for the notion that MSCs from UCB are less liable to undergo adipogenic differentiation. Chang et al. [80] demonstrated that UCB-MSCs had a significantly stronger osteogenic potential but lower capacity for adipogenic differentiation than BM-MSCs [80]. They also showed that leptin, an important regulator of mesenchymal differentiation, was significantly stronger promoter of osteogenesis and inhibitor of adipogenesis in BM-MSCs than in UCB-MSCs. Moreover, core-binding factor alpha1 (Cbfa1) mRNA expression in BM-MSCs and UCB-MSCs was affected to different degrees by leptin during osteogenesis. In contrast, leptin reduced the mRNA expression of adipocyte-specific transcription factor peroxisome proliferator-activated receptor 2 (PPAR-γ2) to the same level during adipogenesis in both types of MSCs. This is in alignment with the findings of Moerman et al. [81], who reported that aging causes a decrease in the commitment of BM-MSCs to the osteoblast lineage and an increase in the commitment to the adipocyte lineage. This is reflected by changes in the expression of phenotype-specific gene markers. The expression of osteoblast-specific transcription factors (Runx2 and Dlx5) and osteoblast markers (collagen and osteocalcin) was decreased in aged BM-MSCs. Conversely, the expression of PPAR-γ2 was increased, as well as a gene marker of adipocyte phenotype, fatty acid-binding protein aP2 [81].

A very rare multipotent MSC subset from umbilical cord blood, named unrestricted somatic stem cells (USSC), has been described that significantly supported proliferation of HSC in an in vitro feeder layer assay [78]. Preclinical studies indicate that USSC can be used as a safe graft adjunct and have an enhancing effect on engraftment of human CD34+ cells [82].

Recent reports suggested that umbilical cord (UC) matrix could serve as an alternative source of MSCs. Some authors have proposed that UC matrix could be even more efficient in generating MSCs and regarded it superior to UC blood as a starting source [83–85]. If confirmed by other authors, UC matrix might represent another valuable source for MSC preparations.

Specific Properties of Adipose Tissue-Derived MSC

Adipose tissues (AT) could be retrieved as a biological waste of cosmetic liposuction in clinics for esthetic surgery. It has been demonstrated that AT contains multipotent mesenchymal stromal cells similar to BM-MSCs, which can be isolated and grown under standard tissue culture conditions and show multilineage differentiation capacity [5, 6, 86]. Some groups also used the term processed lipoaspirate (PLA) cells [87]. Interestingly, it has been described that AT contains MSCs at higher frequencies compared to BM and UCB [6, 21]. de Girolamo et al. [86] compared two distinct

media for osteogenic differentiation and reported a different osteogenic potential, as assessed by increased levels of calcium deposition, alkaline phospatase activity, and osteopontin expression. They concluded that AT-MSCs could efficiently differentiate into osteogenic cell lineages, particularly when cultured in inductive medium supplemented with dexamethasone and ascorbic acid [86]. Lee et al. found that the use of DMEM (Dulbecco's modified Eagle's medium)/MCDB-201 (Sigma-Aldrich) media and low-density plating during cell culture is advantageous for the maintenance of differentiation and proliferation potential as compared to high-density cultures in α-MEM (alpha-modified minimal essential medium). In their study, AT-MSC in DMEM/MCDB maintained their proliferating capacity up to 30 passages, whereas growth of AT-MSCs in α-MEM stopped at 20 passages [6].

AT-MSCs, in contrast to BM- and UCB-MSCs, demonstrated a much lower ability to maintain stemness of HSCs in coculture systems, indicating that AT-MSCs support differentiation but not self-renewal of HSCs [88]. This was reflected in a reduced adhesion rate of HSCs, in the impact on alterations in immunophenotype of HSCs, and above all in a significantly reduced maintenance of long-term culture-initiating cells (LTC-IC) [22].

Conclusions

The notion that adult stem cells might be able to transdifferentiate across germinal boundaries has been severely challenged. A more realistic assessment of their "plasticity potential" has in the meantime prevailed. In the case of MSCs, their differentiation capacity is probably limited to cartilage, fat, and bone tissues. Challenges to their clinical applications, however, include the lack of standardized protocols for their isolation, preparation, and expansion. Above all, MSCs are notoriously heterogeneous.

Several groups have compared the molecular, genetic, and functional features of MSCs derived from BM, UCB, and AT. The appropriate source of MSCs for clinical application may depend upon the indication. If MSCs are needed urgently, e.g., for the treatment of acute graft-versus-host disease, bone marrow from an allogeneic donor would be an appropriate choice. An allogeneic source would also apply for MSCs administered together with HSCs to enhance engraftment after allogeneic HSC transplantation. In case of musculoskeletal tissue engineering or strategies for tissue replacement, MSCs from autologous adipose tissue offer potential advantages. Data from other authors as well as from our group have shown that AT-MSCs may have the highest yield on a cell to cell basis. The availability of UCB will probably remain limited, as the main purpose of UCB banking is to provide a pool of allografts for HSC transplantation. Nevertheless, UCB-MSCs have been applied successfully for the treatment of inborn diseases of metabolisms such as osteogenesis imperfecta [89]. Recent reports have demonstrated that the UCB matrix might serve as an alternative source for preparing MSCs. If confirmed, this strategy might broaden the perspective for new therapeutic approaches.

A prerequisite for the clinical application of MSCs is the development of universal criteria for quality control of the initial cell material as well as for the cell products upon expansion. The International Society for Cell Therapy (ISCT) criteria for the definition of MSCs are an appropriate starting point. In the past 6 years, the START-MSC (Standardization for Regenerative Therapy – Mesenchymal Stem Cells) consortium, funded by the German Federal Ministry for Research and Education (Bundesministerium für Bildung und Forschung), has established optimized protocols for GMP-compliant preparation of MSCs, developed tools and a catalogue of markers for their precise characterization, and defined their differentiation potential in vitro and in animal models. Albeit optimized MSC preparations were able to differentiate into bone, cartilage, and adipose lineages, we found no evidence that the cells can differentiate into hepatocytes in vitro [90]. However, the induction of pluripotency by introducing defined genetic factors into MSCs might open new perspectives, as demonstrated for cardiomyocytes.

Acknowledgments This work was supported by the German Federal Ministry of Education and Research (BMBF) within the supporting program "cell-based regenerative medicine" (START-MSC2) and the German Research Foundation DFG (SFB 873). We thank Anke Diehlmann for excellent technical assistance in cell culture and Dr. Rainer Saffrich for outstanding image acquisition.

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Chapter 12
MSCs in Solid Tumors and Hematological Malignancies: From Basic Biology to Therapeutic Applications

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Abstract Compelling experimental evidence has recently unveiled that the tumor microenvironment plays a crucial role in tumor progression. Importantly, bone marrow-derived mesenchymal stromal cells interact with tumor cells, and these interactions trigger a series of signaling responses that ultimately favor tumor progression and survival. In this chapter, we will describe how the stroma can influence tumor fate and also how we can utilize these stromal cells to deliver antitumoral therapeutic agents based on their innate tropism for tumors and injury sites.

Introduction

In the last few years, the importance of the tumor microenvironment for cancer development, progression, and metastasis has been widely recognized and has become a new focus for cancer research [1]. In hematopoietic malignancies, the interaction between clonally abnormal hematopoietic cells and their microenvironment facilitates proliferation, self-renewal, and differentiation. This rich environment serves as a sanctuary not only for normal and malignant hematopoietic cells but also for epithelial tumor cells that metastasize to the bone, offering protection from chemotherapeutic agents by shared mechanisms. This protection allows tumor cells to

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_12,

survive chemotherapy, resulting in residual disease, thereby increasing the probability of relapse and developing acquired drug resistance. In this chapter, we will review the role of the microenvironment, in particular of mesenchymal stromal/stem cells (MSC), in support of solid and hematopoietic tumors.

The Tumor-Cell Paradigm During Cancer Development

Accumulation of consecutive genetic and epigenetic alterations within the cell genome can lead to phenotypic changes and result in increased proliferation and growth. This multistep process is considered to be one of the main forces driving cancer development [2–6]. In particular, for hematological malignancies, a two-hit hypothesis has been proposed [7]. This view of cancer progression focused on the tumor cell has led to many important discoveries and has contributed to an understanding of cancer. The human cancer genome project is a good example of a systematic use of this hypothesis as an approach to identify new mutations at a rapid pace [8, 9]. However, this tumor cell-centric approach is not capable of comprising the complexity of in vivo tumor growth and metastasis as it is an overly simplistic view modeled on convenient cell culture systems lacking almost all features of the in vivo systems. For about two decades, the concept of tumor "stem cells" has helped to explain how a stemlike parental cell containing increased tumor-initiation potential and capable of generating the bulk of the tumor cells could be sufficient to generate tumors in animal models [10, 11]. Unfortunately, most of stem cell research does not address the complex interplay between the various cell types required to generate tumors, e.g., between tumorigenic epithelial cells and non-neoplastic microenvironmental cells such as those required to generate matrix, vasculature, and the characteristic immune suppression which is a prerequisite for tumor development [12, 13]. Within this context, it is important to consider that regardless of the significant number of genetic alterations that result in development of malignant tumors, it is unclear whether the connective tissue surrounding epithelial tumor malignancies remains consistent. Such a microenvironment was initially considered a passive participant in tumor development and described as a nourishing and supportive neighbor for tumor cells [14]. However, recent experimental evidence has revealed the critical role of the microenvironment in tumor progression and the crucial role of these normal cells of the microenvironment as active participants that shape the frequency and biology of tumors. We now recognize the fact that tumors do not exist in isolation and tumor cells by themselves are not sufficient to generate a malignant tumor. Instead, controlled and specialized interactions between tumor cells and their supportive/permissive stroma are required for tumor progression [15–18]. This concept has been spearheaded by S. Paget's "seed and soil" hypothesis [19, 20] and J. Folkman's insights into the role of angiogenesis in tumor development [21].

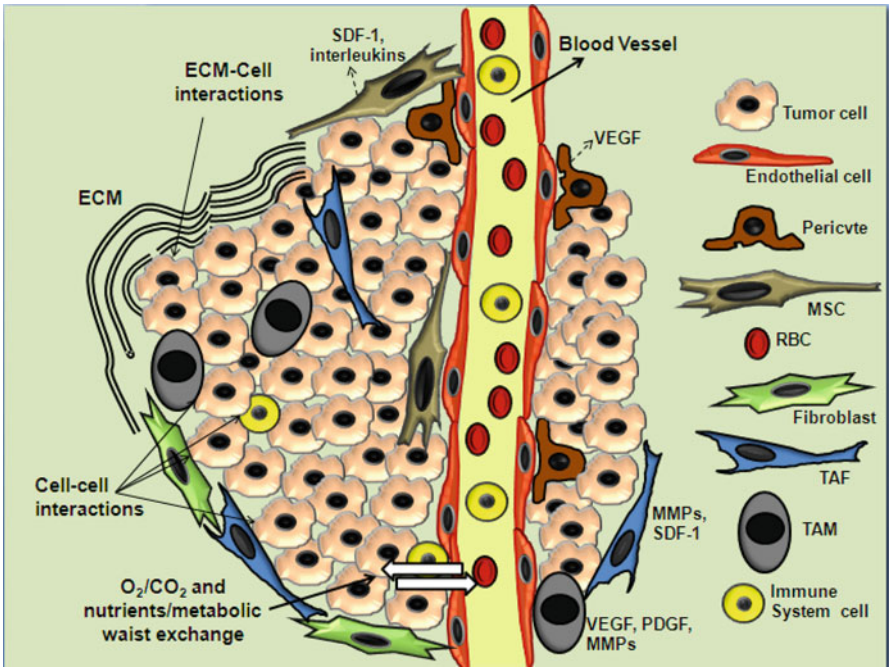

Fig. 12.1 The heterogenous tumor microenvironment. Several cellular components constitute the solid tumor microenvironment. Endothelial cells and pericytes contribute to tumor vascularization. MSCs, fibroblasts, and tumor-associated fibroblasts (*TAF*) contribute to the stromal architecture including extracellular matrix (*ECM*) deposition, matrix remodeling, protein production, and growth factor secretion. Immune cells found in the tumor include dendritic cells, lymphocytes, and macrophages. These cells are often altered from their native state; one such example is the presence of tumor-associated macrophages (*TAM*)

The Role of Stroma in Cancer

Tumor cells exist surrounded by the extracellular matrix (ECM) and nonneoplastic microenvironmental cells (such as fibroblasts, endothelium, and various cells of the immune system), also known as the tumor stroma [22]. Dynamic interactions between the tumor parenchyma (the neoplastic cells) and the tumor stroma appear to be critical for the development and progression of tumors [23–26]. Such stroma responds to signals and factors produced by the tumor cells and provides components necessary for tumor survival, including extracellular matrices, vasculature, and structural support [27, 28] (Fig. 12.1). In the case of the bone marrow (BM) microenvironment, the stroma consists of fibroblasts, bone marrow-derived MSC, endothelial cells, macrophages, adipocytes, and bone-lining cells (e.g., osteoblasts

and osteoclasts). In this context, the observation that most hematological malignant cells die very quickly when cultured in vitro strongly supports the idea that environmental factors are critical for tumor cell survival. This concept is further supported by experimental data showing that hematological malignant cells survive much longer if cultured on stromal cells. Hence, interactions between tumor and BM stroma cells, as well as tumor-ECM interactions, all contribute to tumor cell survival mediated by direct contact. Furthermore, the BM microenvironment provides soluble factors that promote tumor cell growth and survival.

Currently, it is becoming clear that the stroma also acts as a reciprocal-signaling partner in tumor progression and changes in the stromal compartment can often be caused by processes resembling normal inflammatory and wound-healing responses [29]. According to this notion, chronic inflammation or other pathological conditions induce functional and structural changes in the tumor stroma, which can cause dormant tumors to become active and to proliferate or can even induce the genetic alterations that initiate tumorigenesis [30]. This hypothesis is supported by numerous transgenic and knockout animal models in which key paracrine regulators are blocked or altered [31, 32]. For example, a murine model in which the transforming growth factor-β (TGF-β) type-II receptor was conditionally ablated demonstrated that suppression of the TGF-β response in stromal fibroblasts alters stromal-epithelial interactions, disrupting normal mammary development and enhancing tumor growth and cell motility [33]. Reciprocally, it is also known that tumors alter their microenvironments and induce them to recruit additional stromal cells that, once engrafted, remain activated, resulting in further tumor support. As an example, when solid tumors grow larger than a few millimeters in size, oxygen and nutrients required for survival and growth become scarce, and angiogenesis is needed to overcome such limitations. During the onset of angiogenesis, tumor cells and the microenvironment cooperate to recruit several types of stromal cells required for tumor blood vessel formation. These include fibroblasts, endothelial cells, pericytes, macrophages, and inflammatory cells [23]. Under inflammatory conditions, such as during tumor development or wound healing, local fibroblasts are recruited from neighboring tissues and start to divide to form fibrovascular structures [34]. In addition, endothelial cells are recruited and activated to divide and form new vasculature [35]. Two other important factors in tumor angiogenesis are tumor-associated macrophages (TAMs) and tumor-associated fibroblasts (TAFs). Several studies have shown TAM accumulation in areas of hypoxia and neovascularization, as well as high levels of TAM-secreted pro-angiogenic factors including vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) [36]. Additionally, TAMs produce thymidine phosphorylase, an endothelial chemotactic factor. Increased levels of this factor are associated with the formation of new vasculature, invasiveness, metastasis, and decreased patient survival in many human solid tumors [37]. With regard to TAFs, it has been suggested that the tumor microenvironment induces local fibroblasts to assume a TAF phenotype [38] and that TAFs may originate from multiple sources including resident tissue fibroblasts, pericytes, vascular smooth muscle cells, and endothelial

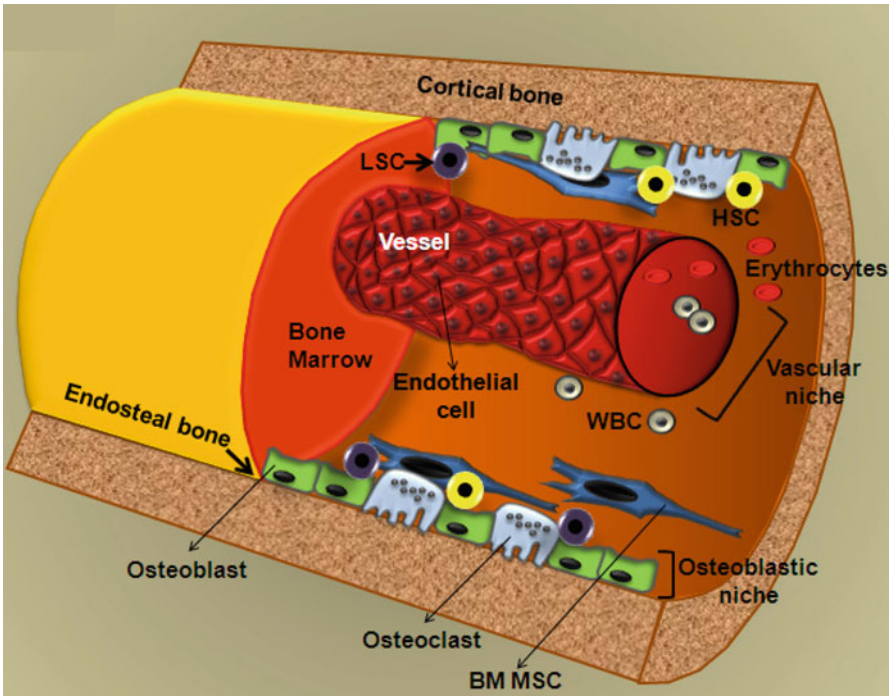

Fig. 12.2 Major components of the BM microenvironment. The BM microenvironment consists of a complex network of cells including osteoblasts, osteoclasts, endothelial cells, and mesenchymal stromal/stem cells (*MSC*) all of which are critical for the regulation of normal and leukemic stem cell maintenance and localization. The normal hematopoietic stem cells (*HCSs*) and leukemic stem cells (*LCSs*) reside in the osteoblastic and vascular niches. Cell–cell interactions as well as soluble fat or mediated signaling between HSCs/LSCs and BM stromal components are critical to determine the fate of normal and malignant hematopoiesis. *WBC* white blood cells

cells [39]. More importantly, recent evidence points to bone marrow-derived MSCs as a source of TAFs [40]. Within this context, it is worth pointing out that TAFs are considered a morphologically homogeneous but functionally heterogeneous group of mesenchymal cells shown to produce large numbers of tumor-promoting growth factors, cytokines, and chemokines. It has also been suggested that they modulate the local immune response against the tumor [41]. During the invasion of cancer cells into the surrounding tissue, considerable destruction and regeneration of intercellular elements take place. A selected group of cells composed of pericytes, fibroblasts, endothelial cells of blood and lymph vessels, and immature myeloid and inflammatory cells (including lymphocytes, granulocytes, and macrophages) become part of the newly synthesized stroma known as "cancer-induced stroma" [39]. TAFs contribute to a pro-invasive environment through several mechanisms including matrix synthesis and degradation [42] and by releasing cytokines and proteinases, more specifically, activate matrix

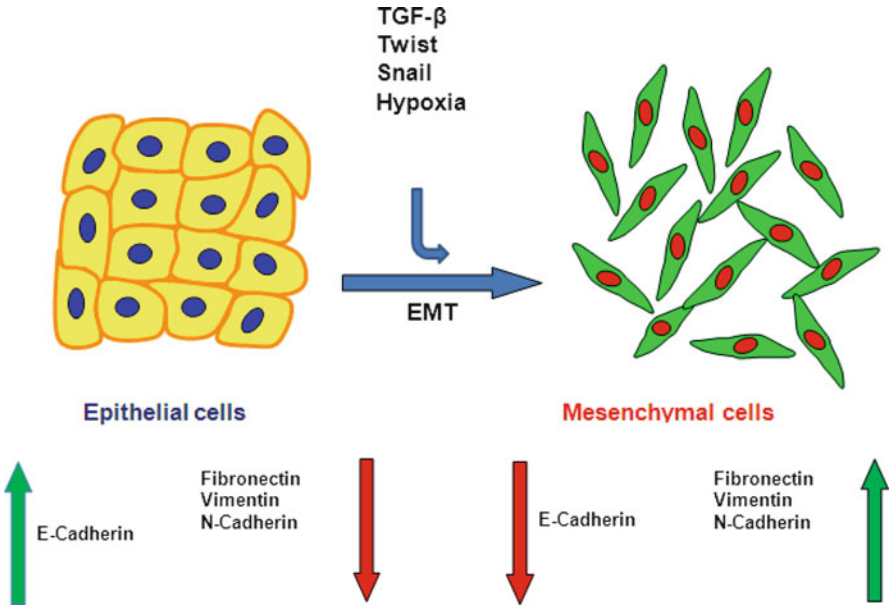

Fig. 12.3 Epithelial to mesenchymal transition (*EMT*). Epithelial cells undergo EMT when exposed to growth factors such as TGF-β or by overexpression of genes such as Twist/Snail or by exposure to extremely low levels of oxygen (hypoxia). During this transformation, epithelial cells lose cobblestone appearance and acquire spindle shapes. At the gene expression level, these cells lose E-cadherin expression and gain mesenchymal markers such as fibronectin, vimentin, and N-cadherin

metalloproteinases (MMPs) [43]. In addition, TAFs are known to release higher levels of stromal-derived factor-1(SDF-1) than normal fibroblasts, as shown in invasive human breast carcinomas [44]. This is also true for the bone marrow microenvironment where it is well known that chemokines, in particular SDF-1 and its cognate receptor CXCR4, play an important role in the homing of hematopoietic cells. This is critical for the development of the hematopoietic compartment as well as in the regulation of normal and malignant hematopoiesis (Fig. 12.2).

Generation of MSC-Like Cells Through EMT

Recently, emerging data have suggested that tumors can generate their own intrinsic mesenchyme through an event termed "epithelial-to-mesenchymal transition (EMT)" [45–47]. EMT is a latent embryonic process that causes epithelial cells to lose their epithelial traits and acquire properties of mesenchymal cells [48, 49]. During EMT, epithelial cells lose cell polarity by downregulating the expression of cytokeratins and cell-cell adhesion molecules such as E-cadherin, and the decrease in epithelial gene expression is accompanied by increased expression of mesenchymal genes, including vimentin and fibronectin [50, 51] (Fig. 12.3). EMT can be

induced by several cytokines and chemokines, including TGF-β, by the expression of several developmentally important transcription factors, including Twist and Snail, or by extremely low levels of oxygen (hypoxia) [52–55]. Importantly, these factors have been demonstrated to also play roles during tumor progression. In addition, it has been reported that when epithelial cells undergo EMT, they not only acquire mesenchymal phenotype but also stem cell properties [56]. Induction of EMT in immortalized human mammary epithelial cells by overexpression of known EMT inducers including Twist and Snail genes enhanced the expression of stem cell markers ($CD44^{high}CD24^{low}$) on these cells. Furthermore, EMT-derived cells showed the ability to form mammospheres, soft agar colonies, and tumors more efficiently, properties that are associated with mammary epithelial stem cells [56]. These findings were further supported by comparison of EMT-derived cells to bone marrow-derived MSCs. Surprisingly, EMT-derived cells exhibited phenotypic and functional properties of MSCs [57]. EMT-derived cells expressed higher levels of MSC markers such as CD44 and PDGF-Rβ (CD140b) on their cell surface compared to their epithelial counterparts. In addition, when cultured under appropriate conditions, these cells differentiated into osteoblasts, adipocytes, and chondrocytes similar to MSCs. Furthermore, EMT-derived cells, but not control cells, invaded and migrated toward MDA-MB-231 breast cancer cells. In vivo wound homing assays in nude mice revealed that the EMT-derived cells home to wound sites suggesting functional analogies with MSCs. In another report, breast cancer epithelial cells cocultured with MSCs showed elevated expression levels of oncogenes (NCOA4, FOS), proto-oncogenes (FYN, JUN), genes associated with invasion (MMP11), angiogenesis (VEGF), and anti-apoptosis (IGF1R, BCL2) [58]. In this study, significant upregulation of EMT specific markers (N-cadherin, Vimentin, Twist, and Snail) was also observed, following coculture with MSCs suggesting that MSCs may promote breast cancer metastasis through facilitation of EMT. These reports suggest that MSC-like cells could be generated by induction of EMT in epithelial cells and that these EMT-derived cells exhibit stem cell properties and may support tumor progression (Fig. 12.4).

Leukemia Cell Homing to the BM Microenvironment

In adult organisms, the bone marrow is the major hematopoietic organ responsible for the production of erythrocytes, granulocytes, monocytes, lymphocytes, and platelets. For hematopoiesis to occur, it must be supported by a unique bone marrow microenvironment able to recognize and retain hematopoietic stem cells and provide the factors (e.g., cytokines and chemokines) required to support proliferation, differentiation, and maturation of stem cells along committed lineages. These HSCs reside within two distinct specialized areas of the bone marrow (BM) microenvironment that have been defined as the osteoblastic (endosteal) and the vascular niche [59] (Fig. 12.2). Within this microenvironment, the BM stromal cells produce cytokines and chemokines and initiate cellular adhesion-mediated

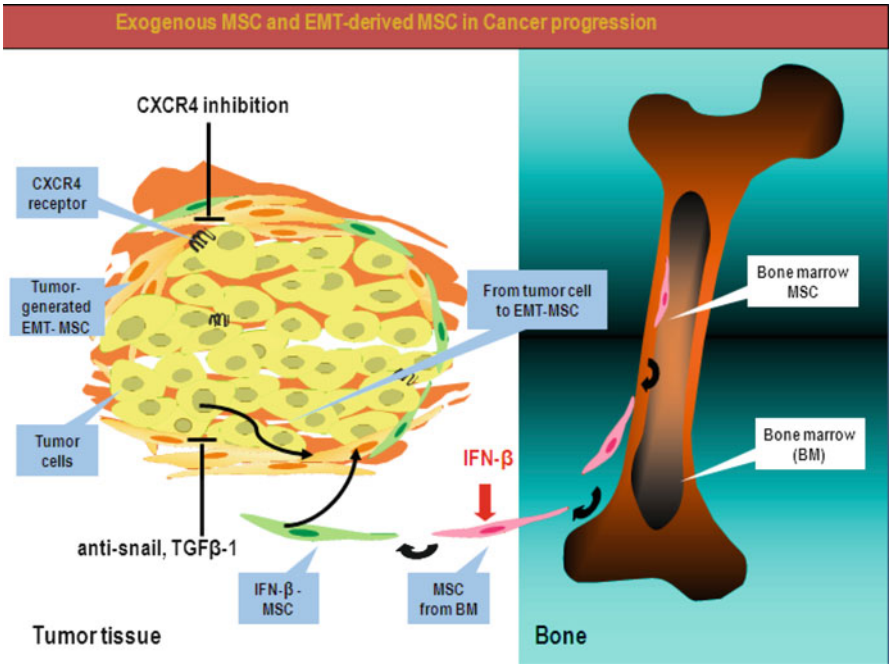

Fig. 12.4 Two sources of MSCs in tumors: "endogenous" MSCs generated by EMT and "exogenous" MSCs attracted from BM and fat tissue

signals that tightly regulate normal and malignant hematopoietic cell development. A variety of cell types, including osteoblasts, osteoclasts, endothelial cells, perivascular reticular cells, and BM MSCs, form part of the BM stromal cells and are critical for the regulation of HSC maintenance and localization [60]. The initial homing and subsequent adhesion of leukemic stem cells (LSC) to the protective areas of BM microenvironment are mediated by leukemia-stroma interactions that are critical steps during the onset of leukemogenesis. This initial homing to the BM microenvironment is facilitated by interaction between SDF-1 and its receptor CXCR4 on leukemic progenitor cells. Bone marrow stromal cells, which are considered to be the main source of chemokines in adults, constitutively express high levels of SDF-1 [61–63]. This high concentration facilitates the recruitment and retention of hematopoietic stem and progenitors cells for growth and differentiation. This is also the case for leukemic stem and progenitor cells. For example, the participation of CXCR4 in mediating tumor cell homing to the bone marrow has been observed in acute and chronic leukemias [64–66], and it has been reported that CXCR4 levels are significantly elevated in leukemic cells from patients with B cell chronic lymphocytic leukemia (B-CLL) [67]. High levels of CXCR4 have also been found in AML and in B cell acute lymphoblastic leukemia (B-ALL) [68, 69], but not in T-ALL [70–72]. More recently, our group and others have shown that CXCR4 levels are highly prognostic in AML [73–75]

and that small peptide inhibitors of CXCR4 could overcome BM stromal cell-mediated resistance to drug-induced apoptosis in AML and primary CLL [64, 76, 77]. In myeloma models, inhibition of CXCR4 blocked migration and homing to the bone marrow as shown by in vitro and in vivo methods [78]. Furthermore, SDF-1 may not only attract tumor cells to the bone marrow but also stimulate cell survival. Burger et al. found that the viability of CLL B cells was enhanced by exogenous SDF-1 in vitro in the absence of supportive bone marrow-derived MSCs [79]. It has been reported that leukemic cells are able to adapt to physiologically low concentrations of oxygen which makes them capable of proliferating even under hypoxic conditions [80, 81]. Concordantly, the expression of the hypoxia-inducible transcription factor-1 alpha (HIF-1α), the master regulator of transcription in hypoxia, has been found upregulated in clusters of leukemic cells in BM specimens from patients with primary ALL [82]. Consistent with the findings that the expression of SDF-1 [81] and CXCR4 [83] is upregulated in areas of hypoxia, we recently reported that CXCR4 expression is highly dependent on the presence of oxygen in acute myeloid leukemia (AML) [84]. In general, leukemic cells expressing CXCR4 are highly responsive to SDF-1. Several groups have shown that SDF-1 enhances very late antigen-4 (VLA-4)-mediated adhesion to the ECM components fibronectin and collagen in hematologic and solid tumors [85, 86]. In this regard, the interaction between VLA-4 on leukemic cells and fibronectin on MSCs has been shown to be crucial for the persistence of minimal residual disease in AML [87]. Following this interaction, the clustering of integrins triggers the activation of prosurvival signaling cascades such as the activation of integrin-linked kinase (ILK) which phosphorylates Akt in aPI3K-dependent manner and promotes survival of leukemic cells [88]. Adhesion of normal and malignant hematopoietic cells is also in part mediated by CD44. It has been demonstrated that LSCs homing to microenvironmental niches and the consequent maintenance of a primitive state is highly regulated by CD44 [89]. Hyaluronic acid, a glycosaminoglycan highly concentrated in the endosteal region, is the main ligand for CD44 and mediates the adhesive interactions between LSC and the BM stroma [90]. CD44 has not only a role in adhesion but can also transduce multiple intracellular signal transduction pathways upon activation with its ligands [91]. Therefore, it is important to consider that inhibition of any of these molecules could be a critical tool not only to block tumor homing and engraftment but also to revert the cell adhesion–mediated drug resistance of tumor cells that reside in the bone marrow microenvironment [92].

Fibroblasts and Stromal Precursors

It has been demonstrated that solid tumor growth cannot be sustained unless the tumor cells attract and stimulate fibroblasts. In this context, activated fibroblasts are a rich source of growth factors, such as TGF-β, IGF-1, and bFGF [93, 94], and provide organization of the tumor stroma by producing extracellular matrix components.

Based on our current understanding of tumor stroma development [95], it can be considered that stroma genesis occurs as a multistep process involving both the local recruitment of neighboring structural cells, like fibroblasts and pericytes, and systemic recruitment of BM-derived stem cells or MSCs through combined regulation of proliferation, pro-invasive, and differentiation signals [96]. These mesenchymal cells phenotypically resemble TAFs and may differentiate into fibroblast-like cells that produce ECM components and/or contribute to perivascular or vascular structures [97]. Further support for this model comes from similarities between reactive tumor stroma and stroma involved in wound repair [29]. Accordingly, many of the biological processes involved in wound repair, including stromal cell acquisition of a myofibroblast phenotype, deposition of type I collagen, and induction of angiogenesis, are observed in reactive stroma during cancer progression [98]. Given their critical role in tumor-stroma construction, one can envision the innate tropism of these cells for tumor and wounding environments.

The Innate Tropism of MSCs for Tumors and Sites of Injury

Numerous studies in a variety of animal models have shown homing of MSCs after systemic or local infusion using a vast repertoire of experimental scenarios [99, 100]. Pereira reported that upon systemic infusion of marker gene expressing MSC in irradiated syngeneic mice, the marker gene expression was detected a month later in 5% of lung cells and 8% of bone marrow cells [101]. The preferential residence homing of MSC to lung and bone when injected systemically could be easily explained by the innate ability of MSC to adhere to matrix components. In this regard, a number of reports have established that under different pathological conditions, MSC selectively homed to sites of injury irrespective of tissue or organ [102, 103]. Many other studies using fluorescent protein [104] or luciferase-labeled MSCs [105] have demonstrated that exogenously delivered MSCs can be found at sites of injury. Confirmation of this innate tropism of MSCs for wounded areas comes from experimental data in wound healing [106], tissue repair and regeneration [107–109], and brain injury [110, 111] models. Our group was first to report that BM-derived MSCs home with high efficiency to multiple tumor types in xenograft models, including melanoma, glioma, and colon and breast cancer [112–114].

In accordance, accumulating evidence now suggests that conditioning regimens prior to cell transplantation (such as chemotherapy and focal or total body irradiation treatment) enhance the efficiency of MSC homing to sites of engraftment [115–118]. It is also known that many factors commonly secreted by tumors and during an injury insult, including interleukin (IL)-1, IL-8, TNFα, TGFβ, EGF, PDGF, and SDF-1, can enhance MSC migration. It can be speculated that higher concentrations of chemoattractant mediators produced at the site of injury are requisite for MSCs to migrate, engraft, and proliferate to replace the damaged niche. In fact, tumors can be regarded as sites of tissue damage or, according to Dvorak, “wounds that never

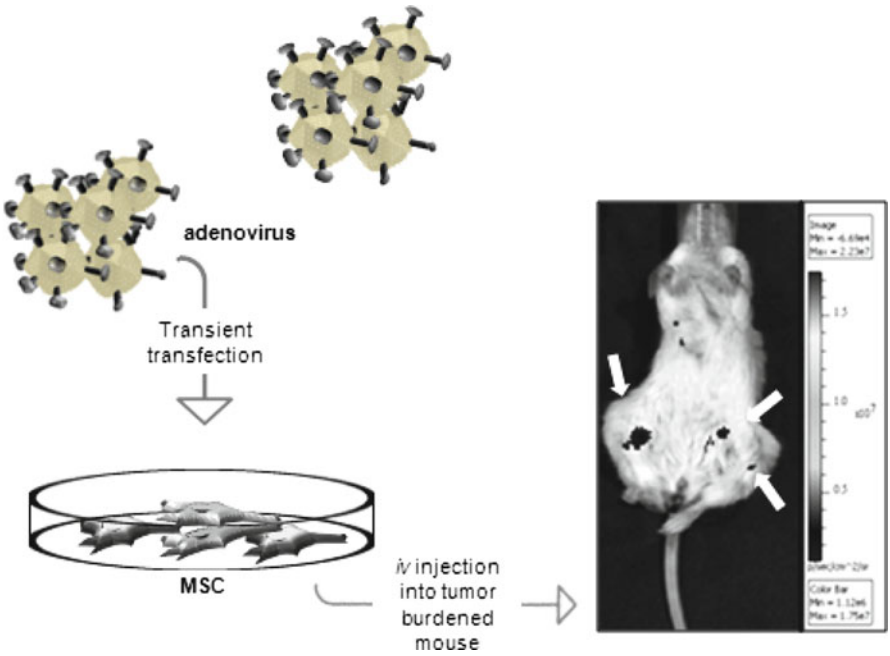

Fig. 12.5 Tracking of murine MSC migration to established bilateral 4 T1 tumors in SCID mice. Adenovirus expressing firefly luciferase is transfected into murine MSC 24 h prior to intravenous injection into the tumor-bearing mouse. MSC can be tracked by bioluminescence imaging 24 h following intravenous injection and is depicted by *white arrows*

heal" [29]. This innate tropism of MSCs for inflammatory sites such as tumors and the ability to home to and engraft in these pathological sites (and deliver therapeutics) strongly suggests the potential use of these cells as anticancer drug delivery vehicles. For example, it has been shown by several groups that MSCs modified by viral transduction methods to stably express tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) home to tumors and reduce tumor burden in multiple zenograft mouse tumor models [119–121]. Currently, in spite of the concerns associated with the use of viral transduction for MSC-mediated gene delivery and an increased risk of oncogenic transformation, the significant amount of favorable in vivo findings in this area encourages the use of this modality for future therapy and will be discussed later in this chapter (Fig. 12.5).

Choosing Stroma Precursor Cells to Target Tumors

As mentioned above, the invasion process of cancer cells is associated with the generation of tumor-specific stroma. Furthermore, fibroblasts are recruited into the stroma and can be converted into smooth muscle actin-positive fibroblasts,

i.e., myofibroblasts or activated fibroblasts. These fibroblasts can produce collagens and extracellular matrix proteins in response to several extracellular stimuli and play important roles in tumor formation and progression [122–124]. In support of these findings, our group has previously demonstrated that the tumor microenvironment preferentially promotes engraftment of intravenously administered bone marrow–derived mesenchymal stromal cells [114]. In addition, Roni et al. have shown that when β-galactosidase-transduced human fibroblasts were injected i.p. into SCID mice along with ovarian cancer cells, these β-galactosidase-positive fibroblasts preferentially localized within the cancer stroma but not within the normal tissue stroma [125]. Elegant work presented by Ischii et al. using a bone marrow transplant model has shown that stroma generated by invasive cancer cells consist of both bone marrow–derived and non-bone marrow–derived activated fibroblasts and that the bone marrow–derived activated fibroblasts are recruited into cancer-induced stroma at a later stage [126] (Fig. 12.6). In light of these findings, it has been suggested that BM-derived myofibroblasts contribute to the cancer-induced desmoplastic reaction and might change the "microenvironment" that influences tumor growth. Furthermore, these findings directly demonstrate the specific contribution of BM-derived cells to the formation of tumor stroma and strongly suggest that BM-derived cells are capable of targeting tumors due to physiological clues initiated by the tumor [127, 128]. It becomes clear that there are specific advantages in using BM-derived cells as cellular delivery vehicles. First, it is likely that most invasive cancers cause a desmoplastic reaction to some extent, thereby providing a common target for the treatment of many types of cancers. Second, the low intrinsic mutation rate of MSC makes them less prone than the genetically unstable cancer cells to acquire a drug-resistant phenotype. Although it has been published that mice implanted with high passage numbers of MSCs can develop sarcoma, there is no report to date of such event in the thousands of patients who have received MSCs mostly in the context of tissue repair studies or for the treatment of graft-versus-host disease [129]. Subsequently several papers reporting the development of tumors in mice transplanted with MSC were retracted because of tumor cell contaminations of MSC cultures [130, 131]. Taking into consideration our present knowledge of MSCs, it is reasonable to suggest the use of BM-derived myofibroblastic progenitor cells as carriers of novel therapies to prevent or inhibit local and metastatic growth of numerous cancer cells.

Cellular Vehicles to Target Tumors

When we think of cells as vehicles to target tumors, we have to consider that an ideal cell-based delivery system for cancer gene therapy must comply with certain important characteristics [132]. For example, the carrier cell should exhibit tumor-selective migratory capacity, that is, it has to recognize a highly specific "zip code" associated exclusively with tumor cells and with no other cell type, or location, in

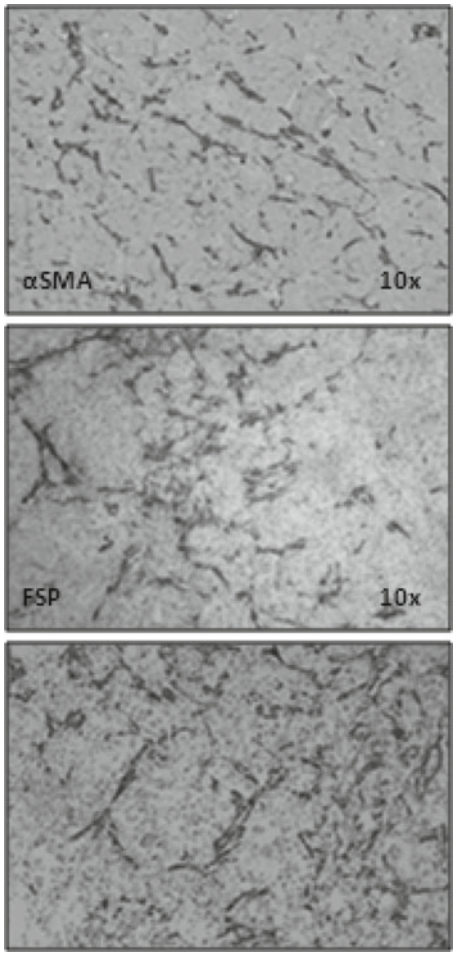

Fig. 12.6 Long-term engraftment of MSCs in tumors establishes fibrovascular networks of stroma and can be defined as tumor-associated fibroblasts displaying a variety of markers associated with activated pathogenic-associated myofibroblasts such as α-smooth muscle actin and fibroblast-specific protein as well as the presence of intermediate filaments like desmin that represent the desmoplastic stroma contributed by the MSCs

the body. In addition, it has to be possible to genetically manipulate these cells *ex vivo* in order to upregulate the expression of therapeutic genes. Lastly, the carrier cell must be capable to carry and deliver the package (the therapeutic gene or agent) to the tumor without triggering a host immune response.

In the past decade, data from several research groups have demonstrated that neural stem cells (NSC) have a unique migratory capacity throughout the brain [133, 134]. Particularly, Aboody et al. demonstrated that NSCs transplanted into animal models of brain neoplasia can migrate and be detected near metastatic tumor beds, far away from the original transplant site. Based on the inherent tumor-tropic properties of these cells, the use of NSCs has been exploited as a tumor-targeting strategy for anti-glioma gene therapy. It has been demonstrated that NSCs engineered to express genes with potent antitumor effects like IL-4

[134], IL-12, IFNβ [135], IL-23 [136], or TRAIL [137] can be very effective in controlling tumor progression. Unlike MSCs, which might be the preferred cell carrier for other organs [138], NSCs are more suitable as therapeutic delivery vehicles for central nervous system malignancies due to their intrinsic characteristics. However, the isolation and expansion of neural stem cells for clinical application is currently technically challenging, and other sources of therapeutic cells need to be considered.

In addition to regenerative and immunomodulatory applications, MSCs can serve as effective gene-delivery vehicles, and many reports from our group and others have proven the efficiency of MSCs as cell carriers for in vivo delivery of various clinically relevant anticancer agents, following engraftment within tumor sites [114, 121, 139–142].

The use of MSCs as gene-delivery vehicles is linked to a series of potential concerns. These are justified because of the lack of knowledge in regard to the homeostatic maintenance of this cell population in vivo and the possibility that MSCs themselves could be transformed and promote the growth of an existing tumor or even initiate one. The potential risk of initiating a tumor by transplanting MSCs is inherent to their self-renewal capacity and the similarities between stem cells and cancer cells. It has been suggested that mouse MSCs are capable of spontaneous transformation in culture but human MSCs are not [143]. In this regard, some studies have demonstrated that post-senescent human MSCs frequently become transformed [144]; however, we have shown that lower passage MSCs do not form tumors in vivo [114].

MSC-Mediated Delivery of Therapeutic Agents

Since our first publications establishing that MSCs engineered to express IFN-β inhibit the growth of malignant cells in vivo and demonstrated that MSCs can produce biological agents locally at tumor sites [114], many reports from other groups have also reported tumor-targeted delivery of therapeutic agents by MSC. Our initial observations have been supported by numerous studies demonstrating that genetically modified MSCs are promising tools for the selective delivery of antitumor agents since they are easy to harvest, isolate, and expand from different sources (i.e., bone marrow, adipose tissue [145], placenta [146], etc.); they are also easy to transduce with viral vectors and have the capacity to selectively home to the tumor microenvironment. Since most models utilize immunodeficient mice, immune responses against gene-modified MSCs are difficult to assess. Therefore, in our most recent work, we have favored the use of electroporation to transfer genes into MSCs, thus avoiding potential immunogenicity against (adeno) viral vectors (Andreeff, unpublished results). In the next paragraphs, we will summarize the various biological agents that have been successfully delivered to tumors using MSC as cellular vehicles.

Interferons

MSCs expressing interferon beta (IFN-β) have been shown to decrease tumor burden and increase animal survival in a number of tumor models. Interferons can mediate direct cytotoxic and immune stimulatory effects, but the systemic delivery of IFN-β or the secretion of it at sites distant from the tumor is ineffective because of its short half-life, suggesting that regional secretion is required [112, 113, 147]. Briefly, using xenograft models of breast cancer and melanoma, we demonstrated that these tumors could be controlled in vivo by weekly systemic injections of IFN-β-expressing MSCs [114]. We failed to achieve this effect by injecting recombinant IFN-β protein. This work provided first proof of principle that MSCs are a valid platform for selective in situ production of biological agents in tumors. Like IFN-β, IFN-α delivered by MSC has yielded similar results. Ren et al. reported that systemic administration of MSC-producing IFN-α reduced the growth of B16F10 melanoma cells and significantly prolonged survival in a lung metastasis model of melanoma [148]. In an aggressive model of blast crisis CML (KBM-5), systematically delivered MSCs homed to all sites of disease, including bone marrow, spleen, and lymph nodes, and produced IFNα which was secreted from a mifepristone (RU486)-activated adeno-associated virus (AAV). The effect of the locally delivered IFN-α resulted in leukemia regression and increased survival, while IFNα injected into the mice was ineffective (Andreeff, unpublished results).

Interleukins

With the rationale of improving antitumor surveillance by activating the host immune cells, the use of MSCs engineered to express interleukins has also become an alternative therapeutic strategy. Matrix-embedded MSCs engineered to secrete IL-12 injected adjacent to tumors have shown to have a significant therapeutic effect [149]. Similar to the results observed with IFN-β, no reduction in tumor growth was observed when the MSC-matrix plugs were implanted in the opposite flank of the tumor reinforcing the notion that regional secretion of the therapeutic agent is required. In addition, several reports have shown that MSCs engineered to secret IL-2 [139, 150] or IL-12 [149, 151, 152], when injected into tumor-bearing mice, elicited an immunological reaction and stimulated inflammatory cell infiltration of the tumor tissue. More importantly, the immunological reaction proceeded without causing systemic toxicity and increased levels of IL-12 in serum and in the tumor area. In contrast, free Ad-IL-12 adenovector administration only increased serum IL-12 levels and induced systemic toxicity [33]. In summary, the local delivery of the therapeutic agent at the tumor site by the MSCs seems to be better tolerated and more effective than systemic administration.

Conditionally Replicating Adenoviruses (CRAds), Retroviruses, and Lentiviruses

Natural and genetically modified oncolytic viruses have been systematically tested as an alternative approach for the treatment of tumors. Unfortunately, insufficient viral delivery to tumor sites and increased systemic toxicities, due to their nonspecific uptake in liver and spleen, are in part responsible for the poor antitumor efficacy observed in clinical trials. In order to overcome these problems, Komarova and colleagues infected MSCs with genetically modified adenovirus and showed that they were not only able to replicate in the carrier MSCs, but they were also efficiently delivered to the tumor site [153]. Using an animal model of ovarian cancer, they showed that the MSC-based delivery of adenoviruses increased the survival of tumor-bearing mice compared with direct viral injection. Similarly, in a SCID mouse xenograft model of breast cancer, Curiel et al. reported that MSCs transduced with CRAds homed to the tumor site, enhanced the oncolytic effects, and increased survival of the tumor-bearing animals when compared with animals treated with CRAds alone [154]. Altogether, these results confirmed that MSCs can mediate CRAds-specific oncolysis by acting as cellular vehicles and transporting CRAds to tumor sites. Our group recently reported that intraperitoneal injection of MSC-CRAds resulted in reduced nonspecific organ infection as compared to systemic injection of CRAds alone [155].

MSCs can also be modified to express therapeutic molecules by retroviral transduction. As an example, the antitumor activity of IL-12 has been evaluated in a mouse melanoma model by injecting human MSCs stably transduced with a retroviral vector expressing IL-12 [156]. Interestingly, the experimental data showed that MSC-IL-12 significantly reduced the formation of lung metastases of B16F10 melanoma cells. The effect was in part mediated by CD8+ T cells, with minor participation of CD4+ T and natural killer cells.

Lentiviral vectors have also been used to transduce MSCs. Human MSCs can be efficiently transduced with lentiviral vectors without affecting their stem cell properties. A recent report showed that transduced MSCs migrated efficiently to tumor sites in a mouse model of human glioblastoma [157]. The authors also showed that MSCs stably transduced to express recombinant TRAIL were resistant to the cytotoxic effects of TRAIL and that secreted TRAIL had a profound antitumor effect in vivo when MSC were implanted into mice-bearing malignant GBM8gliomas. Other groups have also used MSC-TRAIL to deliver high concentrations of TRAIL in tumor-bearing mice with similar results [158, 159].

Growth Factor Antagonist, Chemokines, and Suicide Gene Therapy

Xin et al. reported that murine MSC transduced with an adenoviral vector to express an immunostimulatory chemokine, CX3CL1 (fractalkine) injected into

mice bearing C26 and B16F10 lung metastases, strongly inhibited the development of metastases and prolonged the survival of these tumor-bearing mice [141]. In another report, Kanehira et al. examined the ability of MSC to express NK4, an antagonist of hepatocyte growth factor (HGF), and showed that systemically injected NK4-MSCs only migrated to sites of lung tumors in tumor-bearing tissues but were not found in areas where the tumors were not present [141, 160]. In addition, NK4-MSCs strongly inhibited development of lung metastases in the C26 lung metastasis model and significantly prolonged survival. These effects were mediated by the inhibition of tumor-associated angiogenesis and lymphangiogenesis and by the induction of apoptosis in the tumor cells.

Next, the introduction of suicide genes into MSCs has also been favored by other research groups. Essentially, this type of therapy involves the modification of MSCs to generate a tumor-specific prodrug-converting cellular vehicle for targeted chemotherapy. For example, the fusion gene cytosine deaminase/uracil phosphoribosyltransferase (CD) has been successfully introduced by retroviral transduction into adipose-derived MSCs [161]. CD converts cytosine to uracil and ammonia and the antifungal agent5-fluorocytosine (5FC) into the potent antimetabolite drug, 5-fluorouracil (5FU). In this report, Kucerova and collaborators demonstrated that adipose-tissue-derived MSC expressing the CD gene product (CD-AT-MSC) in combination with systemic administration of 5-FC amplified the cytotoxic effects of 5-FC on HT-29 tumor cells in vitro. Using a tumor xenograft model, they also confirmed the ability of CD-AT-MSC to deliver the CD transgene to tumors and observed pronounced antitumor effects in vivo. Within the suicide gene therapy context, Uchibori and coworkers used retroviral vectors expressing the thymidine kinase of herpes simplex virus (HSV-tk) to transduce MSCs. The systemic delivery in mice of these modified MSCs resulted in enhanced HSV-tk transgene expression in 9L malignant glioma tumors associated with significant suppression of tumor growth [162].

Cell Surface Receptors: CD44

CD44 is a class I transmembrane glycoprotein that has been shown to mediate cell growth, survival, differentiation, and migration [163] and has become one of the well-known markers of a "tumor-initiating cell" particularly in breast cancer. CD44 is associated with drug resistance, apoptosis evasion, and cell survival [164–168]. CD44 is a highly conserved gene that has 12 alternatively spliced exons [169] that make up more than 100 splice forms of the protein. The splice variability occurs in the extracellular portion of CD44. In addition to the splice variants, posttranslational modifications (e.g., glycosylation) account for the differences in CD44 expression between cell types and local environments. CD44 variant expression in cancer cells is often associated with a metastatic phenotype as compared to the primary tumor or normal tissue. This phenomenon pertains to several cancer models including prostate [170], breast [171], and lymphoma [172]. The cytoplasmic tail of CD44 is known to interact with components of the cytoskeleton including ankyrin and the ERM (ezrin, radixin, and moesin) proteins which are thought to regulate cell migration and shape [173, 174].

Known CD44 ligands include osteopontin, hyaluronan, hyaluronan fragments, fibronectin, laminin, and collagen type I [175]. Such ligands are often found in wounded or inflamed environments including the tumor microenvironment, suggesting a role for CD44 in the tumor-tropic migration of MSC.

MSC express high levels of CD44 including several variant forms (unpublished data). Given that CD44 variant expression on tumor cells is associated with metastatic potential, genetic modification of CD44 expression on MSC is a potential tool to alter and enhance the efficiency of the MSC as gene-delivery vehicles. Furthermore, once MSCs have infiltrated the tumor, CD44 expression is also a potential antitumor target. Wallach-Dayan and colleagues published data showing the efficacy of CD44 variants cDNA vaccination reducing the metastatic potential of breast tumors [171]. Others have shown the efficacy of CD44 disruption within the tumor using receptor and oligomer antagonists to show marked decrease in tumor growth including an induction of apoptosis [176, 177]. Furthermore, a number of CD44 antibodies are under investigation as targeted antitumor therapeutics; however, as previously described by Matsuki and colleagues, alterations in glycosylation within the tumor microenvironment may ultimately inhibit the efficacy of antibody binding [178]. In a murine leukemia model, Dick's group reported major antileukemia effects using another CD44-blocking antibody [89]. In conclusion, the expression potential of CD44 is dynamic and could be used to augment the migration efficacy of MSCs toward tumor niches. Conversely, blocking CD44 may disrupt migration and tumor support by MSCs.

Conclusions

In this chapter, we attempted to summarize the role of stroma in tumor development and progression and the importance of MSC as potential cellular carriers of therapeutic agents to the tumor sites. We believe that the tropism of MSC for solid tumors, hematological malignancies, and their microenvironments is based on their innate physiological ability to home to sites of inflammation and tissue repair. It appears that the advantage of using MSCs as cellular vehicles lies in the local delivery and release of therapeutic agents intra-tumorally. It is important to highlight the advantage of using cellular vehicles over other vector systems as cellular vehicles can deliver the therapeutic agent with higher efficacy and much less toxicity and can also serve as protective "coating" for the delivery of replicating onco-selective viruses. Although the use of MSCs in cancer gene therapy is based on their tumor selectivity, strategies to improve tumor homing should be considered if we want to increase the utility of MSCs as tumor-targeted cell delivery vehicles. Their homing ability may be enhanced by manipulating culture conditions to alter the expression profiles of cell surface receptors or by isolating an "enriched" tumor homing MSC population that expresses specific receptor profiles in response to different agents. Additionally, it might be possible to educate the MSC to change their properties during the delivery process resulting in improved tumor targeting. Increased

"wounding" of tumors by local irradiation of systemic chemotherapy can also increase the homing of gene-modified MSCs. Advances in virology, molecular and cell biology, and gene-delivery technologies will hopefully help to optimize the development of targeted cellular vectors. Efforts should be directed toward the development of cell-based delivery systems capable of effectively transporting various therapeutic agents to tumor-specific areas. In conclusion, a better understanding of biology, pharmacology, tissue stem cell engineering, and gene transfer technologies will aid in the development of a safe, effective, and targeted MSC-based cancer therapy that exploits the innate ability of MSCs to migrate to sites of tumors and their metastasis.

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Chapter 13
MSC Studies in Large-Animal Models

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Abstract Mesenchymal stromal cells found within bone marrow, fat, and other tissues are a population of cells with the potential to mediate therapeutic outcomes based on differentiation into multiple cell lineages or through paracrine-mediated mechanisms that influence angiogenesis, apoptosis, or immune response. Currently, mesenchymal stromal cells are being widely investigated for numerous tissue engineering and regenerative medicine applications. Appropriate animal models will be crucial to the development and evaluation of regenerative medicine-based treatments and eventual cures for debilitating diseases. Here, we summarize the ongoing research focused on studying the biological and therapeutic potential of mesenchymal stromal cells in large-animal models.

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_13,

Introduction

The regenerative potential of mesenchymal stromal cells (MSCs), based on their differentiation potential, paracrine effects, immunomodulatory properties, and their abilities to influence and recruit other cells including tissue-resident progenitor cells, makes them an attractive option for treating various diseases and injuries. The potential of MSCs to replace damaged tissue, stimulate endogenous stem cell populations, home to damaged areas and modulate immune cells is promising, but must be proven safe and effective in preclinical animal models before therapeutic use can become a viable option in humans.

Most research on stem cells, including MSCs, has involved cells derived from rodent and human tissues; however, MSCs have been isolated and characterized from large animals including goat [1, 2], dog [3], cat [4], sheep [5–7], pig [8, 9], horse [10, 11], cow [12], and nonhuman primates [13–15]. Several of these animals are used as models for biomedical research. The primary benefit of large-animal models in research is the greater control and precision that the larger size permits for surgical and reagent delivery needs. The similarity in organ size in some animal models allows for better models of surgery and possible future xenotransplantation. Moreover, large animals permit the researchers the ability to collect multiple biopsies and samples and obtain many more cells from one sample because of the ability to draw larger volumes. Another advantage is the ability to noninvasively monitor organs, tissues, track cells, growth, expansion, etc. multiple times without having to sacrifice the animal. Large-animal models also permit the assessment of neuromotor and behavioral testing using many assays that have relevance to humans. It is evident that large-animal models provide an important step in the preclinical testing of potential therapies before their use in humans. The goal of this chapter is to summarize the research done on the application of MSCs for disease treatment in preclinical large-animal models.

MSCs for Orthopedic Applications

Canine Models

The dog is one of the most widely used large-animal models for musculoskeletal and dental research [16]. This is due to the fact that commercially available implants and surgical equipment for canine orthopedic surgery exist and that the organic bone compositions of human and canine bone are very similar [17].

The canine has served as a model for studies in the bone engineering of the cranium using adult stem cells. Mankani et al. [18] studied autologous culture-expanded bone marrow stromal cells (BMSCs) seeded into beta-hydroxyapatite and beta-tricalcium phosphate (HA/TCP) constructs in a critical-sized calvarial defect in a canine model. At intervals ranging from 2 to 20 months, transplants were assessed

using quantitative computed tomography (CT) scans and ultrasound or biopsied/harvested for histological and mechanical analysis. In all animals, BMSC-containing transplants formed significantly more bone than their control counterparts. Moreover, BMSC-infused bone possessed mechanical properties similar to adjacent normal bone, confirmed by both ultrasound, quantitative CT, and ex vivo analysis. The data from this study demonstrate that autologous cultured BMSC transplantation is a clinically feasible method for treating large-sized bone defects and that the transplants can be assessed noninvasively.

Cui et al. [19] studied culture-expanded autologous adipose-derived MSCs (ASCs) differentiated along the osteogenic lineage and seeded on coral scaffolds to repair a bilateral full-thickness cranial bone defect in a canine model, and followed up the outcome for up to 6 months. Three-dimensional CT scans showed that bone formation occurred in the experimental group at 12 weeks post-implantation, while coral scaffolds were partially degraded in the control group. At 24 weeks post-transplantation, radiological analysis determined that an average of 84.2% of each defect volume was repaired in the experimental arm, while controls had only 25.0% of the volume regenerated. Histological examination revealed that the defect was repaired by typical bone tissue in the experimental cohort, while only minimal bone formation with fibrous connection was observed in the control group.

Bruder et al. [20] performed one of the first studies aimed at the repair of segmental critical-size defects (CSDs) of long bones in the femora of adult female dogs by comparing the effect of HA/TCP ceramic constructs or HA/TCP seeded with autologous culture-expanded BM mesenchymal stromal cells (MSCs). The authors verified that radiographic union was established rapidly at the interface between the host bone and the implants seeded with BM-MSCs, whereas in bone transplanted with the empty scaffolds, several fractures occurred during the post-operative period. Arinzeh et al. [21] performed a study with an identical CSD canine model to evaluate the therapeutic effect of allogeneic BM-MSCs loaded onto a hollow ceramic cylinder also composed of HA/TCP without immunosuppressive therapy. No adverse host immune response was detected at any time point by histological analysis, and no antibodies to the donor cells were detected in the serum of transplanted animals. New bone tissue had formed throughout the implant, and implants loaded with allogeneic or autologous cells had significantly greater amounts of bone within the available pore space than did cell-free implants. Jang et al. [22] used a 1.5-cm diaphyseal defect in the radius of Beagle dogs, stabilized with plate and screws for osteosynthesis, to evaluate allogeneic umbilical cord blood-derived mesenchymal stromal/stem cells (UCB-MSCs) mixed with TCP and wrapped with PLGC membrane. This study indicated that the mixture of UCB-MSCs and TCP was a promising osteogenic material for repairing bone defects.

In the engineering of cartilage tissue, Yamazoe et al. [23] evaluated the contribution of BM-MSCs in the repair of a critical osteochondral defect. The results suggested that the autologous transplantation of atelocollagen gel with canine BM-MSCs did not contribute to the repair of the articular cartilage, but instead made significant contributions to the regeneration of the subchondral bone tissue.

Small Ruminant Models

The use of sheep and goats as models for orthopedic research continues to increase in popularity due to similarities with humans in weight, size, joint structure, bone/cartilage regenerative processes, and thus the potential in translational research [24–29]. In the last few years, sheep and goat models have also been applied to adult stem cell studies of bone and cartilage repair with tissue engineering approaches, which permit the testing of therapeutic strategies in large segmental bone and osteochondral defects similar to trauma, inflammation, or tumoral excision injuries in humans [30–32]. The majority of these studies make resource of scaffolds seeded with autologous BM-MSCs, which are directed toward the healing of critical-size defects (CSDs).

Small ruminant animal models have been used for studies of bone regeneration in the head and neck area. An initial study was aimed at the repair of cranial defects in sheep. This study used BM-MSCs within the first three passages and after osteogenic induction was mixed with calcium alginate for implantation in bilateral cranial defects [33]. Histological analysis revealed new bone formation 6 weeks after repair, which became more mature by 18 weeks, and CT scanning showed that the bone defects were almost completely repaired. Chemical analysis showed that the engineered bone defect contained a high level of calcium (71.6% of normal bone tissue), demonstrating a good degree of mineralization. The control defects, implanted with calcium alginate, were maintained almost unrepaired.

For oral and maxillofacial surgery, injectable cells or PRP composites have been used as grafting materials for maxillary sinus floor augmentation and onlay plasty. Xi et al. [34] investigated the feasibility of using natural coral as scaffolds to repair caprine mandibular segmental defect. Autologous BM-MSCs expanded in the presence of recombinant human BMP-2 to enhance osteoblastic differentiation were seeded into coral and implanted into mandibular defect, and the defect was reinforced by titanium reticulum. New bone was observed by histological analysis on the surface and in the pores of coral, whereas in the control group (matrix alone), no evidence of osteogenesis was detected. The results showed that new bone grafts were successfully restored by 16 weeks after implantation.

In the field of periodontal tissue regeneration, a study performed in goats by Marei et al. [35] investigated autologous BMSCs in implant fixtures – porous hollow root-form poly(DL-lactide-co-glycolide) scaffold around the titanium. The results showed that on the experimental side, periodontal-like tissue with newly formed bone was demonstrated both at 10 days and after 1 month, while the control specimens (scaffold alone) showed early signs of connective tissue regeneration around the titanium fixture at 10 days, but was not in the 1-month specimens. The transplanted undifferentiated BM-MSCs were capable of undergoing differentiation into tissues required for periodontal tissue regeneration, namely, cementum, bone, and periodontal ligament.

Sauerbier et al. [36] performed an ovine split-mouth study to compare bovine bone mineral alone and in combination with BM-MSCs regarding their potential in

sinus augmentation to optimize bone formation and osseointegration of dental implants. After 8 and 16 weeks, the bone formation was significantly faster, by 49%, in the transplants composed of bovine bone mineral and the BM-MSCs accelerated new bone formation in this model of maxillary sinus augmentation, which could allow early placement of implants.

Kon et al. [37] evaluated the repair segmental CSDs of long bones using an HA cylinder seeded with BM-MSCs in the tibia of sheep. After 2 months, the bones that received transplants were evaluated by microradiography, bone histomorphometry, and scanning electron microscopy to monitor newly formed bone within and around the implants and the density of mineralization. An identical CSD sheep model was also used by Bensaid et al. [38] to study the tissue-engineered bone regeneration efficiency of a combination of a coral scaffold with in vitro-expanded BM-MSCs and proved that it was possible to develop a weight-bearing bone in large animals. In the study, a massive bone defect was reconstructed by transplanting BM-MSCs expanded in autologous serum combined with a porous scaffold. The authors verified that the BM-MSCs were distributed over the scaffold and enhanced bone formation of the transplant. Due to the high resorption rate of natural coral scaffold, the authors also developed a new porous coralline-based HA scaffold, with lower resorption rate, which was used in combination with BM-MSCs, and they concluded that this was completely replaced by newly formed, structurally competent bone within 14 months.

Lucarelli et al. [39] tested whether the combination of BM-MSCs and platelet-rich plasma was able to increase massive allograft integration in a CSD in the middle diaphysis of the metatarsal bone of sheep. Results of radiographs, mechanical tests, and histomorphometric analysis, including new vascularization, showed substantial new bone formation in the allograft with the BM-MSCs and PRP group.

Zhu et al. [40] studied the bone regeneration in defects created in the femur of weight-bearing goats through the application of expanded and osteogenically induced BM-MSCs seeded to natural coral cylinder scaffolds. At 8 months, the group of animals that received the coral constructs loaded with the induced BMSCs demonstrated newly formed cortexed bone. The tissue-engineered bone segment revealed a similarity to the contralateral femur comparing the bend load strength and bend rigidity. In contrast, the coral cylinders alone of the control group showed no bone formation.

In another study, Nair et al. [41] evaluated a triphasic ceramic-coated hydroxyapatite (HASi) construct seeded with osteogenically differentiated BMSCs for its potential to heal segmental femoral diaphyseal defect in goats. At 4 months, the performance of HASi/BM-MSC was better and faster, as evidenced by the lamellar bone organization of the newly formed bone throughout the defect. On the contrary, with naked HASi, only immature woven bony bridges still intermingled with scattered small remnants of the material in the middle region of the defect were detected.

Niemeyer et al. [42] studied the regenerative capacity of BM-MSCs and ASCs in an ovine CSD model and, based on their immune privileged status, suggested that BMSCs could be used in xenogenic applications. For this purpose, human and ovine

BM-MSCs were cultured on mineralized collagen and implanted into a 3.0-cm segmental sheep tibia bone defect with an allowed postoperative period of 26 weeks. Radiology and histology demonstrated significantly better bone formation after transplantation of autologous ovine BM-MSCs on mineralized collagen compared with unloaded matrices and with the xenogenic transplantation. Nevertheless, no local or systemic rejection reactions were observed after transplantation of human BMSCs and their presence could be demonstrated by human-specific in situ hybridization. The authors concluded that xenogenic transplantation of human BM-MSCs results in poorer bone regeneration than autologous transplantation of ovine BM-MSCs. Another study of Niemeyer et al. [43] compared the osteogenic potential of BM-MSCs and ASCs seeded on mineralized collagen sponges and also evaluated the influence of PRP for the repair of CSD in sheep tibia. Radiographic evaluation revealed a significantly higher amount of newly formed bone in the BM-MSC group compared to both the ASC group at 10 weeks and empty matrix group at 12 weeks post-transplantation. However, the ASC/PRP group had better bone development than the empty control group, but not the ASC-only group. This study indicated that BMSCs may be superior to ASCs in this model.

Rodrigues et al. [44] studied the in vivo performance of cell-scaffold constructs composed of autologous BMSCs seeded in a blend of starch with polycaprolactone (SPCL) fiber mesh scaffolds at different stages of development in the noncritical-size defect in the goat femur. Drill defects alone and defects filled with scaffolds without cells were used as controls. In vivo experiments indicated that bone neoformation occurred in all femoral defects as measured by intravital fluorescence markers and histomorphometric analysis. The results provided important insights about the performance of SPCL constructs seeded with BM-MSCs. However, a more detailed analysis needs to be performed.

Feitosa et al. [45] used an ovine model for osteonecrosis of the femoral head (ONFH) to investigate bone tissue recovery following transplantation of ovine BM-MSCs and human immature dental-pulp stem cells (hIDPSCs). The ONFH model was induced by ethanol through central decompression (CD). The histological results obtained from the experimental group of CD with hIDPSCs suggested that the bone regeneration was better than with the CD-only group apparently favoring bone regeneration of damaged tissues. The results of this study indicate that both types of stem cells were capable of undergoing proliferation within injured region and mediating some recovery of bone tissue.

Boos et al. [46] compared the potential of directly autotransplanted cells (no in vitro expansion) versus BM-MSCs expanded in vitro in the absence or presence of BMP-2. The cells were incorporated in to a large-volume ceramic bone substitute and assessed for bone regeneration in a sheep model. Both, directly autotransplanted and expanded BM-M SCs were constantly proliferating and had decreasing apoptosis over time in vivo. Directly autotransplanted BM-MSCs led to bone neoformation using a beta-TCP/HA matrix comparable to the application of BMP-2 only or implantation of expanded BM-MSCs. The authors concluded that ectopic bone could be generated using directly autotransplanted or expanded BMSCs with beta-TCP/HA granules alone, so BMP-2 stimulation could become dispensable in the future.

MSCs may also serve to mediate a therapeutic benefit in the engineering of cartilage tissue. Guo et al. [47] performed one of the first studies in this field, seeding culture-expanded undifferentiated autologous BM-MSCs into bioceramic scaffold beta-TCP in an attempt to repair articular cartilage defects in a sheep model. Twenty-four weeks post-transplantation, the defects were resurfaced with hyaline-like tissue, and an ideal interface between the engineered cartilage, the adjacent normal cartilage, and the underlying bone was observed.

Mrugala et al. [48] performed partial-thickness lesions in the inner part of the patellae of the posterior legs of sheep. Lesions were filled with autologous BM-MSCs, with or without chitosan and TGFβ-III, in a fibrin clot. At 2 months after implantation, histological analysis revealed chondrocyte-like cells surrounded by a hyaline-like cartilaginous matrix that was integrated to the host cartilage when BMSCs were combined with chitosan and TGFβ-III.

Zscharnack et al. [49] studied the chondrogenic in vitro predifferentiation of autologous BM-MSCs embedded in a collagen type I hydrogel currently in clinical trial use for matrix-associated autologous chondrocyte transplantation to verify if this procedure facilitates the regeneration of a chronic osteochondral defect in an ovine stifle joint. To achieve a chronic defect model, osteochondral defects were created at the medial femoral condyles of the stifle joint, but the implantation of the BMSC/hydrogel constructs was just performed 6 weeks after the defect creation and the evaluation followed after 6 months. The defects treated with predifferentiated BM-MSC-gels showed significantly better histologic scores with morphologic characteristics of hyaline cartilage (e.g., columnarization and presence of collagen type II). The authors concluded that the results suggest an encouraging method for future treatment of focal osteochondral defects to prevent progression to osteoarthritis.

In cell-based therapy for osteoarthritis, Murphy et al. [2] studied the role implanted autologous in vitro-expanded BM-MSCs may play in tissue repair or regeneration of the injured joint following induction of osteoarthritis (OA) in a caprine model. OA was induced unilaterally in the knee joint of donor animals by complete excision of the medial meniscus and resection of the anterior cruciate ligament, and 6 weeks later, the autologous BM-MSCs, suspended in a dilute solution of sodium hyaluronan, were delivered to the injured knee by direct intra-articular injection. The cell-treated joints showed marked regeneration of the medial meniscus, and implanted cells were detected in the newly formed tissue. Degeneration of the articular cartilage, osteophytic remodeling, and subchondral sclerosis were reduced in cell-treated joints compared with joints treated with vehicle alone without cells, but with no evidence of repair of the ligament in any of the joints.

Another study reports a tissue engineering approach with MSCs to enhance bone formation around hip replacements to improve the longevity of the implant and enhance quality of life. Korda et al. [50] investigated the impact of BM-MSCs mixed with an allograft in an ovine hip hemiarthroplasty model. The results indicated by non-decalcified histology that MSCs on an allograft scaffold increased bone formation, indicating that the use of these cells for revision hip arthroplasty may benefit patients undergoing revision surgery in whom the bone stock is compromised.

Sheep are similar to humans in vertebral anatomy, particularly at C3–C4 [51]. This model has been used in spinal conditions, specifically for spinal instability and spinal fusion, such as in the study by Tan et al. [52] with a HA scaffold seeded with autologous BM-MSCs and fibrin. The sheep underwent a posterolateral spinal fusion in which scaffolds with or without BM-MSCs seeding were implanted on both sides of the lumbar spine (L1–L2) and the fusion segments were immobilized using wires. After 3 months, fibrous tissue infiltrated the interconnecting pores of plain HA ceramics of the fusion constructs, indicating inefficient new bone regeneration, while new bone was found surrounding the HA ceramics seeded with autologous cells and fused naturally with the vertebrae. The new bone formed in the sheep was generated by the BM-MSC-encapsulated HA. The authors concluded that incorporation of autologous BM-MSCs improved the effectiveness of HA ceramics for spinal fusion.

Kruyt et al. [53] developed a multiple-condition model focused on the initial process of bone formation from the transverse process and not on a functional fusion obtaining a reliable and highly efficient method to study bone formation in cell-based tissue engineering. The effect of uncharacterized autologous stromal cells expanded to passage one seeded into different porous ceramic scaffolds was investigated with polyacetal cassettes designed to fit on the goat transverse process and house four different ceramic blocks: HA sintered at 1,150° and 1,250°, BCP and TCP. The cassettes were bilaterally mounted on the dorsum of decorticated L2-processes for 9 weeks. To assess the dynamics of bone formation, fluorochrome labels were administered and histomorphometry was focused on the distribution of bone in the scaffolds. The effect of BM-MSC seeding was observed in three of four scaffold types, especially in scaffold regions adjacent to the overlying muscle with the BCP and TCP scaffolds showed generally better osteoconduction and an increased response to stromal cell administration.

More recently, Goldschlager et al. [54] developed an anterior cervical discectomy (ACD) and fusion in sheep since this is one of the most common surgical procedures for cervical radiculopathy and/or myelopathy in patients unresponsive to conservative treatment. The authors confirm that this methodology is useful in preclinical studies, to evaluate different devices and biologics, including stromal cells, for potential clinical application in the surgery of spine.

Periodontal Tissue Engineering

Canine Models

Experimental canine models have been developed in order to reproduce major periodontal diseases (gingivitis, periodontitis) and their pathogenesis as well as to investigate novel surgical techniques [55].

Jafarian et al. [56] used a canine full-thickness alveolar defect model to compare BM-MSC-based bone regeneration in HA/TCP and Bio-Oss matrices. These scaffolds loaded with BM-MSCs were implanted in the masseter muscle and bilateral dog mandibular body defects and evaluated 6 weeks after insertion. Histological analysis of the decalcified scaffold and scanning electron microscopy demonstrated large BM-MSC coverage of the HA/TCP and Bio-Oss related to the control group. The HA/TCP loaded with BM-MSCs demonstrated more bone regeneration than Bio-Oss/cell, but there was no statistically significant difference.

Periodontal disease causes severe destruction of periodontal tissue, including alveolar bone. Ito et al. [57] tested tissue-engineered bone as grafting material for alveolar augmentation with simultaneous implant placement. Dog BM-MSCs combined with fibrin gel and PRP or fibrin only were used as grafting material for alveolar augmentation. The MSC/fibrin/PRP implant resulted in the greatest extent of bone implant at all time points analyzed.

Cardiac and Coronary Ischemia

Since adult human myocardium cannot efficiently regenerate because cardiac muscle cells do not efficiently reenter the cell cycle, the applicability of myoblasts, fetal cardiomyocytes, and BM-MSC-derived cardiomyocytes for replacement of ischemic myocardium has been widely investigated [58–60]. The phenomenon of stem/progenitor cell-induced angiogenesis in acute and chronic ischemic myocardium has been reproduced in several large-animal models [61]. These models are ideal for testing MSCs therapeutically.

Canine Models

Silva et al. [62] induced a chronic ischemia model by ameroid constrictor placement. Thirty days later, dogs received intramyocardial injections of BM-MSCs. The authors observed a trend toward reduced fibrosis and greater vascular density in the MSC-treated group. Moreover, the cells differentiated into smooth muscle and endothelial cells, resulting in increased vascularity and improved cardiac function. Bartunek et al. [63] used a dog chronic MI model generated by ligation of the coronary artery to study the in vivo cardiac differentiation and functional effects of differentiated adult autologous BM-MSCs injected 8 weeks after the infarction. The cells differentiated along the cardiac lineage after culture in the presence of basic FGF, IGF-1, and BMP-2. Infusion of the differentiated cells improved cardiac differentiation in vivo and mediated a functional recovery. The same group performed another study in the same chronic MI model comparing the

therapeutic effect of bone marrow mononuclear cells (BMMCs) versus BM-MSCs [64]. The authors concluded bone marrow mononuclear cell transfer was superior in improving cardiac contractility and regional systolic function and reduced infarct size and the plasma N-terminal B-type natriuretic propeptide level. Functional improvement was associated with a favorable angiogenic environment and neovascularization.

Perin and collaborators assessed the safety of transendocardial electromechanical-guided delivery of BM-MSC therapy in a canine acute myocardial ischemia model [65]. The authors compared intracoronary delivery with transendocardial delivery and concluded that the higher local cell density with transendocardial administration may be more effective.

Ruminant Models

Vincentelli et al. [66] evaluated either autologous BM-MSCs or BMMCs, implanted in a decellularized porcine scaffold to promote in vivo recolonization and limit valve deterioration. Decellularized porcine pulmonary valves were implanted in the pulmonary artery under cardiopulmonary bypass in lambs. At 4 months, valve function evaluated by echocardiography demonstrated that the mean transvalvular and distal gradients were lower in the MSC-transplanted animals than those in the BMMC group. Histological examination showed recolonization and re-endothelialization of explanted valves in both groups with significant valve thickening and inflammatory cell infiltration observed in the BMMC group. In contrast, valves from the BM-MSC group displayed extracellular matrix and cell disposition close to those of native pulmonary valves. The engineered heart valves created from MSCs implanted in a decellularized xenograft scaffold demonstrated satisfactory hemodynamic and histological aspects.

Hamamoto et al. [67] studied the therapeutic effect in damaged myocardium of allogeneic STRO-3-positive mesenchymal precursor cells (MPCs) isolated from male crossbred sheep MPCs were injected in the border zone of the myocardium of female sheep that underwent coronary ligation to produce a transmural left ventricular (LV) anteroapical infarction. Echocardiography was used after myocardial infarction (MI) to quantify LV end-diastolic (LVEDV) and end-systolic volumes (LVESV), ejection fraction (EF), and infarct expansion. Immunohistochemical staining for CD31 and smooth muscle actin (SMA) was performed on the infarct and border zone to quantify vascular density. Compared with controls, MPCs injected at low cell numbers significantly attenuated infarct expansion and increases in LVEDV and LVESV. The EF was improved at all cell doses. CD31 and SMA immunohistochemical staining demonstrated increased vascular density in the border zone only at the lower cell doses. Allogeneic STRO-3-positive MPCs attenuated the remodeling after transmural MI that was associated with vasculogenesis and arteriogenesis within the border zone and infarct.

Nervous System Repair

Preclinical studies in large-animal models for peripheral nerve or spinal cord injury (SCI) and cerebral ischemia for tissue-engineered therapeutic approaches have been developed.

Canine Models

Lim et al. [68] performed the first study to determine the effects of allogeneic UCB-MSCs and human granulocyte colony-stimulating factor (G-CSF) on a canine SCI model after balloon compression at the first lumbar vertebra. The UCB-MSCs were injected directly into the injured site and G-CSF was administered subcutaneously 1 week after the induction of the injury. Functional recovery in the animals was monitored at 4 and 8 weeks post-transplantation. The authors verified that there was significant improvement in the nerve conduction velocity based on the somatosensory-evoked potentials and a distinct structural consistency of the nerve cell bodies was observed in the lesion of the spinal cord on the UCB-MSC and co-treatment groups. The same research team performed another study in a canine SCI model in which they evaluated if implantation of allogeneic ASCs could improve neurological function [69]. The ASCs were delivered by direct injection into the injured site 1 week after induction. Evaluation of pelvic limb function suggested an improvement in neurological function after transplantation of ASCs possibly due, in part, to the neural differentiation of the ASCs. Histopathologically, animals in the ASC group had more Luxol fast blue-positive area, indicating more myelin, as well as implanted cells that stained positive for GFAP, NF160, and Tuj-1.

Lee et al. [70] induced acute SCI by percutaneous balloon compression in dogs in order to test whether percutaneous transplantation of human UCB-MSCs improved neurological functional recovery. The UCB-MSCs were transplanted into the cranial end of the injured segment 7 days after SCI. The dogs that received cellular transplants exhibited gradual improvement in hind limb locomotion from 3 weeks after cell transplantation. The authors also detected UCB-MSCs in the spinal cord lesions at 4 weeks post-transplantation, which reduced cyst and injury size.

Chung et al. [71] examined the effects of human UCB-MSCs delivered through the basilar artery in a canine thromboembolic brain ischemia model. The ischemia was induced through the occlusion of the middle cerebral artery by injecting thrombus emboli. In the UCB-MSC-injected animals, a decrease in the infarction volume after cerebral ischemic induction was observed at 1 week, whereas control animals revealed an increase in infarction volume at the same time point. Transplanted cells had differentiated into neurons and astrocytes. The cells also expressed neuroprotective factors, such as brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) 4 weeks after transplantation. The transplanted

cells demonstrated their efficacy by reducing the infarction lesion volume and through earlier recovery from the neurological deficit.

In 2010, Ding and colleagues investigated peripheral nerve repair with tissue-engineered nerve grafts composed of chitosan/PLGA-based neural scaffold infused with autologous MSCs [72]. The nerve autografts were tested for their ability to bridge 50-mm-long gaps in an injured dog sciatic nerve. Six months after nerve grafting, the transplants were evaluated for the degree of nerve tissue regeneration and reinnervation of target muscle. The results indicated that autologous MSCs implanted in to the chitosan/PLGA-based neural scaffold promoted sciatic nerve regeneration and functional recovery with an efficacy similar to nerve autografting.

Nonhuman Primates

MSCs capable of differentiating into osteogenic, adipogenic, and chondrogenic cell types have been isolated and analyzed from several nonhuman primate species, including rhesus [13, 14], cynomolgus monkey [73], and baboon [74]. In a direct comparison, early passage rhesus BM-MSCs and ASCs had equivalent culture requirements, morphology, growth kinetics, multi-lineage differentiation capabilities, and clonal efficiencies to human BM-MSCs and ASCs [14]. Based on the analogous nature of nonhuman primate and human MSCs, combined with their phylogenetic, anatomical, and physiological relatedness, nonhuman primates are valuable for analysis of cell-based therapies and translational research [75, 76]. Nonhuman primates can especially lend insight to complex immune system reactions or complications associated with allogeneic, autologous, or even xenogenic transplants; however, expense and ethical consideration are considered big challenges [77–79]. Rhesus monkeys are the most commonly used nonhuman primate species in biomedical research. In part, this is because of their ability to reproduce in captivity. Furthermore, the physiology, immunology, genetics, and anatomy of the rhesus monkey have been well characterized. Further, since nonhuman primate colonies are not inbred, they provide genetic diversity in order to study individual variations of characteristics of MSCs in vitro and reaction to cell transplantation in vivo.

Wakao et al. [80] used autologous MSC-derived Schwann cells to treat cynomolgus monkeys with peripheral nervous system injury. In their study, cells were first put into trans-permeable tubes filled with collagen and then transplanted directly to the median nerve, which had been surgically transected immediately prior. Transplantation of cell grafts led to functional recovery with no adverse effects up to 1 year after surgery. However, the cells were not tracked; therefore the specific role of the transplanted cells in the recovery could not be determined.

MSC-derived neurons have also been transplanted to the injured central nervous system of rhesus monkeys with promising results [81]. In this study, spinal cord injury was induced, and 2 weeks later differentiated MSCs were transplanted

directly to the site of injury. Animals receiving the cell treatment showed significant improvement and functional recovery, although, again, the contribution of the cells to the recovery was unclear.

Undifferentiated rhesus BM-MSCs have been injected directly into the central nervous system (CNS) [82, 83]. It was first shown that after direct intracranial administration to young, healthy rhesus monkeys, MSCs could engraft and disseminate throughout the CNS, with no adverse effects on animal health, behavior, or motor function up to 6 months after surgery [82]. The same group later reported higher engraftment levels in infant rhesus monkeys compared to young adults, also with no adverse effects on the health or behavior of the animals up to 6 months [83].

It was recently shown that human MSCs can be neuroprotective in a nonhuman primate cerebral ischemia model [84]. The ischemia model was used to investigate the neuroprotective effect of transplanted human BM-MSCs. Results showed that human BM-MSC transplantation in ischemic tissues improved neurological functions and induced an increase in IL-10 expression, which mediate anti-inflammatory effects in vivo. The levels of neuronal apoptosis and astroglial activity in the peri-ischemic area decreased, and the number of proliferating cells in the SVZ increased.

Kidney Disorders

Ruminants

The applicability of MSCs as an intervention for acute renal failure (ARF) was recently investigated in a sheep model [85]. Autologous BM-MSCs were injected into the renal artery in an ischemia reperfusion injury (IRI) model to assess the effects of timing of administration between injury and cell transplantation on both engraftment and repair. BM-MSCs were transplanted via the renal artery in animals induced to have IRI (via percutaneous transluminal placement of a balloon catheter in the renal artery) or in healthy animals. The authors verified that all the sheep showed renal engraftment by BM-MSCs, in both the tubules and glomeruli. The transplanted cells expressed tubular epithelial cell markers and had a podocyte phenotype. A significant increase in the frequency of tubules was observed when BM-MSCs were injected shortly after the injury, suggesting that the time frame of cell transplantation may be important to an improved outcome. In a second study by the same research team, the effect of autologous MSC transplantation was investigated in an alternative ovine large-animal model of bilateral kidney ischemia-reperfusion injury [86]. Renal bilateral ischemia was induced in sheep, and the animals with ischemia reperfusion injury were treated by the injection of autologous MSCs or with vehicle medium only. Transplanted MSCs were found in glomeruli but not in tubules. However, the cells did not express glomerular cell markers, and functional analysis failed to demonstrate any beneficial effects derived from the MSC infusion. Since morphological and molecular analyses corroborated the functional

results, the authors concluded that MSCs did not repair kidney parenchyma and failed to modulate cell death and proliferation.

Other Studies in Large-Animal Models

Bladder Regeneration

Zhang et al. [87] assessed the potential of passage 1 BM-MSCs seeded into a biodegradable scaffold versus bladder smooth muscle cells (SMCs) for bladder regeneration in a canine model. In vivo, both BM-MSC-seeded and bladder cell-seeded SIS grafts presented solid smooth-muscle bundle formation throughout the graft. The BM-MSCs had a similar cell proliferation, histological appearance, and contractile phenotype as primary cultured bladder SMCs. SIS supported three-dimensional growth of BMSCs in vitro and BMSC-seeded SIS scaffold promoted bladder regeneration in the canine model.

Intervertebral Disc Repair

Several recent studies have focused on the repair of degenerating intervertebral disc in a canine model using stem cells. Hiyama et al. [88] evaluated whether BM-MSC transplantation had an effect on the suppression of disc degeneration in a canine model. The IVD damage was induced by aspiration of the nucleus pulposus (NP). Four weeks after nucleotomy, BM-MSCs were transplanted into the degeneration-induced. Control animals underwent nucleotomy only, without BM-MSC infusion. Twelve weeks post-transplantation, the data indicated that the BM-MSCs effectively promoted the regeneration of degenerated discs. Additional data indicated that the BM-MSCs might contribute to the immune-privileged status of the IVD via differentiation to Fas-ligand (FasL) expressing cells.

Recently, Serigano et al. [89] tried to determine the optimal donor cell number for maximum benefit in a canine disc degeneration model. Autologous BM-MSCs were transplanted into degenerative discs at doses between 10^5 and 10^7 cells/disc. Four weeks prior to transplantation, disc degeneration was induced by NP aspiration. The results showed that abundant extracellular matrix was maintained at the 10^6 BM-MSC dose, while less viable cells were detected in 10^5 dose and a higher frequency of apoptotic cells were present at the 10^7 dose, which suggests that the number of cells transplanted could affect the regenerative capability of BM-MSCs in the canine IVDs.

However, one of the major conclusions obtained from the bone tissue engineering studies performed in animal models, and also in pilot clinical studies, is the absolute

necessity of improving the oxygen and nutrient supply to the cells in the inner part of the implanted scaffolds. This major limitation needs to be overcome, together with additional investigations to adapt adequate scaffold degradation/resorption rates to new bone formation [30].

Cell Biology, Tracking, and Fate Studies

Pre-immune Fetal Sheep Model

The fetal sheep model has been used to assess the in vivo behavior and plasticity of human stem cells in a xenogenic clinically relevant large-animal model. A strength is efficient engraftment of stem cells and the modeling of prenatal treatment [90–93]. This model has the additional advantages of fetus body size and a long life span, which allow assessment of donor cell activity in the same animal for several years after transplantation and an adequate number of human cells to perform secondary transplantation. It has been used to investigate the role of various stem cell populations of stem cells in tissue engineering and regenerative medicine, including hematopoietic stem cells [94–99], MSCs [93, 98, 100], amniotic fluid cells [94], cord blood cells [101, 102], brain, and liver [94, 103].

Several studies have been performed investigating the biological potential of MSCs. Alemida-Porada et al. [100] demonstrated that the cotransplantation of autologous or allogeneic MSCs enhanced the long-term engraftment of human HSCs and higher levels of circulating cells earlier after transplantation during gestation and postnatally. Airey et al. [104] investigated the usefulness of a model of cardiac development in sheep. Both adult and fetal human MSCs were administered intraperitoneally into fetal sheep in utero, and engraftment of human MSCs in the heart was analyzed late in gestation. The analysis indicated that MSCs efficiently differentiated into Purkinje fibers. The fetal sheep model was used to investigate the ability of human MSCs to generate hepatocytes in vivo [103]. MSCs were administered by either intrahepatic or intraperitoneal route. Human origin hepatocytes were detected in all animals; however, the data indicated that intrahepatic delivery resulted in an increase frequency of human cells. Colletti et al. [105] investigated the mechanism(s) by which MSC transplantation contributes to the human cells found in multiple tissue types. The course of events that occurs in the first 120 h after infusion of human MSCs into fetal sheep was investigated. The data from this study indicate upon engraftment, MSCs undergo proliferation and initiate differentiation processes and alter their phenotype into tissue-specific cells. Ersek et al. [106] infused human MSCs isolated from fetal pancreatic tissue in the fetal sheep model. Subsequent to infusion, animals were analyzed between 3 and 27 months for the presence of human cells in the pancreas, and 79% of animals were found to be chimeric while in 50% functional engraftment was noted.

Nonhuman Primates

In addition to safety and efficacy, a central issue in the application of MSC therapy is their ability to avoid immune rejection in mismatched donors and recipients. Thus, cells from a single donor might be able to be transplanted into multiple recipients, and those recipients could receive multiple cell administrations, if necessary. Allogeneic donation could also be an important factor in the timely treatment of injury, when, for example, there is insufficient time for the isolation and expansion of autologous MSCs. The question of immunogenicity and immune compatibility of MSCs has been investigated in nonhuman primates. Devine et al. [107] administered allogeneic MSCs intravenously to baboons and found no toxicity. Further, histoincompatible MSCs were detected in bone marrow biopsies several weeks after infusion, although in no other tissue. It was later shown with more sensitive detection techniques and after longer time intervals that the allogeneic MSCs were distributed to a wide variety of tissues, including nonhematopoietic tissue, after intravenous administration in a manner similar to autologous cells [108]. Subsequent studies confirmed that allogeneic baboon MSCs can engraft and persist in unrelated recipients [109] without affecting overall health or immune status [110] suggesting that histocompatibility of MSCs may not be necessary for successful application.

In addition to their ability to avoid immune rejection, it is likely that MSCs possess immunosuppressive properties. As evidence, a single intravenous infusion of MSCs was able to prolong MHC-mismatched skin graft survival in baboons [111]. MSCs may also prove beneficial for enhancing the engraftment and effectiveness of other cell types. For example, when cotransplanted with islet cells, allogeneic MSCs significantly enhanced islet engraftment and function in diabetic cynomolgus monkeys compared with animals that received islet cells only [112]. MSCs have also been delivered in conjunction with hematopoietic cells [113, 114]. Chapel et al. [113] found that the MSCs homed to sites of injury in cynomolgus monkeys following irradiation and multi-organ failure syndrome. Also, it has been shown in cynomolgus monkeys that cotransplantation with MSCs can improve the engraftment of hematopoietic stem cells after transplantation [114].

Conclusions

MSC-based cell therapy has the potential to treat a variety of diseases and injuries. Their potential ability to replace damaged cells, stimulate endogenous repair mechanisms, and modulate the immune system in vivo, combined with their ease of isolation, expansion, and manipulation in vitro, make them appealing candidates for many therapeutic situations. Further investigation in large-animal models is required to confirm safety and efficacy as well as to establish appropriate dose and modes of administration.

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Chapter 14
Defining the Potential of MSCs with a Prenatal Large Animal Model

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Abstract An experimental model system that allows the assessment of the full differentiative potential of human mesenchymal stromal/stem cells (MSC) under normal physiological conditions, in the absence of genetic or injury-induced dysfunction, could reveal their true capabilities and also provide a valuable tool to dissect the pathways governing differentiation and fate reprogramming. The naturally occurring stem cell migratory patterns, the availability of expanding homing and engraftment sites, and the presence of tissue/organ-specific signals combine to make the developing mammalian fetus an ideal setting for MSCs to display their full biological potential. In addition to these characteristics, the early gestational age fetus also possesses the unique advantage of being relatively immunologically naive, making it possible to achieve engraftment and long-term persistence of MSCs and other stem cells from not only allogeneic but xenogeneic donors as well. In this chapter, we describe the advantages of the pre-immune fetus as a model for studying human MSCs and discuss results we have obtained thus far with a large animal (sheep) fetal model.

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_14,

Introduction

As discussed in detail in other chapters of this book, bone marrow mesenchymal stem cell (MSCs) [also interchangeably referred to as marrow stromal cells and stromal precursor cells] represent one of the most promising stem cell sources for tissue regeneration therapy. From a historical perspective, the presence of a stem cell population within the bone marrow stromal compartment was first suggested in the pioneering studies of Friedenstein over 30 years ago [1, 2], in which it was demonstrated that fibroblastoid cells could be obtained from the bone marrow and that these cells could then be used to transfer the hematopoietic microenvironment to ectopic sites. MSCs make up part of the stromal microenvironment that provides support to the hematopoietic stem cells and drives the process of hematopoiesis. Despite their important role within the bone marrow, MSCs are actually quite rare, representing only 0.001–0.01% of the total nucleated cell population present within the marrow [3]. Unfortunately, while investigators agree that MSCs are definitely negative for hematopoietic markers such as CD45, CD34, CD14, and glycophorin A, to date, there are no markers that specifically identify MSCs, making the isolation of pure MSCs technically impossible [4]. Numerous culture methods and surface markers have been developed/characterized, however, that enable one to enrich for MSCs, with each laboratory preferring its own method of isolation. This lack of uniformity in the methods for isolating cells which are referred to as MSCs has made the comparison of results from one lab to another somewhat complicated. Nevertheless, the vast differentiative capacity of MSCs and their potential value for cellular therapy have been now been established by a number of groups using both in vitro and in vivo assay systems. In this chapter, we provide a brief overview of the various assay systems which have been used to study MSCs and then focus on the fetal sheep model, describing some of the unique opportunities and advantages this model offers and summarizing some of the advances in the understanding of MSC biology and therapeutic potential that have been made by virtue of the unique characteristics of this model.

Models for Studying MSC Potential

In Vitro Models

Arguably, the ideal way to assess the full differentiative potential of MSC and examine their ability to adopt alternate cellular fates would be in vitro, since this would allow careful dissection of the pathways/mechanisms by which the MSCs commit to various lineages and undergo reprogramming. In addition, definitive evidence of reprogramming needs to be answered by demonstrating multipotentiality of a single cell or a clonally derived cell population. Thus, performing in vitro studies with highly purified populations of MSCs from various organs under highly defined culture

conditions would appear to offer distinct advantages over transplantation experiments performed in vivo in which the investigator has little or no control over the conditions in vivo within the recipient. Indeed, in vitro cultures have been pivotal in establishing the concept that marrow-derived MSCs possess a far broader differentiative capacity than originally thought, giving rise not only to the various mesenchymal cell types found within the bone marrow, i.e., bone, cartilage, and fat [5], but also what appear to be functional skeletal muscle cells [6]. By virtue of the fact that these studies were performed in vitro, investigators were then able to study these cells employing microarrays [7–9] to begin shedding light on the molecular mechanisms responsible for commitment to and progression along each of these lineages. These studies provide information vital for developing more efficient means of differentiating MSCs along specific desired pathways. The studies have also revealed some of the genes and signaling pathways important for maintaining MSCs in an undifferentiated state, to help better define this somewhat elusive stem cell population.

Following the demonstration that MSCs could give rise to all cells of the musculoskeletal system subsequent in vitro studies showed, quite remarkably, that MSCs had the ability to give rise not only to cells of mesenchymal derivation, but in fact, to cross what were perceived to be developmental boundaries and reprogram to differentiate into cells of cells of all three germinal layers including neuronal and glial cells [10–13], cardiomyocytes [14–18], endothelial cells [19–21], hepatocytes [22–26], and insulin-producing beta cells [27, 28] under certain experimental conditions. Thus, it is clear that in vitro studies have been pivotal in beginning to define the breadth of differentiative capacity of MSC and hint at some of their wide array of therapeutic uses.

Despite their value, however, in vitro studies are inherently limited by the fact that the mediators required for MSCs to undergo many of the changes in cellular fate observed in vivo (please see subsequent section for details) are largely unknown, making it difficult to reproduce in vitro the conditions present within an organ microenvironment. It is also important to note that in vitro approaches are unable to duplicate conditions that affect the migratory patterns and homing of stem cells to different tissues/organs in vivo, factors which may be important in optimizing therapy with MSCs. Thus, currently the best way to assess the differentiative potential of MSCs is by performing in vivo transplantation studies. However, since ethical and practical considerations prevent limiting dilution studies of highly defined populations of stem cells in humans, investigators have either employed animal stem cells or assessed the ability of human stem cells to engraft/differentiate in xenogeneic animal models.

In Vivo Models

To date, most in vivo models that demonstrate the versatility and therapeutic potential of MSCs have made use of an external stress, such as radiation- or

chemical-induced injury or an experimentally created shortage of a specific cell type in the recipient, to induce the transplanted MSCs to differentiate into the specific missing or injured organ cells [29–49], revealing that MSCs have a seemingly broad differentiative potential if provided with appropriate stimuli. In other studies, rather than inducing an injury to test the reparative ability of MSCs, investigators employed mdx mice, which possess a specific gene defect [50–52], and showed that MSCs also have the ability to engraft and mediate histologic and functional repair in this model of Duchenne muscular dystrophy. Collectively, the results of these studies have provided evidence that surrounding activated cells and/or signaling from organ-specific microenvironments can induce the transplanted cells to divide and differentiate into cells of the injured/deficient organ. However, these studies have, by nature of their design, also restricted the fate of the transplanted cells to the one particular organ/system harboring an injury/defect, thus preventing evaluation of the full potential of the transplanted cell populations. Studies conducted by Verfaillie's group, in which murine so-called multipotent adult progenitor cells (MAPCs; likely a subpopulation of MSCs [53, 54]) were microinjected into mouse embryos, showed that these cells have the ability to give rise to differentiated cells of all three germ layers [55], providing confirmation that previous studies in which investigators had driven the transplanted MSCs to differentiate along a single lineage had underestimated their full differentiative potential. Unfortunately, studies of this nature with early blastocyst injections are not ethically acceptable or feasible with human cells, given the possibility that donor cells could theoretically contribute to the germline in the early-stage embryo. Limiting the transplantation of murine cells into murine recipients makes it difficult to assess outcomes in higher animals and humans.

At present the mechanism(s) underlying the formation of these varied cell types by MSCs is not understood, and the experimental conditions have not enabled researchers to unequivocally determine if the generation of these diverse cell types reflects actual transdifferentiation/reprogramming. An established aspect of these studies, however, is that the microenvironment into which MSCs are placed plays a key role in determining cell fate. Perhaps the most dramatic example of the powerful effect that the microenvironment can exert on cells is the creation of an entire organism, Dolly the sheep, which resulted from the transfer of a nucleus from an adult somatic cell into the cytoplasm of an enucleated egg [56]. The microenvironment, in this case the intracellular microenvironment, enabled an adult cell to overcome the molecular hurdles that control gene expression and reveal its full potential. However, despite the exciting nature of these demonstrations of cellular and molecular reprogramming, an in-depth understanding of the processes controlling lineage specification is needed before the full therapeutic potential of MSCs can be realized.

A number of preclinical animal studies examining the potential of adult MSCs have also highlighted another interesting characteristic of MSCs: their apparent ability to navigate to sites of injury within the body, engrafting and generating tissue-specific cells within the injured tissue, but not to other functionally normal tissues [37, 57–59]. While this selectivity of engraftment within sites of injury/inflammation

raises the exciting possibility that infused MSCs, traffic to, and engraft only within, the tissue(s) in need of repair, it also makes it unlikely that a healthy adult animal model can demonstrate the full differentiative/therapeutic potential of MSCs.

The Fetal Sheep Model

An ideal experimental model would allow transplanted human MSC to participate in the generation of cells from other unrelated tissues under normal physiological conditions, in the absence of genetic or injury-induced dysfunction within a specific organ. Additionally, such a model would also permit formation of various donor-derived tissue-specific cells at adequate levels to allow delineation of the pathways involved in their generation. We hypothesized that it might be possible to exploit the characteristics of the developing fetus to create a novel model to test a variety of adult stem cells. During fetal life, the homing of circulating stem cells to various target organs is possible due to both the vast array of adhesion molecules that are up- or downregulated at specific points of gestation and the expression of related tissue-specific chemokines that provide signaling for the attraction and lodgment of the circulating stem cells. Under the permissive milieu of the target organ, these cells function to produce the requisite cell type(s). The existence of a highly permissive milieu is very likely associated with the continuous need for new cells during fetal development. We felt that transplanting MSCs (or any other stem cell type to be investigated) into fetal recipients at a point in development when all the organs had begun to differentiate, but the need for exponential growth and the formation of large numbers of specialized cell types still existed, would represent an ideal means of performing a rigorous, yet unbiased assessment of their full differentiative potential.

By virtue of the rapid dissemination of MSCs via naturally occurring migratory patterns and the ongoing organ development within the fetus, the transplanted MSCs would be exposed to a host of proliferative/differentiative stimuli enabling the reprogramming cellular fate. The model, however, would not force the cells to adopt a specific fate by damaging/inducing regeneration within a particular organ (as is the case with most other current models). At this stage of development, the transplanted MSCs should thus be provided with the opportunity to find the right stimulus in each organ to give rise to a wide range of organ/tissue-specific cell types, assuming that the transplanted MSCs, in fact, harbor that potential. Furthermore, if the supposition is correct that appropriate microenvironmental influences can induce a cell with a mature phenotype to regress into an undifferentiated state, and/or a primitive stem cell to commence reprogramming toward a new lineage, we reasoned that the fetus should represent an ideal model system in which to examine the full potential of MSCs.

In addition to the presence of a plethora of signals to drive the differentiation of transplanted MSC into various cell types throughout the developing fetus, there are also unique immunological advantages to transplanting cells into a fetal recipient.

If the transplant is timed correctly, immune barriers which normally hinder engraftment of allogeneic or xenogeneic cells after transplantation into postnatal recipients are not fully present. In contrast to other model systems, such as immunodeficient mice, which have been employed for studying the differentiative capabilities and therapeutic potential of human MSCs, fetal recipients have a normal functioning immune system. However, if the transplant is performed at the appropriate time in gestation, this immune system is still largely immuno-naïve. Indeed, early in immunologic development, before thymic processing of mature lymphocytes occurs, the fetus appears to be largely tolerant of foreign antigens [60, 61]. Furthermore, exposure to foreign antigens during this period often results in sustained tolerance, which can become permanent if the presence of antigen is maintained [62–65]. Thus, if cells are transplanted during this immunologic so-called window of opportunity, significant levels of engraftment of not only allogeneic sheep cells, but also xenogeneic human cells, can be achieved in the absence of irradiation or other myeloablative therapies [66–71]. Moreover, transplantation of human cells during this time period creates immunologic tolerance that is specific to the human donor [72], allowing the creation of a lifelong chimera in which a percentage of the cells in various tissues are derived from the transplanted human cells.

This ability to induce donor-specific tolerance provides a unique opportunity, since the transplantation of human MSCs early in gestation during this "pre-immune" stage of development should theoretically condition the fetal sheep recipients to be able to receive additional MSC transplants from the same donor later during the fetal period or even postnatally. Therefore, it becomes possible to perform multiple human MSC transplants at precise developmental stages to target the differentiation of human cells into specific cell types within a particular organ or system. For instance, transplantation of human MSCs in the fetal sheep model with the intent of producing human hepatocytes will likely be best early in gestation when the liver is still growing exponentially and hepatocyte maturation is still occurring (prior to the fetal liver attaining fully developed metabolic functions). At that time the liver still contains hematopoietic niches which are likely conducive to the lodging of bone marrow-derived cells that arrive through the portal circulation after intrauterine injection. In contrast, transplantation of human MSCs at this same time point does not result in production of pneumocytes-I within the lung [73] since pneumocyte-I production only commences within the developing lung much later in gestation, at which time the fetus has achieved immunologic competence. Therefore, by performing an initial transplant early in gestation to induce donor-specific tolerance, it becomes possible to then perform an additional transplant with MSCs from the same donor later in gestation to coax the transplanted MSCs to give rise to pneumocytes within the developing lungs.

In addition to the general advantages of using a fetus as a recipient, sheep specifically possess many added characteristics that make them ideal for developing/testing MSC-based therapies: (1) relatively similar size to humans during development, at birth, and as adults, likely obviating the need for scale-up when attempting to move promising results in the sheep model into the clinical arena; (2) sheep share many important physiological and developmental characteristics with

humans and have therefore been used for decades as a model for studying normal fetal growth and fetal abnormalities [74–79]; (3) sheep are outbred, and thus represent a wide spectrum of genetic determinants of the immune response, as do humans; (4) the development of the sheep immune system is well characterized and closely parallels that of humans [80–89]; and (5) the large size and long lifespan (>10 years) of the sheep enables both the evaluation of donor cell activity in the same animal for years after transplant, allowing questions such as long-term efficacy and safety of novel treatments to be properly addressed, and also makes it possible for the investigator to easily obtain sufficient human cells from the primary recipients to perform detailed molecular studies to define pathways involved in cellular reprogramming or even to conduct serial transplantation studies, if the cells being examined possess such potential. In addition, because of the absence of the need for any myeloablative conditioning, transplanted human cells are forced to compete with the healthy endogenous ovine stem cells for available niches within the bone marrow and other organs, providing a rigorous test of the transplanted cells' potential. These factors combine to make sheep, in our opinion, an ideal model in which to examine the therapeutic potential of MSC and obtain results of high clinical significance.

Results Obtained in Fetal Sheep Model

To begin exploring the in vivo differentiative potential of human MSCs in the absence of injury/selective pressure, we evaluated their ability to give rise to other cell types in vivo in the fetal sheep model. To this end, several clonal MSC populations were magnetically isolated from adult BM using Stro-1, an antibody that reacts with non-hematopoietic bone marrow stromal precursor cells [90]. Although the antigen recognized by this antibody has not yet been identified, we have found that by triple-labeling bone marrow cells with Stro-1, anti-CD45, and anti-GlyA and selecting the Stro-1+CD45-GlyA - cells, we consistently obtain a homogeneous population highly enriched for MSCs. Accordingly, we have used this cell population for all our studies examining MSC differentiative potential. After isolation and expansion, these clonal MSC populations were evaluated for their ability to give rise to donor (human)-derived hepatocytes and other organ-specific cell types.

Analysis of livers of transplanted animals confirmed that clonal-derived populations were capable of giving rise to significant numbers of human hepatocytes (derived from the endoderm during embryonic development) producing albumin and detected by both immunohistochemistry with a monoclonal antibody specific for human hepatocytes (HePar) in situ hybridization using a human-specific probe [91].

Our analyses also show that some of the transplanted MSC clones generated multilineage hematopoietic cells. Whether the ability of human MSCs to give rise to long-term engrafting hematopoietic stem cells is only possible in the setting of the developing fetus is not yet known, but these findings highlight the power of this

unique animal model system to reveal as yet unknown differentiative capabilities of MSCs [91]. Examination of skin from animals transplanted with some of these clonal populations also demonstrated the presence of human keratinocytes expressing human-specific cytokeratin, highlighting the ability of these highly enriched clonal human MSC populations to give rise to cells of ectodermal derivation (El Shabrawy D and Almeida-Porada, personal communication) [92]. Since the nature of the model does not allow the use of single-cell transplantation, the ability of the sheep model to distinguish the differentiative potential and functionality between several identical clones was of the utmost importance. These initial studies thus demonstrated that the fetal sheep model provides the necessary microenvironmental cues to drive the differentiation of clonal populations of BM-derived human MSC into cells of all three germinal derivations.

Although the intraperitoneal (IP) transplantation of clonally derived human MSCs in the fetal sheep model demonstrated that these cells possessed the ability to reprogram to hepatocytes, these cells were only present at levels of a few percent. This led us to hypothesize that human MSCs may not reach the fetal liver effectively after IP injection. We therefore compared two different routes of administration, IP and intrahepatic (IH), in an effort to increase the levels of donor-derived hepatocytes after MSC transplantation.

HEPAR-1 staining revealed that administering the human MSCs via IH injection resulted in roughly five-fold higher levels of hepatocytes than if the same dose of cells was injected IP [91]. Interestingly, the route by which the cells were transplanted influenced not only their levels of hepatic engraftment, but also their distribution of engraftment within the liver. Because there is a preferential synthesis of certain plasma proteins, such as albumin, in hepatocytes localized in the periportal regions of the liver [93–95], it is possible that the hepatocytes generated by IP injection that localized around the periportal area may be better suited to producing albumin than hepatocytes that are generated in another lobular zone. Indeed, these studies showed that animals transplanted with MSCs via the IP route had higher circulating levels of human albumin than those transplanted via the IH route. Placing our results in the context of potential cellular therapy for liver injury/disease, the choice between higher levels of hepatocytes distributed throughout the parenchyma or smaller number of hepatocytes clustered in close proximity to the vasculature will likely be determined by the specific clinical setting, depending upon whether the predominant overall goal of the intervention is to restore the architecture of the liver or to achieve secretion of a particular therapeutic protein into the circulation

Having used this model to establish the ability of BM-derived human MSCs to give rise to cells of multiple tissues/organs in the absence of injury or disease, we next used the sheep model to investigate the differentiative capacity of MSCs derived from tissues other than BM. In similarity to the prior studies with BM-derived MSCs, transplantation of human fetal kidney MSCs resulted in the generation of donor-derived hepatocytes, and human albumin was detected in the serum of the transplanted animals, demonstrating the functionality of the generated hepatocytes [96].

Delineation of Potential Pathways Involved in MSC Reprogramming

Since the prior studies did not address the mechanisms by which the apparent cellular reprogramming was occurring, we conducted studies [73] to elucidate the pathways through which MSCs appear to be capable of giving rise to functional differentiated tissue-specific cells and thus contribute to organ regeneration. Human BM-derived MSCs labeled with either CFSE or DiD were transplanted by IP injection in pre-immune fetal sheep. Flow-cytometric analysis of peripheral blood and peritoneal lavage showed that transplanted cells migrate into the systemic circulation as early as 20 h posttransplant and had all exited the peritoneal cavity by 96 h after injection. Thus, we next evaluated the liver, lung, and brain for the presence of donor $CFSE^+$ or DiD^+ MSCs commencing at 20 h posttransplant until 120 h posttransplant. Transplanted MSCs were first detected in the liver parenchyma at 25 h posttransplant, and their number increased in the next hours, to reach the maximal level of engraftment at 40 h posttransplant. MSCs only reached the lung at 30 h posttransplant, but the number of human MSCs continued to increase at each subsequent time point until the last point of analysis at 120 h. Settlement of MSCs in the brain commenced at 40 h, a later time point than in either liver or lung, and reached its maximum at 60 h.

We next evaluated whether the transplanted cells underwent proliferation upon engraftment and determining whether this proliferation took place before or after the MSCs had initiated differentiation into tissue-specific cell types within the respective tissue. At all time points, 95% of the $CFSE^+$ or DiD^+ cells in each tissue also exhibited Ki67 positivity, demonstrating that many of the engrafted cells either began or continued to proliferate upon lodging within the various organs and suggesting that the higher levels of engraftment observed at later time points were due, at least in part, to the proliferation of the MSCs that had engrafted earlier on and were not solely a result of the continued lodging of transplanted cells within the tissue.

To determine the timeline of MSC differentiation into organ-specific cell types in this model, we examined the different tissues by confocal microscopy using various cell-specific markers that were not expressed by MSCs prior to transplant, looking for evidence of cells that were positive for CFSE or DiD and were simultaneously expressing cell-specific markers for each of the engrafted tissues. α-Fetoprotein was chosen as the marker of MSC induction toward a hepatic phenotype, since during normal liver development, the upregulation of gene transcripts encoding α-fetoprotein and albumin is thought to mark the first evidence of hepatic specification and emergence of the hepatoblast [97, 98]. At 25 h posttransplant, the first time point at which MSCs were detected in the liver, $CFSE^+$ or DiD^+ cells were already expressing α-fetoprotein, demonstrating that MSC rapidly switched to a fetal hepatocyte-like phenotype upon liver engraftment. To evaluate MSC differentiation in the lung, we examined the expression of surfactant protein B, since this protein is expressed in type II pneumocytes early in gestation [99]. We also looked at caveolin-1 expression, since it is a marker of maturation and differentiation of

lung alveolar epithelial type II cells into a type I phenotype [100]. CFSE- and DiD-positive cells were found to express surfactant protein B as soon as they first engrafted in the lung at 30 h posttransplantation and continued to express the protein throughout the evaluation period. By contrast, even at 120 h posttransplant (the last time point of our analysis), caveolin-1 was still not being expressed by the transplanted MSCs. This demonstrates that transplanted MSCs assumed a phenotype consistent with differentiation to a type II epithelial cell, but not the more mature type I epithelial cell.

To investigate the differentiation of MSCs into cells with a neural looking phenotype upon engraftment within the brain, we examined whether CFSE- and DiD-positive cells were also expressing Tau and/or synaptophysin. Tau is widely expressed in the fetal brain during development and correlates with neurite growth and axonal development in neurons and neural cell lines in vitro [101], while synaptophysin is a reliable marker of nerve terminal differentiation [102]. All of the transplanted DiD- and CFSE-positive MSCs expressed Tau promptly at the first time point of 40 h posttransplantation, when these cells were first found in the brain, demonstrating that MSCs quickly upregulated expression of proteins related to neural differentiation. At 40 h posttransplant, synaptophysin was found to be expressed only in approximately 56% of the transplanted MSCs. Nevertheless, by 60 h posttransplant, all engrafted MSCs in the brain expressed synaptophysin.

Taken collectively, these results show that transplanted MSCs in this model, after reaching brain, liver, or lung, rapidly undergo proliferation and differentiation, adopting a phenotype consistent with tissue-specific cell types. To delineate the mechanisms responsible for the observed generation of tissue-specific cells by MSCs, we next investigated whether the rapid expression of tissue-specific cell markers was due to true differentiation of the transplanted MSCs into tissue-specific cells or instead resulted from the transfer of membrane vesicles/mitochondria or fusion of MSCs with resident tissue-specific cells within each organ. To this end, we used confocal microscopy to visualize cells that were positive for CFSE or DiD and performed fluorescence in situ hybridization (FISH) with either a human- or a sheep-specific probe. By examining the species origin of the genomic DNA present within the nuclei of the DiD^+ or $CFSE^+$ cells in each engrafted organ, we were able to determine whether the transplanted MSC had undergone fusion or membrane/mitochondrial transfer. The results of these studies [73] established that $CFSE^+$ cells in the liver, lung, and brain all hybridized exclusively to the human probe, demonstrating that no fusion had occurred between transplanted MSCs and endogenous sheep cells within the examined organs.

Since the majority of studies thus far have focused on the role of mitochondrial transfer from donor cells to recipient tissues as a means of providing respiratory rescue to damaged or injured host tissues/cells and have suggested that this may in fact be the mechanism whereby transplanted MSCs provide therapeutic benefit without the need for actual engraftment [103], we also examined whether membrane vesicles/mitochondria were transferred from the transplanted human cells to the host sheep tissues, again performing FISH with human- and sheep-specific probes and taking advantage of the ability of DiD to label mitochondria and vesicles.

This combinatorial approach demonstrated that, in the absence of disease or injury, MSCs gave rise to tissue-specific cell types without cellular fusion or donor-to-recipient transfer of mitochondria or membrane vesicles. However, it is also possible that exosomes or microvesicles originating from the recipient's neighboring cells influenced the fate of the transplanted MSCs [104].

Using this noninjury fetal model, we showed that human MSCs engrafted in all organs examined, began or continued to proliferate and rapidly underwent differentiation into multiple tissue-specific cell types. These studies also suggest that transplanted MSCs go through a gradual program of differentiation, with the subsequent cells sequentially expressing markers indicative of progressive cell maturation. Because the differentiation process of MSCs into tissue-specific cells occurs promptly upon engraftment, a deeper understanding of how the differentiative process by which MSCs give rise to tissue-specific cells comes about is needed. Furthermore, we have also demonstrated that the formation of tissue-specific cells from transplanted adult human MSCs occurs in several organs in the absence of fusion or donor-to-recipient mitochondrial/membrane transfer. It is likely that in the fetal sheep model system, in the absence of injury or disease, there are sufficient developmental cues present within the physiologic inductive microenvironment to induce upregulation of tissue-specific genes within the transplanted MSCs that then lead to differentiation into cells of the specific desired organ. It is envisioned that further studies elucidating precisely which genes are required for adult stem cells to differentiate into each of the tissue-specific cell types will ultimately lead to the discovery of the means of specifically upregulating only the genes required to produce the cell type needed for benefit in the disease state in question.

Conclusions

In conclusion, we have shown that it is possible to take advantage of the unique proliferative/inductive nature of the fetal microenvironment to reveal the inherent ability of human MSCs to generate tissue-specific cells rapidly in several tissues in vivo following transplantation. This unique xenograft model system also enabled us to conclusively demonstrate that the formation of these cells occurs in the absence of fusion or mitochondrial/vesicular transfer, highlighting the power of this model system. It is important to note, however, that while this model is well suited to defining the mechanisms involved in this process, translation to clinical application will require experiments in adult animals exhibiting a specific defect/disease, to assess the impact the diseased/proapoptotic microenvironment may have on stem cell engraftment and differentiation. Thus, once the requisite gene pathways have been elucidated and the means of circumventing the hurdles that are present in injured/diseased adult tissues have been developed, the stage will be set for beginning to delineate the means of increasing the efficiency of both delivery and selective differentiation of MSCs into desired target cell types, to be able finally to fully exploit the potential of human MSCs for their use in stem cell-based regenerative therapies.

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Part II
From Bench to Bedside

Chapter 15
Bench-to-Bedside Development of MSC Therapies: A Multidisciplinary Approach

Sowmya Viswanathan and Elizabeth J. Read

Abstract There is a worldwide interest in developing mesenchymal stromal/stem cells (MSCs) for a broad and steadily expanding range of potential clinical applications. Whether developed by academia or industry, successful transition from the research bench to the clinic requires a systematic, coordinated, and multidisciplinary approach to execute the many simultaneous, interrelated activities aimed at demonstrating the product's safety and efficacy. Like conventional drug development, MSC product development requires attention to basic and translational science, preclinical safety and efficacy studies in animal models, manufacturing process development, good manufacturing practice, development and validation of analytical methods to characterize the product, quality control and quality assurance, regulatory affairs, and design and execution of clinical trials. Broad expertise is needed to accomplish these activities and includes molecular and cell biologists; scientists and technicians to develop and perform laboratory assays, manufacturing, and imaging; physicians with expertise in the disease application and clinical trials; pathologists; clinical imaging experts; nurses and clinical trials staff; veterinarians; quality officers; regulatory affairs experts, technology transfer officers and patent counsels; and institutional ethics committees. An integrated project management approach can be adapted by both academia and industry to successfully negotiate

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_15,

the complex product development pathway. In this chapter, we review the preclinical, manufacturing, clinical, quality, and regulatory requirements to move MSC products from bench to bedside and highlight the resources needed for success in this endeavor.

Introduction

The first successful allogeneic hematopoietic stem cell (HSC) transplants were performed over 40 years ago for children with congenital immunodeficiency disorders [1–3]. Since then, the field of cellular therapies has grown and evolved substantially. Using a variety of cell and tissue sources and innovative methods for *ex vivo* cell manipulation, cell-based therapies are now in development for a wide range of diseases and for repair and regeneration of tissues and organs. The growth in clinical investigation of mesenchymal stromal cells (MSCs) has been especially dramatic. As of April 2011, over 130 academic- and industry-sponsored interventional studies of therapeutic MSC products were registered on clinicaltrials.gov, a public database that lists clinical trials required by US law to be registered, as well as non-US trials registered electively [4].

Until several years ago, most clinical trials of cell-based therapies were carried out by hematology/oncology investigators, using infrastructure of HSC transplant programs. This infrastructure includes a framework for executing the trials, as well as facilities and expertise for collection, manufacturing, testing, cryopreservation, storage, and infusion of cellular products. Development of novel cell-based therapies by commercial companies and by academic investigators from other medical and surgical disciplines has challenged this model. The practical requirements for bench-to-bedside development of cell-based therapies are complex and demand a multidisciplinary approach that incorporates lessons from both HSC transplantation and conventional drug and biologics development. This chapter outlines those requirements, with a focus on developing clinical trials for MSCs.

MSCs as Investigational Biological Drugs

Unlike conventional hematopoietic stem cell products, most MSC products are considered in the USA, Canada, and Europe as investigational cell therapies, because they require more than minimal manipulation and are typically used in a nonhomologous manner. MSC products therefore fit into the development pathway for investigational drugs and biologics, which have a higher level of regulatory oversight than conventional HSC products. Figure 15.1 illustrates this pathway – from discovery, into clinical trials, and to commercial availability – using regulatory terminology of the US Food and Drug Administration (FDA). In this schema, clinical

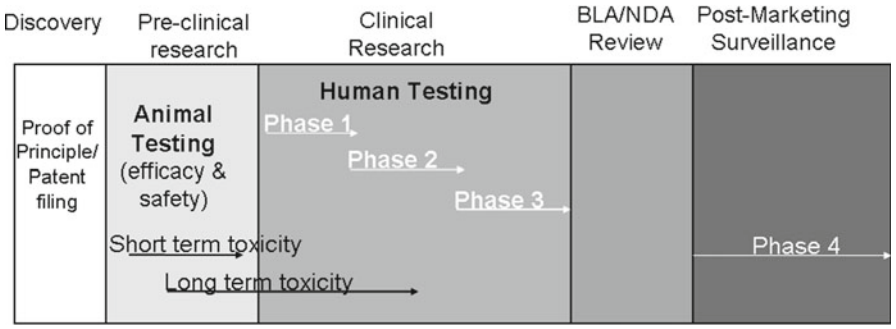

Fig. 15.1 Development pathway for drugs and biologics

trials for drugs or biologics can begin only after review and authorization of an investigational new drug (IND) application, and commercialization occurs only after formal review and approval of a new drug application (NDA) or biologics license application (BLA).

Cellular Therapy Development in Academic Organizations

For development of cell-based therapies, the goals of academia and industry are not identical. Academic physicians are typically motivated to expand therapeutic options for their patients by discovering and developing new therapies that can be tested for proof of principle, sometimes with exploration of biologic correlates, in early phase clinical trials. Alternatively, they may elect to participate in industry-sponsored trials, where the product is being developed and manufactured by a commercial company. Industry's overriding goal is to move a specific product from discovery and preclinical studies through all phases of clinical trials and ultimately achieve regulatory approval for commercialization and use by as many patients as possible.

DiMasi has estimated the average cost of developing a single drug or biologic for commercial use at $800 million [5]; a newer study estimates that this cost may be as high as $2 billion, depending on the therapy or the commercial developing firm [6]. Discovery and preclinical development often entail years of research and optimization studies but do not always meet with success in terms of getting the product into clinical trials. In fact, many product candidates are dropped in the period from discovery through preclinical development, commonly called the "valley of death," because financial realities allow advancement of only the most promising candidates [7].

Academic investigators, therefore, must make a realistic assessment of their goals and the role they will play in development of a novel cellular therapy. The

decision of academic investigators to embark independently on preclinical and clinical development of a novel cell-based product, without industry involvement, depends on their ability to obtain the substantial resources needed to conduct the research and development and to fulfill regulatory obligations as sponsors of clinical trials. Resources to conduct a cell therapy clinical trial are different from those required for drug trials where the drug candidate has been defined and manufactured by a previously standardized process. Although a relatively "standardized" manufacturing process has been established for MSCs, even that process must be developed and established locally, and infrastructure to do so is costly and typically not covered by traditional research grants. Several government funding initiatives in the USA have begun to address these funding gaps and facilitate development of novel cell-based therapies by academic investigators. The National Heart, Lung, and Blood Institute (NHLBI) funds a network of US academic cell therapy facilities to provide product development and manufacturing services to clinical investigators at other institutions, through the PACT (Production Assistance in Cellular Therapies) program [8]. The California Institute for Regenerative Medicine (CIRM), the largest state stem cell agency in the USA, has begun to provide substantial funding for translational studies, IND development, and early phase clinical trials for novel stem cell therapies [9].

Even with adequate funding, one of the most overlooked aspects of cellular therapy development in academia is the need to assemble and manage a team of personnel with appropriate, complementary skill sets. Commercial organizations have long recognized the need for multidisciplinary teams and a project management approach, to efficiently achieve the goals of medical product development. Most academic centers have not fully embraced this approach, for reasons related to organizational mission and culture, and access to infrastructure and resources. Subsequent sections of this chapter describe the expertise and resources needed, as well as a project management approach, to maximize the likelihood that organizations will achieve their MSC development goals.

IND Development for MSC Clinical Trials

IND Requirements

Before implementation of clinical trials for an investigational new drug or biological product, approvals must be obtained from the regulatory agency specific for the jurisdiction, for example, FDA in the USA, Health Canada in Canada, and European Medicines Agency (EMEA) in the European Union. The US FDA regulates investigational drug and biological products under legal authority of the Federal Food, Drug, and Cosmetic Act [10], as well as the IND regulations [11].

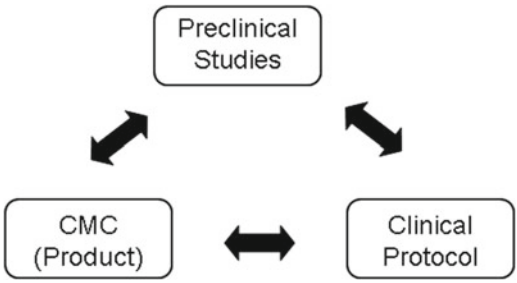

Fig. 15.2 Key IND elements requiring development

IND Definition and Contents

In the USA, an IND application is a formal, detailed written document with defined structure and content that must be reviewed by FDA before the investigational product is administered to human subjects and before a marketed product is used outside the scope of medical practice. A clinical study intended to generate data to support a new labeling indication for an approved product also requires an IND. The contents of the IND application are as follows:

- Cover sheet (Form FDA 1571)
- Table of contents
- Introductory statement and general investigational plan
- Investigator's brochure (not required for investigator-sponsored, single-site studies)
- Clinical protocol and informed consent
- Product information (chemistry, manufacturing, and control)
- Preclinical data and safety testing (pharmacology and toxicology)
- Previous human experience
- Additional information
- Statement of investigator (Form FDA 1572)

During the IND development process, the three major areas requiring development and definition are (1) preclinical animal studies, (2) product information, referred to as chemistry, manufacturing, and control (CMC) information, and (3) clinical protocol. These three sections are interdependent and should be considered and developed simultaneously, rather than sequentially (Fig. 15.2). Additional information regarding IND contents, submission, and review process is available on the FDA/Center for Biologics Evaluation and Research (CBER)'s Office of Cellular, Tissue, and Gene Therapies (OCTGT) web page for industry education [12].

IND Regulatory Process

The regulatory communication process for drugs and biologics in the USA is outlined in the IND regulations and FDA guidance [13]. Formal meetings between the sponsor and FDA include those that are routine for exchange of information during the course of IND development (type B), those reserved for dispute resolution or stalled development programs (type A), and other meetings (type C). Type B meetings include pre-IND, end of phase 1 (EOP-1), end of phase 2/pre-phase 3, and pre-NDA/BLA meetings. Although there is no requirement for the sponsor to communicate through meetings with FDA prior to IND submission, it is strongly recommended that sponsors take advantage of pre-IND meetings to optimize the development process and expenditure of resources and to avoid delays in initiating clinical trials. Informal discussions called pre-pre-IND meetings are not mentioned in either the IND regulations or FDA's guidance on formal meetings, and FDA is not required to hold them. However, OCTGT has encouraged pre-pre-IND meetings for sponsors to obtain early advice on preclinical animal study plans and on CMC/product issues.

Because the IND is a "living" document on file with FDA, updates and reports must be submitted to FDA as amendments to the IND. Amendments with specified content and structure are defined in the IND regulations and include protocol amendments, safety reports, annual reports, and information amendments. For sponsors intending to move forward with commercialization of the investigational drug or biologic product, a New Drug Application (NDA) or Biologics License Application (BLA), respectively, must be submitted following completion of phase 1–3 clinical trials.

Resource Needs for Regulatory Affairs

Personnel with expertise in regulatory affairs (regulatory requirements, process, communications, and strategy, and content and format of regulatory submissions) should be included as part of the multidisciplinary team for MSC clinical trials. Academic investigators intending to sponsor MSC trials are responsible for managing the development, submission, and maintenance of the IND, and associated regulatory communications. For industry-sponsored trials, these functions are handled primarily by the company. In addition to general drug/biologics regulatory expertise, internal or external expertise may be needed for development and written presentation of specific aspects of the IND, especially the preclinical pharmacology and toxicology studies and the CMC information.

Preclinical Animal Studies

Requirements for Preclinical Proof of Concept (POC) and Safety Studies

Before initiating a phase 1 clinical trial, preclinical animal studies must be carried out to establish proof of concept (POC) and safety for the specific product and the specific disease indication. The type, duration, and scope of animal studies depend on the nature of the clinical trial and on specific feedback from the regulatory authority. The International Council on Harmonization (ICH), which develops guidance documents compatible with US, European, and Japanese regulations, has published guidances on preclinical safety studies for biotechnology products. However, preclinical evaluation of cell and gene therapies does not fit perfectly into the recommended formats for conventional pharmaceuticals and biologics [14, 15]. In the USA, OCTGT has provided informal guidance on preclinical animal studies for cell and gene therapy products, through public presentations and through feedback to sponsors in pre-pre-IND and pre-IND meetings. To date, OCTGT has used a case-by-case approach, but it is likely that more formal guidance on preclinical safety assessment will be published in the future, based on recent listing of this topic in FDA's Annual Guidance Agenda [16].

Goals for Preclinical Studies

Preclinical studies should support proceeding to early phase clinical trials by providing

- Scientific rationale or proof-of-concept (POC) for the human studies
- Rationale for a safe starting dose, dosing schedule, and dose escalation scheme for a specific route of administration
- Insight into the timing of the therapy in relation to the onset of disease or injury
- Identification of parameters (endpoints, biomarkers) for monitoring in the clinical protocol
- Data on toxicities and adverse findings
- Identification and exclusion of adverse interactions with other therapeutics to be used in the treatment plan
- Data to support patient eligibility criteria
- Preliminary risk/benefit assessment
- Identification of mechanism of action

Pharmacology, or POC, studies should be done in a biologically relevant model of disease or injury. Ideally, the model will incorporate assessment of the proposed mechanism of action for a specific indication. For example, for the use of MSCs

in stroke, it may be important to show that MSCs can protect neurons from cell death or enhance angiogenesis in the animal model. The model should mimic the clinical scenario as closely as possible and allow optimization of MSC formulation (i.e., cell concentration, volume, excipient solution), dose, and route and timing of administration, and quantification of functional outcomes. Demonstration of a dose-response relationship is important, but translation of dose from animal models to humans may be more difficult for cells than for conventional pharmaceuticals. However, allometric scaling from the animal model(s) is often used in the justification of a proposed starting dose and dose escalation for cell-based therapies [17, 18].

Toxicology (safety) studies include (1) safety assessments that are customized for the cellular product in the specific indication, focused on cell fate in vivo and (2) conventional toxicology assessments common to all drug and biological products, which use standard toxicology endpoints of mortality, clinical observations (weight, appetite), clinical pathology (hematology, coagulation, serum chemistry, urinalysis), and gross/microscopic pathology of target and nontarget organs and tissues. Conventional toxicology studies typically use healthy animals in a biologically relevant species. However, OCTGT frequently recommends use of diseased animals for cell fate/biodistribution assessment (e.g., survival, engraftment, integration, proliferation, differentiation), terminal and nonterminal assessment (e.g., imaging, polymerase chain reaction (PCR), immunohistochemistry, in situ hybridization), and product-dependent endpoints (e.g., tumorigenicity, immunogenicity). Therefore, "hybrid" POC/safety studies, using animal models of disease that allow simultaneous assessment of pharmacology and toxicology endpoints, are often appropriate for cell-based therapies, including MSCs.

Choice of specific animal models depends on the disease indication and the goal of the preclinical study. In general, use of small animals (rodents) is favored for both pharmacology (POC) and toxicology (safety) studies, because of the numbers required and availability of immune deficient, transgenic, and knockout rodent models. However, large animal models may be needed for more realistic assessment of physiological behavior of cells in vivo and modeling of cell delivery and trafficking to a specific vascular bed, organ, tissue, or other anatomic sites. Differences between animal and human cells in biological (e.g., immunogenicity) and physical (e.g., size, deformability) characteristics must be considered when human cellular products are tested in animal models. If human cells are used in large animals, immunosuppression is required, which may impact toxicity or mechanism of action, but provides a better picture of immune-mediated clearance. Alternatively, homologous (same species) cells or a humanized model may be used. Tumorigenicity studies, if required, are more effectively carried out using the human cell product in immunodeficient small animals (rodents) to avoid the confounding effect of immunosuppression on tumor formation. Other important considerations for all preclinical studies include having sufficient numbers of animals and controls for statistical validity, appropriate study duration, and use of multiple endpoints.

Tumorigenicity is a major concern for cell-based therapies derived from pluripotent (embryonic or induced pluripotent stem cells) or even multipotent cells that have been extensively expanded. There is now consensus that MSCs are very unlikely to become tumorigenic for up to 30 population doublings, if culture expansion consists of serial passages, well before confluency and replating at low to medium densities [19]. However, concerns over genetic stability and tumorigenic potential are likely to be raised with products manufactured from MSCs expanded to the point of senescence or by genetic modification of conventionally generated MSCs. Tumorigenicity studies should be performed in an animal susceptible to tumor formation and require longitudinal assessment over the animal's lifespan, typically 9–12 months in rodents. ICH guidelines for other animal toxicity studies including reproductive toxicity [20], genotoxicity [21], and carcinogenicity [22] exist but typically do not apply to MSC products; they are usually restricted to products that have prolonged half-life, likelihood of impact on reproduction and development, or known carcinogenic potential.

Good Laboratory Practice for Preclinical Animal Studies

Preclinical animal studies, especially toxicology studies, should ideally conform to FDA rules for Good Laboratory Practice (GLP) [23]. However, FDA recognizes that this may not be possible for early preclinical studies and requires that deviations from GLP standards be documented and explained in the IND submission [24]. For academic investigators, the most critical aspects of GLP are (1) use of formal animal study protocols with well-defined methods and endpoints, (2) preparation, characterization, and handling of the test article (the cellular product), and (3) preparation of study reports in the appropriate format, with data presented for individual animals and as tabulated results. In the USA, sponsors of cellular therapy INDs are encouraged to review their preclinical safety study plans in detail with OCTGT's pharmacology/toxicology review staff.

Preparation of the MSC product for preclinical animal studies should be as close as possible to the clinical MSC manufacturing process, including methods for cell source collection, expansion, harvest, cryopreservation, final formulation, and combination with structural elements such as synthetic scaffolds. Differences in MSC products prepared by methods that are not identical to the clinical method, and even batch-to-batch differences in MSCs manufactured by the same methods, can affect the quality and in vivo functional characteristics of the MSC product. Therefore, all MSC batches used for preclinical studies must be characterized appropriately: at a minimum, MSC viability, concentration, formulation, morphology, phenotype, and passage number should be quantified and recorded. These data are critical to support arguments that any preclinical versus clinical differences in manufacturing methods, or even batch-to-batch differences unrelated to manufacturing methods, do not impact MSC quality, functionality, or safety.

Resource Needs for Preclinical Studies

A multidisciplinary approach is necessary from the start to plan and conduct preclinical studies. Research scientists, often with cellular biology background, may conduct the initial in vitro and in vivo efficacy studies. For safety/toxicology studies and any hybrid POC/safety studies, scientists will need to interact with qualified veterinarians and pathologists to design and conduct GLP studies. GLP studies are often done by a contract research organization (CRO) and therefore may require technology transfer from the research site, especially with novel and/or complex animal models and methodologies. Typically, if studies are outsourced to a CRO/other party, a service agreement will need to be enacted between the academic institute/sponsor and the CRO/other party and will thus require a contract manager or legal counsel for both parties. Quality assurance officers from the CRO and the sponsor's institute may also be involved in auditing and releasing the data from these studies in the form of an audited formal report, which forms part of the IND submission. As part of a multidisciplinary approach, the research team may also require technicians with appropriate molecular biology expertise (e.g., for measuring MSC biodistribution by qPCR) or imaging expertise (e.g., In Vivo Imaging System or whole body scintigraphic imaging) to track labeled cells. If MSCs are genetically modified, technical expertise in engineering vectors, genetically transducing the cells and assaying the genetically modified cells, is critical. If MSCs are grown in nonconventional ways (e.g., using a bioreactor), additional bioengineering or process engineering expertise may be required.

CMC Development

The goal of CMC development is to define a manufacturing process that will result consistently in MSC products that meet predefined specifications appropriate for the clinical trial. Early initiation of CMC development and interaction of the CMC team with the preclinical and clinical teams will optimize product quality and safety, prevent delays in preclinical GLP toxicology testing, and facilitate planning for Good Manufacturing Practices (GMP) manufacturing and clinical trial implementation.

CMC Requirements

FDA has published detailed guidance on the CMC requirements for cell and gene therapy products. The CMC guidance for somatic cell therapy products is applicable to all investigational MSC products [25]. For MSC products that are genetically modified, the CMC guidance for gene therapy products is also applicable [26]. These two guidance documents overlap substantially; the key difference is the

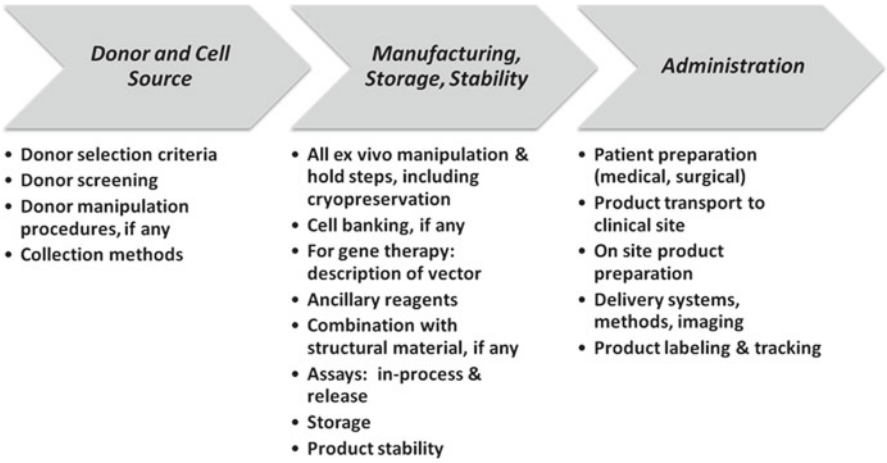

Fig. 15.3 Key considerations for CMC development

additional set of requirements for gene therapies, including detailed description of vector design, manufacturing, and testing.

CMC Development Considerations for MSCs

Categories of information to be described in the CMC portion of the IND are (1) donor, cell source, and collection, (2) manufacturing, testing, and storage of the product, and (3) final product preparation and administration. CMC development is aimed at addressing these considerations in detail before the clinical trial begins (Fig. 15.3).

Definition of the Donor, Cell Source, and Collection Method

For cell therapy products, the starting cellular material is arguably the most important determinant of product quality, efficacy, and safety. Therefore, specifications must be clearly defined for the cell or tissue source. Biologic (donor-to-donor) variability is inevitable and must be evaluated during development, unless a single cell line is the source material for all preclinical and clinical studies. Unrelated allogeneic donors used as the source for an "off the shelf" MSC product are typically normal, healthy individuals who meet criteria similar to those for blood donors but may also be selected by additional investigator-specified criteria (e.g., age, gender, weight/body mass index (BMI)). Even patient-specific (autologous or allogeneic related or

other directed) donors need specifications; both the clinical protocol and CMC should consider the feasibility of manufacturing a cellular product from donors who are not normal and healthy, and alternatives if criteria for the donor or collected cell source are not met.

All allogeneic donors who meet selection criteria are subject to screening and testing measures aimed to prevent transmission of communicable diseases, as required by FDA's donor eligibility rule and guidances [27–30]. In the USA, the relevant communicable diseases and disease agents requiring donor medical history screening and/or testing with an FDA-licensed test kit include:

- Human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2)
- Hepatitis B virus (HBV)
- Hepatitis C virus (HCV)
- Treponema pallidum (syphilis)
- Human T-lymphotropic virus types I and II (HTLV I/II), for donors of viable, leukocyte-rich HCT/Ps
- Cytomegalovirus (CMV), for donors of viable, leukocyte-rich HCT/Ps
- Trypanosoma cruzi (Chagas' disease; T. cruzi)
- West Nile virus (WNV)

In addition, diseases that require medical history screening, but have no current FDA-licensed tests available, include human transmissible spongiform encephalopathy (TSE), vaccinia, and sepsis. For autologous donors, transmissible disease screening and testing measures are recommended but not required.

While bone marrow is the most common source for MSC preparation, other tissue and cell sources, including adipose tissue, placental/umbilical cord blood, and amniotic fluid are in current use. For any source, definition of the collection method should include a standard operating procedure (SOP), quantitative targets for cell counts and volume of the collected cells/tissue, and any anticoagulants or reagents used. It is conventional to collect bone marrow by percutaneous needle aspirations from the posterior iliac crest(s) into syringes prefilled with heparin and/or citrate (acid citrate dextrose solution A, ACD-A) as anticoagulant. For MSCs, the bone marrow volume aspirated is typically no more than 50 mL, about 5% of the volume required for conventional HSC transplant products. The quantity of bone marrow needed to manufacture the product will determine the number of aspirations and aspiration sites; it has been recommended that the volume of each aspiration be limited to 2–5 mL to avoid dilution of the bone marrow with peripheral blood [31]. Specifications for incoming bone marrow intended for MSC preparation typically include appearance (e.g., absence of visible clumps/clots) and number of viable nucleated or mononuclear cells. Although it is reasonable to specify sterility for the incoming cell source, methods to detect bacterial and fungal contamination may require up to 14 days, after MSC culture has already been initiated. Even when using appropriate aseptic methods, bacterial contamination of bone marrow aspirated by the percutaneous route occurs in

a small fraction of cases [32]. It may therefore be useful to establish a baseline of microbial test results on bone marrow aspirates and other sources during development runs.

Manufacturing Process Development

Most MSC manufacturing processes are based on methods developed by research investigators to isolate and expand adherent MSCs from a given tissue source. A standardized protocol for MSC preparation from bone marrow has been published by the Developmental Committee of the European Group for Blood and Bone Marrow Transplantation (EBMT) [33]. This protocol, implemented at academic centers participating in EBMT's phase 2 clinical trial of MSCs for acute graft-versus-host disease, specified the following steps for MSC manufacturing, starting with bone marrow from the donor of the hematopoietic cell transplant:

- Isolate bone marrow mononuclear cells (BM-MNCs) by density gradient (Percoll or Ficoll hypaque) centrifugation
- Wash and resuspend BM-MNCs in Dulbecco's modified Eagle's medium (DMEM) – low glucose, supplemented with 10% fetal bovine serum
- Plate BM-MNCs in 175 cm^2 flasks at cell density of 160,000 cells/cm^2
- Incubate flasks at 37 °C, 5% CO_2, humidified
- When cultured MSCs reach 80% confluence, detach with trypsin and ethylenediaminetetraacetic acid (EDTA) and replate at cell density of 4,000 cells/cm^2
- When total MSC number reaches at least 2×10^6, harvest using trypsin and EDTA, wash and either cryopreserve (in 10% dimethyl sulfoxide, DMSO) or resuspend in saline solution according to local guidelines

For this protocol, release criteria for the final MSC product were absence of visible clumps, presence of spindle-shaped morphology, absence of microbial contamination, viability >95%, and a phenotype showing CD73, CD90, and CD105 expression and absence of CD34, CD45, CD14, and CD3 expression.

Use of a published manufacturing protocol still requires local analysis and verification, that is, the entire process, when performed in the sponsor's manufacturing facility, should be demonstrated to result consistently in products with the desired specifications. Modifications of the standard protocol that have been used, or are currently being investigated, include:

- Use of alternative cell sources, which requires different initial processing steps (e.g., enzymatic digestion and centrifugation for adipose tissue) [34]
- Elimination of the density gradient step to isolate MNCs from bone marrow [35]
- Use of alternative culture media [36]
- Substitution of fetal bovine serum (FBS) with human platelet lysate [37] or use of serum-free media

- Use of multilayered cell factories to allow greater expansion of MSCs in a semi-closed system
- Use of plastic bags to achieve a closed system during culture expansion
- Use of bioreactors to achieve a closed, semiautomated system during culture expansion [38]
- Use of alternative cryopreservation solutions, including lower concentrations of DMSO (5%) or commercial solutions with standardized (but proprietary) ingredients
- Incorporation of more complex manipulations, including gene modification or combination of MSCs with synthetic or natural biomaterial components

The number of MSCs per product and the number of MSC products to be generated from a single donor collection depend on the clinical protocol and whether the intent is to prepare a patient-specific (autologous or allogeneic directed) or an allogeneic, unrelated donor, "off the shelf" product. The process design should specify the optimal and maximum number of cell expansions or culture passages, and whether MSCs will be cryopreserved and stored as final products or in a master cell bank from which future MSC products will be generated. For the latter, the cell bank documentation and testing requirements are more extensive [25, 39, 40]. For clinical trials requiring more than one allogeneic unrelated donor to generate a sufficient number of "off-the-shelf" products, lot-to-lot comparability studies must be done.

Ancillary materials, defined as materials that come into contact with the product during manufacturing but are not intended to be part of the final product, must be qualified for use in clinical manufacturing. It is ideal to identify and qualify clinical-grade materials that can be substituted for research-grade materials as early as possible in CMC development. Substitution of an ancillary material in later stages of development usually requires comparability studies to assess impact on the quality and quantity of the product; these studies can be expensive. Typical ancillary materials for MSC products include reagents (anticoagulants, density gradient media, culture media, FBS, antibiotics, and trypsin) and plastic containers (flasks, bags, tubing, typically single-use, disposable) for transfer, culture, and cryopreservation/storage of the cellular product. The US Pharmacopeia (USP) has published a risk-based approach to qualification of ancillary materials [41]. In this schema, specific qualification and risk-reduction activities are associated with the risk tiers, which are as follows:

- Tier 1: Low-risk, highly qualified materials with intended use as therapeutic drug or biologic, medical device, or implantable material (e.g., heparin, antibiotics, human serum albumin)
- Tier 2: Low-risk, well-characterized materials with intended use as Ancilliary Materials (AMs) produced in compliance with GMPs (e.g., recombinant cytokines, immunomagnetic beads, human AB serum, proteolytic enzymes)
- Tier 3: Moderate-risk materials not intended for use as AMs (frequently produced for in vitro diagnostic use or reagent grade materials, e.g., monoclonal antibodies, tissue culture media)

- Tier 4: High-risk materials, materials not produced in compliance with cGMPs and materials not intended to be used in cell manufacturing (e.g., FBS, some enzymes, human or animal cells used as feeder layers)

At a minimum, the cell therapy product developer should request a certificate of analysis, assess lot-to-lot effect on process performance, assess removal from the final product, and assess stability during storage, for each ancillary material. Additional qualification and/or risk reduction activities are indicated for higher-risk materials. If the manufacturer has submitted a master file of proprietary information on the reagent/material to FDA, IND sponsors may request permission from the manufacturer to cross-reference that master file and thereby allow FDA reviewers access to the proprietary information during review of the IND.

Animal source reagents such as FBS are classified in the highest-risk tier, because of the potential for transmission of TSE and other animal diseases. All animal-sourced reagents should be qualified by documentation of country of origin and assurance that the country of origin is judged as safe with respect to relevant animal diseases; adventitious agent testing for source-relevant viruses is also necessary. In addition to transmissible disease risk, FBS used in production of vectors for gene-modified cell therapies has been associated with delayed hypersensitivity reactions and development of antibodies to bovine apolipoprotein B-100, which appeared after the second infusion [42]. These acquired xenoantigens in the product have been shown to be highly resistant to washing. These observations suggest that FBS should be avoided if at all possible, especially when a repeat dosing schedule is planned. If it is not practical to establish a manufacturing process with human-derived products or serum-free media to replace FBS, then FBS should be selected and qualified according to standards in USP chapters <1,024> and <90>, which include specifications for sourcing, manufacturing, packaging, storage, labeling, characterization, and safety testing [43, 44].

There is a distinction between ancillary reagents and excipients used for drugs and biological products. Whereas an ancillary reagent is not intended to be in the final product and is usually removed by specific processing steps, an excipient is a substance added to a formulation to provide benefits to the processing or protection of the active ingredient and is intended to be present in the final product as an inactive ingredient. FDA does not "approve" either ancillary reagents or excipients but does maintain an Inactive Ingredients Database for those excipients used in approved drug/biological products. For a given product, use of an excipient on that list, in a concentration previously used by another sponsor, is likely to expedite that product's review and approval. FDA does not maintain a comparable list for ancillary reagents.

DMSO commonly used at 5–10% concentration in cryopreservation solutions for MSCs and other cell therapy products can be classified as either an ancillary reagent or an excipient, depending on post-thaw processing steps. DMSO is not listed on FDA's excipient list but is FDA approved as a 50% aqueous solution for bladder irrigation to treat interstitial cystitis. Infusion of DMSO

results in dose-dependent cardiovascular, gastrointestinal, and neurological toxicity; one mechanism identified is DMSO-induced histamine release [45]. DMSO adverse effects have been well documented in recipients of HSC transplant products [46], and recent case reports of severe neurotoxicity in patients with preexisting cerebral disease or history of epileptic seizures raise additional concerns [47–49]. Removal of DMSO from cell therapy products can be accomplished by manual or automated washing methods, either of which can incorporate single-use disposables configured in a semi-closed system. However, post-thaw washing does not always prevent adverse reactions [45]. Residual DMSO can be quantified by high-performance liquid chromatography in cell therapy products, but residual testing for DMSO has not become a routine practice [50]. Physicians typically manage the risk of DMSO adverse effects by administering oral or parenteral diphenhydramine, an anti-histamine, and restricting DMSO in intravenously infused cell therapy products to 1 g/kg/24 h [51]. Additional precautions may be warranted for DMSO-containing products administered by alternative routes.

Procedures for in-process and final product sampling and testing should be evaluated during development of the manufacturing process, with test result acceptance criteria established for intermediate (in-process) and final products (release). Specification of the sample timing, volume, and type (e.g., cells only, cells + supernatant, discarded residual cellular product, or supernatant only) is required in the IND submission. During process design, the need for thorough and reliable assay results must be weighed against the risk of excessive product cell loss due to sampling requirements, especially for patient-specific products.

Specifications developed for the final product formulation should include the cell number per aliquot/container, size and number of product aliquots, type and volume of excipient, and container size/configuration, and specify if any pooling of stored aliquots will be done. The excipient should be FDA approved whenever possible. The original MSC product as formulated may require further manipulations such as thawing, dilution, washing, sampling, pooling, or transfer to a different container to prepare it for clinical use. Standard procedures need to be developed for these steps and require specification of where and when these steps will be performed and who will perform them.

Impact of the Clinical Protocol on CMC Development

The clinical protocol's description of how the product will be administered to the patient must be aligned with the CMC description of the product's formulation, dose, identity, potency, safety/purity, stability, and labeling. The logistics of getting the product into the patient are often ignored until the late stages of CMC development but should be considered early. Optimal and predictable timing of product administration requires not only clear definition of the route of

administration but also detailed understanding of both the patient's overall clinical regimen and the location of the patient care unit in relationship to the site(s) of product manufacturing, storage, and final preparation steps. These factors will bear on decisions regarding final product formulation. Stability should be assessed on the MSC product after final preparation steps, whether freshly harvested or thawed after cryopreservation. Based on stability studies, specifications should be developed for MSC recovery, viability, and function after a defined interval that allows for reasonable transport time, and the possibility that the patient's clinical status may cause unforeseen delays in product administration.

Assay Development

CMC development is not complete without development of assays for product safety, identity, potency, purity, and dose [24, 52]. Planning and evaluation of product assays should be considered early in IND development, because assay results will inform the design of the manufacturing process and must be established for preclinical (GLP) and clinical (GMP) product manufacturing and for assessment of product stability and lot-to-lot consistency.

During CMC development, it is useful to evaluate a greater range and frequency of assays than what will ultimately be done during GLP/GMP product manufacturing. This serves to identify the most appropriate battery of assays and to optimize sample specifications and timing. All assays, even those considered standard, should be evaluated with in-house samples and qualified, that is, demonstrated to meet performance specifications for each proposed sample of a given size, cell content, and suspension medium (including anticoagulant). For example, most automated cell counting and blood culture systems consist of standardized kits and instrumentation designed to test patient samples and have not been validated for cell therapy product testing; these systems should therefore be qualified for cell therapy product samples. Assay development typically continues beyond IND submission, through clinical trials, to meet the progressively stringent cGMP requirements for assay validation by phase 3. Full validation of an assay requires thorough evaluation and description of the assay's parameters, including precision, specificity, linearity and range, system suitability, and robustness [53, 54].

For MSC and other cell therapy products, process development studies should establish performance criteria for critical manufacturing steps (e.g., density gradient separation, culture expansion, cryopreservation). These criteria are dependent on well-defined assays, which at minimum include cell counts and viability to allow calculation of viable cell recovery. Other assays (phenotype, function/bioactivity) are often desirable to define process performance criteria. Assays for the presence of residual ancillary reagents may need to be carried out during manufacturing process development or even as a final product release assay during cGMP manufacturing. Stability studies on cryopreserved products typically

include cell counts, viability, function/bioactivity assay, and sterility, all measured pre-freeze, immediately post-thaw, and on the thawed product after a defined timeframe to demonstrate that the product retains attributes important for efficacy and safety.

Typical assays for MSC products are presented in Table 15.1. It is conventional to specify in the CMC that a given assay on a given sample will be used for either in-process testing or product release testing. Assays that establish identity of the cellular product as MSCs include cell counts, cell surface phenotyping, and light microscopic cell morphology. Additional identity testing may be required to establish that a given MSC product is distinct from other products prepared in the same facility, for example, human leukocyte antigen (HLA) testing or DNA fingerprinting. Extensively expanded MSC product should have karyotype analysis to document that MSCs have not acquired chromosomal abnormalities. Cell counts and phenotyping also function, in combination with viability, as dose-defining assays. Flow cytometric phenotyping of a relatively pure population of MSCs after culture expansion will show expression of CD73, CD90, and CD105 in >90–95% of cells, and little or no expression of antigens associated with peripheral blood or bone marrow hematopoietic cells, that is, CD45 (pan-leukocyte marker), CD34 (hematopoietic cell marker), CD14 or CD11b (monocyte/dendritic cell markers), and CD19 or CD79α (B cell markers) [55].

Assays that address product safety include microbiological assays for bacteria, fungus, and mycoplasma. Product testing for adventitious viruses is required only for master cell banks, working cell banks, and as a one-time test on end of production cells from a cell bank [25]. Safety is also addressed by purity assays, where purity is defined by FDA as "freedom from extraneous material in the product, whether or not harmful to the patient or deleterious to the product." Purity assays include endotoxin and residuals, that is, residual ancillary reagents or residual cells that are irrelevant or unwanted [25].

Development of potency assays for MSC products is not straightforward, because MSCs have a wide array of potential biological activities. Although many potency assays are functional assays, the term "potency assay" is not synonymous with "functional assay." The concept of product potency is focused on defining measurable product attributes that will predict that a product will result in the desired effect in vivo [56]. A validated potency assay is required for product licensure, but not required for early phase clinical trials. However, FDA usually recommends that sponsors start considering potential potency assays during early clinical development. While it is ideal for the potency assay to represent the product's mechanism of action (i.e., relevant therapeutic activity or intended biological effect), many cell-based products have multiple or poorly defined mechanisms of action, such that it is difficult to define a single attribute most relevant to potency. A potency assay can be either a biological assay or an analytic assay that serves as a surrogate for bioactivity. For example, for a HSC product, the number of CD34+ cells, calculated from flow cytometric phenotyping and automated cell counting values, might serve as the potency assay, if that assay has been demonstrated to predict clinical efficacy. MSC product potency assessment for a given clinical application might be based on quantifying

Table 15.1 Assays used for MSC manufacturing process development and GMP manufacturing

Assay	Assay category	Methods	Purpose	Expected results and comments
Cell counting and viability	Identity and dose defining	Hemocytometer and light microscope	Enumerate viable and nonviable MSCs and other cells	In-process and final product targets defined for each application
		Trypan blue exclusion to assess viability		% Viability typically specified as >70% [25]
Cell surface phenotyping	Identity and dose defining	Flow cytometry to quantify cells with expression of MSC and other cell markers	Identify and quantify MSCs and other cells	MSCs express (≥95%) CD73, CD90, and CD105
				MSCs do not express (≤2%) CD45, CD34, CD14 or CD11b, and CD19 or CD79α [55]
				Other markers (e.g., HLA-DR) may be desirable for some protocols
Cell morphology	Identity	Light microscopy	Confirm MSC morphology	Spindle-shaped cells, no aggregates [55]
HLA typing or DNA fingerprinting	Identity	Molecular methods	Confirm identity of product	HLA antigen typing by PCR
			For patient-specific product, confirm match of product to patient	DNA fingerprinting by STR or VNTR
Karyotyping	Identity	Cytogenetic methods including FISH	Confirm genetic stability of MSCs after culture expansion	Normal (46 XX or 46 XY) karyotype, with no chromosomal abnormalities
Trilineage differentiation	Identity	Histochemical staining after in vitro culture to promote MSC differentiation	Confirm multipotency of MSCs	Trilineage differentiation to adipocytes, chondroblasts, and osteoblasts [55]

(continued)

Table 15.1 (continued)

Assay	Assay category	Methods	Purpose	Expected results and comments
Bioassays related to in vivo mechanism of action	Potency (functional, bioactivity)	Protocol-specific	Measure MSC product attribute that predicts therapeutic effect in vivo	Application specific Potency assay is not necessarily a bioactivity/functional assay and is not required for early phase studies [56]
Sterility (bacterial and fungal)	Safety	Culture by CFR/USP method, or commercial automated blood culture method (e.g., BacT/Alert, Bactec)	Detection of bacterial and fungal organisms	No growth of organisms If antibiotics used, may need bacteriostasis and fungistasis testing Method should comply with 21 CFR 610.12 or USP <71>, or be qualified/validated [26, 57]
Mycoplasma	Safety	Culture (may include biochemical indicators) PCR for antigen detection	Detection of mycoplasma organisms or antigens	No growth in culture, or no mycoplasma antigens detected Sampling recommended at time points with highest likelihood of detection [26]
Pyrogenicity (Endotoxin)	Safety/purity	Endotoxin limulus amebocyte lysate (LAL) method (gel clot, chromogenic, or kinetic) or alternative	Detection of endotoxin in comparison to an endotoxin standard	For LAL method, upper limit of endotoxin is 5 EU/kg patient weight/h for parenteral administration [26]
Residual reagents	Safety/purity	Various analytic methods, e.g., HPLC	Quantify residual reagents	Application specific[26, 58]

MSCs with a defined flow cytometric phenotype but for another application might include a functional assay such as a cytokine secretion profile. The potency of a gene-modified cell therapy typically incorporates both a measure of gene transfer (e.g., vector copy number per cell) and the biological effect of the transferred gene [56].

Resource Needs for CMC Development

A coordinated multidisciplinary approach is needed to ensure that CMC development can proceed in concert with preclinical studies and development of the clinical protocol. Bone marrow for use in research and development studies can be purchased from commercial vendors with capability for appropriate consenting, selection, and qualification of normal donors. If these functions are performed in-house, the staff to identify, administer consent to, and qualify normal donors of research cells are the same as staff that would perform these functions for clinical manufacturing. Bone marrow collection from normal donors requires an Institutional Review Board (IRB)-approved clinical protocol with appropriate informed consent. Donor screening may be performed by nursing or medical staff using a blood donor medical history questionnaire and supplemented with additional screening questions, if appropriate. Infectious disease testing is best accomplished by a laboratory qualified to test blood donors, using appropriately licensed/approved test kits. Collection of bone marrow for MSC production typically requires only local anesthesia but should be performed by a licensed physician, usually a hematologist or oncologist qualified for this procedure by experience. Technical staff usually participate in the bone marrow collection procedure by preparing sterile, anticoagulant-filled syringes, and ensuring that the anticoagulated bone marrow is labeled with appropriate identifiers and transported to the processing lab.

The range of personnel and laboratory resources for manufacturing process development should parallel those required for cGMP product manufacturing, in that development and scale-up experiments should progressively mimic conditions that would be used in cGMP manufacturing, that is, use of aseptic technique, appropriate controls, and well-defined laboratory methods. However, development runs do not require adherence to the extensive cGMP facility, quality, and documentation requirements. Because conventional research labs are not often designed or staffed for process development studies, some developers have established "pre-GMP" facilities, with specially trained technical staff to conduct these studies before transfer of the process to a cGMP environment. cGMP facility resources are presented in Quality and Requirements for Manufacturing, cGMP and cGTP Requirements below.

CMC development also requires trained technical staff and equipment for standard cell counting, viability, and flow cytometric phenotyping. Molecular testing such as HLA or DNA fingerprinting is best performed by laboratories that focus primarily on those techniques. Sterility (bacterial, fungal, mycoplasma)

and endotoxin testing may be set up in-house or contracted to specialized laboratories. For development of more complex or novel bioassays, special expertise may be needed, especially for validation of a potency assay prior to a phase 3 trial. These assays are often developed in-house and then transferred to a contract research organization (CRO) for qualification and validation. This involves working closely with the technicians and quality personnel at the CRO to ensure that the assay is properly transferred and can produce reliable and robust results. For products used in GLP preclinical studies, assay results should be compiled in proper report format and reviewed by an independent quality assurance officer.

Clinical Protocol Development

Regulatory Requirements for Clinical Protocols

The clinical protocol is a formal written document whose format and content are guided by requirements of the regulatory authority and the local institution and, in some cases, the funding agency. As research involving human subjects, clinical trials must be reviewed and approved by an ethical committee or IRB. Multi-institutional studies typically require IRB approval for each clinical site. In the USA, IRBs operate locally but are guided by the federal Protection of Human Subjects regulations [59], administered by the Office for Human Research Protections (OHRP) of the Department of Health and Human Services (DHHS). These regulations overlap substantially with FDA's IRB and informed consent regulations [60, 61].

Clinical Protocol Elements

The key elements of the clinical protocol that need to be established during the protocol development process are shown in Table 15.2.

In addition to the actual clinical study protocol, practices and documents to ensure appropriate informed consent, privacy, and confidentiality of subjects are required and are typically reviewed by the IRB.

The clinical protocol should be defined primarily by experts in the clinical disease discipline who have an in-depth understanding of the disease, patient population, and outcome (endpoint) measures, and of adverse events likely to occur in the patient population. Definition of the most appropriate patient population for a given clinical trial should take advantage of published data on the disease's natural

Table 15.2 Key elements of clinical study protocols

Study element	Description and comments
Study objective	Clear statement of the study's purpose
	May include multiple objectives, but each should be designated as primary or secondary
	For phase 1 study, primary objective is to evaluate safety but may have secondary objectives that address efficacy
Study rationale	Clinical protocol must be supported by a rationale based on preclinical animal studies and previous clinical studies
	Should include rationale for dose, dosing schedule, and route of administration
Study phase and design	Designate study phase (1, 2, or 3)
	Include description of randomization, stratification, use of placebo controls, sample size, and cohorts for dose escalation
Study population	Include disease state, stage of disease, performance status, key inclusion criteria, key exclusion criteria
Administration of study drug	Include dose, route, schedule, and use of repeat dosing
	For phase 1 study, describe dose escalation between cohorts, number of subjects per cohort, definition of dose-limiting toxicity, and how maximum tolerated dose will be determined
	For phase 2 and 3 studies, include dose modification criteria for specific known adverse events
Concomitant therapies and schedule of interventions	Describe subject's ongoing therapies that may be continued or should be discontinued during study
	Present detailed schedule of interventions, including therapies, routine and special laboratory testing, radiologic studies, and clinical evaluation, including recording of adverse events
Outcome (endpoint) measures	Outcome measures should be aligned with study objectives
	Provide qualitative and quantitative description of outcome measures (clinical, laboratory, imaging)
	For phase 1 study, outcomes will focus on safety/toxicity but may include efficacy outcomes
	For phase 2 and 3, efficacy outcomes should be well defined and provide clear definition of a positive response
Analysis plan	For phase 2 and 3 studies, analysis plan should include outcome to be measured, definition of a positive response, population to be analyzed, method of analysis, and treatment effect that can be detected for a given study size
	Include information on stopping rules for individual subjects and entire study
	Indicate nature and timing of safety reviews or interim analyses by a data safety monitoring board or committee
Logistics	List study sites
	Describe how study sites will interact with coordinating center and with each other

(continued)

Table 15.2 (continued)

Study element	Description and comments
Safety monitoring	Present definitions for adverse events, serious adverse events, including description of those expected with study interventions
	Define criteria for reporting adverse events to IRB, FDA, and other parties if required
	Include statement about collection of adverse events in patients who discontinue participation before completing study
Termination criteria	Present criteria for discontinuing the study, based on patient outcomes and adverse events

history, prognostic factors, and responses to therapy in previous clinical trials. Conventional study designs may need to be modified for certain disease states. For example, the strong placebo effect frequently observed in clinical trials of cardiovascular disease has prompted use of placebo control arms, even for phase 1 studies evaluating safety [62]. Adverse event classification and grading guidelines used in cancer clinical trials are detailed in NCI's Common Terminology Classification for Adverse Events (CTCAE) and may be applicable to other disease disciplines too [63]. FDA has published guidance for cell-based therapies for specific product classes in specific disease states, including cell-scaffold products for knee repair and replacement, pancreatic islets for diabetes, therapeutic cancer vaccines, cord blood for hematologic malignancies and other diseases, and cell therapies in cardiac disease [64–68].

Input from the product manufacturing team and experts in the practical details of product administration are important for a successful clinical protocol. Issues commonly overlooked or misunderstood by clinical investigators unfamiliar with cell-based therapies are:

- The need to coordinate timing of final product preparation with administration to the patient
- Reasonable infusion volumes and rates for cellular products
- Expected adverse events related to infusion of fresh or cryopreserved cellular products [51]
- The need for premedication/histamine blockade in patients receiving products containing DMSO

Resource Needs for Clinical Protocol Development

Clinical protocols are developed primarily by clinical investigators with expertise in the disease under study. During IND development for a novel cellular therapy,

clinical investigators should interact extensively with preclinical scientists to ensure that the rationale for the clinical trial will be well supported by preclinical animal studies, especially with regard to cell dose, dosing schedule, and route of administration. It is also critical for the clinical team to interact with the product/CMC team to ensure a common understanding of product characteristics (identity, dose, potency, purity, and safety), which will impact definition and measurement of clinical efficacy and safety endpoints. For patient-specific (autologous and family-related allogeneic) MSC products, it is especially important for clinical protocol development to incorporate an understanding of donor variability, methods for qualification and screening of the donor source, and timing of collection, product manufacturing, and final product preparation in relation to administration of the product to the patient. Clinicians who are not from conventional hematology/oncology/bone marrow transplantation backgrounds will need to interface with experts on the practical clinical requirements for administration of, and commonly expected adverse events of, cell-based therapies. Clinical protocol development also requires expertise in biostatistics and study design and may require expertise in bioethics, especially for novel stem cell therapies with unknown risks. Finally, if assessment of clinical endpoints requires development or customization of specialized assays (e.g., to assess immune response) or clinical imaging methods, this expertise should be included on the multidisciplinary team.

Quality and Implementation Issues for MSC Clinical Trials

Quality and GxP

Quality system requirements for the conduct of preclinical studies, product manufacturing, and clinical trials are contained in FDA regulations and guidances for GLP, GMP, and Good Clinical Practice (GCP), respectively [23, 69, 70]. These requirements are often referred to, collectively, as the "GxP" requirements, because they all share common quality system elements. The focus of GxP requirements is adherence to, and documentation of, well-defined practices to ensure consistency and integrity of results in preclinical studies, product manufacturing, and clinical trial. These practices include quality control measures that prospectively prevent and/or detect defective products or services and quality assurance measures that retrospectively review critical aspects of the system and data output. Because GLP requirements for preclinical studies have been addressed above, the discussion below is focused on GCP and GMP requirements.

Quality and Requirements for Clinical Trials

Implementation of clinical trials for investigational drug and biological products is dictated by GCPs, OHRP regulations, and the organizational policies and practices of the trial's sponsor and investigators.

Good Clinical Practice Requirements

GCPs are international standards for ethics and scientific quality in the conduct, monitoring, audit, analysis, record keeping, and reporting of clinical trials, as presented in the International Council for Harmonization (ICH) E6 guideline [70]. GCPs are also captured in the Federal FD&C Act of 1938 [10], Title 21 of the Code of Federal Regulations [11], and guidelines at federal and state levels. The purpose of the GCPs is twofold: (1) to ensure the protection of human subjects and (2) to ensure the integrity of clinical trial data, so that future patients will be protected.

Key parties with obligations in clinical trials, as defined in GCPs and the IND regulations, are the sponsor, the investigator, and the IRB. The responsibilities of each of these parties are presented in Table 15.3. The sponsor is the party who takes overall responsibility for, and initiates, a clinical trial. The investigator is the person who conducts the clinical trial. A dual role called sponsor-investigator is common in many academic clinical trials. The IRB, defined in the IND regulations as one type of independent ethics committee (IEC), is a review panel responsible for ensuring protection of human subjects involved in a clinical investigation. Industry-sponsored trials frequently engage a contract research organization (CRO), defined as a person, company, or agency that serves as an independent contractor for the sponsor and assumes one or more of the sponsor's obligations.

In addition to IRB review and approval, each clinical protocol must have a Data Safety and Monitoring Plan (DSMP). NIH requires use of a Data Safety and Monitoring Board (DSMB) for phase 3 trials that it supports but allows alternative monitoring plans for phase 1 and 2 studies. Alternatives include use of the IRB, an independent monitor, a designated medical monitor, the principal investigator, or an internal committee with explicit guidelines, to monitor the clinical trial.

Resource Needs for Clinical Trial Implementation

Based on GCP requirements, the resources needed to implement a clinical trial of autologous or allogeneic MSCs include:

Table 15.3 Responsibilities of sponsor, investigator, and IRB in clinical trials

	Sponsor	Investigator	Institutional review board (IRB)
Definition	Party initiating the clinical trial	Party conducting the clinical trial	Review panel responsible for ensuring protection of human subjects
Primary responsibilities	Overall responsibility for clinical trial's ethical, scientific, and regulatory obligations	Ensure the rights, safety, and welfare of human subjects in the clinical trial	Protect the rights, safety, and welfare of human subjects in the clinical trial
Specific or additional responsibilities	Ensure that trial is adequately supported by nonclinical and clinical information Provide scientifically sound, clear protocol Ensure that protocol is approved by IRB Ensure that decisions made on behalf of study subjects are made by qualified physician Ensure that all clinical study tasks are performed by qualified personnel Ensure that all clinical trial information is recorded Ensure that informed consents were obtained and freely given Ensure protection of confidentiality of records Ensure preparation of investigational product under cGMP Ensure that quality systems have been implemented Report unanticipated adverse events	Adhere to protocol and its procedures Ensure proper conduct of trial at sites under his/her authority, in compliance with signed agreement, investigational plan, protocol, and applicable regulations Prepare timely and accurate reports to IRB, sponsor, medical monitor, and FDA Maintain accurate records Control the investigational product Ensure that patients sign informed consent form before study procedures begin Maintain appropriate and scheduled communication with study sponsor, medical monitor, and IRB	Ensure that risks to subjects are minimized and reasonable in relation to anticipated benefits Ensure that patient selection is as equitable as possible relative to demographics and the clinical trial's purpose Review the protocol, informed consent form, and investigator brochure Ensure that a pediatric clinical trial is conducted according to current regulations Provide initial and continuing review of ongoing clinical trial Request more information, if required Approve, disapprove, and terminate the clinical trial

- Investigators responsible for implementing the trial at each site, including a PI who will lead the overall effort
- Staff to establish agreements/contracts with each clinical site and to train investigators and clinical staff at each clinical site
- Staff at each site to recruit subjects, conduct the informed consent process, conduct preenrollment screening (medical history, physical examination, lab assessments), enroll subjects, conduct follow-up visits, and complete the case report forms
- Resource and mechanisms for randomization of subjects, creating placebos, and blinding of treatment assignments, if necessary; for example, subjects may receive instructions from an interactive voice response system (IVRS)
- Technical and/or nursing staff who will be responsible for shipping/receiving of the MSC product, final formulation steps, and maintaining a log of receipt, distribution, destruction, and return
- Clinical staff to administer the product to, and monitor the subjects, to conduct assessments, and to report adverse events
- Independent staff (monitors) who will ensure compliance to GCP and evaluate source documents for completeness and data integrity
- Qualified staff to plan and execute the DSMP, that is, to analyze clinical trial progress, safety and efficacy data, and to make recommendations to continue, modify, or terminate the study. At a minimum, this should include medical and biostatistical expertise and may need broader expertise if a formal DSMB is required

Quality and Requirements for Manufacturing

cGMP and cGTP Requirements

Academic investigators are frequently confused about the applicability of cGMP to early phase clinical trials due to misunderstanding of the difference between statutory and regulatory laws. cGMP is a standard based on the US Food, Drug, and Cosmetic Act (a statute) and applicable to all drugs, biological products, and devices [71]. The cGMP regulations in the Code of Federal Regulations (CFR) apply to all drugs and biologics in phase 2 clinical trials and beyond, but phase 1 trials are considered exempt from those regulations [69, 72]. However, FDA expects phase 1 trials to be compliant with statutory cGMP and has the authority to regulate phase 1 investigational agents by IND reviews and inspections. This is the case for MSC products in phase 1 clinical trials.

In addition to cGMP, MSC manufacturing should comply with current good tissue practice (cGTP) regulations for human cells, tissue, and cellular and tissue-based products (HCT/Ps) [73]. In fact, the cGTP requirements overlap substantially with cGMP requirements in their approach to ensuring product quality and safety but address more specifically the prevention and detection of

communicable disease and tracking of the product from donor source through administration to the patient.

Recent FDA guidance on cGMP for drugs and biologics in phase 1 presents approaches for all key aspects of cGMP and highlights a number of technologies and resources that may facilitate cGMP compliance, including use of disposable equipment and supplies, use of commercial prepackaged sterile materials, use of closed processing systems to alleviate the need for stricter air quality classification, and use of shared cGMP manufacturing and testing facilities [74]. For cell and gene therapy products in phase 1, the following measures are specifically recommended:

- Cleaning and procedural controls (line clearance, component and product segregation, use of unique product identifiers) to prevent cross-contamination and mix-ups
- Control of the manufacturing process in conjunction with in-process and release testing and retention of product samples for subsequent analysis and comparison, to ensure consistency in product quality
- Internal reviews of manufacturing performance when multiple batches are produced, to ensure product safety and quality
- Use of appropriate cleaning and environmental testing procedures and use of dedicated equipment and/or disposable parts (e.g., tubing), to prevent contamination
- Use of manufacturing controls to ensure aseptic processing, including use of an aseptic workstation, process simulations to demonstrate that a sterile product can be produced in the manufacturing environment, and environmental monitoring; use of appropriate sterility testing methods; training of personnel in aseptic technique; and ensuring that the product is not released before review of records indicates that aseptic procedures were followed and that acceptable results of sterility testing were obtained [75]

The cornerstone of cGTP and cGMP is a comprehensive quality program to prevent, detect, and correct deficiencies in manufacturing. All manufacturing staff should be trained in cGMP and cGTP requirements, including the need to follow standard operating procedures and to concurrently document all critical manufacturing steps. Quality should be integrated with operational activities and their management. Specific staffing should be established to perform and oversee defined quality functions, as well as management of the cGMP facility and oversight of staff. Quality control (QC) functions are prospectively applied practices that typically include equipment monitoring, management of incoming materials (reagents, containers, supplies), regular monitoring of the facility environment, concurrent review of critical steps in manufacturing and testing, and release of the product after review of its conformance with lot release criteria. Although small facilities may not have adequate staffing to identify separate personnel for all QC functions, it is critical that cumulative manufacturing record review and release of final product lots be assigned to a person who was not directly involved with manufacture of the product. Quality functions applied retrospectively (often called quality assur-

ance) consist of audits, which are independent inspections by staff internal to the organization, or external, that is, by accreditation or regulatory bodies. Internal audits are often focused on specific aspects of the operation and may be done on a regularly scheduled basis or in response to deviation reports. External audits of cell therapy facilities are typically comprehensive and performed by accreditation organizations such as the Foundation for Accreditation in Cellular Therapy (FACT) or American Association of Blood Banks (AABB) but can also be outsourced to independent specialists in quality compliance. Audits should encompass all aspects of product collection, manufacturing, and testing, and should include contract manufacturing and testing facilities, if used. The quality program should incorporate mechanisms for documenting, analyzing, reporting, and correcting problems or deviations that occur.

Resource Needs for Quality in MSC Manufacturing

Resources to ensure quality in MSC manufacturing consist of well-qualified and trained personnel for manufacturing and quality functions. Ideally, an overall operations manager should be designated to oversee facility and manufacturing operations and to ensure that the quality program is integrated with operations. Depending on the size of the operation, manufacturing staff may be assigned, as either part-time or full-time "quality operations" staff, to specific QC functions described above. Cumulative manufacturing record review and release of a given final product lot should be performed by a person not directly involved with manufacturing of that lot. Although these functions may be performed by a staff member within the operation, it is ideally handled by a designated quality officer who is independent from manufacturing. An independent quality officer is also required to perform internal audits and audits of contract facilities. Additional duties that may be performed by a quality officer or an overall operations manager are maintenance of personnel training and competency records and preparation of the facility/operation for external audits. Record keeping functions may require in-house information technology support and/or outsourcing to a contractor.

Project Management and Multidisciplinary Teams for MSC Clinical Trials

A project management approach is necessary to develop the multidisciplinary team and guide the project from basic research, through development and to manufacturing and the clinical trial, regardless of whether the sponsor is based in

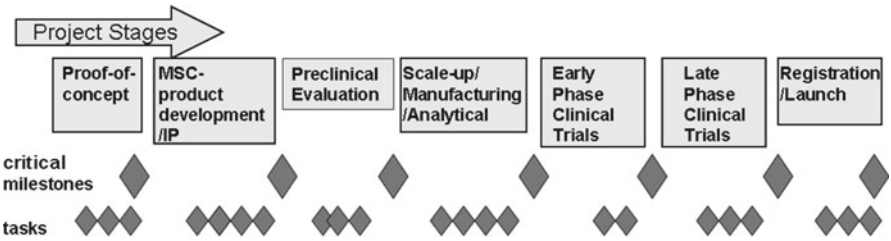

Fig. 15.4 Project stages during the product life cycle

academia or in industry (Table 15.4). This approach requires that the diverse tasks be assigned to people with appropriate expertise and allows for all aspects of the development process to proceed simultaneously and in concert with one another.

In the pharmaceutical and biotechnology industries, a stage-gated process has been successfully employed to transition projects through the different stages of discovery and development (Fig. 15.4). With this approach, each stage consists of more than one goal and is managed by a multidisciplinary team, resulting in a work matrix. Passing through a gate means achieving a critical milestone (usually composed of sub-milestones) and planning and committing for the next stage. The decision to move to the next stage, also called a go/no-go decision, is based on reviewing critical information (e.g., proof of concept, preclinical toxicity, manufacturing feasibility) against a set of predetermined criteria. All relevant information is typically judged by a committee of reviewers, which could include an executive management committee, principal investigators and collaborators, or a grant funding review panel. Gate decisions do not usually impede project progress unless critical milestones have not been met, or the decision-makers believe the project should not progress to the next stage due to resource limitations. Sometimes milestones are not fully met in a stage, and there may be opportunities to allow the project to proceed with the caveat that unmet milestones are addressed in the project's next stage. However, acknowledgment of early failure can prove to be more cost-effective than delaying the go/no-go decision, in that it allows the sponsor to focus on other projects with greater chance of success.

A project leader is required to keep the project on track and monitor its progress, resource use, and multifaceted requirements, including basic research, preclinical safety, manufacturing, quality, regulatory, intellectual property, and budget. Project leaders at the discovery stage are usually the principal investigators or scientists working in the field of their expertise and tend to be focused on the scientific data, rather than the many diverse elements bearing on product development. Project leaders at the development stage should be detail oriented but able to analyze and assess the development status of the project, comfortable with project tracking and

Table 15.4 Multidisciplinary approach to drug and biologics development

		Key personnel resources needed to accomplish project goals based on sponsor		
Project stage	Major goals	Academia sponsored (single site)	Academia sponsored (multiple sites)	Industry sponsored
Proof of concept (discovery and early translational research)	Identify and assess potential product candidate Assess intellectual property and assign development and commercialization rights, if appropriate	Principal investigator (PI), technicians, students and fellows, technology transfer office	Same as academia, single site	R and D scientists, managers, marketing, contracts department, patent counsel, legal department, human resources
MSC product (manufacturing process) development	Design, develop, scale up, and evaluate manufacturing process for proposed cellular product	PI and/or project leader, translational research scientists, process scientists and/or bioengineers, technicians, technology transfer office	Same as academia, single site Likely to be centralized at one site	Same as academia, single site, but likely to include analytical groups, manufacturing, marketing, and regulatory
Preclinical evaluation	Design and execute preclinical animal studies to assess safety, efficacy, and feasibility of using proposed cellular product, in clinical trials	PI and/or project leader, translational research scientists, technicians (lab, animal facility, imaging), veterinarians, pathologists, molecular biologists, quality assurance (QA), regulatory, animal use review board, CRO for GLP studies	Same as academia, single site May be centralized at one site or performed at multiple academic sites or CRO	Same as academia, single site
cGMP manufacturing	Design and implement cGMP manufacturing process for cellular product	PI and/or project leader, cGMP facility operations manager, cGMP technical staff (manufacturing, testing, storage), quality control (QC) and QA, regulatory	Same as academia, single site May be centralized at one site or performed at each site using common SOPs	Same as academia, single site, but manufacturing may be done either in-house or by contract manufacturing organization (CMO)
Final product preparation	Perform final product preparation steps and ensure prompt delivery to clinical site	Technical staff to perform final product preparation steps, QC/QA	Same as academia, single site, with each clinical site needing this staffing	Same as academia, multiple sites

Assay development	Design, develop, evaluate, and validate assays for identity, dose, potency, purity, and safety of cellular product	PI and/or project leader, translational scientists, assay scientists, clinical pathologists, assay technicians, research associates, QC/QA	Same as academia, single site	Same as academia, single site
		Some assays may require CRO(s)	Actual testing may be performed at each clinical site or centralized at one site or CRO	Often involves in-house analytical department and/or CRO(s)
Phase 1 clinical evaluation	Evaluate product safety	Sponsor, PI, associate investigators, project leader, study coordinator/ nurse, study monitor, technical staff to perform final product preparation steps, hospital clinical services department including medical/ nursing staff to administer product, hospital and/or contract clinical laboratories, clinical imaging department, QC/QA, regulatory, IRB, DSMB	Same as academia, single site, with each site needing this staffing (except sponsor, who will be at one site but responsible for all sites)	Same as academia, multiple sites
			If product is shipped from centralized manufacturing site to multiple clinical sites, will need contracts and technology transfer offices, and technical staff for shipping/receiving	Also typically involves chief medical officer/medial director, clinical department, marketing, contracts manager, legal department, scientific advisory board
Phase 2 clinical evaluation	Evaluate product efficacy and expand evaluation of product safety	Same as phase 1	Same as phase 1	Same as phase 1 but will typically refine cGMP product manufacturing and testing
Phase 3 clinical evaluation	Evaluate product efficacy in comparison to standard therapy and expand evaluation of product safety	Not typically done	Not typically done	Same as phase 2 but expanded medical and clinical department staff to execute and monitor large trial

reporting tools, and capable of understanding and communicating all elements of the project. Several project management software tools are available to assist with monitoring project progress, cost, resource allocation, time, and risk through the project life cycle.

Such a systematic and integrated project management approach is needed to guide and manage the multidisciplinary and parallel aspects of MSC product development from the early discovery stages, preclinical studies, to product manufacturing, analysis, release, and finally clinical evaluation and regulatory approval.

Conclusions

Development of MSCs for clinical applications is a multidisciplinary effort requiring expertise of many individuals working in a coordinated manner to develop the cellular product and implement clinical trials. As the field of cell-based therapies evolves, approaches to preclinical studies, CMC development, and clinical trials for these therapies are likely to be more standardized globally. The NIH's Center for Regenerative Medicine has already taken a lead on such initiatives. However, an integrated, multidisciplinary approach for the bench-to-bedside process will continue to be critical for success.

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Chapter 16
cGMP Production of MSCs

Derek J. Hei and David H. McKenna Jr.

Abstract Over the past decade, mesenchymal stromal/stem cells (MSCs) have evolved into an important cell therapy demonstrating potential utility in a range of clinical applications, including bone and cartilage repair, cardiac repair, and immune disorders. MSCs can be isolated from a variety of tissue sources, including bone marrow, adipose tissue, dental pulp, and placenta. Groups have developed different manufacturing processes with a goal of improving the quality of clinical-grade cells and the overall efficiency of the manufacturing process. Variations in cell source and manufacturing process may have a significant impact on the efficacy of the final MSC product. Moreover, this variability in cell source and manufacturing processes has made it challenging to compare the resulting MSC products and associated results from clinical trials that have been conducted to date. The development of consistent, well-controlled manufacturing processes along with the implementation of thorough quality control testing, including rigorous potency assays, will insure high quality and may help to clarify the impact of cell source and manufacturing process on the resulting MSC product. In addition to providing an overview of the current good manufacturing practice (cGMP) methods for MSC production, this chapter summarizes key FDA regulatory requirements, including those related to cell source, raw materials, and quality control testing.

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_16,

Introduction

Mesenchymal stromal/stem cells (MSCs) are adherent, fibroblast-like cells that are characterized by the expression of certain cell surface markers and the potential to differentiate into bone, fat, and cartilage [1, 2]. Although bone marrow (BM) is the most common source of starting material, cells with characteristics similar to BM-derived MSCs have been isolated from other tissue sources, including adipose, umbilical cord blood, placenta, and dental pulp [3–6]. Given the ability of MSCs to differentiate into adipocytes, osteoblasts, and chondrocytes, initial clinical applications focused on the use of MSCs to regenerate tissues using engineered bone constructs [7]. However, MSCs are excellent candidates for other applications due to several characteristics, including their ability to migrate to the site of injury/inflammation, the potential to stimulate proliferation and differentiation of resident progenitor cells, and the propensity to promote recovery of injured cells and/or modulate the immune system through secretion of growth factors [8–15]. Recent clinical applications have focused on utilizing the immunomodulatory properties and paracrine effects of MSCs in cardiovascular disease, neurological disorders, and immune dysregulation disorders. MSCs have demonstrated encouraging clinical results in Crohn's disease [16] and graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell (HSC) transplantation, and these studies have now advanced to phase III clinical trials [17].

Many of these initial clinical trials have demonstrated significant promise in using MSCs as a therapeutic. However, efforts to repeat clinical observations have resulted in variable success. One major hurdle in comparing results from clinical studies is the potential variability in cell quality and characteristics between clinical sites. Due to the complex nature of cell therapeutics, it is important to recognize that the manufacturing process will likely have a significant impact on important cell properties that impact *in vivo* efficacy. In addition to variability in starting cell source, there is a wide range of cell culture media and culture practices that are currently employed in producing MSCs for clinical applications. It is therefore critical to establish a panel of quality control (QC) test methods that can be used to assess the impact of these variables on the safety and potency of the final MSC product. This chapter provides an overview of important considerations when producing MSCs for clinical applications. In addition to a brief overview of regulations for clinical production of MSCs, this chapter provides an overview of a number of manufacturing and testing considerations.

FDA Regulations and cGMP Compliance

A thorough understanding of applicable regulations and industry standards are essential when developing biotherapeutics. Regulatory requirements will often drive key decisions for manufacturing process development, selection of raw materials,

and development of QC testing plans for raw materials and final product. This section provides a brief overview of regulations that are applicable to MSC-based therapies in the USA.

In the USA, cell therapies are regulated by the Center for Biologics Evaluation and Research (CBER) division of the Food and Drug Administration (FDA). Although the regulatory requirements for cell therapies are expected to evolve as new therapies move through human clinical trials toward approval, the FDA has provided guidance documents and regulations covering several key areas of production and testing.

In May 2005, Part 1271 of Chapter 21 of the US Code of Federal Regulations became effective. Part 1271, Human Cells, Tissues, and Cellular and Tissue-Based Products provides the basis for regulation of human cellular and tissue-based products (HCT/Ps). In addition to providing regulations for Donor Eligibility (Subpart C), Subpart D outlines Current Good Tissue Practices (cGTP). The regulations in 21 CFR 1271 Subpart D cover a broad range of requirements, including quality system, personnel, procedures, facilities, environmental monitoring and control, equipment, supplies and reagents, process changes/validation, and product labeling/storage/tracking [18]. The HCT/P regulations outlined in 21 CFR 1271 and cGMP regulations (21 CFR 210, 211, 610) are intended to be applied in a progressively more strict manner as therapeutics move toward the eventual filing of a Biologics License Application (BLA) [19]. However, the FDA expects that certain key requirements of the cGTP/cGMP regulations even will be met during early-stage human clinical trials [20].

In addition to the regulations outlined above, the FDA has issued several guidance documents that are applicable to HCT/Ps. The FDA issued a guidance in March 1998 that provides an overview of manufacturing and testing requirements for human somatic cell therapy and gene therapy products including procedures for cell collection, cell culture, cell banking systems, and release testing requirements for cellular therapy products [21]. The *International Conference on Harmonization* (*ICH*) has also issued several guidance documents that provide further details on testing requirements for cell therapeutics [22, 23]. Guidance documents are also available for issues related to the sourcing and testing of the initial cell material including donor eligibility determination and addressing xenotransplantation issues for cell therapeutics that were previously cultured *ex vivo* with live nonhuman animal feeder cells [24, 25]. Since HCT/Ps typically cannot undergo a terminal sterilization step, HCT/Ps must be manufactured following aseptic processing methods. Several documents are available providing general guidance for validation and cGMP compliance for aseptic processes [26]. In addition to guidance from the FDA, AABB (formerly the American Association of Blood Banks) and the Foundation for the Accreditation of Cellular Therapy, or FACT, have established standards to assist with meeting regulatory requirements [27, 28]. Several groups from academia and industry have published documents that provide guidelines for moving HCT/Ps into human clinical trials [29–31].

Cell Source

MSCs were originally isolated as an adherent cell population derived from bone marrow (BM) [1]. Subsequent studies have found that similar populations can be isolated from other adult and perinatal tissues, including adipose tissue (AT) [6], skeletal muscle [32], synovium [33], dental pulp [34], placenta [35], amniotic fluid [36], and umbilical cord blood (UCB) [37]. Several studies that have compared the properties of the cells derived from these diverse sources have found that the cells demonstrate very similar characteristics including cell marker expression, differentiation potential, and immunological properties [38–40]. However, a study that compared the gene expression profiles of MSCs derived from BM, AT, and UCB found that while MSCs derived from different donors using the same source material and expansion protocol exhibited consistent and reproducible profiles, MSCs from AT, BM, and UCB display differences in the transcriptome [41]. The impact of these differences on *in vivo* efficacy remains unclear. However, the results serve to highlight potentially important differences between MSCs derived from different sources. This section provides a brief overview of MSCs derived from BM, AT, and UCB. In addition, information is provided on donor screening and eligibility requirements that apply to all sources of starting cell material.

Bone Marrow

The starting BM for MSC production is typically obtained from a 25–100-mL BM aspirate from the posterior superior iliac crest of the donor. The procedure is performed in a clinical setting allowing for sterile harvest of the BM aspirate. In addition, donors typically go through a full medical screening process (see Donor Screening below) and a rigorous informed consent procedure, very similar to that of a blood donor.

Several important factors regarding the BM donation may have a significant impact on the quantity and quality of MSCs derived from the BM. The age, sex, and health of the donor, including factors such as smoking, may impact the quality of the BM harvest [42, 43]. Donor-to-donor variation has also been observed in the profile of cytokines and chemokines that are secreted by MSCs in response to stimulation with proinflammatory cytokines [44]. Freezing of BM prior to MSC isolation was also reported to have a negative impact on both MSC yield and immunosuppressive properties of the MSC in mixed lymphocyte cultures [45]. Finally, as discussed in section "MSC Isolation from Bone Marrow," the method that is used for isolating the mononuclear cell fraction from the BM may have a signification impact on the resulting MSCs.

Adipose Tissue

Although the bulk of the published literature concerns BM-derived MSCs, AT is also considered to be an easily obtainable source of starting cells for MSC production. AT-derived MSCs have been used in a few small clinical trials for Crohn's disease [46], steroid-refractory acute GVHD [47], enhancement of HSC engraftment [48], and as salvage therapy for refractory pure red cell aplasia after major ABO-incompatible HSC transplantation [49]. The procedure for producing MSCs from AT involves red blood cell (RBC) washing steps similar to BM processing with the density-gradient step essentially replaced by a collagenase digestion step. A number of factors including donor characteristics and anatomical location of AT harvest can impact the characteristics of the resulting MSCs [50].

Umbilical Cord Blood

UCB is the most recently established source of hematopoietic stem cells for clinical utility [51]. Although some investigators have had limited success [52, 53], it is also now generally accepted that UCB is a suitable starting material for MSC isolation and expansion [3, 54]. With efficiency of isolation varying among research groups with success rates in the range of 24–63% [3, 55], an effort has been made to optimize cell processing [55]. In general, the approach is very similar to that of marrow-derived MSCs. The mononuclear cell (MNC) fraction is isolated using a density-gradient centrifugation and then seeded into culture flasks (e.g., 1×10^6 MNC/cm^2). Within 24 h the non-adherent cells are removed, and the remaining adherent cells are carried through culture much like MSCs from other sources. Interestingly, one group demonstrated UCB-derived MSCs to have a greater proliferation capacity, becoming senescent later than adipose- and marrow-derived MSCs [56]. The same group was unable to show adipogenic potential of UCB-derived MSCs, though others have been able to demonstrate in vitro differentiation to fat cells [3, 54]. In fact, some researchers have isolated MSC-like cells from UCB and succeeded in coaxing to cell types representative of all three embryonic lineages [57–59]. The potential value of UCB-derived MSCs over other types remains to be determined, though their unique qualities suggest there may be some advantages [56].

Donor Evaluation

Donor evaluation is an important requirement for cell therapeutics derived from human tissue sources. Requirements for donor evaluation are outlined in the HCT/P regulations (21 CFR 1271 Subpart C) and FDA guidance documents on donor

Table 16.1 Examples of current FDA-licensed kits for Donor Testing[a]

Test	Methodology	Manufacturers
HBsAg	EIA	Bio-Rad Laboratories
	EIA	Abbott Laboratories
	ELISA	Ortho Clinical Diagnostics
Anti-HBc (IgG+IgM Ab)	EIA	Abbott Laboratories
	EIA	Ortho Clinical Diagnostics
HBV nucleic acid	PCR	Roche Molecular Systems
	TMA	Gen-Probe, Inc.
Anti-HCV	EIA	Abbott Laboratories
	EIA	Ortho Clinical Diagnostics
HCV nucleic acid	PCR	Roche Molecular Systems
	TMA	Gen-Probe, Inc.
Anti- HIV-1/2	EIA	Bio-Rad Laboratories
	ChLIA/EIA	Abbott Laboratories
HIV nucleic acid	PCR	Roche Molecular Systems
	TMA	Gen-Probe, Inc.
Anti-HTLV I/II	EIA	Abbott Laboratories bioMerieux
Treponema pallidum[a]	Olympus PK TP System Anti-TP(IgG & IgM)	Fujirebio Inc.
CMV antibody[a] (IgG+IgM Ab)	Solid phase red cell adherence	Immucor
	Solid phase EIA	Abbot Laboratories
West Nile virus Nucleic acid	PCR	Roche Molecular Systems
	TMA	Gen-Probe, Inc.

[a]See the FDA website for specific testing requirements and additional approved tests (www.fda.gov/BiologicsBloodVaccines/SafetyAvailability/TissueSafety/ucm095440.htm)

eligibility [25]. A comprehensive donor evaluation is typically performed by a physician with expertise in the collection procedure at the time of initial evaluation. The donor evaluation typically consists of three components: donor questionnaire, medical examination, and testing for infectious disease markers. All potential donors fill out a questionnaire that screens donors for transmissible diseases on the basis of history [60]. The donor's medical history is reviewed including information on transfusion history, surgical history, pregnancies, vaccination history, family history, social history, and health habits including smoking, alcohol, and recreational drug use. A general medical examination is performed prior to donation and typically includes routine laboratory testing (CBC with differential and platelet count, PT/INR, standard blood chemistry panel, and ABO/Rh type).

A blood sample is also taken from the donor at the time of initial donor assessment and, if needed, at the time of collection for infectious disease testing as required in 21 CFR 1271 Subpart C FDA Donor Eligibility. Infectious disease testing is performed using FDA-licensed test kits as summarized in Table 16.1. The results from donor testing, donor eligibility assessment, and the informed consent for tissue donation are typically retained in a file that is coded to protect patient confidentiality while maintaining traceability of the final MSC product back to the original tissue source.

MSC Production Methods

Along with advances in clinical applications for MSC-based therapies, strides have been made in several key technical areas related to production, testing, and banking of MSCs. Producing MSCs for clinical applications requires addressing several key issues [61, 62]. In addition to addressing regulatory compliance issues, manufacturers of MSC for clinical applications must address issues related to source material, cell culture conditions, and media source/quality. Several studies have been performed to determine the optimal conditions for culturing MSC for clinical applications [63–66]. New media formulations that avoid the use of FBS have been described recently [67, 68]. Efficient procedures for MSC cryopreservation and conditions for transporting and holding cells for transplantation have also been evaluated [66, 69].

In addition to developing well-defined and reproducible manufacturing procedures, quality control (QC) test methods must be established to characterize and evaluate the final cell product. Characterization assays are especially critical for MSC products given the diversity of starting material, isolation methods, and culture methods [70]. Several groups have published reports on QC test methods that are currently used for both in-process testing and testing MSC products intended for human clinical trials [71, 72]. This section provides a brief overview of a typical manufacturing process for BM-derived MSCs including discussions regarding key process steps and parameters that potentially impact the quality and efficacy of the final MSC product.

Overview of MSC Manufacturing Process

A typical MSC manufacturing process consists of the following steps: isolation of MNC fraction from BM, MSC seed/master cell bank (MCB) production (optional), MSC expansion, and cryopreservation. Final formulation may take place prior to or after cryopreservation following the thaw. The overall process is depicted in the process flow diagram presented in Fig. 16.1. Different seeding and passaging strategies can be used in the MSC production process. For example, a low seeding density of MSCs may be subjected to a single expansion step without production of an intermediate cell bank. Seeding density and passaging schedule have an impact on the final MSC population and this is discussed in section "MSC Culture Method."

Raw Materials

Raw materials that are used in cGMP manufacturing processes should be sourced from vendors that have been audited for compliance with cGMPs or other appropriate quality standards. QC testing and documentation should be maintained for each

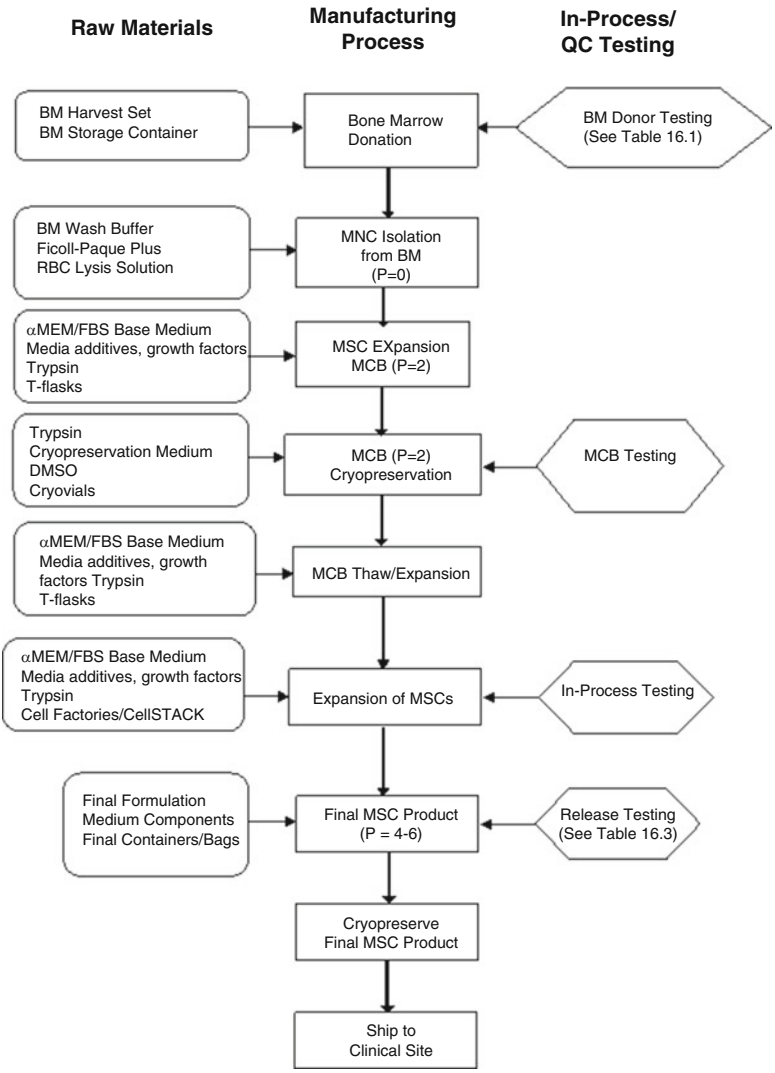

Fig. 16.1 Process flow diagram for MSC production from bone marrow

raw material, and traceability from final MSC product back to all raw materials should be maintained for each production lot. Raw materials should be reviewed to identify potential risks that may be introduced, for example, through the use of animal-derived raw materials. Table 16.2 provides an overview of common raw materials used in MSC production with recommendations for QC testing and documentation requirements.

Table 16.2 Biological source raw materials common for MSC production

Raw material	Use	Special QC testing and documentation considerations	Risk mitigation
Bone marrow	Starting material	Donor testing, medical history, and informed consent for donation	
Fetal bovine serum	Media component	9CFR 113 bovine pathogen testing	Consider irradiation or viral filtration
		Country of origin certificate	Consider moving to serum-free media
		Bovine source from low TSE-risk country	
Growth factors (e.g., FGF-2, TGF-β, PDGF)	Media supplement	Review manufacturing process for recombinant proteins to assess risk from mammalian cell lines and animal-derived materials	Utilize GFs produced in microbial expression systems or well-characterized mammalian cell lines
Porcine trypsin	Cell detachment	9 CFR 113 testing for porcine pathogens	Consider moving to recombinant enzyme
Human serum albumin	Final formulation	Derived from human serum collected from tested donors collected, complies with 21 CFR 640.80	Recombinant HSA
Final container	Product storage	Sterility, endotoxin – USP <161>	
		Extractables – USP <661>/USP <88>	

MSC Isolation from Bone Marrow

MSCs are present in the mononuclear cell (MNC) fraction of the BM. The MNC fraction is enriched from the BM using density-gradient centrifugation. This step is typically performed using Ficoll-Hypaque density-gradient medium. cGMP-grade versions of density-gradient medium are commercially available. Following enrichment, the cells are washed with PBS or Hank's Balanced Salts Solution (no phenol red, calcium, or magnesium) prior to initial plating. Studies have demonstrated that modifications to the MNC isolation step can have a significant impact on the yield and quality of the resulting MSC product. For example, MNC isolation using 1.073 g/mL Ficoll produced an MNC fraction that was lower in CD45+ cells resulting in about a twofold increase in MSC yield after four passages with higher expression of CD90, CD146, and GD2 [73].

MSC Seed Bank Production

Following enrichment of the MNC fraction by density-gradient centrifugation, the washed cells are typically plated (passage 0) in cell culture flasks and incubated at 37°C with 5% humidified CO_2 using the selected MSC culture media (see section "Media Selection"). Twenty-four to forty-eight hours later, the non-adherent cells are removed (suctioned out) and the adherent cells are expanded in culture with media changed every 3–4 days.

At this point in the manufacturing process, the MSCs may either be expanded directly to final product or expanded to an intermediate stage (e.g., $P=2$) where they are harvested and cryopreserved to create a seed bank for future production trials. The creation of MSC seed banks allows future production campaigns to be performed with a starting cell source that has undergone testing for key attributes such as growth characteristics and biological activity. This allows for more uniform production campaigns and can be used to address key issues such as donor-to-donor variability in MSC properties. Cells from the seed bank ($P=2$) are typically expanded through several additional passages to generate the final MSC product ($P=4$–6) to be used in clinical trials. It should be noted that this product will be several passages older than MSCs that are expanded directly without creating an intermediate seed bank. While there are advantages to such an expansion strategy from a time/yield and logistics perspective, the overall impact of time in culture and passaging on cell quality and potency remains to be established. Limited studies suggest that there is an impact of time in culture on MSCs possibly related to the age of the donor [74, 75]. Other studies have shown that moderate time in culture (4–7 passages) does not affect the immunosuppressive activity of MSCs [45]. It is advisable, however, that investigators qualify their chosen MSC manufacturing approach for the intended clinical use (see section "Potency Assays").

Media Selection

Currently there is no standard method of culturing MSCs from any source/starting material, and there is no consensus among the investigators on the most efficient approach to producing MSCs. This is important since proliferation rate, differentiation potential, and immunophenotype of cells could change depending on the culture method. Nevertheless, clinical trials based on the use of MSCs generated at different academic centers have all showed that infusions of these cells are safe and potentially efficacious. The most commonly used media for MSC production appears to be fetal bovine serum (FBS)/alpha-minimum essential medium (αMEM). Considerations for the use of FBS in MSC culture along with several media options are presented below.

Fetal Bovine Serum

FBS has traditionally been utilized to expand human MSCs for both research and clinical applications. FBS is often added to alpha-MEM base media supplemented with glutamine with the FBS concentration ranging typically from 5 to 17%. Lot-to-lot variability is typically observed in the ability of FBS to support MSC expansion requiring screening of FBS lots and highlighting the potential impact on MSC quality and potency. Interestingly, the concentration of FBS can affect the subpopulation of MSCs that grow out in culture with serum deprivation resulted in selection of an Oct-4-positive early progenitor population [76]

The use of FBS in the production of cellular therapies generates several potential concerns including the introduction of the risk of transmission of zoonotic agents, bovine spongiform encephalopathy (BSE), and the introduction of antigens of animal origin that may be incorporated into the cell therapeutic (e.g., Neu5Gc) or present from residual contaminating FBS [77, 78]. The risk associated with BSE transmission may be reduced by selecting a FBS source from countries classified by the World Organization for Animal Health [Office International des Epizooties (OIE)] as negligible BSE risk or Geographical BSE-Risk (GBR) I, as classified by the European Food Safety Authority (EFSA) [79]. The potential risk of bovine pathogen transmission may also be mitigated by using only FBS that has undergone screening for bovine pathogens (9 CFR 113) and that has additionally undergone a viral inactivation step such as gamma irradiation.

The potential risks associated with BSE, pathogens, animal antigens, and variability drive the desire to identify other potential media for MSC clinical production. Alternatives to FBS that have been investigated include serum-free media, autologous serum, fresh-frozen plasma, and human platelet lysates [65, 68, 80–82].

Platelet Lysates

Among the current alternatives for FBS, media based on human platelet lysate have been studied the most extensively, including evaluation in human clinical trials [83]. One advantage of platelet lysate media is that it can be sourced from normal healthy donors that have passed all infectious disease testing. Platelet lysate media is typically produced using platelet concentrates collected from single donors by apheresis. The platelets are frozen, thawed, and then heat inactivated at 56°C for 30 min. After removal of the remaining platelets by centrifugation, the resulting platelet lysate is frozen in aliquots for future use in MSC culture. Despite its clear advantages, preparation of platelet lysate media requires additional time, and it may result in donor-to-donor (i.e., lot-to-lot) variability in MSC growth characteristics and potentially cell quality due to variability in growth factor content (e.g., platelet-derived growth factor – PDGF) [84].

Serum-Free Media

Several groups have developed serum-free media formulations that have demonstrated promise in MSC production. Meuleman et al. found that commercially available medium supplemented with a serum substitute demonstrated a significant increase in MSC yield compared to standard FBS/αMEM. In addition, the resulting MSCs were similar with respect to cell marker expression, differentiation potential, and the ability to support the growth of hematopoietic progenitors [85]. Chase et al. described development of a proprietary serum-free media that also demonstrated enhanced MSC growth over FBS/αMEM when the medium was supplemented with fibroblast growth factor-2 (FGF-2), transforming growth factor-beta (TGF-β) and PDGF [86]. MSCs produced using this medium showed similar cell surface marker expression by flow cytometry, differentiation potential, and gene expression profile relative to MSCs produced using standard FBS/αMEM. Although this initial version of media contained animal-derived components, a new xeno-free version is now commercially available [87, 88]. Additional *in vitro* potency studies and animal studies are needed to demonstrate whether the use of these serum-free media will have a significant impact on the *in vivo* efficacy of the MSCs. In addition, these media are proprietary formulations that contain undisclosed components. Care should be taken to identify potential risks from media components such as growth factors or other animal-derived components. For example, some growth factors may be produced using mammalian cell lines such as rodent cell lines (e.g., CHO, NS0) that inherently introduce the risk of retrovirus and retrovirus-like particle contamination [89]. Growth factors that are derived using such mammalian expression systems should utilize tested cell lines and have purification processes that have been validated for clearance of viral pathogens.

MSC Culture Method

MSCs are typically grown as adherent cells using standard tissue culture plasticware. Initial cultures of MSCs from the enriched MNC fraction or seed bank are typically expanded in T-flasks. Cells from T-flasks are then used to seed large-scale cell culture devices such as Cell Factories (Nunc) or CellSTACK (Corning). Cell Factories have demonstrated utility in producing MSCs for clinical applications [90]. Cell Factories/CellSTACK provide a convenient format for large-scale culture of adherent cells. Media may be prepared in disposable bioprocess containers, and bags and tubing sets can be used to allow the entire feeding and harvesting steps to be performed in a single-use, disposable, closed system. This format, therefore, provides the added benefits of decreased contamination risk and elimination of the need to perform cleaning validation as would be required for multiuse bioreactors.

Beyond the impact of donor characteristics, MNC isolation method, and media selection discussed above, a number of factors in MSC culture can impact the final

MSC characteristics. Seeding density is one important major factor that has a significant impact on the MSCs. Low seeding densities (10–50 cells/cm^2) have been shown to promote the growth of a subpopulation of MSCs that appears to represent early progenitors [91]. The resulting MSCs have an increased growth rate, thin spindle-shaped morphology, and have increased adipogenic potential relative to the later developing MSCs that have a wider morphology and greater chondrogenic potential.

Although several scalable formats including Cell Factories (Nunc) and CellSTACK have been used for MSC production, the relatively large doses ($0.4–9 \times 10^6$ cells/kg) [92] of MSCs that are required for many indications suggest that other scalable production methods may be needed for future applications. Bioreactors offer a potential solution for large-scale production of cell therapeutics with the opportunity to provide greater control over cell growth conditions and potentially over cell quality. Most of the work aimed at growing MSCs in bioreactors is recent with a focus on growing MSCs on novel and commercially available microcarriers [93–96]. Although results to date have demonstrated modest levels of expansion, further optimization of seeding parameters, media formulation, feeding strategies, and bioreactor conditions will likely lead to further improvements in cell yield and manufacturing efficiencies.

Final Formulation and Cryopreservation

Following the final harvest, the MSCs are typically centrifuged, washed, and changed over to a formulation that is compatible with cryopreservation and administration to the patient. One formulation that has been used in previous clinical trials is PlasmaLyte A (Baxter, Deerfield, IL, USA) containing 5–10% human serum albumin and 10% DMSO. Alternative cryoprotectants have been evaluated with some success in reducing the required levels of DMSO by utilizing PEG and albumin [97]. The dose range for MSCs is typically $2–8 \times 10^6$ cells/kg or $1–6 \times 10^8$ MSCs/dose that is formulated as 25–100 mL of cells in a bag that is suitable for low-temperature storage. Bags of cells are typically frozen using a controlled rate freezer (−1°C/min) and stored in liquid nitrogen freezers in liquid or vapor phase at temperatures below −150°C.

MSCs have been thawed and immediately infused; however, they are often thawed and washed or diluted with an appropriate solution (e.g., Dextran 40, 5% human serum albumin) and then infused. Studies should be conducted to ensure that time limits are established for holding the final thawed product under defined conditions prior to administration. Previous studies have demonstrated a range of acceptable hold times depending on the formulation and hold temperature [66].

Manufacturing Controls

Cleanroom Environment

A key aspect of manufacturing cell therapeutics for clinical applications is the inability to perform a terminal sterilization step. This necessitates that the product be manufactured under strict aseptic conditions through the entire production process. The FDA has issued a guidance document that outlines key issues for aseptic manufacturing processes [98]. Key areas of focus that should be addressed for an aseptic manufacturing process include: clean room design, clean room cleaning practices, environmental monitoring practices, personnel gowning, personnel monitoring, and validation of aseptic processing methods. For cell therapy production, the clean room environment should, at minimum, meet class 10,000 (ISO class 7) clean room rating with a biosafety cabinet or other class 100 (ISO class 5) zone for performing open manipulations. Strict gowning practices, cleaning practices, and environmental monitoring (viable and nonviable) are critical for ensuring that the manufacturing environment is maintained in a controlled state during clinical production.

Process Qualification

Process validation is defined by the FDA as the "collection and evaluation of data, from the process design stage throughout production, which establishes scientific evidence that a process is capable of consistently delivering quality products" [99]. Initial process (performance) qualification (PQ) trials are typically conducted at the end of the initial process development studies and prior to initiating clinical production campaigns. Preclinical PQ trials typically consist of performing trials (3–5 runs) of the cGMP manufacturing process with full documentation and testing, including in-process testing. These trials allow final details to be worked out for manufacturing procedures and documentation and demonstrate that the manufacturing process is capable of producing material that will meet release testing requirements for clinical trials. Material from these initial PQ trials can typically be used as reference standard for future QC testing or for use in preclinical animal studies.

Process validation typically occurs throughout the product life cycle with data collected during production runs and process design experiments. The goal of this stage is to identify key process parameters and material attributes (e.g., donor variability) that impact process variability and product quality. Studies are then performed to demonstrate that the manufacturing process is capable of producing acceptable product within the limits established for these key operating parameters. Comprehensive process validation studies are required to be completed prior to commencing commercial distribution of the therapeutic [99].

Aseptic Processing Qualification

As discussed above, maintaining aseptic conditions during manufacturing is a critical aspect of clinical production for cell therapeutics. Demonstrating the ability to maintain aseptic conditions during the manufacturing process, especially during critical steps such as open manipulations, is therefore a critical component of process qualification. Media simulation studies are typically performed to validate aseptic processes [98]. These studies are performed using microbial growth media (e.g., soybean casein digest (SCD) medium) in place of cell culture medium with simulation of a full production run. Critical steps in cell production including seeding, feeding, harvest, and dispensing of the product into the final container should be included in the simulation runs. The final product containers containing SCD medium are incubated for 14 days, typically at two temperatures, with observation for any signs of microbial growth.

Quality Control Testing

Quality control testing is a critical component of the clinical production program. QC testing is typically performed at multiple points in the manufacturing process, prior to production (i.e., including donor material, raw material), cell (seed) bank, in-process samples, and final product release testing. Specifications are typically set for donor, raw materials, and final product based on key safety and performance requirements. In addition, data from PQ trials are used to establish process capabilities and set specifications for both in-process testing and final product release testing. Specifications are expected to address key attributes including identity, strength, quality, purity, and potency. Typical testing for donor tissue (see Donor Evalution) and raw materials (see Raw Materials) is discussed above. This section will cover QC testing requirements for the final MSC product.

Release Testing

Each lot of final MSC product will undergo testing to demonstrate that it meets preestablished specifications prior to release for clinical trials. Quality assurance is responsible for reviewing all production records, including QC testing, prior to release of final product. A summary of the typical final QC release testing performed on each lot of MSCs is provided in Table 16.3.

Identity Testing

Identity testing is typically performed using either short tandem repeat (STR) testing or human leukocyte antigen (HLA) testing. The identity tests create a genetic

Table 16.3 Quality control testing for MSCs

Characteristic	Test method	Specification
Identity Testing	Short tandem repeat testing HLA – high-resolution mapping of HLA-A, HLA-B, HLA-C, and HLA-DRB1	STR/HLA profile matches donor
Viable cell count	Viable count – Trypan Blue or 7-amino-actinomycin D (7-AAD)	>70%
Microbial and fungal contamination	21 CFR 610.12 sterility testing including bacteriostasis and fungistasis	No contamination detected
Mycoplasma	In vitro in Vero cells with culture method consistent with FDA PTC document	No contamination detected
Endotoxin	LAL kinetic turbidometric method – USP	<5 EU/kg/dose
MSC Antigen Expression	Flow cytometry for MSC markers: Positive: CD105, CD73, CD90 Negative: CD34, CD45, CD14, CD19, HLA-DR	 ≥95% expression ≤2% expression
Karyotype	G-band, 20 metaphase spreads	No clonal abnormalities
Residual FBS	ELISA assay for residual bovine proteins (e.g., BSA, transferrin)	Report level 6-log reduction
Bovine pathogens[a]	Testing for specific bovine pathogens according to 9 CFR 113	No contamination detected
Porcine pathogens[a]	Testing for specific porcine pathogens according to 9 CFR 113	No contamination detected
Human pathogens[a]	PCR or other appropriate assays for human pathogens – HIV-1 and HIV-2, HTLV-1 and HTLV-2, HBV, HCV, CMV, EBV	No contamination detected
Potency testing	Testing based on intended clinical indication	Specification to be established

[a]Testing is preferably performed on the raw material or human donor sample

fingerprint that can be used to relate the cell source back to the original donor. This is especially important if multiple cell lines are being produced in the same facility. STR testing is typically performed using commercially available kits [100]. HLA testing is performed by high-resolution sequencing of the HLA-A, HLA-B, HLA-C, and HLA-DRB1 loci. This technique is becoming more efficient as techniques utilizing next-generation sequencing methods are developed [101].

Viable Cell Count

Viable cell counts are typically performed by staining cells with reagents such as Trypan Blue or acridine orange (AO)/propidium iodide (PI) and performing manual counts with a hemacytometer or using an automated cell counter. Alternatively,

viable counts can be performed using 7-AAD or PI staining in conjunction with flow cytometry analysis of MSC cell maker expression [102].

Microbial and Fungal Contamination

Sterility testing is typically conducted using the direct transfer method in accordance with 21 CFR 610.12. The test article is inoculated into SCD and FTM and incubated at 20–25 and 30–35°C, respectively, for 14 days. Alternative strategies such as use of automated testing systems (e.g., BACTEC, BD, Franklin Lakes, NJ, USA) commonly used in the clinical setting may be employed if agreeable by FDA. Bacteriostasis and fungistasis testing described in United States Pharmacopeia (USP) <71> is also performed on the product at a minimum with the PQ to insure that the product components, or residual antibiotics if used in initial isolation, do not interfere with sterility testing.

Mycoplasma

Mycoplasma testing is conducted on both the cells and supernatant from the final product as well as the master cell bank, if that manufacturing approach is taken. Although PCR or chemical testing can be used as a screening assay for mycoplasma, the Points to Consider (PTC) culture method is preferred for release testing. The PTC method, which takes 28 days for completion, includes both a direct assay and an indirect assay [103]. The direct assay involves cultivation of the test article in agar and broth media under conditions suitable for growth of cultivatable mycoplasmas. The indirect method involves culturing the test article in Vero indicator cells followed by staining with a DNA-binding fluorochrome (Hoechst stain) to detect nuclear and extranuclear fluorescence. Appropriate positive controls are included in each arm of the assay.

Endotoxin

Endotoxin testing that is performed on the final production should conform with USP <85> Bacterial Endotoxins Tests. Testing is typically based on the Limulus amebocyte lysate (LAL) assay utilizing commercially available reagents and test kits (e.g., Endosafe, Charles River). Testing should include inhibition and enhancement test controls. A typical recommended specification for endotoxin is <5.0 EU/kg/dose.

MSC Antigen Expression

Flow cytometry is performed on the MSC seed bank and final product to verify appropriate expression of MSC markers. Most groups use the guidelines as proposed by the Mesenchymal and Tissue Stem Cell Committee of the ISCT [2].

This group defined criteria for MSC identification to include presence of CD105, CD73, and CD90 as positive markers (≥95%), and absence of CD45, CD34, CD14/CD11b, CD19/CD79α, and HLA-DR as negative markers (≤2%).

Karyotype

Karyotyping is typically performed using standard Giemsa/Trypsin/Leishman (GTL) banding (or simply G-banding) on 20 metaphase spreads. Analysis is performed in compliance with the Clinical Cytogenetics Standards and Guidelines published by the American College of Medical Genetics. Chromosome counts are performed on 20 cells with full band analysis performed on 5–10 cells [104].

Residual FBS

Levels of residual FBS in the final product are typically determined based on the level of residual bovine serum albumin (BSA). Levels of BSA can be measured using a commercially available ELISA kit. A typical target of reduction for therapeutics is <1 ppm residual FBS. However, acceptable specifications should be based on process capabilities and potential risk to the patient population. Cross-reactivity of the ELISA with human serum albumin will be a primary consideration if the final product is formulated with HSA. In that case, other components of FBS can be exploited to determine residual amounts of FBS in the final product (e.g., bovine transferrin) [105].

Potency Assays

The FDA requires that biological products meet requirements of safety, purity, and potency for biologics license application approval. A potency assay must be established prior to initiating phase III trials, and it must be validated before BLA submission. Potency is defined by FDA as "the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result" [21 CFR 600.3(s)]. The regulations allow potency assays to be *in vitro*, *in vivo*, or both as long as the assay(s) is designed specifically for the given product to assess potency as described above [106].

Since MSCs are used for a variety of clinical applications, the intended effect will undoubtedly vary. MSCs may be administered for an immunomodulatory effect (e.g., graft-versus-host disease), tissue or organ repair (e.g., meniscal repair), enhancement of engraftment following blood/marrow transplant, etc. Table 16.4 lists a few resources for potency testing of MSCs for various medical applications. Some approaches are more developed than others. Cytokine-based analysis is listed

Table 16.4 Examples of potency assays for MSCs

MSC application	Potency assay	References
GVHD treatment	MLR-based, cytokine-based	[107, 108]
Acute lung injury	*Ex vivo* lung model, cytokine-based	[109]
Crohn's disease	MLR-based	[110]
Connective tissue repair	PCR-based, trilineage potential	[111, 112]
Engraftment post-BMT	CFU-/LTCIC-based	[113, 114]
Cardiac regeneration	*In vitro* support of cardiac stem cell proliferation	[115]

below as a possible potency assay, and this approach is expected to grow given the expansion of research in this area [116].

There are several advantages to establishing a potency assay as early in the developmental pathway as possible. These include evaluating multiple candidate assays, evaluating the impact of media and production methods, generating data to support lot release specifications, and establishing a stability program. In the 2008 guidance, the FDA provides more practical benefits to early work on potency testing, as well as direction toward relevant biologics and cGMP regulations for consideration of potency assays [106].

Conclusion

MSCs can be produced from a variety of different cell sources with many variations in the initial isolation, cell expansion, and formulation/cryopreservation procedures. In addition, a variety of different test methods are used by groups to assess the quality of MSCs. This chapter provides a brief overview of some of the more common methods that are used for producing and testing MSCs for clinical applications. Clearly one of the major challenges facing the field of MSC-based therapeutics is the need to develop better analytical methods, including potency assays, to better assess how differences in production methods impact cell quality and *in vivo* potency.

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Chapter 17
MSCs: The US Regulatory Perspective

Adrian P. Gee

Abstract In the USA, the Food and Drug Administration (FDA – the Agency) regulates cellular therapies, primarily through the Center for Biologics Evaluation and Research (CBER) Office of Cellular, Tissue and Gene Therapies. The rapid expansion of these therapies has prompted the Agency both to determine the applicability of existing regulations and to develop specific new laws. The strategy that has evolved is based upon perceived risks to the donor and recipient of the cell product and to the product itself by *ex vivo* manipulation during the manufacturing process. Mesenchymal stromal cell (MSC) products are considered to be more-than-minimally manipulated, due to the requirement for expansion of the cells in culture. As such, the product must be manufactured under current Good Manufacturing Practices (cGMP) and clinical trials carried out under an Investigational New Drug (IND) application. The development of this regulatory strategy and the factors involved in cGMP manufacturing and applying for an IND are reviewed in this chapter.

Introduction

The resurgence of interest in cellular therapies has excited the attention of national regulatory authorities. Their concerns primarily relate to the potential development of commercial products and services associated with the new therapies, the rapid expansion of novel technologies, and the risk of blurring the boundary between research activities and billable clinical therapies.

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_17,

Regulation of Somatic Cell Therapies in the USA

In the USA, regulatory responsibility falls to the Food and Drug Administration (FDA – the Agency) and, more specifically, to the Office of Cellular, Tissue and Gene Therapies in the Center for Biologics Evaluation and Research (CBER). Prompted by the use of cells in a variety of therapeutic applications, the Agency has worked to develop a regulatory strategy to encompass these diverse and developing therapies and products while ensuring the safety of patients and donors.

When seeking to regulate a new area, the FDA will usually review existing regulations to determine if they could be applied and whether they require supplementation. The Agency identified applicable regulations within the United States' Public Health Service (PHS) Act of 1912 and the Federal Food, Drug and Cosmetic Act of 1938. In 1993, they summarized which existing regulations could be applied to somatic cell and gene therapies in the Federal Register [1]. This document served to define somatic cell therapy products and to categorize them as biological products subject to the provisions of the PHS Act but noted that they also fell within the definition of drugs. As such, cellular therapy products would be subject to regulation under Investigational New Drug (IND) laws and would be manufactured under current Good Manufacturing Practice (cGMP) regulations. They would also be subject to establishment and product licensure.

The Agency recognized, however, that the existing regulations were insufficient to address current activities in a comprehensive manner. The solution has been to develop a unifying strategy for regulation based upon the potential risks [2, 3]. These include risks to the donor of the cells, risks posed by *ex vivo* handling, and risks posed to the intended recipient(s) by administration of the cellular product.

Manipulation

The risk-based regulatory strategy placed particular emphasis on the hazards posed by *ex vivo* handling of the cells. This was considered to be related to the degree to which the cells were manipulated. Manipulation was subdivided into two categories, "minimal manipulation" which posed a lower risk than the second category "more-than-minimal manipulation." Attempts were made to define how various *ex vivo* procedures should be classified, and after some initial confusion, a definition was developed, which was published in 1997 by the FDA [4]. Minimal manipulation was processing that did not alter the original relevant characteristics of the cells. More-than-minimal manipulation would include processing such as expansion, encapsulation, activation, or genetic modification. Cell selection, by contrast, was eventually considered not to be more-than-minimal manipulation [5]. Subsequently, more-than-minimal manipulation was broadened to include cells that were used in a nonhomologous manner, that is, were not being used in the recipient to perform the same basic function as they did in the donor. Examples would be marrow-derived cells that were being administered to treat cardiac or neurologic

diseases. By defining these two categories of manipulation, the FDA determined the regulations to be followed during product manufacturing. More-than-minimally manipulated cells would fall under cGMP, and clinical trials using these cells would require an IND.

Further information on the regulation of cell and gene therapies was provided in March 1998 by publication of the Guidance for Human Somatic Cell Therapy and Gene Therapy [6]. This provided guidelines for characterization and release testing of cells for cell-based and gene therapies, including information on preclinical studies and gene vectors. This guidance is particularly valuable for investigators developing therapies using genetically modified MSCs, since it describes the preparation and testing of cell and virus banks used to manufacture the vector and testing on the final transduced cell product. When using gene-modified cells, the clinical protocol will require testing of the recipients for the presence of replication-competent virus.

For some time, it was not clear which manufacturing regulations applied to minimally manipulated cells. This was clarified in 2005 with publication of the current Good Tissue Practices (cGTP) regulations [5]. These closed the loop by providing a regulatory framework for these types of cellular products (Fig. 17.1). cGTP regulations were published as Subpart D of a new part (Part 1271) of Title 21 of the Code of Federal Regulations. This established the regulations regarding human cells, tissues, and cellular and tissue-based products (HCTPs). Specifically excluded from HCTPs are vascularized organs for transplant, whole blood and blood components, secreted or extracted human products, and minimally manipulated bone marrow for homologous use and not used in combination with another article, for example, scaffold or matrix. Part 1271 described the general provisions of the regulations (Subpart A), including the requirement to register your establishment annually with the FDA and to list the activities performed and products manufactured (as described in Subpart B) and to determine the eligibility of donors to provide cells (described in Subpart C). Subpart D describes in detail the cGTP regulations to be followed when handling minimally manipulated cell products. In essence, these are a "light" version of the cGMP regulations, containing many similar elements. Subpart E addresses enforcement of Part 1271.

MSCs require *ex vivo* expansion before clinical use. This places their manufacture into the more-than-minimal manipulation category and subject to the cGMP regulations and clinical use under the IND mechanism. This position has been legally challenged (unsuccessfully) by a commercial entity involved in MSC-based therapy [7].

Investigational New Drug Applications

The IND application provides the FDA with a summary of the preclinical data generated (including animal studies where performed); the details of manufacturing, testing, and criteria for release; and labeling of the cellular product (contained in the

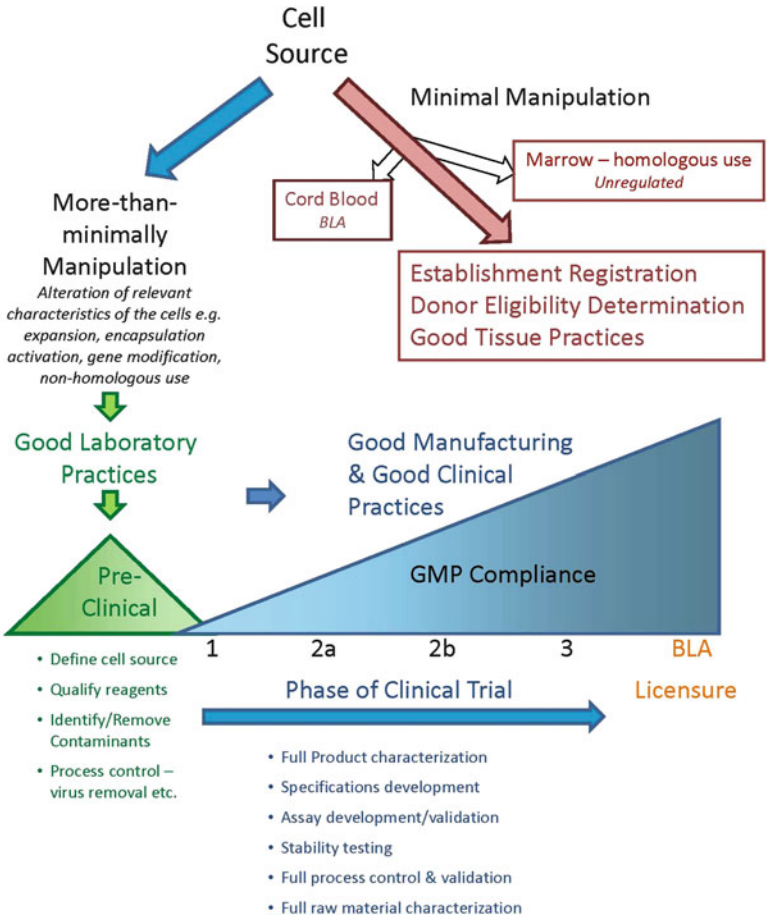

Fig. 17.1 Summary of FDA regulatory pathways for cellular therapy products. This figure summarizes the pathways for regulation of cellular therapy products based upon risk. The major differentiation is based upon the degree of manipulation of the cells *ex vivo*. Minimally manipulated cells are subject to manufacturing under current Good Tissue Practices, whereas manufacturing of more-than-minimally manipulated cells falls under current Good Manufacturing Practices (*cGMP*). As products move to later phases of clinical trials, the cGMP regulations become applicable with increasing stringency. *BLA* biologic license application

Chemistry, Manufacturing and Control (CMC) section of the application), the clinical trial design, and the evaluation criteria, including stopping rules. The most comprehensive assistance for preparation of an IND is found on the FDA webpage at www.fda.gov/Drugs/DevelopmentApprovalProcess/HowDrugsareDevelopedandApproved/ApprovalApplications/InvestigationalNewDrugINDApplication/default.htm and in the guidance "Content and Format of Investigational New Drug Applications (INDs) for Phase 1 Studies of Drugs, Including Well-Characterized, Therapeutic, Biotechnology-Derived Products" [8]. When preparing to submit an IND application, the investigator

is strongly advised to initiate a "pre- or pre-pre-IND meeting" with the FDA. A pre-pre-IND meeting is usually a general discussion of the purpose and structure of the proposed study to gauge the initial response of the FDA to the intention to submit an IND. The pre-IND meeting provides the opportunity to address areas of confusion and to clarify questions that may have arisen during the preparation of the application. The investigator should make a written request for the meeting and provide the Agency with a list of specific topics that are to be addressed. Within 60 days, the FDA will arrange a conference call that will be attended by selected representatives of the Agency with expertise in the areas to be covered. The call will be of specified duration and provides the investigators with an excellent opportunity to resolve problems and amend the application accordingly. The value of these types of initial interactions cannot be overstated. Carefully structured pre-IND meetings can greatly expedite the review and approval of the final IND application. The types and scope of meetings that can be held with the Agency are described in the 2009 Guidance document "Formal Meetings Between the FDA and Sponsors or Applicants" [9].

It is important that the preclinical experimental and toxicity data submitted in the IND are generated using a product manufactured under the same conditions as those proposed for the clinical trial. Where possible, the product proposed for the trial should be available as a single lot, or the manufacturing process should have been sufficiently validated to show lot equivalence where more than one lot will be used. These types of issues are frequently on pre-IND meeting agendas.

The formal IND application will proceed more smoothly if the CMC section is carefully written. A template for this section is available from the FDA "Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Somatic Cell Therapy Investigational New Drug Applications (INDs)" [10]. Although intended for reviewers of IND applications, it provides a stepwise approach to constructing the CMC section to contain all of the required information in a format that is familiar to the Agency. The main elements include description of the origin of the cells and the reagents and excipients that will be used during collection and manufacturing. This is usually presented in tabular form. Wherever possible, media, reagents, and additives should be of clinical grade. Where this is not possible, the purest available alternatives should be proposed, and certificates of analysis (CsofA) from the manufacturers should be submitted to indicate the level of testing that is performed. The Agency may require additional testing prior to the use of such materials for product manufacturing.

The procedure for manufacturing is provided in detail, including the timeline for production and details of any in-process storage and the final formulation that will be used for administration. The manufacturing process must have been qualified to provide assurance that different batches of cells can consistently meet specifications. A detailed listing is required of the tests that will be performed on the product to demonstrate identity, purity, residual contaminants, endotoxin, and freedom from microbiological agents. Potency testing is listed but is not formally required until initiation of phase 3 clinical trials. Cell dose, viability, and stability testing results

should be provided. For the latter, it is advisable to include the anticipated stability under cryopreservation and the stability of the product once thawed. A draft CofA should be provided. This lists the tests to be performed (and their sensitivity or limit of detection), the identity of the testing laboratory, and the specification for release. Release will be based on results that will be available prior to administration of the product. Additional testing may be performed (and required by the FDA) for which the results will be received post administration. A procedure should also be submitted for dealing with out-of-specification test results that are received after administration.

A system for tracking and tracing the product between collection and administration should be described. It is advisable to provide a copy of the proposed label for the final product, ensuring that it contains the required FDA terminology. A description of the product container is required together with the proposed route for administration. In cases where catheters will be used for delivery, a validation of the delivery system should be provided to demonstrate that the product is not altered or adversely affected by the means of administration.

Standard operating procedures should be referenced for procedures not described fully in the body of the CMC. It is usually not necessary to submit copies with the IND application, although the investigator may subsequently be asked to provide selected examples. It is important to coordinate the CMC section contents with information in the remainder of IND application, which is frequently multiauthored by researchers, clinicians, statisticians, regulatory staff, and manufacturing technologists.

Once the application has been filed, the FDA has 30 days in which to reply. If there are no issues, the application will be approved. More frequently, it will be put "on hold" pending answers to questions raised by the Agency. These are generally provided by the investigators in a written reply which carefully and specifically addresses the issues raised. An approval may include "non-hold" issues that allow the trial to be initiated but point out that additional information will be required subsequently, for example, by the start of the phase 3 studies [9]. All communications with the FDA during the application process should be documented to ensure that there is a written record of interactions. Follow-up written confirmation of important points raised during telephone calls should be copied to the Agency to avoid misunderstandings.

GMP Manufacturing

New investigators often misinterpret cGMP manufacturing requirements. A common misconception is that a clean room facility is required [11]. Such facilities are now commonplace in larger academic institutions but are not a prerequisite. For phase 1/2 studies, the FDA is primarily concerned that the product is safe and manufactured by a reproducible procedure. The cGMP infrastructure is designed to provide this [12, 13], predominantly in the form of documentation. The regula-

tions require that there be adequate space, personnel, equipment, etc., and these must be described. There must be documentation of staff training and competency, control of environmental conditions (when specified), written manufacturing procedures, a quality program, methods for handling reagents and materials, procedures for release of the product, etc. At first glance, the regulations may appear intimidating but, with familiarity, become routine in even a small manufacturing facility [11]. The Agency has recognized that not all components of cGMP are appropriate at the start of clinical trials. Full cGMP compliance is "phased in" as part of what has been called the cGMP continuum, such that by the initiation of phase 3 studies, all of the major regulations must be followed (Fig. 17.1). To assist investigators performing phase 1 studies, the FDA published in 2008 a guidance "cGMP for Phase 1 Investigational Drugs," which outlines the Agency's expectations for compliance. The "c" in cGMP indicates "current" and updates to the regulations can be found on a special FDA web page at www.fda.gov/AboutFDA/CentersOffices/cder/ucm095412.htm.

Mesenchymal Stromal Cell Products

The Agency tends to look for specific items when reviewing the CMC section of an IND application. The GTP regulations are based on risk, including that posed to the cells during manufacturing of the product; this same philosophy can be applied to more-than-minimally manipulated products manufactured under cGMP regulations. Potential risks and methods for their elimination or avoidance should be addressed in the CMC section.

For MSC products, the investigator should propose eligibility determination of the donor within 7 days of collection of the product. The collection method should be described in detail, stating the source of the material, the collection method, and precautions taken to protect both the donor and the cells. Wherever possible, functionally closed systems should be used for cell handling. These include the use of disposable bags, culture systems, and tubing sets that can be sterile connected. In some cases, especially when starting with small numbers of cells, this is not possible and "open" culture systems are initially used. Under such circumstances, the investigator should describe precautions taken to prevent contamination and cross-contamination of the products during handling. Where multiple products are handled in a facility, a procedure should be described for changeover between handling of cells from different donors. Reagents used during cell culture should be described in detail, and CsofA submitted in the IND application. Where the materials are not of clinical grade, justification for their use should be provided, and the CofA included for the proposed source. Antimicrobial agents should be avoided if possible, and where their use is justified, evidence should be provided to indicate the maximum residual amount that could be present in the product at the time of administration. It is also advisable to demonstrate that final sterility testing of the product is not adversely affected by the presence of residual antibiotics or other additives

that may interfere with the sterility assay. This is accomplished by performing a bacteriostasis/fungistasis assay in which the product or excipient is examined for its ability to suppress or stimulate bacterial and fungal growth.

A major question that arises when products are manufactured by *ex vivo* expansion is the use of serum in the culture medium. In an ideal situation, the culture medium would consist of salt solutions containing non-proteinaceous supplements, but successful cell growth under such conditions is difficult to achieve. Ideally, the MSC culture medium should be free of animal sera [14]. In reality, attempts to come up with such formulations have met with varying success [15, 16]. Substitution with human AB [15] or autologous serum is an option. Pooled serum requires the appropriate screening for infectious agents and usually needs to be sourced carefully to minimize batch to batch variation. Autologous serum may be difficult to obtain in sufficient quantities and will often show subject-specific variability. The FDA has accepted protocols using media containing animal, pooled human, autologous sera and platelet lysate. The responsibility for justification of the serum/protein type lies with the investigator, in showing that the chosen source is essential to manufacture products with the required characteristics, and that alternatives which potentially are of lower risk are not capable of producing the same results. It should be appreciated that the type of serum and culture conditions chosen may have an important effect on the composition, phenotype, and function of the resulting MSC cell product [17, 18]. As clinical trials progress toward licensure, there may need to be substitution of previously acceptable supplements.

Attention should also be paid to the use of cytokines. The use of each should be justified. It is not acceptable in a proposed manufacturing procedure to add a "cocktail" of growth factors without demonstrating that each component is required. This evidence can be provided in the preclinical section of the IND application and/or published justifications provided.

In MSC therapeutic regenerative medicine applications there should be justification that the cells in the final product either retain the ability to differentiate along multiple pathways or have been primed toward a particular lineage. This is most frequently achieved by the use of multi- or unipotential colony-forming assays. Although of limited value for demonstrating therapeutic potential, these assays remain an important indicator of cell function and will normally be expected to be on the list of release tests. In addition, retention of multi-potentiality and replicative capacity may diminish with time in culture [19] and, thereby, limit the degree of expansion possible if, for example, aiming to generate a large bank of MSC. In this context, the use of colony-forming assays coupled with gene expression studies may be invaluable. Colony information provided on the final product will only be available after clinical administration, and in-process testing may offer useful supplementary information. If large numbers of MSCs are to be generated for an allogeneic bank, in addition to the question of how many times can the cells be passaged, are the issues of when a "cell bank" is considered to have been generated (requiring more complex and extensive testing) and the effects of cryopreservation and thawing on the cells.

When any cell with ability to differentiate along multiple pathways is proposed for therapeutic application, a major concern is that of aberrant differentiation and mutagenesis. Cells administered with the intent of differentiation into myocytes could potentially grow into bone, or senescent cells could reactivate and mutate into tumor. Initial reports described the development of malignant cells in MSC cultures [20, 21]. In at least one such report, the findings were eventually attributed to cross-contamination of the cultures with malignant cells [22]. This reinforces the importance of developing manufacturing procedures that eliminate the potential for cross-contamination and also for thorough screening of cell donors. A recent review of the risks associated with MSC therapy concluded that "the conditions for safe expansion of MSC without generating tumorigenic cells are now well documented" [23]. This concern may be additionally addressed in appropriate preclinical animal models using cells of the type proposed in the clinical trial. This does not, however, provide indisputable evidence for cell fate, due to the well-known vagaries of these models. As described above, the in vitro colony assays may provide some additional evidence, and investigators have examined the genotype and morphology of cultured cells during the manufacturing process to detect changes. A major problem is that genotypic changes occur with varying frequency during cell culture and their potential clinical significance is not always completely understood. The value of these assays and the relevance of their results to the clinical study plan are excellent points for discussion with the FDA at the "pre-" or even "pre-pre-" IND meeting. The earlier these issues are discussed with the Agency the better, as the answers will affect preclinical studies, manufacturing, and trial design.

The weak immunogenicity of MSCs has led to their use in immunomodulation [24] and for allogeneic regenerative medicine studies [25]. Immunosuppressive activity of MSCs on a mixed leukocyte reaction may be evaluated as a release criterion in these applications. HLA matching has, therefore, not been a major stumbling block when using MSCs clinically. It has been reported, however, that during differentiation in vivo, allogeneic MSCs may provoke an immune response in the recipient [26]. Similar responses can also occur to MSC culture constituents [27]. Many of these differences are due to the multiple methods for generating MSCs, and it is clear that a variety of cell types have initially been used under this name. In attempt to address this issue, the International Society for Cellular Therapy developed minimal criteria for defining multipotent MSCs, based on immunophenotype, plastic adherence, and trilineage (osteoblasts, adipocytes, and chondroblasts) differentiation capacity [28]. The abbreviation MSC itself has been redefined over time as representing mesenchymal stem cells, mesenchymal stromal cells, and, finally, multipotent mesenchymal stromal cells, further indicating the complexity and variety of the cell types under study.

Under such circumstances, each cell product essentially stands alone when it comes to regulatory interpretation. Where there is clear and indisputable identity between a cell type proposed for study and one that is already in clinical trials, it is helpful to ask the principal investigator (PI) of the clinical trial for permission to cross-reference his or her IND. This provides the Agency with additional information

on the cell type under study and its use in clinical studies. One potential drawback is that any product-related adverse events on the existing or new trial may result in both studies being placed on hold.

In the absence of such cellular identity, the investigator must provide the regulatory authority with a stand-alone submission. This may cross-reference other studies with similar cells types but provides independent data on the characteristics, manufacturing, and proposed clinical use of his or her specific MSC product.

Conclusions

As our understanding of the identity, properties, and clinical applications for MSC populations grows, the regulatory requirements and procedures for manufacturing, release, and administration are likely to change. This chapter can, therefore, only provide a general overview. This is especially true for a cell type with plasticity and with seemingly multiple applications. Investigators wishing to start a new clinical study should always revisit the regulations and talk with the Agency to determine the current regulatory strategy for their specific MSC product.

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Chapter 18
MSCs: Clinical Applications and European Regulatory Aspects

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Abstract Mesenchymal stromal/stem cells (MSCs) are used to treat a wide variety of diseases. The conditions for which MSCs are administered are often rare and provide an unmet medical need. The biology of MSCs, however, is still not fully understood, and the risks associated with administration of such products are not fully defined. MSCs are in most cases classified as advanced therapy medicinal products (ATMPs) based either on the substantial manipulation of the cells during the manufacturing process or their heterologous use. MSC-based ATMPs pose certain challenges for developers and regulators, including reliable characterisation/identity of cells, microbial and viral safety, biological activity/potency, relevant animal models, biodistribution and genetic stability. This review highlights the scientific and regulatory challenges of MSC-based ATMPs.

Introduction

Mesenchymal stromal cells (MSCs) play an emerging role in the field of advanced therapy medicinal products (ATMPs). Residing quite close to haematopoietic stem cells in the bone marrow, they were initially discovered and identified as a heterogeneous fibroblast-like cell population [1]. MSCs can be expanded in vitro where they could differentiate into a variety of cell types, for example, osteoblasts, chondrocytes,

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_18,

and adipocytes. Beside their multipotency, MSCs show an immunomodulatory effect onto their direct cellular environment [2], for example, the slowdown of an overshooting inflammatory response that may impede tissue repair. Another characteristic of MSCs is their ability to support intrinsic tissue regeneration processes, for example, by secretion of cytokines [3]. Considering all these attributes, MSCs could serve as therapeutic agents for immune modulation, tissue repair and regenerative medicine, even though their mode of action has not been fully elucidated yet.

While the first MSCs were isolated from bone marrow, MSC-like cells can be isolated from a variety of other tissues [2–5] as well. Usually, MSCs are selected and cultured in vitro in the presence of fetal bovine serum (FBS), human serum, serum-free media, or human platelet lysate [6]. For some indications, MSC-based therapies have been already explored under the prerequisite of clinical trials, and several clinical trial applications have already been filed in European Union (EU) member states. The number of indications reflects the large capacity of the cells for self-renewal and immunomodulation and range from treatment of bone fractures and large bone defects through graft versus host disease to critical limb ischemia and cardiac ischemia.

The Landscape in Europe for Clinical Studies and Marketing Authorization

To achieve harmonised market availability within the EU, the European Commission (EC) has established a dedicated ATMP Regulation (Regulation (EC) No. 1394/2007) [7], which came into force on December 30, 2008. The Regulation is supported by an amendment of the medical code (Directive 2001/83/EC), which contains updated definitions of gene therapy and cell therapy medicinal products. This amendment also contains specialised requirements for the marketing authorisation of ATMPs and provides a definition for tissue-engineered medicinal products (TEP), which are now defined as a class of ATMPs.

With implementation of the ATMP Regulation, the centralised marketing authorisation application (MAA) procedure becomes mandatory for ATMPs. To take into account the innovative character of these medicinal products, a new Committee for Advanced Therapies (CAT) was established at the European Medicines Agency in London (EMA). The CAT comprises members with specific expertise in the area of ATMPs and scientifically evaluates their MAA preparing a draft opinion to be transmitted to the Committee for Medicinal Products for Human Use (CHMP) for adoption.

While the MSC-based ATMPs are mandated to undergo a central marketing authorisation procedure, the approval of clinical trials resides within the responsibility of each member state and is therefore regulated at a national level. However, since the scientific expertise of the EMA committees and working parties is provided by experts of the national competent authorities (NCAs), a basis for networking and transparency is built up within the European community. As a prerequisite for clinical trials and for authorisation, a manufacturing authorisation for the

medicinal product under investigation is required before entering phase I clinical trials. The manufacturing authorisation is within the remit of the competent agencies in the member state of manufacture and has to comply with European Good Manufacturing Practice (GMP) requirements.

Innovative Biomedicines and European Regulatory Considerations on MSC-Based ATMPs

For the MSC-based TEPs and CTMPs, which had been legally on the national markets before the ATMP Regulation entered into force, a transitional period was granted before having to comply with the new legislation and undergo a marketing authorisation procedure: ATMPs other than tissue-engineered products had to comply with the legislation by December 30, 2011, while for TEPs the transition period ends by December 30, 2012 (Article 29, transitional period). The exemption from this rule is the so-called hospital exemption (Article 28). The hospital exemption is applicable to all ATMPs that are prepared on a nonroutine basis according to specific standards and that are used within the same member state in a hospital under the exclusive professional responsibility of a medicinal practitioner, in order to comply with the conditions for an individual medical prescription for a custom-made product for an individual patient. The authorisation on the basis of the hospital exemption is in the remit of the corresponding member state.

The classification of MSC-based medicinal products as ATMPs is based on the condition of the cells being "engineered," which requires the fulfilment of one of the following two conditions: (i) The cells have been subject to substantial manipulation or (ii) the cells are not intended to be used for the same essential function(s) in the recipient and the donor (nonhomologous use). This is to account for the fact that in both cases, the cells, even if autologous in origin, will face a new physiological microenvironment after application, either because the cells have been changed or the environment has been changed, and their behaviour in this new environment may not be predicted from their former behaviour. A substantial manipulation is defined as one that alters biological characteristics, physiological functions or structural properties, relevant for the intended regeneration, repair or replacement. One well-established example is the long-term in vitro expansion and/or in vitro differentiation of cells. In particular, the manipulations listed in Annex I of the ATMP Regulation shall not be considered as substantial manipulations.

MSC-Based ATMPs Are Setting New Challenges in Comparison to Small Molecule Medicinal Products

The novelty, complexity and extreme diversity of MSC-based ATMPs demand new regulatory tools to allow an appropriate balancing of the risks and benefits. This can be illustrated by the five parameters described in Table 18.1.

Table 18.1 Differences between conventional small molecule medicinal products and MSC-based ATMPs

Parameter	Small molecule medicinal products	MSC-based ATMPs
Size	Small (<500 Da)	Large, with a considerable size range (from single cells to tissues)
Manufacture	Highly controllable synthesis process	Biological or biotechnological manufacturing and subsequent purification or cell procurement, manipulation and expansion
Characterisation: structure and impurities	Precisely defined structure of the active substance, clearly defined impurities	No precisely defined structure; product may also contain scaffolds or matrices, purity of cell population may differ, activity may display high variability depending on the donor
Metabolism	Products are metabolised within the cells and excreted renally, via the lungs or the skin	Products may be integrated into the body, cells may differentiate, grow, migrate within the body, biodegradation/apoptosis may occur
Species specificity	Limited species specificity	Products often have a species specificity

The manufacture of MSC-based products cannot be controlled as precisely as that of a chemically synthesised small molecule. Characterisation of the structure and the (cellular) impurities pose a particular challenge, since MSCs are a heterogeneous population that lack a unique cellular marker. Moreover, their mechanism of action for most applications are not well established, and therefore, it is difficult to decide whether any subgroup of cells is critical; Also, the way cellular therapies interact in vivo may differ significantly from that of conventional medicines, that is, they are not metabolised but may be integrated into or rejected by the recipient. Therefore, pharmacokinetic studies such as metabolism and excretion studies become less relevant, and classical carcinogenicity is not expected. Instead, the biodistribution of MSC-based ATMPs needs to be addressed in more detail to predict migration and differentiation patterns and the persistence of cells in the patient. Biodistribution may help to predict the long-term efficacy of the product as well as the potential for ectopic effects, which may have impact on safety.

Conventional animal studies may not be reliable because the species specificity of MSCs may result in markedly different interactions between the cells and their environment in animal models compared to patients. Thus, relevant animal models, that is, models in which the test material is pharmacologically active so that the response leads to meaningful conclusions for the intended clinical indication, should be used wherever possible (as highlighted below).

In conclusion, special regulatory requirements are necessary to evaluate MSC-based therapies using more product-adapted assessments in a case-by-case approach. This does not prevent the manufacturers of such products from adhering to the principles of the community code relating to medicinal products for human use (Directive 2001/83/EC) as laid out in Annex I part 1. Rather, these specific

regulatory requirements permit justified deviations. Nevertheless, the production processes for MSC-based ATMPs need to be in accordance with GMP, as described in the Commission Directive 2003/94/EC and more precisely in an overview of the GMP requirements for MSCs [7, 8]. The current GMP requirements for the manufacturing of biological medicinal substances and products for human use (comprising cell-based ATMP) are reflected in the new Annex 2.

Several major scientific issues need to be considered in the development of MSC-based ATMPs. These issues are under discussion by regulatory authorities, as described in the "Reflection paper on stem cell-based medicinal products" adopted by the Committee for Advanced Therapies (CAT) in January 2011. From a regulatory perspective, the current most important scientific challenges during the development of MSC-based medicinal products concern aspects of characterisation / identity of the cells, microbial and viral safety aspects, biological activity/potency, relevant animal models, biodistribution and genetic stability of the cells.

Characterisation/Identity of MSCs

MSCs are routinely purified by their selective adherence to plastic, but MSC cultures, especially early passages, can contain impurities like hematopoietic cells, epithelial cells, fibroblasts and others. The characterisation of MSC-based medicinal products in terms of cell composition (purity of the MSCs and identity of contaminating cells) is therefore a key issue for the standardisation of the manufacturing process to ensure the efficacy and safety of the medicinal product.

Currently, no single-cell surface marker is available for the unambiguous identification of MSCs. Therefore, these cells are mainly characterised by the combination of three criteria [9]: (i) growth on plastic surfaces, (ii) capacity to differentiate into osteogenic, chondrogenic and adipogenic cell lines in vitro [9], and (iii) the expression of a set of different surface proteins (CD, cluster of differentiation): $CD73^+$, $CD\ 90^+$, $CD105^+$ or absent: $CD34^-$, $CD45^-$, $CD14^-$ or $11b^-$, $CD79\alpha^-$ or $CD19^-$, and human leucocyte antigen (HLA)-DR^-. More recently, a range of surface markers including SSEA-4, STRO-1 and CD271 [10–12] have been employed to prospectively isolate primary uncultured MSCs. Battula and colleagues have recently isolated MSCs from human BM by flow cytometry, using antibodies directed against the surface antigens $CD271^+$, mesenchymal stem cell antigen-1 ($MSCA\text{-}1^+$) and $CD56^+$, and have identified novel MSC subsets with distinct phenotypic and functional properties [13]. Platelet-derived growth factor receptor-beta (PDGF-RB; $CD140b^+$) has also been identified as a selective marker for the enrichment of clonogenic MSCs [10], and other reports have demonstrated an enrichment of MSCs in human BM cells with prominent aldehyde dehydrogenase activity [14]. Usually, only a small portion of cells is tested by fluorescent-activated cell sorting (FACS) analysis, and only a subset of the surface markers, which in any case do not usually correlate with the relevant biological activity of the cells, is employed. As a consequence, identification of MSCs and contaminating cells still remains difficult. Currently, the product is characterised according to the best available scientific

information for purity (% MSC and % contaminating cells), efficacy, and safety and the results are correlated with clinical outcome. The purity of MSCs directly impacts on the product safety in an animal model: The transplantation of unpurified bone marrow cells in a rat model did lead to intramyocardial calcification, not observed using purified MSCs [15].

Microbiological and Viral Safety of MSCs

Since MSCs are usually administered parentally, aseptic manufacturing is essential for safe medicinal products [16]. A range of tests is available to detect microbial pathogens or contamination with microorganisms (e.g., mycoplasma), but as for living cells in general, extensive and rapid testing for microbial pathogens is not feasible. For this reason, donors should be tested for bacterial, viral, and fungal diseases and regular sterility tests should be performed during the entire manufacturing process. In future, it will be necessary to develop enhanced and validated testing systems with shorter incubation times to detect infections sensitively and rapidly.

Due to the risk of viral transmission by MSCs, comprehensive pre-screening of the donor by diagnostic testing and consideration of medical history is needed. The transmission of viral disease needs to be addressed in an autologous setting as well, since manufacturing and manipulation could potentially lead to (i) concentration of (patient's own) viruses, (ii) cross contamination derived from medicinal products handled at the same time, and (iii) infections from the personnel involved in the production process. Special attention should be given to the potential contamination of excipients, especially of animal origin (e.g., bovine serum, porcine trypsin, proteases, etc.). This concerns viruses of human or animal origin and transmissible spongiform encephalopathies. Replacing excipients of animal origin with recombinant enzymes and using serum-free media may be a strategy to minimise these risks. However, where such a replacement is not possible, complex testing of such excipients may be necessary [17].

Biological Activity of MSCs: Potency Assays

Testing the biological activity of a medicinal product is the most reliable way of ensuring consistent efficacy, and therefore, a potency assay should always be part of the release specifications of the medicinal product. The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guideline Q6B [18] defines potency as the qualitative measure of biological activity based on the attribute of the product. Ideally, biological activity should correlate with the clinical response. Thus, a reliable potency assay should be based on a thorough understanding of the mechanism of action of the product. However, in the case of MSC-based ATMPs, the establishment of a potency assay can be challenging, due to a limited understanding of the mode of action and the lack of an appropriate animal model (as discussed below).

While it is not always feasible to study the relevant biological activity in an animal model [18], it may nonetheless be possible to test surrogate markers to predict product efficacy. Indeed, an in vitro assay with a reliable surrogate parameter is usually faster and less expensive than the use of animal models. In general, the following procedures are available to measure the biological activity of MSCs: animal-based biological assays when relevant experimental animal models are available; cell culture-based biological assays to measure physiological responses at a cellular level; and biochemical assays to measure enzymatic activities or biological responses induced by immunological interactions. For instance, a biological assay may be used to measure the amount of IL-2 secretion by T cells in mixtures containing varying amounts of MSCs in vitro.

The in vitro tests currently available may not fully reflect the activities responsible for efficacy in vivo and validated tests need to be developed as part of clinical studies. Until such assays become available, it is important to focus on the quality of the product and on the clinical studies aiming at quantifying efficacy as a basis for regular risk benefit assessment. Biological assays are still difficult to standardise and generally not accepted as surrogates for MSC potency to date.

Relevant Nonclinical Models for MSCs

The Guideline on Human Cell-Based Medicinal Products [19] suggests performing nonclinical studies in relevant models of the disease or injury. Relevant models should react to medicinal products in the same way as patients do, that is, its responses should be predictable, including adverse events. Relevant animal models may include genetically modified animals (receptor knock-in or knock-out animals or specifically humanised animals) or homologous models using animal cells of the respective species instead of using human cells. The route of administration and the dosage tested should reflect the intended clinical use in humans, and their physical, mechanical, chemical and biological properties should be considered. A scientific justification of the use of the model, based on its relevance and limitations, should be provided. In cases where a relevant animal model cannot be generated, data generated by tissue culture assays may compensate for the lack of animal data. The scientific justification for the nonclinical strategy and for the animal model employed needs to be provided.

Biodistribution of MSCs

The conventional requirements for pharmacokinetic and toxicological testing, including adsorption, distribution, metabolism and excretion (ADME) studies, are not appropriate for MSC-containing ATMPs. More appropriate are biodistribution studies, which should focus on cell survival, growth and differentiation potential using a product composition as close as possible to that used in the clinical trials.

An alternative is to set up a "worst case scenario" situation by injecting the MSC-based ATMPs to follow both, their distribution and the possible resulting adverse events on unintended target organs. Several preclinical studies of the biodistribution of MSCs have been published. MSCs injected into the veins of baboons were distributed to the lung, thymus, bone, skin, cerebellum and the gastrointestinal tract [20]. In a rat model, MSCs primarily relocated to the lung and secondarily to the liver after both intra-arterial and intravenous injection [21]. While such data may help to predict target organ toxicity, the specific manipulation of the cells may change their behaviour. Hence, data on cells manipulated in a certain way can support only a particular cell product.

Genetic Stability of MSCs

In cases, where MSCs are substantially manipulated by the means of culture expansion and/or *ex vivo* manipulation (sometimes including genetic modification), the substantial manipulation may increase the risk of mutations, which can lead to loss of function and maybe even tumourigenesis. The current method to detect genetic instability is karyotyping, although this technique only allows detection of large chromosomal rearrangements of more than ten mega bases. More refined methods such as fluorescence in situ hybridisation (FISH) that may identify rearrangements at a single-gene level are in most cases restricted to a limited number of candidate gene rearrangements and, thus, do not lead to the detection of unexpected genetic instabilities. However, this limited approach may be considered as a secondary test method for the confirmation of identified chromosomal abnormalities by karyotyping. For the karyotyping, the GC-banding technique still remains the gold standard. Therefore, the data generated from GC banding together with functional assays and tumour formation study results may provide an adequate basis for the risk evaluation of cultivated MSC-based ATMPs.

Furthermore, new techniques under development may be used to characterise the whole genome, as does karyotyping but with higher sensitivities. One example is spectral karyotyping (SKY). This technique takes advantage of the FISH principle – the hybridisation of labelled complementary DNA probes with target sequences – but is based on the simultaneous hybridisation of painting probes that are specific for each chromosome labelled with different fluorochromes. This method allows the detection of some of the chromosomal aberrations and rearrangements that cannot be identified by G-banding karyology alone [22]. Another method is comparative genomic hybridisation (CGH) [23]. In this approach, fragmented genomic DNA is hybridised against special microarrays that represent the whole genome as overlapping oligonucleotides. Thereby, the hybridisation signals allow to control whether any genomic sequence is still present and within the right context. Although this technique has not been established yet on a diagnostic level, it may be an option for the future. Currently, reduction of passages and karyotyping remain the best strategy to detect genetic instability.

Conclusions

MSC-based ATMPs are used in clinical/experimental settings, targeting many conditions with unmet medical needs. However, the biology of MSCs is still not fully understood, and the risks associated with such products are currently not fully elucidated.

Therefore, as long as clear evidence of efficacy is lacking, ATMP therapies should be undertaken solely in the context of clinical studies that require approval by competent national authorities and should adhere strictly to GCP recommendations.

Numerous challenges arise from the derivation and nature of MSC-based medicinal products. In this context, current guidelines are intended to support academic research groups and pharmaceutical companies to foster the development of safe and effective medicinal products. For a successful development of ATMPs, an early dialogue between the scientific / clinical community and regulatory agencies at national and European level is of utmost importance. This way, we can expect to overcome the challenges and foster an informed regulatory environment supporting the rapid development of safe and efficient medicinal products for the treatment of patients with otherwise unmet medical needs.

Disclaimer The views expressed here do not necessarily reflect the views of the Paul Ehrlich Institute (PEI).

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Chapter 19
Stem Cell Treatments Around the World: Boon or Bane?

Alok Srivastava

Abstract Stem cell-based therapies hold immense possibilities for treating diseases. While responsible scientists and physicians attempt to develop such treatments in a scientific and ethical manner, many others around the world are already offering unproven therapies for a wide range of diseases. Interestingly, they often use cells that have been known and accessible for decades. It has resulted in large numbers of vulnerable patients being exploited around the world. This chapter will address the reasons for this phenomenon, the impact that it has had on the field, the harm that can come from them, and the potential solutions to this problem.

Introduction

While academic scientists and physicians struggle to unravel the biology of stem cells and work together to take them to the clinic in a systematic and phased manner for the treatment of many currently untreatable diseases [1–3], there are others who offer stem cells for the treatment of a host of diseases as though they are already established therapies [4, 5]. This phenomenon is not restricted to any single cell type or disease nor is it confined to a few locations but seems to be an increasingly pervasive problem that transcends international borders [6]. Why did this situation arise? What is driving it? What is its magnitude? What good or harm can come from it? How should it be controlled? This chapter will attempt to address these issues.

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_19,

The Unmet Needs in Medicine

As we celebrate the advances in medical science and its impact on human health, it is important to remember that there are still many unmet needs where patients are desperately waiting for relief, if not cure. These needs are a result of two types of limitations. The first is the limitation of available therapies themselves with conventional therapeutics such as drugs to improve the function of organs severely damaged by ischemia, inflammation, or degeneration. There is little that current medicines can offer many of these patients. While organ transplants rescue some of these patients, logistical and technical limitations prevent the vast majority from availing such therapies [7]. Neurological diseases remain outside the ambit of transplantation, in any case. The second of these limitations is related to the problem of access to and availability of existing therapies to patients around the world. Most drug therapies have been developed by big pharmaceuticals that control registration and prices in different markets and therefore access [8]. Patients and physicians outside of developed countries have at times waited for years before being able to access certain drugs. In some countries, many patients have never been able to use certain drugs as they have not been able to afford them.

Added to these issues are other inadequacies of health-care systems which leave patients dissatisfied [9]. For chronic incurable conditions, many patients receive no or grossly insufficient attention and support from their health-care providers. The cost of long-term rehabilitation and the social support needed by families to deal with such patients are also not provided in many places [10]. In fact, it is often conveyed to them that there is very little hope for any significant relief for them in the foreseeable future.

Why Stem Cells?

It is not difficult to imagine then that central to the excitement of the first report of the generation of human embryonic stem cells (ESC) in the late 1990s was the presumption that such cells could be differentiated into different types and tissues that could then be used to regenerate or replace failing organs [11]. While the science of ESCs has been mired in ethical controversies along with logistical and technological limitations [12], what has gained prominence is the relatively softer science of the plasticity and potential of some of the easily accessible bone marrow-derived adult stem cells in regenerating diseased organs [13] even though these concepts have been challenged [14].

Bone marrow- and cord blood-derived hematopoietic stem cells (HSCs) had been used for decades to treat hematological diseases by allogeneic and autologous transplantation [15]. Their use had remained relatively controlled, not by intellectual property rights or access to the required stem cells, but simply by issues related to histocompatibility as well as the medical, technological, and financial challenges of this treatment. The situation changed greatly when in the early 2000s, there were

clinical data to suggest that myocardial damage after infarction could be contained and recovery enhanced with autologous HSC infusions into the heart [16]. More reports followed on the ability of the HSC to transform to cells of different non-hematopoietic lineages and therefore their potential to regenerate them [17, 18]. Notwithstanding the fact that subsequent large randomized clinical trials have failed to establish clear efficacy and superiority of hematopoietic cells in the treatment of ischemic myocardial damage over other conventional options [19–21], the use of adult stem cells for organ regeneration had become a real possibility.

Mesenchymal Stromal/Stem Cells: Even Better!

In the domain of finding easy options of cells for regenerating organs, mesenchymal stromal/stem cells (MSCs) have acquired a special position. Though they received limited attention for almost two decades after their discovery in the early 1970s, interest in MSCs was reignited in this era of cell therapy and regenerative medicine [22]. Not only could these cells be expanded in large numbers under GMP conditions, but they could also be differentiated in vitro well beyond the classical mesodermal lineages of chondrocytes, osteoblasts, and adipocytes [23]. Furthermore, it was also discovered that they were not only immunologically naive allowing their use across HLA barriers, but could in fact have an immunomodulatory effect, adding a new dimension to their potential uses [24]. To top it all, here was a cell that could be grown from other easily accessible tissues, such as the umbilical cord, placenta, adipose tissues, and many more [25]. In some ways, the MSC, if it could actually regenerate organs, was almost the ideal candidate.

The understanding that allogeneic MSCs can be prepared in large batches and used across HLA restrictions has also helped expand the use of these cells in regenerative medicine, unlike all other current options that require HLA compatibility and therefore need to be prepared specifically for selected patients. This also adds to their potential for commercialization as a ready-to-use product, if found safe and effective [26]. This field therefore attracts investor interest and funds for development but then also leads to an expectation of return on investments in an environment not yet ready for it.

The Scientific Path to Stem Cell Therapies

The classical approach to developing safe and effective therapies with stem cells is outlined in Fig. 19.1. It would involve reasonable characterization of the cell of interest and documentation in vitro, and then in suitable animal models, if available, of the functions it is expected to perform in treating human disease, followed by evaluation of safety in preclinical animal models and ultimately followed by clinical trials in a phased manner [2]. This would need to be combined with production of

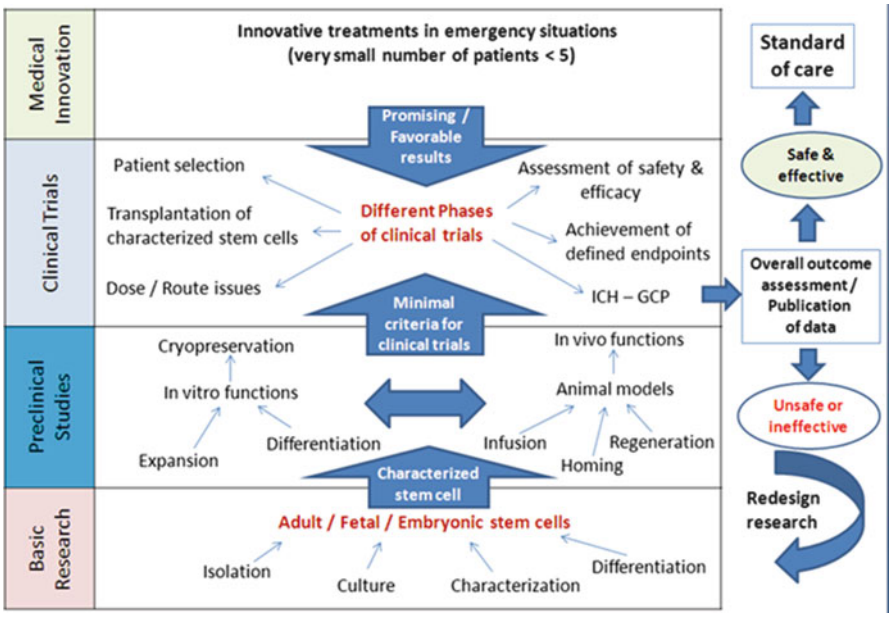

Fig. 19.1 Steps in translation of stem cell research – from bench to bedside. (1) The first step in basic research to identify, culture, characterize, and develop protocols for differentiation of the cell of interest; (2) the next step has two parts – first is to test function in appropriate laboratory studies and infusion, homing and regenerative abilities in suitable animal models, if available, and second is to develop methods for large-scale clinical grade expansion and storage of these cells for clinical trials. (3) The third step would involve actual clinical trials in phases to test different doses and routes of administration in the appropriate patient populations as per current GCP guidelines. If judged to be safe and effective, such therapies may go on to become the standard of care. Otherwise, it will need to go back to preclinical studies for further research. (4) It must also be recognized that at times, instead of going through this classical path, a few patients (<5) may be treated with innovative cell therapies in emergency situations. However, if the result of such treatment is found to be encouraging, such therapies will still need to be taken through phased clinical trials before becoming standard of care

adequate quantities of cells under GMP condition in facilities that have regulatory approval [27]. The data obtained from these studies will need to be reviewed by independent monitoring committees and then published in peer-reviewed journals. It would also be expected that the initial report be followed up with another that would provide data on longer follow-up on the same patients for both efficacy and adverse events. Finally, it should be possible to reproduce the results by other groups using similar treatment. Once these stages have been achieved, the particular treatment may go on to become the standard of care, if confirmed to be superior to other available options [28, 29].

This approach is certainly the scientific, safe, and ethical way to develop therapies. However, it also can be very slow, taking years to go through the different steps [30, 31], and requires major investment of funds to conduct the appropriate studies. In fact, if we look at the current situation, in spite of over 5 years of concerted efforts

at finding effective stem cell-based therapies for different diseases, very few have even reached the stages of advanced clinical trials, and none has established unequivocal efficacy [32].

An alternative approach, often described as "medical innovation," is also possible without going through this long process [33]. Physicians may justify the use of stem cells in conditions where there are no options and the patient's condition is critical. While this path is also accepted in the International Society for Stem Cell Research (ISSCR) guidelines for clinical translation [2], what has been suggested is that after establishing feasibility and showing safety and early efficacy in a few patients (usually less than 5), such therapies should be evaluated through formal clinical trials before being offered as standard therapies. Unfortunately, this has not always been the case [34]. In fact, based on soft assessment of outcomes, mostly on patient testimonies alone and no objectively documented parameters, many clinics have moved on to offering treatment with stem cells to large numbers of patients with different diseases [6, 35].

Proliferation of Clinics Offering Stem Cell Treatment

Given the circumstances described above, it is not difficult to understand why clinics have mushroomed in different parts of the world offering treatment with stem cells. The reasons are obvious. There are huge unmet medical needs all over the world, and here is an opportunity to offer hope, if nothing else [36]. The combination of easily accessible stem cells, often autologous from the bone marrow or adipose tissue, and the hype of potential of stem cell therapies from the scientific community is enough to lead scores of patients to such clinics. It would be difficult to attribute any true service motives to these clinics. They must be recognized for what they are – centers that mostly exploit vulnerable patients with half-truths and lies, offering unproven stem cell treatments and charging large sums of money for doing so [37]. Needless to mention, these treatments have little scientific basis, and it is very unlikely that the physicians involved in such work are not aware of that [38].

It is also important to emphasize that such clinics also target the local population, particularly so in developing countries [39, 40]. Of course, they do also advertise themselves well on the Internet and through liaison offices in different countries giving rise to what has been called "stem cell tourism."

Stem Cell Tourism

The flight of a large number of Western patients to clinics, mostly in developing countries offering stem cell treatments for a variety of diseases, has been called "stem cell tourism" [41, 42]. The vast majority of clinics tend to use autologous stem cells, but some also offer cord blood-derived and even embryonic stem cells.

The approach is not individualized with the same treatment being offered to most patients. They are often combined with other treatment options such as physical therapy and acupuncture. Outcome is recorded largely in terms of the patient's testimony which is then made available to other patients as proof of success. Such data are almost never published in any scientific journal as a peer-reviewed article.

Like any other marketing plan, the treatments are sold at what these clinics have determined patients can pay, knowing very well that the margins of their profits are huge. These costs vary between $5000 and $20,000 per treatment in most cases [37, 41]. While these are immense cost for patients in developing countries and not insignificant even for those coming from the West, they still look affordable compared to the cost of other therapies in general or to long-term supportive care. More than anything else, they provide hope and, blatantly untrue as the claims may be, it is human nature to go for them.

What adds to the enthusiasm of Western patients in particular going to these destinations for stem cell treatments is the raging controversy around stem cell research, especially in the USA [43, 44]. The average patient does not understand that this controversy is mostly around ESC research and not so much with the use of adult stem cells. The media adds to this feeling of slow progress within the medical and scientific community in the West, in terms of translational research and clinical trials, by over-portraying issues related to ethical controversies, scientific dilemmas, and inadequate funding while lauding the so-called advances in stem cell treatments in entrepreneurial clinics in developing countries [45]. The harm from such clinics is not restricted to overseas patients coming to them but affects local patients in even larger numbers.

It should be recognized that not all medical tourism is necessarily bad. When patients travel anywhere for a specific treatment of proven efficacy considered the standard of care but not available in their home country for reasons of cost or capacity, then that serves an important human need. This is necessarily very different from the situation described above where patients are lured to receive unproven and potentially harmful treatments.

Maverick Physicians and Entrepreneurial Clinics: What Harm?

It would be a travesty to assign any motive other than financial gain to the clinics offering completely unproven and often baseless stem cell treatments to desperate patients [46]. The financial loss suffered by these patients is not insignificant in any part of the world. Many patients even from Western countries struggle to put together the required funds that need to be paid to the clinic in addition to the cost of travel [47]. Notwithstanding the financial loss, even the joy from what appears to be the gift of hope in the beginning [36] is bound to turn to anger and frustration when at some stage in future these patients realize the deliberate fraud to which they have been subjected.

Apart from this indirect harm, there has been direct harm as well. The now famous cases of the child from Israel with ataxia telangiectasia who developed multifocal neural tumors years after treatment with fetal neural stem cells in Russia [48], another child from Romania, who died following injection of stem cells in the brain [49], and the patient in Thailand who was found to have "strange" lesions in the kidney at post-mortem examination after stem cell treatment show that seemingly innocuous so-called adult stem cells [50] also can result in serious adverse effects. In fact, these problems may represent gross underreporting because most patients treated in such clinics are not closely followed up or monitored.

Such examples should also put an end to the argument of "no harm in trying" to justify these nonscientific attempts at treatment. The least that must be demanded from these clinics is a detailed report of the treatment provided, objective and verifiable assessment of efficacy, and a serious effort at follow-up for adverse effects. If they want to be true to their declared objective of developing innovative stem cell treatments, then they should also provide follow-up instruction to the patient's original physician. Unfortunately, this is unlikely to ever happen as this will remove the cloak under which such practices thrive at present unless there are laws that can enforce these requirements.

Further justification is provided by these clinics, particularly in developing countries, for the treatment they provide. They boast that for once, they are not shackled by patents and intellectual property rights issues of multinational companies, as they can produce adult stem cells of different types and inject them into patients. Such efforts are often also praised by the local media.

Unregulated Stem Cell Treatments: Is There a Way Forward?

It is clear from the above description that a large number of facilities around the world provide stem cell-based treatments to many patients. While they may be deemed unscientific, unethical, and even harmful by academic physicians and scientists, they certainly are making an impact on the vulnerable and desperate patients they claim to serve. Their numbers are also increasing. Thus, on one side is the traditional path to clinical translation based on sequential progression from bench work to animal studies to phased clinical trials, and on the other is the maverick approach of direct administration to patients of relatively "safe" stem cells to patients based on theoretical potentials but very little scientific evidence. The success and prowess of this group of service providers in pursuing and achieving what they have set out to do should not be underestimated. On the face of it, they are treating large number of patients who are apparently satisfied with the care provided [36], but do we really know the actual situation? Are there any authentic data on what these patients think or feel 6–12 months later when they may have a better understanding of the true value, or the lack of it, of the purported treatment given to them? [51].

Stopping the Misuse of Stem Cells in Clinics: What Are Potential Options?

What then can be done to reduce the menace of clinics offering unproven and potentially harmful stem cell treatments from proliferating globally? Several options exist for proactive steps to be taken [52], which are discussed below.

The option that may seem most obvious is to ensure that such clinics are immediately banned by the regulatory authorities in the countries where they function. Logical as it may sound, this has not been practical for several reasons. The most important of these is the fact that most of these countries lack specific laws under which such activities can be controlled or stopped [53]. It is also often not possible to locate the clinics. They tend to operate through many intermediaries in the same or different countries. In some countries, it is also not clear which regulatory authority is responsible for them. While drug control authorities regulate traditional drugs and devices and organ transplantation laws regulate use of allogeneic organs from live or deceased donors, there are no laws that define and control the use of autologous cells in some countries. Indeed, this is the gap in the system that these physicians and clinics are exploiting. A government order banning all unregulated stem cell treatments has recently been introduced in China. However, it seems that in the short term, it has not had the impact that was intended [54].

The solution therefore is to bring in suitable strategies and laws that will help regulate stem cell treatment offered by these clinics [55]. While the latter approach will ultimately be needed, in the interim, several other possibilities exist:

I. The medical and scientific community should be proactive in discussing these options with patients and helping them understand what is actually being offered. This may also help to maintain communication with these patients. There is a need to have dedicated personnel within scientific societies and medical institutions to provide this service so that it is not left to the medical staff who are often overburdened with providing regular medical services [56].
II. If the patient does choose to go for treatment to such a clinic, they should be encouraged to return for follow-up not only to assess efficacy but also to monitor for adverse effects. Such data can be systematically analyzed and reported. Furthermore, if patients are provided examples of specific adverse effects that are possible or have occurred in others treated similarly, they may be more willing to come back for follow-up after their treatment.
III. A third possibility which may be much more difficult to accept is to actually attempt some of these treatments in limited number of patients within the mainstream medical institutions, particularly with autologous stem cells, in reasonably designed and powered studies to prove, if nothing else, that such simplistic transplantation or infusion of stem cells does nothing to treat those diseases or objectively improve those patients. This will also put an end to the arguments of those who wish to give the benefit of doubt in the absence of incontrovertible proof of the likely futility of most of these treatments.

IV. It is imperative that scientists and physicians learn to exercise greater restraint when reporting early scientific results [57, 58]. It is almost the norm for top scientists to discuss the potentially far-reaching medical and social impact of their very early findings reported in the scientific literature. The media overplays the latter, and the lay public then wants to believe that a miracle is around the corner. We need to ask ourselves the following – when aspiration of bone marrow and isolation of mononuclear cells or CD34 cells or culture of MSCs was well known for more than three decades, why is it that only in the last decade that they have been misused? Where did these ideas originate? Academic scientists and physicians need to be a lot more restrained and circumspect when highlighting their research as should those not directly involved in the research when providing media commentary.
V. The media too need to be more responsible in not misleading people [59]. Perhaps they need to be counseled and regulated through appropriate agencies. Just as false advertisement is wrong, irresponsible journalism that leads to people to believe half-truths and then expect miraculous cure is also wrong.

Conclusions

In this rapidly evolving field, the potential of developing innovative stem cell-based therapies is indeed very attractive. However, while such therapies evolve in a scientific and ethical manner, unregulated stem cell treatments are already being offered by many clinics around the world. This has hurt both the local people as also those who travel long distances to avail them. It is also likely to harm the long-term development of this field by turning away public opinion when they realize that so many patients have been cheated by unproven therapies [60]. It should be recognized that such practices are primarily driven by the greed of unscrupulous scientists, physicians, and entrepreneurs often inadvertently supported by the actions of their academic counterparts. A concerted effort at controlling this menace through a multipronged approach can significantly reduce its harmful impact while promoting good translational research with stem cells to truly help those patients who have no options and very little hope at present.

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Chapter 20
Safety Issues in MSC Therapy

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Abstract Multipotent mesenchymal stromal cells (MSCs) have enormous therapeutic potential because of their unique biological properties related to proliferative potential, differentiation capability, lack of immunogenicity, immunosuppressive nature, homing ability, tissue repair, and cytokine production. However, the very properties that drive enthusiasm for MSCs as cellular therapy could potentially impact safety. Most troublesome is the risk of promoting malignancy demonstrated in animal models. Culture conditions (media, passage length), donor (autologous versus allogeneic), source (marrow, cord, placental, or adipose tissue), and therapy indication (transplant versus cardiovascular versus tissue regeneration) are additional variables that may potentially influence the safety profile.

Introduction

Multipotent mesenchymal stromal cells (MSCs) have many unique properties justifying their clinical exploitation in allogeneic hematopoietic stem cell transplants, autoimmune disease, inherited disorders, and regenerative medicine. Infusion of human MSCs has a long track record of safety; low doses of MSCs, comprising 0.1% of the content a marrow graft, have been given without *ex vivo* expansion for decades in the context of allogeneic bone marrow transplantation. The cells are easy to isolate, expand *ex vivo*, and infuse without immediate toxicity. However, the very features that make MSCs therapeutically useful such as proliferative potential, migratory capacity, cytokine/chemokine production, paracrine stimulation, and

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_20,

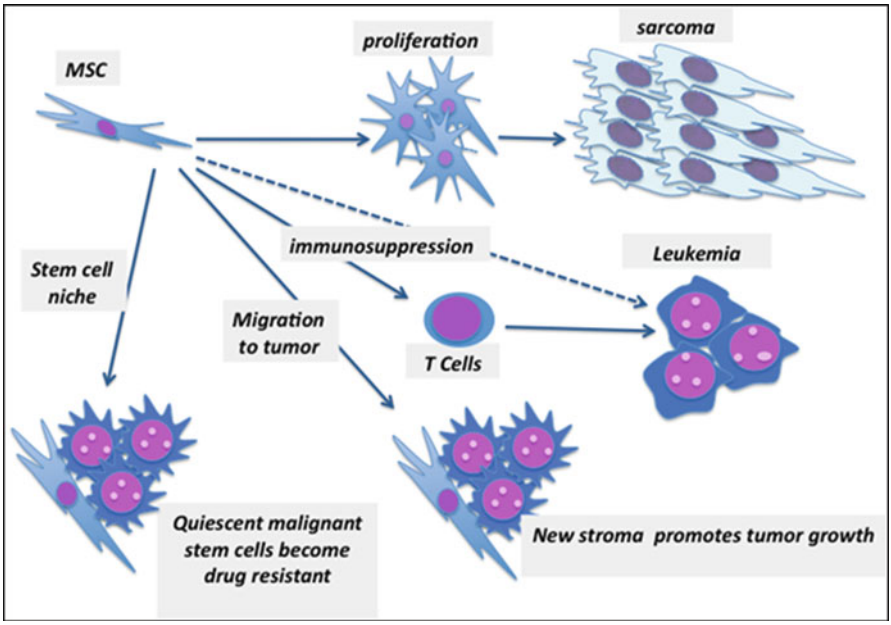

Fig. 20.1 Potential of MSCs to promote malignancy

immune-evasion have aroused concern over the potential for harm. The broad areas for most concern are the risk of tumorigenesis, *ex vivo* cell culture issues and the heterogeneity of the cell sources, and conditions being treated (Fig. 20.1).

Replicative Senescence and the Risk of Intrinsic Transformation

Immortality is the critical characteristic of a normal stem cell and malignant tumor cell, alike. Since MSCs are stem-like progenitors, intrinsic transformation to malignancy has been a concern and has been examined in vitro, in animal models and in the clinical setting of human bone marrow transplantation.

Exuberant proliferation in the process of *ex vivo* expansion subjects MSCs to considerable replicative stress. Cultured MSCs derived from several laboratory mouse strains have been shown to frequently develop the recurrent cytogenetic abnormalities typically seen in sarcomas. The infusion of cultured murine MSCs may lead to spontaneous sarcoma formation. Cytogenetic clonal evolution in cultured murine MSCs also occurs in vivo after infusion, and the sarcoma can be propagated by second transplants [1].

While murine experiments have shown clear evidence of tumorigenic potential, this has yet to be encountered with human MSCs. One reason is that, in contrast to the cytogenetic instability of murine MSCs with prolonged in vitro passage, human MSCs enter replicative senescence after serial culture. Cultured human

marrow MSCs became senescent after 20 passages and 120–140 population doublings [2]. MSC senescence is accompanied by a decline in plasticity as well as in proliferation rate, thereby stopping the risk of transformation [3, 4]. The probability of cultured human marrow MSCs emerging from senescence has been quantified as being less than 10^{-9} [2, 5]. Escape from senescence with human MSC cultures has been described but subsequently disputed by laboratories with the greatest experience [6].

Risk of Promotion of Non-hematologic Malignancies

Immunodeficient murine models are often used to study stroma-tumor interactions to exclude influence from the immune system, which may be suppressed by the MSCs. Such models have demonstrated the potential of MSCs to promote or to inhibit malignancy. Homing to sites of tissue injury to suppress inflammation and to induce repair is a physiological role for MSCs. Tumors provide a similar inflammatory environment described in wound healing and provide a site for preferential MSC migration. Bone marrow derived MSCs have chemotactic capabilities similar to immune cells and are capable of selective homing to integrate into tumor stroma [7, 8]. Tumor stromal elements have been shown to be of marrow origin [9, 10]. Indeed, engineered MSCs have been utilized as a homing platform for delivery of anticancer therapy [11]. After incorporation into the tumor stroma, MSCs could potentially provide structural support, vasculature, extracellular matrix, and nourishment to the tumor while suppressing immune responses. Despite the tropism of MSCs for tumors, the net effect of MSCs on tumors is based on the relative balance of stimulatory versus inhibitory factors.

Djouad et al. have shown that the immunosuppressive consequences of MSC infusions aid tumor growth in allogeneic animals [12]. Yu et al. have shown that human adipose tissue-derived MSCs promote tumor growth in a nude mouse xenotransplant model, indicating that additional mechanisms independent of immunosuppression are involved [13]. The contextual signals from MSCs that migrate to tumor stroma are being discovered. MSCs produce therapeutic angiogenesis in chronic limb ischemia models by secretion of proangiogenic factors (such as VEGF, PDGF, FGF) [14–16]. Tumor stromal-cell interactions enhance the invasiveness of human uterine cervical carcinoma cells by augmenting the expression and activation of matrix metalloproteinases [17]. Bone marrow-derived MSCs greatly increased metastasis from a co-injected human breast cancer cells in a xenograft model. A reversible paracrine feedback loop was generated with breast cancer cells inducing CCL5 (RANTES) release from MSCs and in turn enhancing motility, invasion, and metastasis of the breast cancer cells [18].

On the other hand, several experiments have shown the potential of MSCs to inhibit tumors. Maestroni et al. described inhibition of tumor growth and metastasis of Lewis lung carcinoma, and B16 melanoma lines upon co-injection with marrow-derived MSCs in syngeneic mice [19]. MSCs also inhibited the outgrowth of rat

colon carcinoma upon subcutaneous co-transplantation in a gelatin matrix [20]. MSCs also impaired growth in a rat glioma model [21]. Co-injected human MSCs inhibit Kaposi Sarcoma, a highly inflammatory tumor, in athymic nude mice by a contact-dependent inhibition of Akt activity [22].

Ultimately, the interaction of MSCs with tumors may have several consequences that may be deleterious or beneficial based on their context, requiring the careful consideration of their use in the setting of malignancy [23]. Several variables are capable of influencing the outcome of tumor-MSC interactions: the nature of the primary tumor, MSC source, propagation and dosing, and the diversity of the in vivo models. To further confuse the issue, paradoxical effects with changes in MSC dose have been reported. In a syngeneic murine model utilizing Renca adenocarcinoma and B16 melanoma, the co-injection of MSC doses greater than the cancer promote tumor formation, while lower doses mediate rejection [24].

Safety may be enhanced in the setting of malignancy by the introduction of a suicide gene such as the inducible caspase 9 (iCasp9) system that is activated by specific chemical inducers of dimerization (CID). Transduced MSCs preserve their surface phenotype and differentiation potential. iCasp9 engineered MSCs and their differentiated progeny can be selectively eliminated within 24 h by the addition of CID, thereby providing an additional level of safety for high-risk clinical applications [25].

In summary, the risk of tumorigenesis remains the most controversial safety aspect of MSC therapy. Clearly, avoidance of MSCs in patients with active malignancy is desirable outside the context of carefully designed clinical trials.

Risk of Promotion of Hematologic Malignancies

MSCs have a proven physiological role in forming the hematopoietic stem cell (HSC) niche that supports normal HSCs as well as leukemic stem cells [26, 27]. Supraphysiological doses of MSCs have been used in clinical trials in the setting of allogeneic stem cell transplant because preliminary evidence suggests that they favor HSC engraftment [28] and inhibit graft-versus-host disease [29, 30]. Infused MSCs are capable of homing to the marrow and survive for more than a year, at least in large animal models [31]. Since allogeneic stem cell transplant is usually indicated for hematologic neoplasms and MSCs provide a niche for normal and malignant stem cells, the impact of MSCs on hematologic malignancy deserves attention. The marrow microenvironment, which includes MSCs, is a proven sanctuary for hematological neoplasm, contributing to survival of minimal residual disease and drug resistance [32]. In vitro, MSCs support proliferation of hematopoietic malignancy, and in vivo enhance malignant proliferation, i.e., in NOD-SCID mice. This lends support to the possibility that MSCs in vivo may contribute to a leukemia stem cell niche [33]. Roorda et al. have shown promotion of tumor growth in a Daudi lymphoma xenograft model that is dependent on cell contact and, to a lesser

extent, on soluble factors. Signal transduction pathways were activated in the malignant cells [34]. Similar effects have been shown for CML [33].

In the right setting by optimizing culture and passage conditions or by engineering, MSCs may be able to serve as therapeutic platforms for therapies that target the marrow. Lentiviral vectors efficiently transduce MSCs, and efforts have been made to target Raji Burkitt Leukemia/Lymphoma [35] and myeloma [36].

A frequent use of MSCs has been in the setting of allogeneic stem cell transplantation, to improve engraftment, to control or treat GVHD, and to repair organ injury. Several small phase II trials have been reported with clinical response endpoints that do not provide a holistic view of safety. The two large randomized control trials that have been conducted for acute steroid refractory GVHD and for first-line therapy of GVHD did not meet their primary endpoints.

The theoretical risk of promoting malignancy in the allogeneic stem cell transplant setting was reported by Ning et al. In a small randomized controlled trial of MSC co-transplantation in HLA-identical sibling transplantation for hematologic malignancies, a higher relapse rate (60% versus 20%) was seen in the patients who received MSCs. Although the incidence of GVHD was reduced, disease-free survival at 3 years was only 30% in those patients receiving MSCs versus 66.7% in those who did not receive MSCs [37]. The conclusions have been criticized for paucity of information regarding relapse likelihood in the two groups and the variable nature of the graft source being either marrow, peripheral blood, or a mixture [38].

Cell Culture Risks

European and US regulatory agencies have issued guidelines for preparation of cellular products with regard to identity of the cell type, purity, and potency [39, 40]. With adherence to guidelines, there are only minor safety considerations which pertain to the MSC manufacturing process.

Traditional techniques of *ex vivo* MSC expansion/culture have relied upon the presence of fetal bovine serum (FBS), a xenogeneic protein. This has raised concerns about the transmission of BSE, other infectious complications, and host immune reactions. Even immunosuppressed patients can generate antibodies against FBS which bind to MSCs but no alloantibodies; however, these antibodies against FBS have not been shown to be clinically significant [41].

Awareness of potential risks with FBS has fueled investigation into alternative culture supplements. Since PDGF is a critical growth factor for MSCs, newer culture techniques have replaced FBS with platelet lysate [42] or allogeneic human serum [43], which then will involve risks related to blood component therapy.

Apart from the standard infectious agents that are routinely screened, there is an additional concern about the possibility of persistence of human parvovirus B19. MSCs express the erythrocyte P antigen, the B19 receptor. One in 20 healthy donors was found to have MSCs harboring the virus responsible for erythema infectiosum

and transient aplastic crises [44]. Immunocompromised recipients with low immunoglobulin levels are potentially susceptible.

Far more relevant than the specifics of culture technique is the final biological property because the critical safety issues reside in the inherent nature of the *ex vivo* expanded MSC product. MSC products vary significantly in their biological potential. Human MSC properties are greatly influenced by the extent of *ex vivo* expansion [45]. Late passage cells have reduced residual in vivo expansion potential, which translates into a shorter life span after infusion. For instance, the utilization of late passage MSCs in the Prochymal phase III trials for graft-versus-host disease could have been the cause for failure to meet primary endpoints. In EU trials for patients with GVHD, 1-year survival was 75% in patients who received early-passage MSCs in contrast to 21% using later passage MSCs ($P < .01$) [46]. Heterogeneity in origin (autologous versus allogeneic), tissue source (marrow versus adipose versus cord blood), and growth media (serum versus serum free) also impact the biological attributes of MSCs. Certainly, different characteristics are desirable for different indications, and there is no need to have a single manufacturing process. Nevertheless, there is an urgent need for standardized potency assays for MSCs which examine multiple attributes such as proliferative potential, differentiation ability, immunomodulatory nature, cytokine secretion, and surface immunophenotype to define the characteristics of each MSC product used in clinical trials.

Safety Aspects for Individual Indications

Given the powerful biological properties of MSCs, unanticipated safety implications may arise in specialized settings. For instance, in cardiology investigations, concerns have been raised about increase in artherosclerotic lesion size [47] and proarrhythmic potential [48]. Larger clinical trials in acute myocardial infarction with 6-month follow-up have not uncovered safety issues [49]. Donor source may be important with allogeneic MSCs having greater potential for risk than autologous MSCs for the purpose of tissue regeneration.

In the allogeneic transplant setting, the biggest risk is one of inhibiting disease control by the immunosuppressive effect of MSCs [37]. However, relapse risk is less relevant for a patient with severe GVHD because the patient has already derived the full benefit of GVHD as protection against relapse. For similar reasons, mismatched transplants having a more powerful antimalignancy effect but with a higher risk of GVHD could benefit from MSC infusion. For other post-transplant indications where MSCs are being investigated, such as marrow failure or organ injury, the risk of relapse needs to be balanced against the severity of the indication.

There is widespread interest in using MSCs as carriers of therapeutics/genes to home to sites of inflammation or malignancy to deliver a therapeutic benefit. Studies involving gene modification impose additional considerations for safety.

Clinical Trial Design with Emphasis on Safety Outcomes

Clinical trials for MSCs have mostly been uncontrolled, small exploratory studies with considerable heterogeneity in MSC source, manufacture, and dosing with short follow-up [50]. It is cautionary to note that the only two large randomized control trials of an MSC product (Prochymal®, Osiris therapeutics) for indications of new onset GVHD and steroid-refractory GVHD failed to meet their primary endpoint despite encouraging responses. It is frustrating that after several years of clinical trials in the field of transplantation, it is impossible to know for certain if MSCs help or induce harm. Also, given the potential impact of MSCs on both GVHD as well as relapse of malignancy in allogeneic transplant, the assessment of GVHD alone as a single endpoint for MSC trials is potentially misleading without a thorough analysis of competing risks. An analysis of descriptions of current phase III trials in MSCs from Clinicaltrials.gov (accessed on Nov, 2010) shows that long-term safety is not being comprehensively addressed (Table 20.1).

Proposed guidelines to enhance information from clinical trials with MSCs:

1. Standardize the MSC origin, manufacturing process, dose, and administration for all patients on a single study.
2. Establish standardized potency assays for MSC products. Establish and encourage standards for reporting of MSC characteristics (such as culture conditions, immunophenotype, passage, and population doublings) in publication.
3. Full safety, especially in the setting of malignancy, cannot be fully evaluated without randomized clinical trials with long-term follow-up and a comprehensive reporting of clinical endpoints. In the setting of allogeneic hematopoietic stem cell transplantation, multivariable modeling using competing risks for relapse, graft failure, and nonrelapse mortality is necessary.
4. A control arm should be used whenever possible. If a historical control is unavoidable, then it must be well defined.

Conclusions

MSCs have enormous therapeutic potential, but their unique biological attributes may cause harm in certain settings. The greatest known risk is one of enhancing a malignant condition. Unanticipated risks may arise which are unique for specific settings where MSCs are infused. Consideration should be given to suicide gene incorporation or selecting late passage cells with reduced survival, for indications where harm is possible. Efforts must be made for a GMP-grade standardized manufacture process, particularly for multicenter trials. There is an urgent need for standardized potency assays. Careful clinical trials, preferably with a randomized control arm, adequate duration of follow-up with multiple endpoints are required, and a thorough analysis of competing risks (where applicable) is necessary. Clinical trial data should include specifics on the method of generation and the biological attributes of the MSCs used.

Table 20.1 Safety analysis of ongoing/completed phase III MSC trials from clinicaltrials.gov

No.	Indication	MSC source	Culture conditions	Country	Sponsor	*N*	Primary outcome measure	Secondary outcome measure	Study duration
1.	Diabetic foot	Autologous marrow	Plastic adherence, autologous serum, 3 week culture	China	Third Military Medical University	80	Not listed	Not listed	180 days
2.	Type 1 DM onset	Autologous marrow	Plastic adherence, autologous serum, 3 week culture	China	Third Military Medical University	80	C-peptide release	Not listed	24 months
3.	Articular cartilage defects	Autologous marrow	Plastic adherence, 3 week culture	Egypt	Cairo University	25	Clinical/radiological joint scores	Not listed	12 months
4.	Newly diagnosed acute GVHD	Third party allogeneic marrow	Prochymal ®: Prolonged passage	USA, Australia, Canada	Osiris Therapeutics	190	Clinical response at 90 days	Clinical efficacy measures	12 months
5.	Steroid refractory acute GVHD	Third party allogeneic marrow	Prochymal ®: Prolonged passage	USA, Australia, Canada	Osiris Therapeutics	240	Clinical response at d100	Clinical efficacy measures	180 days
6.	Treatment-resistant moderate-to-severe Crohn's disease	Third party allogeneic marrow	Prochymal ®: Prolonged passage	USA/Canada	Osiris Therapeutics	270	Disease remission at 28 days	Clinical improvement/ QOL at 28 days	28 days
7.	Treatment-resistant moderate-to-severe Crohn's disease	Third party allogeneic marrow	Prochymal ®: Prolonged passage	USA/Canada	Osiris Therapeutics	120	Disease remission at 6 months	Clinical improvement/ QOL/SAEs at 6 months	180 days
8.	Knee articular cartilage injury or defect	Allogeneic umbilical cord blood	Cartistem ®: Cultured, mixed with semisolid polymer	Korea	Medipost Co Ltd.	104	Cartilage Repair at 48 weeks	Various clinical efficacy measures at 48 weeks	48 weeks
9.	Ischemic heart failure	Autologous marrow	C-Cure®: Culture expanded and differentiated	Belgium/Serbia	Cardio3 BioSciences	240	Change in LVEF at 6 months	Standard cardiovascular endpoints at 6 months	180 days

Measures of safety assessed are indication-specific and based on public information listed on clinicaltrials.gov in Nov, '2010. Results have not been published on any of these trials. Safety has concentrated on acute infusional toxicity. Follow-up is uniformly too short, even on registrational phase III trials, to conclusively determine long-term safety for MSC therapy. Trials #1–3 and 8–9 had no safety outcomes listed. Trials 4 and 5 in allogeneic hematopoietic stem cell transplant have inadequate follow-up duration for evaluation of risk of malignancy relapse. Trials 6 and 7 for Crohn's disease have extension phases which are more likely to answer long-term safety concerns

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Chapter 21
In Vivo Imaging of MSCs

Jeff W.M. Bulte

Abstract Clinical applications of mesenchymal stromal/stem cells (MSCs) to treat a myriad of diseases are currently being initiated. For MSC therapy to become common practice, basic questions about the fate of MSCs once administered need to be addressed. These include the biodistribution, survival, and differentiation of MSCs. In this chapter, several imaging techniques are described that can be used to interrogate these questions in a clinically applicable manner, and several examples are discussed. Single-photon emission computed tomography (SPECT)/radioisotope imaging and magnetic resonance imaging (MRI) cell-tracking techniques are the two modalities that likely will become mainstays in clinical practice.

Abbreviations

BLI	Bioluminescent imaging
FDA	Food and Drug Administration
FHBG	9-(4-[18F] Fluoro-3-hydroxymethylbutyl) guanine
FIAU	1-(2-deoxy-2-fluoro-1-D-arabinofuranosyl)-5-iodouracil
GFP	Green fluorescent protein
HSV-tk	Herpes simplex virus thymidine kinase
MI	Myocardial infarct
MSC	Mesenchymal stem cell
MRI	Magnetic resonance imaging

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_21,

PET	Positron emission tomography
SPECT	Single-photon emission computed tomography
SPIO	Superparamagnetic iron oxide

Introduction

Cell therapy with MSCs now pursued clinically for a variety of ailments, has generated considerable excitement in the field as to where this will eventually bring us. There is no question that non-invasive in vivo imaging will play a critical role in future clinical implantation of MSCs beyond initial phase I/II clinical trials. The overall and ultimate goal of non-invasive in vivo imaging is to be able to deliver and track MSCs administered to patients. Major clinical questions need to be addressed in order to ensure that cell therapy can be performed successfully in a more mainstream setting beyond the few academic centers where it currently takes place. Ideally, noninvasive stem cell tracking should address the following key issues outlined in Table 21.1: (1) Are the MSCs delivered/injected at the right place, in particular for small sites located deep within parenchymal tissue? (2) How many MSCs have been successfully delivered at the target site, or how many MSCs homed successfully to the target site when injected remotely/systemically? (3) How long and how many MSCs survive? (4) Do undifferentiated MSCs keep proliferating following administration and form neoplasms? (This is extremely unlikely and has only once been reported to occur for sarcoma, see section "Optical Imaging.") (5) Do MSCs remain undifferentiated, or do they follow downstream lineages and if so, when do MSCs differentiate and into what cell type? (6) What are the morphological, physiological, or functional changes of the host tissue indicative of therapeutic success or failure following MSC administration? At the present time, there is no single imaging technique that can address all of these questions. In the following sections, representative studies for each separate imaging technique will be discussed.

Optical Imaging

Optical imaging is a broadly defined term and encompasses all imaging techniques that rely on capturing emitted photons. This includes the detection of fluorescent and near-infrared agents, as well as bioluminescence. For noninvasive imaging purposes, bioluminescence imaging (BLI) has now become one of the most widely used techniques for tracking stem cells. Unlike the other imaging techniques described here, it cannot be used clinically due to the limited penetration depth of photons in tissue. Even when used in mice, animals either need to be white or shaved. In black mice, their pigmented skin absorbs photons and decreases sensitivity.

BLI relies on transfecting cells with a bioluminescent reporter gene, that is, firefly luciferase, which was identified as early as the 1940s [1]. Unlike fluorescent

Table 21.1 Questions in a clinical setting that need to be answered using noninvasive imaging

Question	MRI	MRI/PET	PET	PET/CT	SPECT	SPECT/CT
Are MSCs injected correctly during procedure?	X	X	–	–	–	–
Number of MSCs homing to target site?	–	–	–	–	X	X
How long do MSCs survive?	–	X	X	X	–	–
When and what do MSCs differentiate into?	–	X	X	X	–	–
Do MSCs proliferate and form tumors?	X	X	X	X	–	–
Does host tissue show therapeutic effect?	X	X	X	X	X	X

Indicated with X are the modalities that have the ability to answer these questions. Note that, due to its inability to perform whole-body imaging, optical imaging is not included. The column listed for SPECT also includes other nuclear imaging modalities that use radioisotopes

proteins, luciferases are enzymes that require the injection of a substrate for light generation, which is D-luciferin for the firefly luciferase. The peak emission wavelength is about 560 nm. Due to the attenuation of blue-green light in tissues, the red shift (compared to the other systems) of this emission makes detection of firefly luciferase much more sensitive in vivo compared to conventional fluorescent proteins. Wild-type firefly luciferase is a 62-kDa monomer, which, when expressed in mammalian cells, is targeted to peroxisomes [2] and has a short half-life due to thermal instability [3]. A mutated firefly luciferase with increased expression in mammalian cells, better thermal stability, and no peroxisomal signal has been generated [2, 4]. In addition, similar to work on GFP, genetic engineering has provided red- and green-emitting firefly luciferases [5].

Renilla luciferase (from the sea pansy) requires its substrate, coelenterazine, to be injected as well. As opposed to luciferin, coelenterazine has a lower bioavailability (likely due to MDR1 transporting it out of mammalian cells). Additionally, the peak emission wavelength is about 480 nm. Red and green luciferases from the click beetle (*Pyrophorus plagiopthalamus*), as well as a green Renilla luciferase, are being tested in mice [6]. This study has shown that firefly luciferase was comparable to click beetle red luciferase and that both outperformed Renilla and click beetle green luciferases with respect to tissue penetration. It also clearly underscored the advantages of imaging at wavelengths above 600 nm.

A third type of luciferase, bacterial luciferase, has an advantage in that the lux operon used to express it also encodes the enzymes required for substrate biosynthesis. This system has not yet been adapted for mammalian cell expression but is widely used for developing bioluminescent pathogens. This luciferase reaction has a peak wavelength of about 490 nm.

Currently, one has the option of using fluorescence or bioluminescence or a combination of both for optical tracking studies of MSCs in vivo. In addition, dual and triple reporter vectors that allow for multimodality imaging, for example, positron emission tomography (PET, see below), fluorescence, and bioluminescence, are in use. Fluorescent protein-based optical tracking of cells in mice has the advantage of not requiring a substrate. The need to administer substrates or targeting

molecules complicates the imaging process in that the biodistribution and pharmacokinetics of such agents must be considered, and thus, doses, as well as dose timing, need to be optimized [7]. If these reagents do not reach the cells to be imaged or are too diluted at the target cell, false negatives occur. In addition, post-mortem fluorescence microscopy facilitates fluorescent cell location and eliminates the need for staining cell-specific markers that might have been lost during, for example, the course of metastatic transformation. On the other hand, the optical properties of tissue have limited most GFP-based imaging to superficial lesions. Firefly-based bioluminescence penetrates mammalian tissue to a greater depth than GFP fluorescence [8, 9]. In addition, unlike GFP, where background auto-fluorescence is a problem, there is no bioluminescent background [9], which is also minimized with fluorescence imaging at wavelengths above 600 nm. For optical imaging to be a comprehensive photon-based imaging modality, tomographic reconstructions will be needed. The development of such reconstruction technologies has been pursued in various laboratories [10, 11] and is in progress.

Kidd et al. transfected MSCs with luciferase in order to determine under which conditions they selectively engraft in sites of inflammation [12]. The inflammatory insult model included cutaneous needle-stick and surgical incision wounds, as well as xenogeneic and syngeneic tumors. It was shown that in normal animals, MSCs initially reside in the lungs, then egress to the liver and spleen, and exhibit decreased signal over time. In wounded mice, however, human MSCs engraft and remain detectable only at sites of injury. Similarly, in this study using syngeneic and xenogeneic breast carcinoma-bearing mice, bioluminescent detection of systemically delivered MSC revealed persistent, specific co-localization with sites of tumor development (Fig. 21.1). This pattern of tropism was also observed in an ovarian tumor model in which MSC were intraperitoneally injected. Thus, using BLI, the investigators were able to identify conditions under which MSC tropism and selective engraftment in sites of inflammation can occur [12].

In another study, human pancreatic carcinoma cells, PANC-1, expressing Renilla luciferase were orthotopically implanted into immunodeficient mice and allowed to develop for 10 days [13]. Firefly luciferase-transduced MSCs were then injected intraperitoneally weekly for 3 weeks. Mice were monitored by BLI for expression of Renilla (PANC-1) and firefly (MSC) luciferase. It was demonstrated that MSC can selectively home to sites of primary and metastatic pancreatic tumors and can inhibit tumor growth when acting as delivery vehicles for interferon-beta for the treatment of pancreatic cancer. Tropism of MSCs for other forms of cancer has been demonstrated, that is, gliomas, using BLI to assess their overall biodistribution pattern [14]. By incorporating the thymidine kinase suicide gene into MSCs, it was further shown that tumors could be suppressed following administration of ganciclovir once the MSCs had successfully localized to the tumors.

BLI can also be used to probe the survival of MSCs and to compare outcomes to other cell types, for example, as shown for a murine myocardial infarct model [15]. In vivo BLI revealed acute donor cell death of MSCs within 3 weeks after transplantation, which did not occur in bone marrow mononuclear cells where BLI signals were

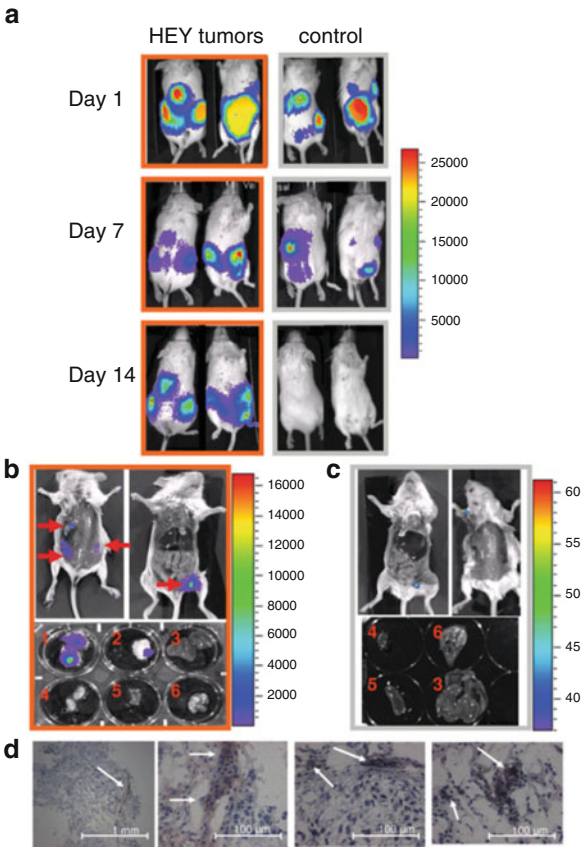

Fig. 21.1 MSC tropism for HEY ovarian carcinoma. SCID mice were i.p. injected with HEY cells ($n=3$, *orange outlined boxes*) or phosphate-buffered saline ($n=3$, *gray outlined boxes*). 15 days later, luc-hMSCs were i.p. injected into tumor-bearing and control mice (*day 1*). (**a**) Images were acquired at days 1, 7, and 14, indicating initial dissemination throughout the peritoneal cavity, followed by specific localization in tumor-bearing animals and disappearance in control animals. On day 14, the mice were sacrificed and bioluminescent activity was localized to sites of visible tumor development in the open cavities and dissected organs—(*1*) ventral tumor, (*2*) dorsal tumor, (*3*) liver, (*4*) kidney, (*5*) spleen, and (*6*) heart and lungs—of HEY-bearing mice (**b**) but not control mice (**c**). (**d**) Immunohistochemistry for Luc on tumor sections from the HEY-bearing mice confirmed the presence of hMSC (magnification as indicated) (Reproduced, with permission, from Ref. [12])

still present after 6 weeks in the mononuclear cell group. This translated into more robust preservation of cardiac function for mononuclear cells compared to MSCs.

To study the biodistribution of allogeneic MSCs in vivo in irradiated mice, cells were transfected with firefly luciferase and DsRed2 fluorescent protein [16]. BLI signals were shown to be increased between weeks 3 and 12. Interestingly, some

mice with the highest luciferase signals died, and all surviving mice developed foci of sarcoma in their lungs. Further studies revealed that primary MSCs derived from the bone marrow of both BALB/c and C57BL/6 mice showed cytogenetic aberrations after several passages in vitro; in these studies, sarcomas were found to evolve from MSC cultures. It is not generally believed that multipotent adult stem cells such as MSCs can develop tumors, but the use of BLI here enabled researchers to prove that this is not always the case in murine models.

SPECT and Nuclear Imaging

Radiotracer labeling of cells is the oldest technique for tracking cells. The use of ^{111}In-oxine labeling of autologous white blood cells for use as diagnostic agent for imaging of inflammation and infection is, since the mid-1980s, the only Food and Drug Administration (FDA)-approved cell-tracking technique as of today. Radiotracer labeling is not completely without toxicity, and some say that it would not have been approved today with the FDA's current more stringent approval processes. Labeling cells with ^{111}In-oxine is straightforward, with the lipophilic complex readily diffusing through the lipid bilayer of the cell membrane followed by complexation to intracellular macromolecules. Some studies have used ^{111}In-tropolone instead [17, 18].

Nuclear imaging using radioisotopes is particularly useful for whole body distribution studies, as there is no background signal. It has therefore been frequently used with labeled cells administered systemically, for example, intraperitoneally, intravenously, or intra-arterially [19–21]. Most MSC imaging studies have focused on systemically administered MSCs to treat myocardial infarction [17, 20, 21]. In the early stages (the first 24 h), MSCs are nonspecifically trapped in the lungs, with subsequent redistribution in the next 24 h toward the liver and spleen. In a canine myocardial model, it was shown that MSCs arrive in the infarcted area as soon as 48 h (Fig. 21.2) [21]. As there is no background signal, and the gamma emission is not hindered by deeper tissues, it is possible to quantify the amount of radiolabel (corrected for half-life, which is 2.8 days for ^{111}Indium) and hence the amount of cells. For a very precise determination, organs can be excised, and tissue radioactive counts performed using a liquid scintillation counter. In the study by Kraitchman et al. [21], it was calculated that of the approximate 1×10^8 cells injected, about 8×10^4 cells homed to the area of the MI at day 7 postinjection (day 10 post induction of myocardial infarction). Other studies used technetium 99m and showed a similar whole body distribution [22].

An alternative, relatively new way of labeling MSCs is with the sodium iodide symporter (NIS), in which the transfected end encodes a protein that entraps the radiolabel following administration of (99m) Tc-pertechnetate [23]. As both indium and technetium are already clinically used, it is likely that imaging of radiolabeled cells will become one of the primary imaging techniques in humans.

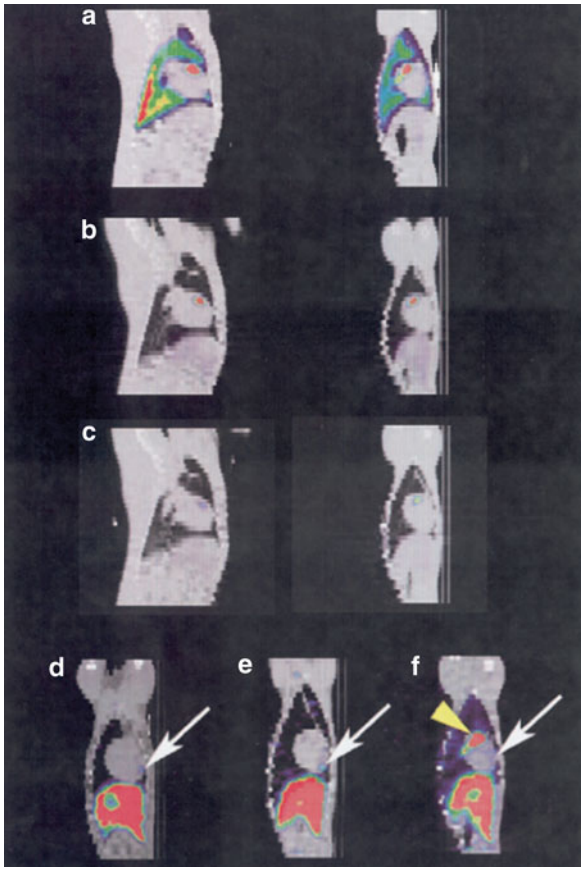

Fig. 21.2 Sagittal (*left*) and coronal (*right*) view of fused SPECT/CT images on days 1 (**a**), 2 (**b**), and 7 (**c**) in an animal that demonstrated focal uptake in the anterior midventricular region of the heart. (**d–f**) At the last imaging time point (days 5–8), an anterior apical region of MSC uptake (*arrow*) is shown in three representative animals in the coronal view. This more anterior apical distribution was present independent of whether an early focal hot spot was observed (*yellow arrowhead*, **f** only) (Reproduced, with permission, from Ref. [21])

PET Imaging

Reporter genes entered the arena of nuclear medicine in the early 1990s with the development of the thymidine kinase (tk) enzyme derived from the herpes simplex virus (HSV) [24]. Upon administering positron-emitting substrates, such as ^{18}FIAU or ^{18}FHBG, the phosphorylating enzyme is responsible for prolonged retention of the radioactive probe in transfected cells. It has now been widely used for imaging of stem cell [25] and T cell [26, 27] trafficking, and also recently for MSCs [28, 29]. In the study of Willmann et al. [29], HSV-tk-transfected MSCs were injected in the ventricular wall of swine (Fig. 21.3). It was shown that when they were co-implanted with a matrigel scaffold, retention of the injected PET substrate ^{18}FHBG was significantly increased compared to injection of cells only.

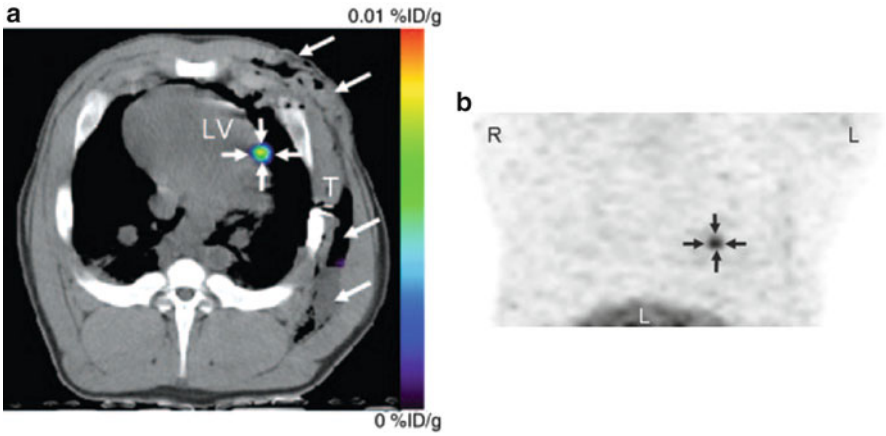

Fig. 21.3 PET-CT imaging of intramyocardial reporter gene expression in a large animal model (swine). (**a**) Transverse non-enhanced PET-CT fusion image reconstructed at the level of the left ventricle (*LV*) after direct open-chest administration of transduced human MSCs. The image was acquired 4 h after intravenous FHBG administration. A distinct imaging signal (*small arrows*) can be delineated at the intramyocardial injection site of human MSCs. *T* = beveled part of chest tube. (**b**) Coronal reconstruction of PET data set of the thorax and cranial upper abdomen in the same animal demonstrates high FHBG uptake at the level of intramyocardial injection site (*arrows*), with low background signal intensity in all other intrathoracic anatomic structures. Note high tracer accumulation in the liver (*white L*) due to biliary FHBG excretion. *R* right proximal foreleg, *L* left proximal foreleg (Reproduced, with permission, from Ref. [29])

Single-photon emission computed tomography (SPECT) can also be used with the HSV-tk reporter when using (^{131}I)-FIAU [30]. Alternatively, the sodium iodide symporter gene has been employed to trap ^{131}I and ^{99}Tc, also allowing visualization by SPECT imaging [31]. Currently, several variants of suitable reporter genes are being developed for nuclear medicine applications. There are some recent reports indicating that the immunogenicity of HSV-tk may be an issue for clinical translation; however, efforts toward using the human variant of HSV-tk are underway. A recent clinical HSV-tk cellular imaging trial using cytotoxic T cells [32] is encouraging for the further clinical translation of PET imaging of MSCs.

MR Imaging

MRI cell tracking is an indirect technique for detecting cells based on loading cells with metals, in particular superparamagnetic iron oxides (SPIOs). These nanoparticles affect the magnetic field on a microscopic scale, which leads to dephasing of protons and a loss of proton signal on the MR images [33]. The first magnetic labeling report on MSCs appeared about a decade ago, using a SPIO preparation termed magnetodendrimers [34]. Since then, numerous studies have

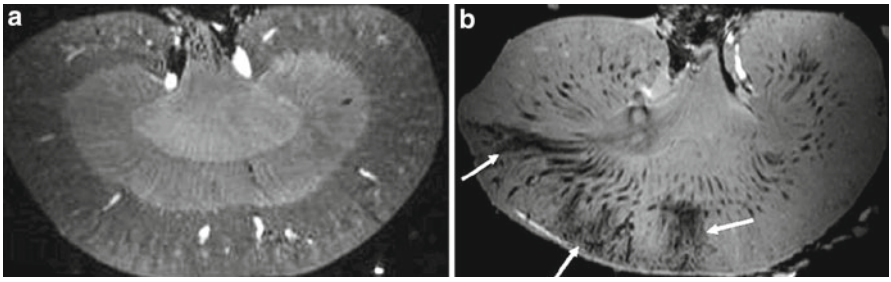

Fig. 21.4 *Ex vivo* sagittal three-dimensional T2*-weighted (110/15.7, flip angle of 30°) 9.4-T MR images of (**a**) control and (**b**) pathologic kidney 6 days after intravenous injection of 1×10^7 Feridex®-labeled MSCs (the upper pole is oriented left). In (**a**), no signal intensity decrease is noted. In (**b**), the cortico-medullary differentiation is absent and distinct areas of cortical signal intensity decrease are present in the superior and superior midportion (*arrows*) poles (Reproduced, with permission, from Ref. [41])

described SPIO-labeling of MSCs; although the use of transfection agents significantly enhances cellular uptake, it has been widely reported that MSCs are able to take up SPIO without further modification steps. SPIOs used for tracking purposes include Bang's particles [35], Resovist® [36, 37], and Feridex® [38–40]. For clinical translation, the lack of a second agent is an important consideration from a regulatory perspective, although the first MSC clinical MRI cell-tracking study was carried out using the addition of poly-L-lysine as a transfection agent [40]. Preclinical MRI-MSC-tracking studies have been carried out in animal models of nephropathies [41, 42], myocardial infarct [38, 43, 44], stroke [39], perinatal brain injury [45], experimental autoimmune encephalomyelitis (a mouse model for multiple sclerosis) [46], and brain tumors [47]. An example of the excellent high-resolution soft tissue contrast can be seen in Fig. 21.4 from the study by Hauger et al. [41].

As of the timing of this writing, seven clinical MRI cell-tracking trials have been published. The first four, from 2005 to 2008, have been reviewed elsewhere [48]. The latest three studies include the first MSC-tracking trial [40], carried out by Karussis et al. at Hadassah Hebrew University Hospital in Jerusalem in a collaborative effort with my lab at the Johns Hopkins University School of Medicine. In this study, the feasibility, safety, and immunological effects of intrathecal and intravenous administration of autologous MSCs were evaluated in patients with multiple sclerosis and amyotrophic lateral sclerosis. MSCs were labeled with Feridex® and poly-L-lysine, and MR images were obtained at 1–2 days and again at 1–3 months after injection. In nine patients, hypointense signals in T2-weighted images, indicating the presence of Feridex®-labeled cells, were detected in the meninges of the spinal cord and nerve roots and in the spinal cord parenchyma (Fig. 21.5).

All MRI clinical tracking trials published so far have been performed outside USA, perhaps as a result from the more rigorous review process for off-label use of

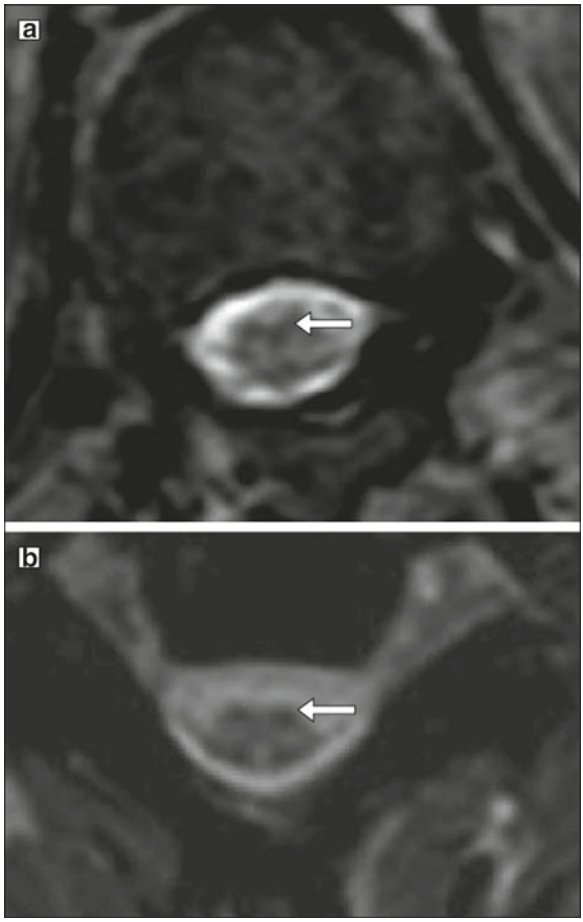

Fig. 21.5 MR imaging after injection of Feridex®-labeled MSCs. (**a**) Axial T2-weighted gradient echo scan through the inferior thoracic cord shows a hypointense pial signal coating the cord characteristic of (Feridex®)-labeled cells. (**b**) Axial T2-weighted gradient echo scan through the cervical cord shows hypointensity of the dorsal roots and their entry zone and a similar hypointensity of the ventral root entry zones, suggesting the presence of Feridex®-labeled MSCs (Reproduced, with permission, from Ref. [40])

clinical MRI contrast formulations by the local institutional review boards and the FDA. In six of these seven studies, the FDA-approved SPIO formulation Feridex® was used, sold in Europe under the name of Endorem™. As for safety concerns, the total dose of SPIO iron introduced into the human body is of the order of about 1 mg, or 0.025% of total body iron, calculated for a dose of 1×10^8 cells and 10 pg Fe/cell. SPIO does not affect viability or proliferation of MSCs in culture nor differentiation of labeled cells into adipocytes or osteocytes. Through an as yet still unexplained mechanism, several reports have described a SPIO-dose dependent inhibition of chondrogenesis and glycosaminoglycan production [37, 49–51]. However, this has not been considered a concern when performing cell therapy trials with undifferentiated MSCs outside of chondrocytic applications.

It is quite unfortunate that the Feridex®, Endorem®, and Resovist® SPIO formulations, originally developed as a liver contrast agent for in vivo labeling of

Kupffer cells and cancer staging, have been taken off the market. It is uncertain if similar clinical products will come back any time soon, but if it does, MRI cell tracking is likely to become an integral part of MSC therapy both for delivery and homing assessment purposes.

Conclusions

MSCs have been labeled and imaged with all currently available techniques that are applicable to cellular imaging. Clinically, SPECT, PET, and MR imaging are suitable imaging techniques that each have their strength and weaknesses. For systemically injected, whole body distribution studies, SPECT and PET are the techniques of choice, while for real-time image-guided parenchymal injections, MR imaging will be used. However, there is no reason why these techniques cannot be combined in the same clinical setting.

Acknowledgment The author is supported by grant MSCRFII-0161-00.

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Chapter 22
National Heart, Lung, and Blood Institute Support of Cellular Therapies Regenerative Medicine

Traci Heath Mondoro and John Thomas

Abstract Scientific advances have provided new and unprecedented opportunities for the therapeutic use of stem, progenitor, and differentiated cells for the future treatment of heart, lung, blood, and sleep disorders. Stem and progenitor cells have the potential to replace cells that are damaged or diseased, restore vital functions, and offer the promise of curing disease and ending disabilities. The potential for safe new treatments can only be realized if preclinical and clinical research programs provide the scientific and clinical basis to establish new therapies for regenerative medicine. NHLBI seeks to catalyze translational efforts in this area by supporting key efforts needed for the field's development. This chapter discusses NHLBI support for cellular therapies and illustrates this support with descriptions of two key programs, one for research centers and the other providing key resources.

Introduction

Scientific advances have provided new and unprecedented opportunities for the therapeutic use of stem, progenitor, and differentiated cells for the future treatment of heart, lung, blood, and sleep disorders. Stem and progenitor cells have the potential to replace cells that are damaged or diseased, restore vital functions, and offer

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_22,

the promise of curing disease and ending disabilities. The potential for safe new treatments can only be realized if preclinical and clinical research programs provide the scientific and clinical basis to establish new therapies for regenerative medicine.

Cell-based therapies may involve a variety of cell sources. Bone marrow, peripheral blood, and cord blood stem cells have been used to treat serious blood disorders, malignant disease, and inherited diseases. These therapeutic uses are based on the presence of hematopoietic stem cells in each of these cell sources with the ability to give rise to all blood cells. Putative cardiac stem cells have been identified in the human heart and ongoing research seeks to define their regenerative capacity. It has also hypothesized that lung progenitor cells exist in some locations, and further studies are being conducted to determine their role in lung development and regeneration.

Bone marrow and cord blood are also being used as a source of endothelial progenitor cells for exploratory treatments for cardiac, limb ischemia, and for the regeneration of the microvasculature and precapillary arterioles in experimental pulmonary arterial hypertension (PAH). Mesenchymal stem cells from bone marrow can differentiate into a variety of tissues including bone osteoblasts, chondrocytes in cartilage, and adipocytes in fat tissue. Mesenchymal stromal cell (MSC) preparations containing mesenchymal stem cells are being explored as a cellular intervention for the treatment of myocardial infarction. Interesting data also suggest that MSCs may promote hematopoietic stem cell engraftment and foster tolerance thus facilitating the transplant of cells or tissue from allogeneic donors.

NHLBI has a long-standing interest and investment in the study of stem cells. The institute has demonstrated its commitment by setting aside funds through institute-initiated programs as well as supporting investigator-initiated grants. This chapter will describe some of the NHLBI programs focused on cellular therapeutics and their origins.

NHLBI Specialized Centers for Cell-Based Therapy (SCCT)

Origins of SCCT

In August 2001, the National Heart, Lung, and Blood Institute (NHLBI) formed the cell-based therapy group in order to address stem cell and cell-based therapy issues, to track human embryonic stem cell applications and grants, and to formulate a strategic plan for the development of research programs leading to new cell-based therapeutics. The Cell Therapy Group formulated an institute-wide implementation plan for integrating basic and translational research programs to support the development of new cellular therapies formulated in part following special working groups devoted to stem cell research and cellular therapies.

The NHLBI Cell Therapy Group organized a working group of 24 investigators on May 2002 to define the scientific state-of-the-art regarding cell-based therapies, discuss the implications of that knowledge for research and medicine, and identify opportunities and obstacles to successfully exploit cell-based therapies for repairing or replacing damaged, diseased, or defective tissue with new, functional tissue. The working group's report and executive summary are available on the NHLBI Web site [1]. Key recommendations are listed in Table 22.1.

Following the completion of the working group report, NHLBI undertook a series of initiatives supporting stem cell research, including participation in the NIH Human Embryonic Stem Cell Research Resource Infrastructure Enhancement Award issued in December of 2003 (NOT-HL-04-102), release of the NHLBI Cellular and Molecular Imaging of Cardiovascular, Pulmonary, and Hematopoietic Systems announcement in August of 2003 (RFA-HL-04-003), and the announcement of NHLBI Specialized Centers for Cell-Based Therapy (SCCT) for Heart, Lung, and Blood Diseases on May 10, 2004 (RFA-HL-04-17, [2]). In addition, NHLBI continued its support of stem cell research using animal models by renewing its participation in the program announcement on Novel Approaches to Enhance Animal Stem Cell Research reissued on July 8, 2004 (PA-04-125). In January 2005, program announcements were released to support research on "Directed Stem Cell Differentiation for Cell-Based Therapies for Heart, Lung, and Blood, and Aging Diseases" using the NIH Exploratory/Developmental Research Grant Award (R21), Small Business Innovation Research (SBIR), and Small Business Technology Transfer (STTR) mechanisms (PA-05-043 & -044). A more recent working group convened on September 22–23, 2008 focused on Enhancing Translational Research and Early-Phase Trials for Cellular Therapy for Blood Diseases. An executive summary from this working group is available [3], and the challenges and recommendations identified by this group are summarized in Table 22.2. These working groups and NHLBI's strategic planning process have provided useful input into the efforts to convert stem cell research results into future cell therapies. While as listed above, NHLBI has had a number of efforts in these areas, the next section will focus on the establishment of the NHLBI's Specialized Centers for Cell-Based Therapy or SCCT program, its accomplishments to date, and future directions for cell therapy research.

The SCCT program utilized a NIH center mechanism and cooperative agreements to fund three centers and one coordinating center. Each center was tasked with conducting one or two basic research projects and each center was required to conduct two or more early-phase clinical studies involving cellular products. A six-person steering committee was formed consisting of the principal investigator (PI) from each center, the PI from the coordinating center, the NHLBI program officer, and an external NHLBI-appointed chairperson. The steering committee along with other center and NHLBI staff members held semiannual meetings to review ongoing basic research projects, to discuss the opening and accrual of clinical studies, and to review any new basic or clinical studies proposed by the program. The group

Table 22.1 NHLBI Working Group (2002): Cell-based therapies for regenerative and reparative medicine – vision, scope, and directions

Recommendation	Comments
Basic research on stem cell biology	Recommended a strong, continued emphasis on the support of basic stem cell research as the basis for future preclinical and clinical advances for new cell therapies
Definition of the stem cell niche both structurally and functionally	Recommended new projects on the influence of the stem cell niche or environment on the differentiation of the cells. It was felt that tools are available to structurally and functionally define the stem cell niche including stem cell-stromal cell interactions
Identification of regeneration mechanisms at both the cellular and tissue level	Recommended new studies of natural cellular or tissue mechanisms for the regeneration of cells, tissues, and organs involving resident stem, precursor, and differentiated cell types
Understanding the immunogenic response to cells intended for use as cell-based therapies	New research needs to be initiated on the immunogenic potential of stem and precursor cells. This will address the question of tolerance or the development of histocompatibility for engrafted allogeneic cells
The development of improved noninvasive imaging techniques to track cells in vivo	Unanimously indicated new and improved methods are needed to track cell fate in vivo for animal studies and for clinical research. Group felt advances in these techniques would greatly accelerate the pace of research
An original effort promoting lung stem cell research	Identified pulmonary stem and progenitor cell research as key areas in need of development. New areas recommended: (1) Studies to identify currently unidentified lung stem cells; (2) Research on factors that control growth and development of lung and lung vasculature; (3) Development of lung cell clonogenic assays; (4) Tools for lung functional Genomics, e.g., gene inactivation technology; (5) a lung genome project modeled after hematopoietic stem cell database; (6) a lung resource project to generate cDNA libraries from embryonic and adult lung cells; and (7) preclinical animal models and translational studies investigating stem cell repair of lung injury
New research on cardiomyogenic potential of stem cells	New area of interest due to its clear clinical relevance. To accelerate this area, group recommended preclinical research in key areas: (1) assessment of the cardiomyogenic differentiation potential of various stem cell sources, (2) determination of the extent and basis of physiologic improvement as the result of cell therapy, and (3) determination of the mechanism of improvement and heart repair whether due to incorporation of cardiomyocytes or due to alternate mechanisms

Table 22.2 NHLBI Working Group (2008): Enhancing translational research and early-phase trials for cellular therapy

Challenge	Recommendations
1. *Preclinical cellular therapy research funding*: Current grant mechanisms and existing standing study sections are not well suited to evaluate grant applications containing applied, preclinical studies. However, these studies are necessary for IND applications utilizing cellular therapies	Support preclinical studies, including scale-up and validation of cellular products for clinical trials, via grant mechanisms that utilize review criteria that do not require hypothesis-driven research Implement a separate preclinical mechanism, perhaps a planning grant application leading to a subsequent clinical trial (R34)
2. *Clinical cellular therapy research funding*: Current grant mechanisms do not fit early-phase cellular therapy trials, and existing study sections lack the expertise necessary to evaluate these applications	Support clinical studies by constituting a specialized review panel with expertise necessary to evaluate early-phase trial studies. Membership should include regulatory, statistical, cell manufacturing, and clinical expertise Introduce a funding mechanism similar to the National Cancer Institute's "Quick Trial" to reduce the time from submission and review of a grant application to funding Permit multiple funding sources for the same trial by facilitating both private-public partnerships with Foundations and public-public partnerships with either State or other Federal funding agencies
3. *Timelines*: Timelines for funding are too long. The review and approval process for clinical trial protocols is cumbersome and often duplicative. For example, protocols are sequentially reviewed by an NHLBI-appointed Protocol Review Committee (PRC) and a Data and Safety Monitoring Board (DSMB). In addition, protocols also undergo several, additional reviews, such as institutional scientific reviews, NIH's Recombinant DNA Advisory Committee (RAC) for trials including gene therapy, the FDA, and an institutional review board	Eliminate the sequential reviews by the NHLBI-appointed PRC and DSMB by having ad hoc content-specific experts provide their scientific review to the DSMB when the protocol is reviewed by the DSMB. Combining the PRC review with the DSMB review would shorten the review process by eliminating the requirement for a separate PRC meeting
4. *Investigator recognition*: Key cellular therapy team contributors are excluded from recognition as needed for promotion. This includes cell processing scientists and mid-level clinical investigators	Establish a new "R" award for clinical investigators that provides protected-time and recognition of their contribution Publicize the importance of "Team Science" contributions to facilitate a change in academic promotions policies
5. *Contract delays*: Contract preparation and execution is frequently the rate-limiting step for the formation of academic-industry partnerships necessary for clinical trial initiation	Encourage leaders from academia and industry to develop and utilize contract templates for academic-industry partnerships Foster academic-industry partnerships

(continued)

Table 22.2 (continued)

Challenge	Recommendations
6. *Best models and assays*: The most suitable animal models are uncertain, and diverse assays are used to provide data for IND applications evaluating cellular therapies.	Cosponsor consensus conferences with industry partners to define the most appropriate in vitro assays and suitable animal models for evaluating cellular therapies. Companies may welcome this opportunity to standardize methods. IND applications using agreed-upon standards would expedite FDA review and approval
7. *Clinical trial experience*: Many investigators with excellent new therapy ideas are unable to implement and conduct early-phase clinical trials. They may lack clinical trial experience and may not understand the process leading to a successful IND application	Leverage existing NIH resources, such as the Clinical and Translational Science Awards (CTSA), to provide training in clinical trials methodology, GMP compliance, and other regulatory issues Establish a regular training course on IND preparation that is widely available. Encourage investigators to communicate with the FDA as early as possible during the discovery/preclinical phase Sponsor a consensus conference to define the most appropriate study designs for proof-of-concept and early-phase studies evaluating cellular therapies. Many protocols have been based on cancer drug study models that may not be appropriate for evaluating cellular therapies Foster novel early-phase trials using an existing clinical trials network infrastructure, such as the Blood and Marrow Transplant Clinical Trials Network (BMT CTN)
8. *Patient accrual*: Multiple factors including inadequate funding or infrastructure and the paucity of eligible patients at clinical sites lead to slow accrual to early-phase clinical trials evaluating cellular therapies	Leverage existing resources by allowing investigators with innovative concepts to conduct early-phase studies evaluating novel cell therapies under the umbrella of existing relevant NHLBI-supported programs, such as the BMT CTN. In addition to facilitating accrual, a network's expertise and infrastructure could assist protocol development and early-phase trial execution hastening the transition into future definitive trials of promising cellular therapies Continue support for NHLBI's hematopoietic stem cell therapy programs, such as the BMT CTN, as this provides the potential to fast-track promising cell-based therapies into large multisite trials using an existing infrastructure experienced with large multisite trials and cell therapy
9. *Capturing trial outcomes*: Outcomes, in particular long-term outcomes from early-phase cellular therapy trials, are not easily captured in data registries; rare, long-term effects may not be systematically captured	Encourage existing organizations, e.g., Center for International Blood and Marrow Transplant Research and American College of Cardiology, to collaborate and to encourage investigators to collect and compile outcome information critical to understanding the long-term safety of cellular therapy for new indications, such as heart disease

(continued)

Table 22.2 (continued)

Challenge	Recommendations
10. *Infrastructure*: Infrastructure and support for gene and cell therapy is incomplete. Specialized centers can provide a wide array of preclinical and clinical resources and support to program investigators. However, the institute's Gene Therapy Resources Program (GTRP) supports only lentiviral and AAV vectors, but not other vectors are being developed for clinical application	Continue the institute's collaborative cellular therapy programs that focus on translation and early-phase trials, such as the Specialized Centers for Cellular Therapy (SCCT) Enhance the available resources for cell and gene therapy, such as resources available through the institute's Production Assistance for Cellular Therapies (PACT) and GTRP resource programs
11. *New indications for cell therapies*: Studies for cellular therapy for lung diseases lag behind other clinical areas despite the urgent need to manage pulmonary conditions and the availability of supporting preclinical findings	This affords a unique opportunity to implement the strategies outlined in these recommendations prospectively and to fast-track clinical research in this important area

was strongly encouraged to share expertise across disciplines and, when clinical studies reached a roadblock, to share assistance across centers to identify solutions and new approaches. The original program initiated in September 2005 was funded until August 2010 when the basic research projects were concluded. However, given the time needed to formulate trials, complete multiple review steps, and initiate sites for clinical protocols, it was realized that centers would require additional time to complete their clinical protocols. For this reason, a 2-year no-cost extension period was granted to allow for the completion of clinical trial accrual and follow-up.

NHLBI Expectations of SCCT

NHLBI expected SCCT to carry out basic research to yield new leads for translation and preclinical research to support the development of clinical ideas. The key component, though, was conducting the early-phase clinical trials designed to begin the clinical translation and potentially leading to future cell therapies. A coordinating center was built into the program to assist with clinical design, statistics, and execution, adding clinical sites and with data collection and data analysis. There was a strong emphasis on having this program successfully complete these early clinical trials, and to this end clinical funds were restricted and could only be used to initiate and carry out the clinical protocols. As noted above, additional time from a 2-year

extension provided a total of 7 years for preclinical data acquisition, protocol planning, regulatory filings, protocol and site initiation, patient accrual, and follow-up.

With the initiation of clinical studies in the area of novel cell-based therapies, there was a special concern for patient safety as well as data integrity. Observation of current regulations along with having policies and procedures in place to monitor studies was considered critical, and all clinical studies were reviewed by an NHLBI Protocol Review Committee (PRC) and monitored by an NHLBI data safety and monitoring board (DSMB).

The Research Centers

To mark the beginning of the SCCT program, NHLBI Director Elizabeth G. Nabel, M.D. noted "Recent advances in stem cell biology and transplantation have set the stage for the next level of research emphasis: a program that emphasizes the translation of knowledge about cell-based therapy into clinical practice" [4]. The SCCT program began with three Research Centers: one at Baylor College of Medicine, one at Johns Hopkins University, and one at Massachusetts General Hospital. During the course of the program, investigators originally at Johns Hopkins University relocated to the University of Miami and to Cedars-Sinai Medical Center resulting in the final SCCT composition shown in Table 22.3. These changes of institution expanded the original Johns Hopkins site to a mini-consortium with three sites. The original sites and these two additional sites were all encouraged to collaborate through exchange of personnel or reagents related to the basic projects as well as through the contribution of ideas to each other's clinical studies.

Center cooperation also assisted clinical trial accrual. Clinical studies planned for the Johns Hopkins site in Baltimore benefited from having additional accrual at sites at the University of Miami in Miami or from the Cedars-Sinai site in Los Angeles. The Massachusetts General Hospital site also benefited from having enlisted other accrual sites for its trials, namely, Beth Israel Deaconess Medical Center, Boston, MA, Dana-Farber Cancer Institute, Boston, MA, and MD Anderson Cancer Center, Houston, TX. This group formed another mini-network of sites, and some of these sites also participated in the accrual of patients for the Baylor Center's clinical trials. The Baylor Center also began with additional clinical sites at Texas Children's Hospital, Houston, TX, and at the Methodist Hospital, Houston, TX.

SCCT Research Activities

The proposed research activities for the SCCT are listed in Table 22.3. A summary of SCCT activities is also available on the program's Web site [5]. In most cases, the basic research activities were directly related to the clinical projects and provided

Table 22.3 SCCT centers

Center	Proposed research projects
Baylor College of Medicine, Houston, TX (*Research Center site*)	Validation of suicide strategies for cellular therapy
	Multipathogen-apecific CTL for the immunocompromised
	Cardiac tissue repair with adult stem cells
Johns Hopkins Univ., Baltimore, MD/University of Miami, Miami, FL/Cedars-Sinai Medical Center, Los Angeles, CA (*Research Center sites*)	MSCs for treatment of post-MI ischemic cardiomyopathy
	Properties of human and porcine cardiac stem cells
	Large animal and initial clinical trials of human cardiac stem cells
Massachusetts General Hospital, Boston, MA (*Research Center site*)	PTH and wnt signaling in osteoblasts
	Defining the bone marrow stem cell niche
	Applying stem cell, stem cell niche interactions to clinical use
	Clinical trials with PTH for bone marrow transplantation
EMMES Corporation, Rockville, MD (*Coordinating Center site*)	Provide administrative and communication support
	Facilitate research planning including study design
	Provide meeting support
	Monitor center performance
	Provide regulatory guidance on federal requirements
	Establish database for cell products and clinical data
	Assess existing databases for development of common cell therapy data elements
	Ensure high-quality databases through quality checks and education of centers

preclinical data from animal studies as required for regulatory submission such as an Investigational New Drug (IND) filing. In the case of the Baylor Center, the proposed preclinical research sought to open up new research avenues by exploring the use of hematopoietic stem cells for the repair of cardiac tissues. Prior research had suggested that hematopoietic stem cells might contribute directly to heart regeneration; however, subsequent work by the Baylor group and others indicated these effects were due to secondary mechanisms such as paracrine factors rather than cellular integration. The basic studies at the Johns Hopkins University and later at the University of Miami and the Cedars-Sinai Medical Center were in direct support of the planned clinical trials. Preclinical data on MSCs and cardiospheres was obtained in laboratory studies to establish clinical cell production methods and in animal studies to provide safety data. These preclinical data supported separate IND filings

for two different cell products, one containing mesenchymal stem cells and the other containing cardiac stem cells. The Massachusetts General Hospital's basic research studies on the bone marrow stem cell niche sought to improve hematopoietic stem cell homing, engraftment, and other niche interactions as an approach to improving clinical protocols for hematopoietic stem cell transplant. Their preclinical studies indicated that parathyroid hormone (PTH) injections increased the number of stem cells and improved survival after bone marrow transplant in murine models.

SCCT Clinical Activities

The clinical research trials conducted by the SCCT are listed in Table 22.4. Each center initiated two or three clinical trials. The Baylor Center completed its first trial using virus-specific cytotoxic lymphocytes (CTLs) for the treatment of adenoviral infections in patients who had received an allogeneic stem cell transplant. The CTLs used were of autologous origin, meaning each line needed to be custom derived from the transplant patient, requiring additional time before treatment. This study demonstrated that CTLs to a single virus, either adenovirus or Epstein-Barr virus, could be generated and safely administered to transplant patients [6]. Early initiation of this trial enabled the Baylor group to conduct a second CTL trial with several key changes. In this second trial, CTLs were derived with reactivity against three common viral targets—adenovirus, Epstein-Barr virus, and cytomegalovirus—and the CTL's lines were derived in advance from other donors and matched to the patient's HLA type. Thus, this second trial is examining the safety of HLA-matched allogeneic CTLs and looks at potential effect on three viral infections common in patients with compromised immune systems. This second CTL study, CHALLAH, will finish its accrual in 2011 and follow-up in 2012; an abstract of preliminary results from this study have been presented [7]. The third Baylor trial looks at key question in cellular therapeutics. Cells introduced into a patient could potentially have a very long persistence. In this event, how could a cellular side effect be limited or terminated, if needed? To address this issue, an inducible suicide gene based on the caspase death pathway was developed and introduced into T cells. The T cells have been depleted to reduce cells reactive to other target sites while increasing the potential number of cells targeted to the tumor. This strategy enables the administration of increasing doses of T cells to a patient, while the suicide gene strategy provides control of potential side effects, such as graft-versus-host disease, through the administration of a small molecule dimerization agent that initiates T cell death. This study, CASPALLO, will be completed in 2012.

The Johns Hopkins University/University of Miami/Cedars-Sinai Medical Center focused on the use of cellular therapies for the treatment of heart disease. The PROMETHEUS study introduced an autologous cell product containing mesenchymal stem cells into the heart around the infarcted zone by injection while the patient was undergoing cardiac artery bypass graft (CAGB) surgery. The patient's own

Table 22.4 SCCT clinical trials

Center	Clinical trials
Baylor College of Medicine, Houston, TX	Virus-specific cytotoxic T lymphocytes (CTLs) for adenovirus infection following an allogeneic stem cell transplant NCT00111033—*this study has been completed*
	CASPALLO: Allodepleted T cells transduced with inducible caspase 9 suicide gene NCT00710892—*this study has been completed*
	Most closely HLA-matched allogeneic virus specific cytotoxic T lymphocytes (CTL) (CHALLAH) NCT00711035—*this study has been completed*
Johns Hopkins Univ., Baltimore, MD/ University of Miami, Miami, FL/ Cedars-Sinai Medical Center, Los Angeles, CA	Prospective randomized study of mesenchymal stem cell therapy in patients undergoing cardiac surgery (PROMETHEUS) NCT00587990—*this study has been completed*
	The percutaneous stem cell injection delivery effects on neomyogenesis pilot study (the POSEIDON-Pilot Study) NCT01087996—*this study has been completed*
	Cardiosphere-derived autologous stem cells to reverse ventricular dysfunction (CADUCEUS) NCT00893360—*this study has been completed*
Massachusetts General Hospital, Boston, MA	Study of parathyroid hormone following sequential cord blood transplantation from an unrelated donor NCT00393380—*this study has been completed*
	Reduced intensity, sequential double umbilical cord blood transplantation using prostaglandin E2 (PGE2) NCT00890500—*this study has been completed*

expanded mesenchymal cells were directly injected into the damaged region of the heart. However, since the cells first needed to harvested and expanded in the laboratory for several weeks prior to injection, this protocol was an option only for patients who could delay their CABG procedure, while the mesenchymal cells were being expanded. Twelve patients were enrolled on the PROMETHEUS protocol, but this study has been closed, as the need to expand cells for several weeks prior to reinfusion slowed enrollment. However, a second related study has been opened called POSEIDON. By using allogeneic mesenchymal cells, the cells for this second trial can be prepared in advance, and the cell product is administered by intracoronary infusion. The POSEIDON study tests the safety of using allogeneic instead of autologous mesenchymal cells and also examines cell dose effects. POSEIDON is expected to be completed in 2012. The third study in this center, CADUCEUS, tests a new cell source, cells obtained from a biopsy of the heart and expanded in culture.

The patient's own heart cells are expanded in culture to generate a spherical cell mass, termed a cardiosphere, harvested and administered by intracoronary infusion a few weeks after myocardial infarct. This initial study will test the safety of this new cell preparation at two cell doses and set the stage of future work to look at the effect of this intervention. This study has completed enrollment and should complete follow-up in 2012.

The center at Massachusetts General Hospital began with a study of parathyroid hormone (PTH) in the setting of cord blood transplantation. Preclinical data had shown that PTH-enhanced hematopoietic stem cell engraftment two- to four-fold suggesting an important new clinical application for this hormone with known calcium regulatory effects. This study was stopped because of toxicity concerns, and proposed follow-up studies with PTH were not pursued. The center considered alternate preclinical leads and proposed a study to examine prostaglandin E2 (PGE2) based on a zebra fish screen and subsequent murine model data from a laboratory at the Harvard Stem Cell Institute. This led to the design of a second protocol in the cord blood transplant setting where the drug, PGE2, is not administered to the patient, but used to treat the cord blood cells prior to infusion of the cells. The preclinical data indicated that this pretreatment can increase cell engraftment. This study is ongoing and is expect to be completed in 2012.

The SCCT's basic research activities have been mostly completed, and final research publications are still in progress. As of October 18, 2010, the SCCT had reported 47 publications and 22 abstracts for the program [5]. Many of the centers' clinical protocols are still in progress, and following completion, meeting abstracts and manuscripts are planned. As described above, four studies are still accruing patients, and a fifth trial is monitoring patient follow-up. Results from the trials will be published after the follow-up studies are completed in a year or two. Additional information on the clinical trials is available at ClinicalTrials.gov. The US National Clinical Trials (NCT) identifier number and the link to ClinicalTrials.Gov site are listed in Table 22.4. The SCCT complete clinical protocols are also listed on the SCCT Web site [5].

Future Directions

The basic research studies and the clinical studies supported by the SCCT program have advanced cell therapies within areas of the NHLBI mission. The studies have addressed some of the limitations of hematopoietic stem cell transplantation, including viral infections in immune compromised patients or graft-versus-host disease or limited cord blood cell dose. Studies have also addressed the adverse effects of myocardial infarction using first autologous and then allogeneic MSCs as well as a new cell source, expanded cardiac cells obtained from cultured cardiospheres. These early studies supported in the SCCT program were designed to examine if a new cellular intervention was safe and, if so, to set the stage for future studies to examine the efficacy of these cell-based treatments.

In addition to the SCCT program, other NHLBI efforts are making important contributions. The basic research foundation for future work is supported foremost by NHLBI-funded investigator-initiated research project grants, notably R01 grants. Institute-initiated programs also play a role, particularly the NHLBI Progenitor Cell Biology Consortium (PCBC, [8]). The institute has also funded an initiative to "Characterizing the Blood Stem Cell Niche," an area needed to advancing cellular therapeutics for blood diseases [9]. A related initiative for the "Translation of Pluripotent Stem Cell Therapies for Blood Diseases" will support new technology needed to generate transplantable blood stem cells [10]. Research on differentiation of human pluripotent stem cells or cellular reprogramming will be supported to produce sufficient number of transplantable cells for clinical use.

For more advanced clinical studies, NHLBI has research networks for cell therapies, such as the Blood and Marrow Clinical Trials Network (BMT CTN, [11] and the Cardiovascular Cell Therapy Research Network (CCTRN). For early-phase trials, a need is seen for mechanisms that provide researchers with expertise related to clinical protocol development and to review panels with clinical expertise, points recommended by our working groups (Tables 22.1 and 22.2). NHLBI has assembled a set of resource programs to provide for cell therapy protocols with clinical-grade cells (PACT program—this article and [12]), clinical-grade gene transfer vectors (GTRP, [13]), and clinical-grade biologics (SMARTT, [14]). Building on this base of resource programs, it is hoped that the funding opportunity announcement (FOA) for Early-Phase Clinical Trials for Blood Cell Therapies (R01) (PAR-11-204) will provide cell therapy researchers the opportunity to submit early-phase clinical protocols that can be evaluated with the appropriate expertise and thus to foster the development of future cell therapy treatments [15].

NHLBI Production Assistance for Cellular Therapies (PACT)

Origins of PACT

As the field of cellular therapy moved beyond mononuclear cell transfusion and bone marrow transplantation, it became apparent that the preparation of the cellular product was becoming a science unto itself. The isolation and identification of the desired cell population could take months, and the optimization of this process could take a year or longer. This did not include the development and validation of potency assays. All of this work is expensive and labor intensive and requires staff qualified to perform at the highest technical level. Even though these tasks are vital to the production process, they are not hypothesis-driven so this type of work does not meet the requirements for NIH research project grants such as R01s and R21s.

In 2002, the National Heart, Lung, and Blood Institute (NHLBI) held a working group to discuss immune reconstitution and cell therapy after stem cell transplantation. The workshop addressed the biology of immune reconstitution after transplantation, methods to enhance immune recovery, and regulatory and practical

Table 22.5 Recommendations to NHLBI to advance cellular therapies

Recommendation	Comments
Support facilities for cell processing	Develop a comprehensive program to include space and facilities to perform work ranging from basic science through animal studies and proof-of-principle that could be made readily available to many investigators, rather than having each one creating his or her own GMP-level, quality assurance facility
Support infrastructure to produce clinical-grade reagents, such as cytokines and monoclonal antibodies	NHLBI could support a small business initiative or programs of excellence, with core facilities, educational components, animal model capabilities, and support for translational efforts, could be funded to provide these reagents
Training for regulatory issues, quality control, and quality assurance	Support training to develop knowledge of regulatory, quality control, and quality assurance requirements and oversight. Most research scientists are not familiar with the FDA guidelines and requirements for preparing cellular therapeutic products and standardized reagents for clinical trials
Education and recruitment assistance	Initiatives should be developed to facilitate the transition of junior investigators from career development awards to R01 grant support in the area of cell therapy clinical studies
Develop initiatives to solicit basic and clinical research of cellular therapies	NHLBI and other NIH Institutes should co-fund initiatives involving the collection, expansion, preservation, and stimulation of specialized cells for immune therapy and stem cells for transplantation

issues involved in implementing new clinical strategies to enhance immune reconstitution [16]. At the conclusion of the workshop, a think tank discussion was convened with the purpose of identifying areas in need of support that would lead to cellular therapy clinical studies involving transplantation and transfusion medicine. Several recurrent recommendations emerged and are listed in Table 22.5.

In response to these recommendations, NHLBI designed the Production Assistance for Cellular Therapies or PACT program. This program first focused exclusively on manufacturing cells. It was not until 2010, that an NHLBI program [Science Moving towArds Research Translation and Therapy (SMARTT)] would be created to assist in the manufacture of cytokines and monoclonal antibodies. SMARTT will begin accepting requests in 2011 [14].

The initial PACT program (2003–2009) included three cell processing facilities and an administrative center to manufacture cellular therapy products and assist investigators in completing clinical studies involving these products. A steering committee, consisting of representatives from each manufacturing facility, the administrative center, NHLBI, and external NHLBI-appointed co-chairs, was established to formulate and implement policy decisions related to the conduct of the project.

An external review panel was convened by NHLBI to independently review and annually evaluate the PACT program and to advise NHLBI regarding the adequacy of progress toward its goals. For an in-depth review of the first 5 years of the PACT program see reference [17].

The NHLBI charged the PACT facilities with manufacturing a clinical-grade product for requesting investigators. These investigators may lack access to or expertise in clinical-grade manufacturing or sufficient funds to manufacture their cell therapy product. In the beginning PACT was envisioned as a manufacturing service only. However, it was quickly discovered that the products were not yet ready to be used in clinical trials. Some of the most frequent deficiencies encountered were the lack of interaction with the FDA, lack of scale-up data, incomplete potency data, and the presence of lot-to-lot variation. In an effort to address these issues, PACT was therefore tasked with establishing an effective educational program to assist investigators in regulatory affairs. The educational program consisted of public workshops providing hands-on laboratory experience and didactic sessions as well as webinars that were available free of charge to the public.

In addition to assisting investigators, PACT services resulted in the generation of publications. The PACT Web site continually updates the bibliography, but in the first iteration of PACT, there were 19 abstracts and 29 manuscripts. For the sake of brevity, two publications are highlighted here for illustrative purposes. Stem cell transplant recipients are highly susceptible to posttransplant infection from potentially lethal viruses such as cytomegalovirus (CMV), Epstein-Barr virus (EBV), and adenovirus. One therapeutic strategy is the production and infusion of virus-specific cytotoxic T lymphocytes (CTLs) to protect the transplant recipient. It was assumed that three distinct cell lines would be required because the dominant antigens of each virus would compete for presentation to effector cells which would lack multivirus specificity. PACT services led to the generation of CTLs specific for CMV, EBV, and adenovirus. These cells can expand in response to viral challenge after administration and produce clinically relevant effects. Eleven stem cell recipients received these CTLs, all of which expanded in vivo, reduced the viral titer, and resolved disease symptoms in those with evidence of active CMV, EBV, or adenovirus infection [18]. Another cellular therapy of wide interest is T regulatory cells (T-regs). T-regs participate in the prevention of acute graft-versus-host disease (GVHD) by suppressing CD8+ effector cell expansion in the GVHD target organs. PACT produced T-regs from umbilical cord blood which were used in a phase I dose escalation trial to examine the safety of this therapeutic strategy in subjects with advanced or high-risk hematologic malignancies. Twenty-three subjects received T-regs with no infusional toxicities and circulating cells persisted for 14 days. When these subjects were compared to identically treated controls (who did not receive T-regs), there was a statistically significant reduction in grade II–IV acute GVHD [19].

As the first 5 years of PACT were drawing to a close, NHLBI began an in-depth analysis of the program and the needs of the field. NHLBI staff consulted PACT members and customers, the PACT External Review Panel, NIH working groups, and even held an internal retreat to assess the evolving needs of cellular

therapy investigators. The result was to hold a competitive renewal of the PACT program with an expanded scope of work which would include services identified by the groups listed above. The additional services included providing support to proof-of-principle animal and early translational research and diversifying the cell therapy expertise within the program by increasing the number of manufacturing facilities. It was noted that adding facilities would also have the benefit of making cell therapy products that could not be shipped long distances more widely available.

A mini-retreat was conducted on February 5, 2008 with attendees from the heart, lung, and blood divisions. After presenting background information on the current PACT program, a discussion on the cell therapy manufacturing needs of the NHLBI scientific community ensued. The recommendations were as follows:

Continue to support scale-up of cell lines and manufacture of cellular products for use in phase I/II clinical trials
Increase the number and geographic distribution of PACT manufacturing centers to increase diversification and easier distribution of products that cannot be shipped long distances
Make available a centralized laboratory testing for novel cellular therapies to allow for standardization of results and costs savings, e.g., flow cytometry panels or functional assays such as assays related to cell homing or migration
Have PACT facilities serve as a master bank for cell lineages derived by the Progenitor Cell Biology Consortium
Provide cell manufacturing, trial design, regulatory consultation, and a mechanism to enable implementation of cell therapy phase I/II trials when a special need is demonstrated
Support cell manufacturing needs for preclinical studies including basic and translational (animal models) work. [For example, a source of human and mammalian cells including bone marrow or cord blood mesenchymal stem cells, lung biopsy expanded progenitor cells, and progenitor cells delivered onto bioscaffolds]
Provide genetically engineered cells for therapy of lung and sleep disorders such as narcolepsy and cystic fibrosis
Provide defined human hematopoietic stem cells for preclinical testing of gene transfer vectors

Thus, in 2009, NHLBI released a request for proposals to competitively renew the PACT program. The statement of work called for an expansion of the program to include the recommendations listed above. To accompany the new scope of work, the PACT program was expanded to include more cell processing facilities. Six contracts were awarded. The PACT Coordinating Center is the EMMES Corporation of Rockville, Maryland. The current five PACT cell processing centers are located at the following institutions:

- Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, Texas
- Center for Human Cell Therapy, Children's Hospital, Boston, Massachusetts
- Molecular and Cellular Therapeutics, University of Minnesota, Minneapolis, Minnesota
- Center for Applied Technology Development, City of Hope, Duarte, California
- Waisman Clinical BioManufacturing Center, University of Wisconsin, Madison, Wisconsin

Table 22.6 PACT review criteria

Criteria	Description
Objective evidence of scientific merit and evidence that the product can be manufactured	Preliminary data should be provided along with any details of peer-reviewed funding
Adequate funding to complete clinical study	The proposed clinical study must be supported with sufficient funding prior to initiation of manufacture
Appropriateness of study design	Areas such as statistical design, safety, and efficacy endpoint data for the product will be considered along with preliminary product characterization information and the potential for enrollment in the trial based on the targeted patient population
Regulatory status	The regulatory status is assessed in order to determine the stage from which the facility will be developing the product. Regulatory support and assistance in filing the IND will be provided by PACT
Ability of a PACT facility to manufacture the product	The facilities will determine if the capacity and capability exists for the product
Relevance to the NHLBI scientific mission	Clinical studies must fall within the mission of NHLBI. Translational studies may be within any disease area

The PACT Application Process

The application process consists of two stages with each requiring review and approval by the steering committee. Applications are accepted on a rolling basis. The preliminary application is designed to be brief and easy for the applicant to complete but provides sufficient detail to allow the steering committee members to determine whether the application is scientifically sound. If the steering committee approves the preliminary application, the applicant is invited to submit a full application that provides in-depth information regarding the scientific, technical, and logistic details required to manufacture the requested product. Once the applicant is approved to submit a full application, a technical liaison from one of the PACT facilities is assigned to work with the applicant for the remainder of the process. An important part of the full application involves the clinical trial protocol, access to patients for the trial, a plan for evaluating the biologic (as well as clinical) effect of the novel cellular product, and the funding and resources for these activities. The full application is reviewed by outside peer reviewers who evaluate the strengths and weaknesses of preliminary data, manufacturing feasibility, significance to the field, degree of innovation, and overall scientific merit of the application. The independent review is then submitted to the steering committee, to assist in the final decision to approve or reject the submission. For review criteria, see Table 22.6.

When a full application is approved for product manufacture, the technical liaison begins to work with the applicant to develop the timeline, milestones, budget, and a contract between organizations if one is needed. The staff at the appointed manufacturing facility works closely with the applicant to understand the product characteristics, methods of delivery, and other requirements.

Manufacturing is performed under a formal contract between the organizations based upon a mutually agreed-upon template. Figure 22.1 Contractual agreements between organizations may also include confidentiality agreements, material transfer agreements, and agreements regarding intellectual property rights. In the beginning of the PACT program, the issue of indemnification was encountered. PACT was committed to manufacturing cells for investigators located anywhere in the United States, but each PACT facility's institution needed a method to transfer the responsibility of any adverse clinical events resulting from the infusion of the PACT product to the institution where the clinical study was occurring. The cell processing facilities were all located at academic institutions; however, one was a public university (University of Minnesota), one was a private university (Baylor College of Medicine), and one was funded with a combination of public and private funds (University of Pittsburgh). The level of institutional insurance varied greatly among the three institutions. A Material Transfer Agreement template was developed by the NHLBI Technology Transfer Office and the PACT institutions that individual institutions could modify by adding any required state-specific language. Once the customer investigator's institution accepted the PACT product, the associated responsibilities were also transferred.

By the end of the fifth year of the PACT program, 65 preliminary applications were received of which 45 were approved for product manufacture. A variety of cell therapies were manufactured including T regulatory cells, natural killer cells, adipose-derived stem cells, cardiac progenitor cells for cardiac disease, hematopoietic progenitor cells (HPCs) for central nervous system applications, cytotoxic

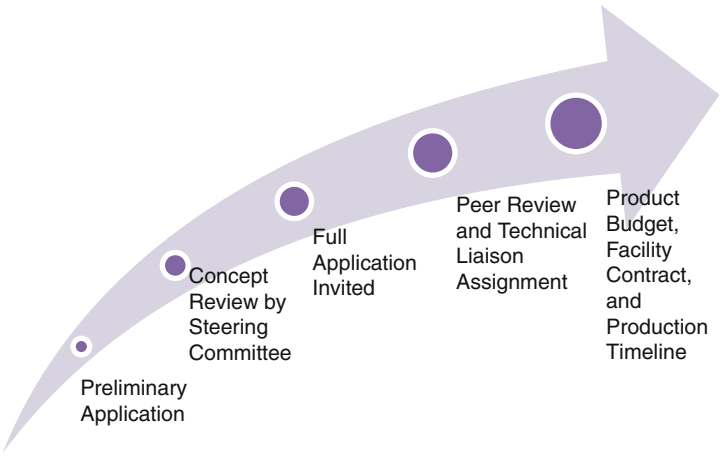

Fig. 22.1 The PACT application process

T lymphocytes, and dendritic cells. A total of 169 products were administered under the purview of 12 applications. Fourteen peer-reviewed publications and 15 abstracts resulted from the first 5 years of the PACT project along with a cell therapy textbook. In the renewed PACT program, many of the same products are still being produced along with some new ones requiring translational services. For a partial list of PACT-manufactured products from the past and renewed program and their indications, see Table 22.7.

The new PACT contracts were awarded January 15, 2010. In addition to the expanded scope of work, there is one other major change which involves the scientific area of the cell therapy projects. Translational applications to PACT can be in any scientific area, even those outside the scientific interest of NHLBI. Clinical applications to PACT must be in the programmatic interest of NHLBI. The main point is that each application will be considered on an individual basis. The criteria listed on the PACT website are based on the NHLBI Referral Guidelines which are used to decide which grant applications will be accepted and which will be referred to other NIH Institutes. It is anticipated that the biggest area of overlap will be with the National Cancer Institute (NCI) in the area of hematopoietic stem cell

Table 22.7 Examples of PACT products

Cell product	Indication
Cardiac stem/progenitor cells	Cardiac regeneration in acute myocardial infarction
Mesenchymal stem cells	Repair cardiac damage in patients following myocardial infarction
	Sickle cell disease
	Bronchiolitis obliterans
Autologous CD34+ stem cells	Intermittent claudication
Autologous bone marrow mononuclear cells	Cardiac repair
	Stroke
CMV, adenovirus, and EBV-specific cytotoxic T lymphocytes	Prevent and treat posttransplantation viral infection
Autologous mature apoptotic dendritic cells with HIV-1	Therapeutic autologous vaccine
Allogeneic natural killer cells	Posttransplantation residual viral infection
Allodepleted donor leukocytes	Enhanced immune reconstitution
Multivalent virus-specific cytotoxic T lymphocytes	Immune reconstitution of cord blood transplantation patients
Allogeneic T cell-depleted progenitor cells	Improvement of posttransplant immune reconstitution
Cord blood derived hematopoietic progenitor stem cells	Bone marrow reconstitution
CD4+/CD25+ T regulatory cells	Prevent graft-versus-host disease and maintain graft-versus-leukemia effect
Skeletal myoblasts	Translational development (cardiac)
Stem cells (adipose-derived)	Translational development (ischemia)
Pancreatic islet cells	Translational development to improve the digestion process in a closed system (diabetes)

transplantation for malignancies. PACT will follow the guidelines that NHLBI uses to determine which grant applications can be assigned to NHLBI and which ones to send to NCI. PACT will accept clinical applications for studies that examine the process or effects of transplantation such as graft-versus-host disease and infectious complications of transplantation. NHLBI also accepts applications on new approaches to transplantation including mini-transplants, donor lymphocyte infusions, and regimens to induce allochimerism. The new PACT program is currently accepting translational and clinical applications. There are no receipt dates, and the applications are reviewed on a rolling basis. The PACT facilities have experience in working with mesenchymal stem cells and are capable of assisting investigators by providing and characterizing these cells for the multiple therapeutic applications described in this book.

Conclusions

In summary, NHLBI is firmly committed to supporting basic, translational, and clinical studies focused on the use of all cellular therapies including MSCs. The institute accepts investigator-initiated grant applications, and NHLBI program staff members are willing to speak with investigators to answer questions related to the grant's process or to assist in the navigation of the NIH system. While the budgetary environment can place limits, NHLBI staff is continually attempting to put forward programs for the advancement of the field of cell therapy. The SCCT and PACT programs are examples of productive research and resource programs, and we hope they are the forerunners of many more to come.

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Part III
Clinical Applications

Chapter 23
MSC Therapy of Inborn Errors

Edwin M. Horwitz

Abstract Mesenchymal stromal cells are ideally suited as cell therapy for inborn errors. While there are several potential mechanisms of therapeutic activity, in all studies yet, MSCs seem to exert their effects through the release of soluble mediators. This vast secretome, together with the capacity to modulate gene expression of the MSC therapeutic product through optimal selection of tissue source and *ex vivo* expansion protocols, suggests the potential to develop broadly applicable therapy for a wide array of disorders. However, the clinical experience to date is limited, most likely due to our unrealized historic view that MSCs were stem cells that could regenerate tissue. A pilot study in children with metachromatic leukodystrophy showed that MSCs may be able to increase nerve conduction velocity, suggesting that the cells may be stimulating remyelination of peripheral nerves. In osteogenesis imperfecta, MSCs unambiguously stimulate growth of the children who exhibit the characteristic severe growth deficiency. As our understanding of the fundamental biology of MSCs continues to improve, enthusiasm to assess MSCs in patients with inborn errors is also growing. The coming decade promises a swell of clinical trials and likely important breakthroughs.

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_23,

Introduction

Mesenchymal stromal cells (MSCs) are spindle-shaped plastic adherent cells isolated from bone marrow, adipose, and placenta as well as many other somatic tissues. Many investigators term these cells mesenchymal stem cells [1]; however, we prefer the designation of mesenchymal stromal cells as stem cell characteristics have yet to be demonstrated in vivo [2]. Nonetheless, the cell population designated by both terminologies is precisely the same. The initial interest of using MSCs to treat genetic disorders, that is, inborn errors, was based on the established use of bone marrow transplantation as therapy for these maladies. Indeed, bone marrow transplantation has long been accepted as an effective therapeutic modality for genetic diseases of the hematopoietic system. This cellular therapy is based on the principle that bone marrow contains hematopoietic stem cells that can repopulate the blood. Over two decades ago, investigators recognized that bone marrow is a rich source of MSCs. Together with the prevailing view of the time, that MSCs were stem cells that could regenerate a wide array of mesenchymal tissues, investigators proposed bone marrow transplantation, as a means of transplanting both hematopoietic and mesenchymal cells, to treat disorders of mesenchymal tissues as well as those of the blood. Subsequently, the notion arose that intravenous transplantation of MSCs alone may be effective cell therapy for mesenchymal diseases [3]. Limited clinical success, however, dampened enthusiasm for MSCs in this field. Recently, new insights into the mechanism of therapeutic activity of *ex vivo* expanded MSCs has rekindled great interest in MSC therapy for inborn errors.

MSC Biology and Potential Mechanisms of Therapeutic Activity

While the biology of MSCs is discussed elsewhere in this text, a brief overview specifically in relation to inborn errors is important to fully appreciate the early investigations and the future potential applications.

MSCs were originally considered to be stem cells [4] with the capacity to regenerate an assortment of mesenchymal tissues [5]. Undoubtedly, the developing field of regenerative medicine was excited about the prospects of MSC therapy [6]. Accordingly, many inborn errors were considered candidates for investigational therapies. Over the last decade, convincing data to support the idea that MSCs are stem cells has not been forthcoming; thus, the notion that MSCs can rebuild diseased or damaged tissues lost prominence [2] and interest in using MSCs as agents for inborn errors waned. We now understand that after intravenous infusion, the mechanism of MSC activity in most, or perhaps all, cases seems to be through the release of soluble mediators that act on target host tissues [7]. This is not simply an academic issue; rather, the mechanistic basis for MSC therapy has substantial

implications for the design of clinical protocols which directly impacts the likelihood of a successful outcome. MSCs should be given to patients at the time, relative to other therapies, when the secreted mediators will be active. Moreover, combining MSC therapy with other cell or pharmacologic therapies may offer novel approaches to diseases that currently have no curative options. Thus, once again, momentum is building for the study of MSCs as therapy for inborn errors.

There are five categories of mechanisms of MSC activity. First, the above discussion notwithstanding, MSCs may be able to differentiate to mesenchymal and even some non-mesenchymal tissues in vivo [8], especially after local implantation into the diseased organ. Second, MSCs may be able to fuse with host tissue providing the missing protein that is the cause of the specific genetic disorder. Third, missing enzymes could be transferred from transplanted/implanted donor MSCs into host somatic tissues genetically lacking the essential enzyme, a process termed cross-correction. Fourth, MSCs could secrete cytokines, growth factors, or other humoral mediators of host target tissues. Finally, MSCs can modulate the immune response which could attenuate metabolic disorders in which inflammation plays a prominent pathogenetic role.

Direct differentiation of MSCs to host tissues, once thought to be the primary mechanism of action, does not seem to be a major pathway of therapeutic benefit for any systemic application of MSCs. However, MSCs can differentiate to bone and cartilage in vitro, and studies of local bone/cartilage regeneration to facilitate healing of nonunion fractures have been presented. However, given that inborn errors are systemic disorders, it seems unlikely that MSCs will be used to build healthy tissue to replace the genetically deficient tissue for any disorder.

Fusion refers to the merging of healthy donor cells with diseased or damaged host cells leading to a normal functioning combined cell. This concept was demonstrated in 2002 in cell culture when Terada et al. [9] and Ying et al. [10] simultaneously demonstrated that murine bone marrow cells and brain cells, respectively, obtained from green fluorescent protein (GFP) transgenic mice can fuse spontaneously with murine embryonic stem cells in vitro. The resulting cell displayed an undifferentiated stem cell phenotype carrying the transgenic marker. However, both studies of cell fusion used embryonic stem cells, a unique cell population distinctly different from MSCs or other somatic cells and employed prolonged in vitro cell culture, a cell processing procedure not commonly used in clinical cell processing. Thus, the relevance to clinical MSC therapy is unclear. Nonetheless, MSCs may, in principle, act by cell fusion [11]. Moreover, when the goal is to develop effective cell therapy, a fusion mechanism, in contrast to a differentiation mechanism, does not detract from the treatment strategy if the resulting cells are fully functional and physiologic correction of the defective tissue is achieved.

Cross-correction is the process by which proteins, usually enzymes, are transferred from normal cells to genetically defective cells resulting in the correction of the diseased tissue. First recognized in 1981, investigators demonstrated the direct transfer of lysosomal enzymes from lymphoid cell to enzyme-deficient fibroblasts [12]. Cross-correction was soon shown to be a property of some but not all lysosomal enzymes [13], indicating that each new genetic disorder would require a

specific study. The lysosomal enzyme alpha-mannosidase was then shown to be capable of transfer from lymphocytes to enzyme-deficient fibroblasts obtained from a patient during in vitro culture [14]. Subsequently, a feline model of alpha-mannosidosis was treated by bone marrow transplantation. Functional enzyme was found in the neurons, glial cells, and the cells associated with blood vessels providing direct evidence of the potential of bone marrow-derived cells to cross correct nonhematopoietic tissues [15]. Although clinical evidence has not yet been reported, genetically intact MSCs can transfer ALDP, the missing protein in adrenoleukodystrophy (ALD), to fibroblasts carrying the ALD mutation.

MSCs have an enormous capacity for secretion of soluble mediators. MSCs secrete stromal-derived factor-1 (SDF-1) [16], which plays a critical role in the homing of hematopoietic stem cells to the marrow niche [17]. In vitro, MSCs constitutively secrete interleukin (IL)-6, IL-7, IL-8, IL-11, IL-12, IL-14, IL-15, macrophage colony-stimulating factor (M-CSF), Flt-3 ligand, and stem cell factor (SCF). Upon IL-1α stimulation, MSCs are induced to further express IL-1α, leukemia inhibitory factor (LIF), granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) [18, 19]. MSCs also can secrete several chemokine ligands including CCL2, CCL4, CCL5, CCL20, CX_3CL1, and CXCL8 [20]. Importantly for the treatment of inborn errors, MSCs express high levels of β-hexosaminidase A, total β-hexosaminidase, arylsulfatase A, and β-galactosidase, each of which is defective in a specific neurometabolic disorder [21]. Finally, MSCs secrete mediators outside of immunohematology. For example, MSCs secrete proangiogenic factors such as vascular endothelial growth factor (VEGF) and neural growth factors such as nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), and brain-derived neurotropic factor (BDNF).

The notion that this remarkable secretome underlies most therapeutic activity is especially important given the fact that the gene expression profile of MSCs varies according to the tissue source of origin as well as the specific conditions of *ex vivo* expansions [7]. Thus, MSCs from particular tissue sources may be uniquely suited for therapeutic application of a given disorder. Moreover, culture conditions, in theory, can be developed to promote the expression of the needed protein.

MSCs have a potent immune modulatory capability. First recognized as the ability to suppress T cell proliferation in a mixed lymphocyte culture assay [22], MSCs have now been successfully used to treat graft-versus-host disease after bone marrow transplantation [23] providing unequivocal evidence of immune suppressive activity. Murine and human data often conflict in this arena, but murine and human MSCs seem to suppress the immune effector cells by distinct mechanisms, the mouse by nitric oxide [24], and the human by indoleamine 2,3-dioxygenase [25], suggesting data may not necessarily be extrapolated across these two species. Moreover, the immune suppressive effects seem to be induced [24] and are not universally operative [26, 27] so that failed clinical studies may be due to the tissue source or *ex vivo* expansion protocol that did not generate immune suppressive MSCs. The relevance is that in select inborn errors of metabolism, an inflammatory response seems to play a role in the tissue destruction that is a component of the

pathophysiology and contributes to the clinical symptoms. If the inflammatory response could be partially or at least temporarily controlled, then the disease may be treated. Thus, MSCs could serve as primary or adjunct cell therapy of inborn errors by an anti-inflammatory mechanism.

Clinical Applications

There are limited clinical data, case reports aside, of MSCs for the treatment of inborn errors. This is likely due, in part, to early disappointment as investigators recognized that MSCs did not home to and regenerate mesenchymal tissues. Additionally, there were substantial competing interests from research efforts to develop enzyme replacement therapies as well as small molecule (pharmaceutical) therapy that could alter gene expression. As our understanding of the biology of MSCs continues to grow, greater emphasis will likely be placed on these diseases which, other than possibly bone marrow transplantation, have no therapeutic options with intent to cure.

Hurler Syndrome

Hurler syndrome is an autosomal recessive genetic disorder which results from the lack of α-L-iduronidase expression. This enzyme deficiency results in accumulation of heparan sulfate and dermatan sulfate in lysosomes. If untreated, progressive hepatosplenomegaly, cardiac dysfunction, severe skeletal abnormalities, hydrocephalus, and mental retardation result in early death. Allogeneic bone marrow transplantation can appreciably halt disease progression and improve survival in these patients. The proposed mechanism of therapeutic benefit is host tissue infiltration of donor hematopoietic stem cell-derived macrophages that express α-L-iduronidase and transfer of normal enzyme into defective cells by endocytosis, the so-called cross-correction. However, despite improvements in the liver and heart, the neural pathology and skeletal deformities persist and often progress resulting in death despite complete engraftment of donor hematopoietic cells.

In an effort to improve the outcome of Hurler syndrome patients, a pilot study was undertaken of MSC therapy after hematopoietic cell transplantation. Five children who previously underwent successful bone marrow transplantation from an HLA-identical sibling were infused with $2–10 \times 10^6$ MSCs/kg, which were isolated from the original bone marrow donor and expanded *ex vivo* [28]. There was no toxicity of the therapy, but there was also no measurable improvement in the children's mental or physical development. While the bone mineral density was either maintained or slightly improved, the overall skeletal manifestations were not markedly improved.

Metachromatic Leukodystrophy

Metachromatic leukodystrophy (MLD) is an autosomal recessive disorder due to the deficiency of arylsulfatase A. The lack of this enzyme leads to an accumulation of sulfatides causing demyelination of the central and peripheral nervous system. Patients develop severe gait disturbances, spasticity, mental regression, and eventually absence of any voluntary function rapidly leading to death. Similar to Hurler syndrome, bone marrow transplantation can arrest the progression of the disease and improve patient survival. The mechanism of clinical benefit is also thought to be similar to that of bone marrow transplantation for Hurler syndrome, namely, cross-correction of the missing enzyme, arylsulfatase A. This idea is especially intriguing given that complete donor hematopoietic engraftment does not always result in clinical success and MSCs are known to express high levels of arylsulfatase A [21].

Since slowing or absence of nerve conduction correlate with peripheral nerve demyelination, a pilot clinical trial of MSC therapy was undertaken to improve nerve condition as an indicator of improved peripheral nerve myelination. Six children who previously underwent successful bone marrow transplantation from an HLA-identical sibling were infused with $2–10 \times 10^6$ MSCs/kg, which were isolated from the original bone marrow donor and expanded *ex vivo* [28]. In four of the six children, there were significant improvements in nerve conduction suggesting improved nerve myelination (Fig. 23.1). Neither long-term follow-up of these patients nor a larger efficacy study (e.g., Phase II) have been reported; these early results are encouraging. Given our growing understanding of the biology of MSCs, these cells seem to hold great promise as adjunct therapy with bone marrow transplantation for children with MLD.

Osteogenesis Imperfecta

Osteogenesis imperfecta (OI) is a genetic disorder of mesenchymal cells, characterized by bony fractures and deformities, short stature, and often a reduced life expectancy [29, 30]. The underlying defect in most cases is a mutation in one of the two genes, *COL1A1* and *COL1A2*, that encode collagen type I, the major structural protein in bone. There is a wide variety in the severity of the phenotype of the affected children. In the more severe forms, Type II and Type III according to the Sillence classification [29], spontaneous, new, autosomal dominant mutations are typically found within one of the exons so that structurally defective protein is expressed at reasonably normal levels. The protein disrupts bone structure by a dominant negative mechanism. Children with Type II OI exhibit 60% mortality in the first day of life and >99% mortality in the first year. Children with Type III OI, the most severe form to routinely survive infancy, have numerous painful fractures, severe bony deformities, and markedly shortened stature. The life expectancy of these patients, historically, was quite short; however, with improved awareness and improved supportive medical/surgical care, many such patients will live a relatively long life.

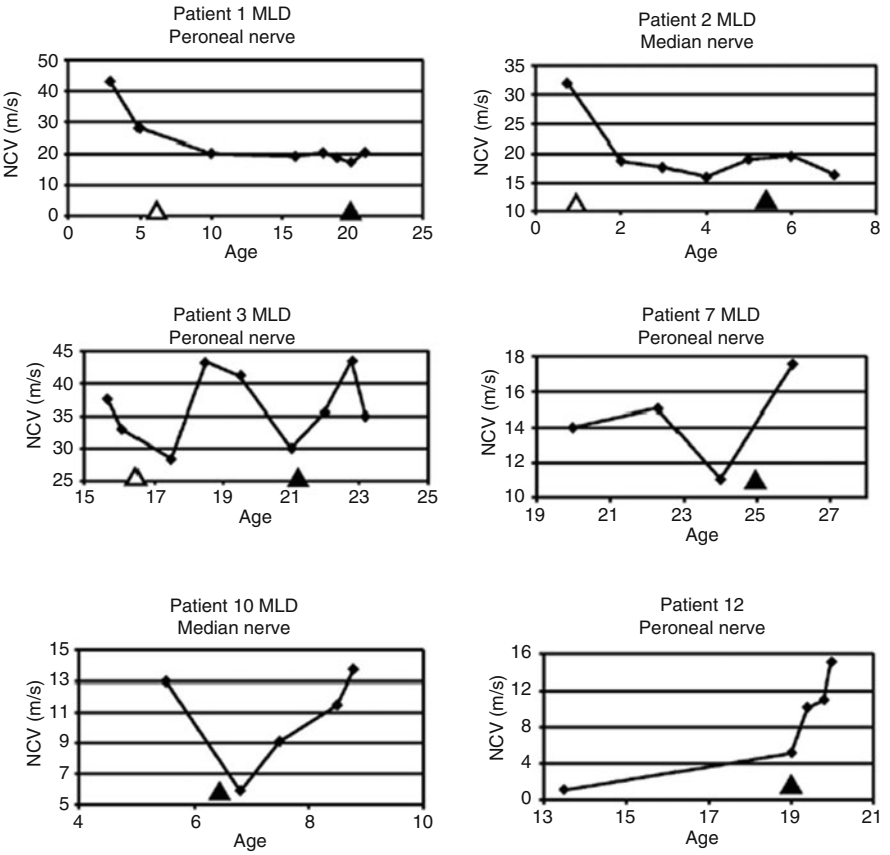

Fig. 23.1 Nerve conduction velocity measurements over time in MLD patients after MSC therapy. *Open arrows* indicate time of allogeneic BMT. *Solid arrows* indicate time of donor allogeneic MSC infusion (Taken from Ref. [28], with permission)

When we began our work, OI was the first inborn error to be considered for mesenchymal cell therapy. The selection of OI as a model disorder was based on several considerations. First, there were no alternative therapies other than supportive care. Second, bone marrow was known to be a rich source of MSCs which were viewed, at that time, as stem cells that could engraft and robustly differentiated to bone. Third, the defective osteogenic environment, may promote engraftment of donor-derived cells. Finally, the notion of intravenous transplantation of MSCs ameliorating the symptoms of OI was supported by animal studies and bone marrow transplantation as a means of transplanted MSCs (in addition to hematopoietic cells) without the risk of rejection seemed logical. Thus, our research team began a series of studies to develop MSC therapy for children with severe OI.

Our work on mesenchymal cell therapy of OI has consisted of two clinical trials (Fig. 23.2). The first protocol was designed to investigate whether we could

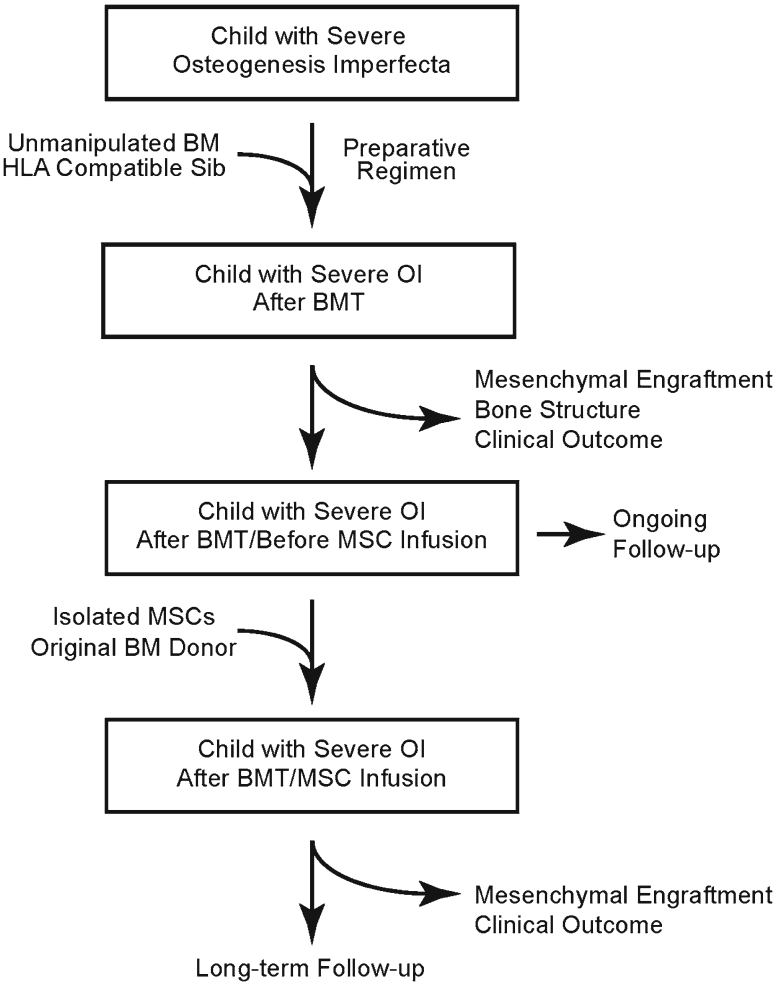

Fig. 23.2 Schematic overview of the clinical trials of mesenchymal cell therapy for children with severe osteogenesis imperfecta

transplant unmanipulated bone marrow and attain mesenchymal engraftment in the bones of the children with severe OI [31]. Then, we assessed whether this marrow cell therapy benefited the children clinically [32]. This study was the first prospective trial of marrow transplantation focused on cells other than the hematopoietic cells. In the second protocol, we investigated whether we could transplant isolated marrow MSCs harvested from the original bone marrow donor and recreate the effects of the original bone marrow transplantation [33]. This later study was the first clinical trial of allogeneic bone marrow mesenchymal cell transplantation.

MSC Transplantation

In our first study, we demonstrated osteopoietic engraftment of donor-derived cells and differentiation to osteoblasts in the patients' bones. This engraftment was associated with an improvement in the microscopic structure of the bone, increased bone mineralization, and decreased rate of fractures [31]. Most importantly, all children showed a remarkable growth acceleration in the first 6 months after transplantation [32].

At the conclusion of this trial, we considered the marrow MSCs to be the cellular source of all clinical benefit. In an effort to enhance these benefits, we developed a clinical study to test the hypothesis that isolated, allogeneic marrow mesenchymal cells could be safely infused after allogeneic BMT and would benefit children with severe OI. To unequivocally identify the marrow mesenchymal cells infused in this trial (compared to cells that may persist after the original bone marrow transplantation), we "gene marked" the cells by transduction with a retroviral vector. Furthermore, to investigate whether marrow mesenchymal cells could be expanded *ex vivo* and retain their biologic potential, we used a double gene marking strategy in which minimally processed cells and expanded cells were each transduced with a unique retroviral vector.

Marrow Mesenchymal Cell Processing

After the mesenchymal cells were isolated from bone marrow by adherence to plastic, the cells were divided into two fractions, and each was transduced with one of the two retroviral vectors that may be distinguished by a PCR-based assay. One fraction was allowed to remain in culture for the minimal time required for isolation and transduction, while the other was expanded over three passages. The minimally maintained cell preparation was infused into the patients, without a chemotherapy-conditioning regimen, at a dose of 1×10^6 cells/kg, and the expanded mesenchymal cells were infused at an intended dose of 5×10^6 cells/kg after about 2–3 weeks, again without a conditioning regimen.

Engraftment

About 6 weeks after the cell infusions, we obtained a biopsy of bone, and skin, and an aspirate of bone marrow and isolated osteoblasts, skin fibroblasts, and marrow stromal cells. We then used our PCR assay to assess for engraftment of each cell population. In five of the six patients, we were able to identify marked mesenchymal cells in at least one of the tissues studied. Both minimally processed cells and expanded cells engrafted. *Ex vivo* expansion may diminish the osteogenic engraftment and/or differentiation potential of marrow mesenchymal cells; however, the limited data in this trial preclude a definitive conclusion of the effect of *ex vivo* expansion.

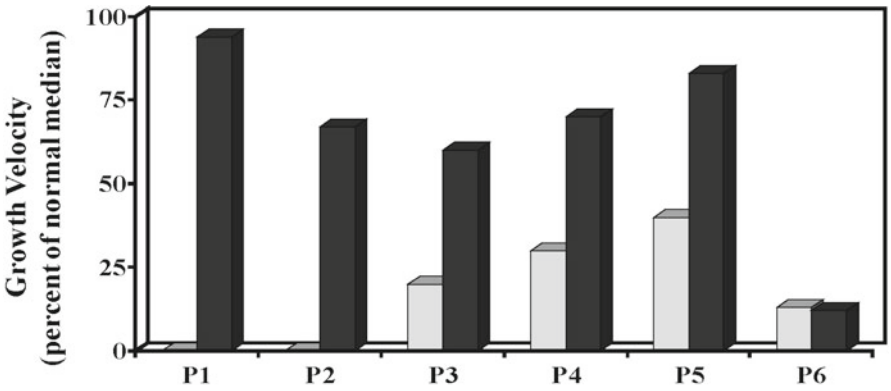

Fig. 23.3 Growth stimulation after MPC engraftment. Growth velocity of the patients during the 6 months immediately before (▒) and after (■) the first MPC infusion. The values are percentages of the median growth of unaffected children of the same age and gender [43]. *P* patient (Taken from Ref. [33], with permission)

Clinical Outcome

All five children in whom we documented mesenchymal cell engraftment showed an acute acceleration of their growth velocity in the first 6 months after the cell infusions compared with the 6 months immediately preceding the infusions (Fig. 23.3). The outcome was most significant for patients #1 and #2, who did not grow prior to the cell therapy, but accelerated their growth velocity to 94 and 67%, respectively, of the predicted growth velocity for age- and gender-matched children. There was not an unambiguous improvement of the total body bone mineral content (TBBMC) after the mesenchymal cell infusions. Since a chemotherapy-conditioning regimen was not given to the children prior to the cell infusions and the cells were relatively pure compared to unmanipulated marrow (although still quite heterogeneous), the growth velocity data, TBBMC data notwithstanding, formulate a compelling argument supporting the therapeutic potential of marrow mesenchymal cells.

Immunology

Marrow mesenchymal cells have been reported to be immunologically privileged [22, 35–37]. In our trial, we used two retroviral vectors, one that expressed neomycin phosphotransferase (neo^R) and one that did not express the encoded sequences. Interestingly, in all the patients, we found only cells marked with the nonexpressing vector. This suggested that the neo^R-expressing cells were immunologically attacked when they were infused into these immunocompetent patients. In one patient, we were able to demonstrate, using a chromium release assay, cytotoxic T-cell activity against neo^R-expressing mesenchymal cells in contrast to mesenchymal cells that

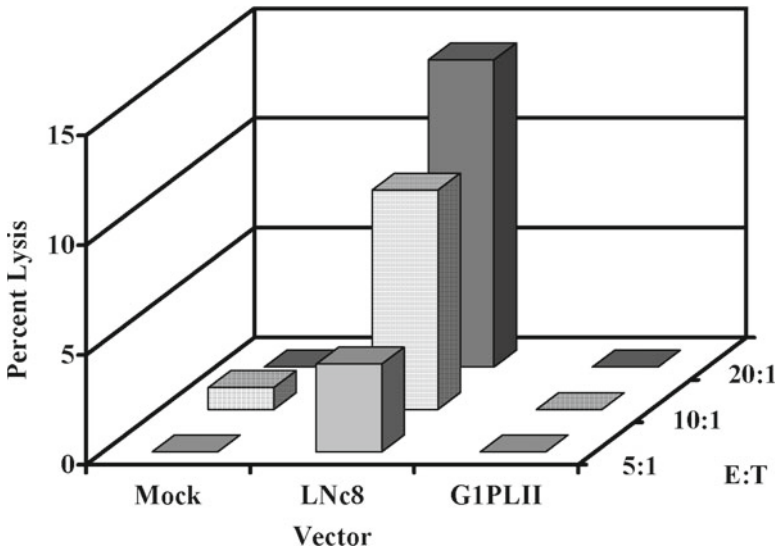

Fig. 23.4 T cell response against transduced MPCs. CTL-mediated lysis of donor MSCs transduced with either the LNc8 or G1PLII retroviral vector or mock supernatant only. E:T designates the effector-to-target cell ratio. Each bar represents the mean of triplicate determinations (Taken from Ref. [33], with permission)

were transduced with the nonexpressing vector (Fig. 23.4). Mesenchymal cells, therefore, seem to be subject to an immune response when expressing a foreign protein.

We also evaluated the patients for the antifetal bovine serum (FBS) antibodies, since FBS was a component of the media throughout the retroviral transduction and *ex vivo* expansion procedures. Using an ELISA assay, we demonstrated a greater than 100-fold increase in anti-FBS antibody titers in post-infusion serum compared to the pre-infusion serum in the patient who did not show engraftment nor a clinical response (Fig. 23.5). The remaining patients did not show a change in anti-FBS antibody titers after the infusions were completed. Although the lack of evidence of engraftment must be considered inconclusive as detailed above, these observations taken together suggest that this child had anti-FBS antibodies that attacked the marrow mesenchymal cells, which precluded engraftment and thereby any clinical response. This data further suggests that mesenchymal cells are subject to an immune response when presenting a foreign antigen.

Developmental Outcome

Although the clinical outcome parameters discussed above are critically important in improving the life of children with severe OI, the capacity to enhance their motor development would also be of great benefit and currently, there are no therapeutic options. Bisphosphonate therapy increases bone mineral density and decreases

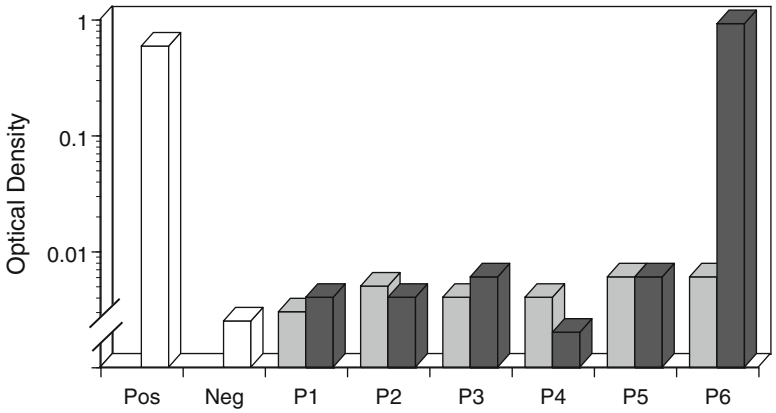

Fig. 23.5 Antibody response against fetal bovine serum proteins. ELISA assay measuring antifetal bovine serum antibodies in the sera of patients before (▒) and after (■) both MSC infusions. Each bar represents the mean of triplicate determinations. *P* patient (Taken from Ref. [33], with permission)

fractures, but does not facilitate growth [38] and may have long-term, as yet undescribed, consequences for bone metabolism [39, 40]. Surgical correction of bony deformities and placement of intramedullary rods is advocated by many caregivers and seems to be rather beneficial, but this intervention does not decrease the fracture rate nor increase the capacity to walk, which we consider a quite useful skill to improve the quality of life.

For children with OI, several studies have shown that the ability to sit without support at 9 or 10 months of age predicts the patient's ultimate ability to walk [41–43]. In our cohort of six patients, none could sit without support at 10 months of age. Although these children are not yet at physical maturity, three children became ambulatory for a few years: two walked independently and one "cruised" around his home. However, all three children lost the capacity for independent ambulation within a few years, possibly indicating the duration of MSC activity in OI.

Ongoing MSC Research

The outcome of our first study suggests that MSCs are of value to children with OI, but the benefits of a single cell treatment are not permanent. We are currently conducting a new clinical trial to investigate whether repeated infusions of MSCs can stimulate growth and maintain the accelerated growth velocity. While the trial is ongoing, our preliminary results at the time of this text are very encouraging. Moreover, we have discovered that MSCs stimulate growth in children with OI by the secretion of a soluble factor which initiates a biochemical pathway resulting in overall growth. Strategies are now being developed to enhance the growth-promoting effect of MSCs, which is the only therapy currently under study which seems to promote growth in all severely affected OI children.

Conclusions

Inborn errors are a group of disorders that gene therapy was destined to remedy. However, such lofty goals have not been realized. While MSCs are unlikely to be the magic bullet to cure all inborn errors, the outstanding safety profile of these cells allows for a wide array of cell therapy research in order to identify the most readily treatable disorders. Investigators must bear in mind that different tissue sources and *ex vivo* expansion protocols will significantly impact the biology and therapeutic potential of the processed MSCs; hence, MSC processing is of prime importance. Additionally, we must avoid the pitfall of too much hype and too little data. To be sure, "home runs" are not likely. The development of broadly applicable MSC therapy for inborn errors, as with most cell therapy, will require carefully designed iterative clinical trials closely aligned with laboratory-based investigation. Although all of these disorders are rare, given the limited options and generally unsatisfactory outcomes with current approaches, MSC therapy may make the greatest impact in this arena.

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Chapter 24
MSCs for Enhancement of Hematopoietic Progenitor Cell Engraftment and Poor Graft Function

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Abstract Engraftment failure is a rare but life-threatening complication of hematopoietic cell transplantation (HCT). Newer approaches to HCT, including use of haploidentical donors, umbilical cord blood (UCB) transplant, nonmyeloablative and reduced-intensity conditioning regimens, appear to have an increased risk of graft failure. Multipotent mesenchymal stromal cells (MSCs) are essential bone marrow components that have the potential to differentiate in vitro into tissues along mesenchymal lineages, including bone marrow stroma. This regenerative potential, coupled with the capability to secrete cytokines and growth factors, suggests that MSCs would facilitate and promote hematopoiesis. Moreover, MSCs have immunoregulatory properties and thus could have an additional application in the setting of HCT by reducing both graft rejection and graft-versus-host disease. Initial trials have demonstrated the safety and feasibility of infusion of ex vivo-expanded autologous and allogeneic MSCs. Results from these early trials suggested MSCs may enhance hematopoiesis when infused at the time of HCT; however, subsequent trials have not yet provided confirmation that MSCs accelerate hematopoietic recovery when given shortly after HCT. Ongoing research initiatives include use of MSC infusions for patients who have some evidence of regenerating marrow but have delayed or incomplete hematopoiesis.

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_24,

Introduction

Hematopoietic cell transplantation (HCT) is a potentially curative therapy for a variety of benign and malignant hematologic diseases. In benign disorders, such as bone marrow failure syndromes, hemoglobinopathies, immunodeficiencies, and enzyme deficiencies, infusion of the normal donor-derived hematopoietic progenitor cells (HPCs) can reconstitute normal hematopoiesis and provide immunocompetent and metabolically intact progenitor cells. In malignant disorders, the autologous or allogeneic HPCs rescue patients from the myeloablative effects of escalated doses of chemotherapy; further, allogeneic immunocompetent cells may confer the added benefit of a "graft-versus-malignancy" (allogeneic) effect. The full clinical effectiveness of HCT is hampered by several barriers that relate both to the choice of the graft source as well as the type of conditioning regimen. In the autologous HCT setting, delayed, poor, or absent engraftment may result from infusion of insufficient numbers of HPCs. Umbilical cord blood (UCB) represents a valuable source of HPCs in patients who do not have available sibling-matched or matched unrelated donors, but slow engraftment of red cells, neutrophils, and platelets often is the norm. Other strategies include use of haploidentical donors (parents or children of the patient) that may be associated with an increased risk of engraftment failure [1]. In these situations, prolonged neutropenia carries a markedly increased risk of opportunistic infection. A prolonged time to restore production of red blood cells and platelets is associated with the dangers of increased alloimmunization, iron overload, and transmission of infections, in addition to the inconvenience of frequently receiving blood products and the associated significant financial cost.

Myeloablative conditioning regimens are fraught with added danger when administered to frail or elderly patients and to those with significant comorbidities. Further, graft-versus-host disease (GVHD) with or without opportunistic infection remains a significant complication of the posttransplant state. Reduced-intensity conditioning (RIC) and nonmyeloablative conditioning have been used with increased frequency to facilitate allogeneic HCT in patients who cannot tolerate the significantly higher-dose myeloablative chemotherapy. These approaches rely predominantly upon the donor effector cells in the graft to exert the therapeutic allogeneic or immune effect. Graft failure, however, is an important complication of RIC and nonmyeloablative allogeneic HCT, with rates reported between 18 and 42%, depending on the regimen used [2]. Finally, in vitro T cell depletion of the graft, for a variety of reasons, may be associated with engraftment failure states [3].

Multipotent Mesenchymal Stromal Cells (MSCs)

Multipotent mesenchymal stromal cells (MSCs) are essential components of the bone marrow microenvironment. These cells provide a supporting physical matrix and elaborate a variety of cytokines and other factors that have been shown in vitro and in vivo to support hematopoiesis [4]. As a result, over the last two decades,

MSCs have been the object of intensive research [5, 6]. These cells have the capacity to differentiate in vitro along mesenchymal lineages and give rise to multiple tissues, including bone, cartilage, adipose tissue, and bone marrow stroma; they also exhibit intense paracrine activity, secreting bioactive molecules with trophic and immunomodulatory capacity [7]. When infused, MSCs home to tissue sites of active inflammation [8] and participate in tissue repair [7]. The tissue regenerative potential has prompted interest in using MSCs to provide stability and restore function in organs such as the heart and nervous system, using intravenous as well as intrathecally administered MSCs [9–11]; the immunomodulatory or immunosuppressive activity of MSCs has led to novel research initiatives in autoimmune conditions [12] and in the treatment of GVHD. The combination of these characteristics, e.g., the capacity to enhance regeneration of the hematopoietic process and the bone marrow stroma, the immunomodulatory properties [13], and the secretion of bioactive agents [14], has led to the study of the role of MSCs in facilitating HPC engraftment and the prevention and treatment of graft failure after HCT.

Biologic Properties of MSCs

First identified by Friedenstein and colleagues in postnatal bone marrow more than 40 years ago [5, 15], MSCs have been isolated from a wide variety of adult organs, including periosteum, muscle connective tissue, perichondrium, and adipose tissue [16–19]. These cells also are present in fetal bone marrow, lung, liver, and spleen [20, 21]. Amniotic fluid and placenta are rich sources of MSCs, the latter containing both maternal and fetal MSCs [22, 23]. Their isolation from UCB has been reported with variable success [24]. The proportion of MSCs in bone marrow is small and varies among species, representing 0.001–0.01% of all nucleated marrow cells. Moreover, their number decreases with age, ranging from 1 MSC per 10,000 nucleated marrow cells in newborns to 1 MSC per 250,000 nucleated marrow cells in adults [25]. This paucity of MSCs in bone marrow and other tissues, along with the lack of specific markers, has made study of directly isolated cells difficult, and thus, little is known about the primary progenitor cell in vivo. On the other hand, MSCs have a remarkable capacity to undergo expansion in ex vivo culture settings, which has led to two important developments. First, most of the information regarding MSC function and phenotype is based on cells expanded in vitro. Secondly, as a small bone marrow sample can be expanded to yield hundreds of millions of cells, clinical use of culture-expanded MSCs has been made possible, and thus, clinical studies have been performed before a reliable preclinical animal model was readily available [26].

The wide variety of tissues from which MSCs can be harvested and the multiple available methods for isolation and expansion of cells prompted the International Society for Cellular Therapy (ISCT) to define minimal criteria for the definition of MSCs [6]. These criteria include the adherence to plastic under standard culture conditions; a specific phenotype (≥95% of the cells expressing CD105, CD73, and

CD90 and ≤2% of cells expressing CD45, CD34, CD14, CD11b, CD79a, or CD19 or HLA Class II); and the capacity to differentiate into osteoblasts, chondrocytes, or adipocytes under standard in vitro differentiating conditions. The population of plastic-adherent cells, however, is heterogeneous and only a small proportion of these cells can generate fibroblast colonies in vitro [27]. In 2005 the ISCT clarified the nomenclature for "mesenchymal stem cells," a term popularized in early 1990s by Caplan [25], to the current "multipotent mesenchymal stromal cells." This designation conveys the multipotentiality and tissue-regenerating capacity without ascribing to them the homogeneous quality of stem cells [25].

The range and mechanisms of the immunomodulatory properties of MSCs have not yet been fully elucidated. MSCs have been shown to suppress T cell proliferation and cytokine production in response to alloantigens and nonspecific mitogens [28, 29]. These cells appear to exert their influence both by soluble factors (IFN-gamma and nitric oxide appear to have a central role [30, 31]) as well as by direct cell-cell interactions [32]. MSCs also inhibit the proliferation, differentiation, and cytokine secretion of dendritic cells [33–35] and natural killer (NK) cells leading to decreased cell-mediated cytotoxic activity [36]. B cell proliferation, chemotaxis, antibody production, and terminal differentiation also are suppressed by MSCs [37–39].

In addition to their immunomodulatory properties, MSCs appear to be immunologically privileged cells [40]. Culture-expanded human MSCs express major histocompatibility complex (MHC) class I proteins, are negative for MHC class II proteins, and appear to lack expression of the costimulatory molecules CD80, CD86, and CD40 [41–44]. MHC class I expression and upregulation in the presence of increased interferon-gamma make MSCs less susceptible to NK cell lysis. MSCs can suppress the proliferation of CD4+ and CD8+ T lymphocytes [32] while selectively promoting proliferation of CD4+ CD25+ T regulatory lymphocytes [45]. When cocultured with allogeneic lymphocytes, MSCs did not induce lymphocyte proliferation [46]; and in animal models, infusion of allogeneic mismatched MSCs did not induce an immune response [8]. Moreover, the immunosuppressive effect of MSCs appears to be independent from MHC compatibility status [29]. Such data provide a scientific basis for undertaking investigations of infusing MSCs obtained from unrelated or mismatched donors for immunomodulation and promotion of engraftment in the setting of HCT.

Clinical Studies

Early Studies

As a result of differences in the immunoregulatory properties of MSCs between species, no adequate preclinical animal models are available to predict the in vivo function of MSCs in the HCT setting [47]. The establishment of human MSC expansion methods, however, has facilitated their clinical use. The consequence has been

a unique situation in which clinical trials of MSC infusion were initiated before complete understanding of the in vivo properties of these cells [48].

In 1995 Lazarus and colleagues [49] published the first clinical trial using MSCs. This pilot study examined collection, ex vivo expansion, and reinfusion of autologous bone marrow-derived MSCs obtained from 23 hematologic malignancy patients. The procedure, demonstrated to be safe and feasible, was followed by a phase I–II trial of autologous, culture-expanded bone marrow-derived MSC infusion after high-dose chemotherapy and autologous peripheral blood progenitor cell transplantation in 28 breast cancer patients [50]. No toxic effects directly attributable to MSC infusion were observed and hematologic recovery was rapid. Blood neutrophil engraftment (neutrophils > 500/μL) occurred at a median of 8 days and untransfused platelet count exceeded 20,000/μL in a median of 8.5 days. Unfortunately, no firm conclusions regarding the benefit in hematopoietic engraftment could be drawn due to the nonrandomized nature of this trial.

Frassoni and coworkers [51] retrospectively examined a matched-pair analysis of 31 hematologic malignancy patients undergoing HLA-identical, sibling-matched HCT procedures at the US and European centers. Patients received culture-expanded bone marrow-derived MSCs harvested from the HLA-identical HPC donors. Compared with historic controls, study patients had a statistically significant lower incidence of acute and chronic GVHD and superior survival rates after 6 months of follow-up [51]. No observations were reported regarding engraftment rates. These initial trials spearheaded the use of human MSCs, demonstrating the safety and feasibility of their use, both in the autologous and HLA-identical allogeneic setting, and suggested these cells may have therapeutic potential in the HCT setting, as engraftment enhancers or prophylaxis of GVHD.

Infusion of MSCs for Enhancement of Hematopoietic Engraftment

Recently, several trials have been undertaken to evaluate the coadministration of bone marrow-derived, culture-expanded MSCs along with varied sources of HPCs during allogeneic HCT for the purpose of correcting or preventing engraftment failure. The Karolinska University clinical group [52] reported seven patients who underwent cotransplantation of HPCs and MSCs. Three patients were treated for a previous graft failure or rejection. The remaining four were part of a pilot study designed to enhance hematopoietic engraftment in which haploidentical MSCs were given. Despite remarkable variability in the patient population, source of HPCs and MSCs, and HLA compatibility status of donors, all patients had hematopoietic engraftment, with median time to neutrophil engraftment ($ANC > 0.5 \times 10^9/L$) of 12 days (range 10–28 days) and median time to platelet engraftment ($platelets > 30 \times 10^9/L$) of 12 days (range 8–36 days). While these are encouraging results, the patient population, underlying diagnoses and transplant settings were markedly heterogeneous, making difficult a generalization of the findings on the effects of MSCs.

Ball and colleagues [53] cotransplanted MSCs and HPCs obtained from haploidentical related donors into 14 children who had hematologic malignancies, immune deficiencies, and nonmalignant disorders. HPC engraftment was demonstrated in all patients. Mean MSC dose was 1.6×10^6 MSC/kg (range 1–3.3×10^6 MSC/kg). When compared to historic controls ($n = 47$), the 14 study patients had comparable platelet and neutrophil recoveries yet faster attainments of a total blood leukocyte count $> 1 \times 10^9$/L. Acute and chronic GVHD rates were comparable with historic controls.

Macmillan and colleagues [54] infused ex vivo-expanded MSCs in conjunction with an UCB transplant. Fifteen children with hematologic malignancies received UCB grafts; 8 subjects also received parental haploidentical MSCs infusions on the day of UCB transplant, while 3 received repeat MSC infusions 21 days later. A second MSC infusion could not be given to the remaining five patients secondary to insufficient growth of parental MSCs. The median MSC dose on day 0 was 2.1×10^6 MSC/kg (range 0.9–5×10^6 MSC/kg). Haploidentical MSC infusions at the time of UCB transplant were shown to be safe, but tempo to recovery of neutrophil and platelet engraftment as well as the rates of acute GVHD were similar to those of historic controls.

A recent multicenter European trial explored the coinfusion of culture-expanded parental MSCs in 13 children who received UCB grafts obtained from related or unrelated donors [55]. Median MSC dose was 1.9×10^6 MSC/kg (range 1–3.9×10^6 MSC/kg). The incidence of graft failure or the rate of neutrophil and platelet engraftment did not differ statistically from that observed in 39 historic controls matched for the diagnosis and type of UCB donor. Overall survival rates also did not differ. On the other hand, there were no cases of severe acute GVHD (grades III–IV) in patients who received MSC coinfusion, compared to 10 (grade III, 7 cases and grade IV, 3 cases) of 39 patients in the historic controls. The authors postulated that in the UCB transplant setting, graft failure is more a function of the low numbers of infused HPCs rather than an immune-mediated mechanism; hence, MSC coinfusion may not be justified in the UCB transplant setting. However, MSCs may enhance hematopoietic engraftment via additional, non-immune-mediated mechanisms, such as cytokine release and reconstitution of the bone marrow stroma, effects that may be of particular importance in cases with borderline hematopoiesis.

Gonzalo-Daganzo et al. reported [56] a phase I–II study in which nine hematologic malignancy patients received third-party MSCs 1–24 h after coinfusion of UCB grafts and third-party HPCs (UCB/HPC). The median (range) MSC dose was $1.18\ (1.04–2.22) \times 10^6$ MSC/kg. No adverse effects of MSC infusion were observed. Hematopoietic engraftment and achievement of full UCB chimerism appeared to be delayed in the study group when compared with 46 controls that received UCB/HPC coinfusion alone, although the differences were not statistically significant. The incidence of acute GVHD also did not differ statistically from that observed in control patients. Two patients who received additional subsequent MSC infusions for treatment of corticosteroid-refractory GVHD attained complete responses. This study showed that MSC infusion at the time of cotransplantation of UCB/HPC is

safe and well tolerated, but the small number of patients included precludes further conclusions from being drawn regarding the effect of MSCs on engraftment and GVHD incidence.

Finally, Baron and colleagues [57] reported a recent study using cotransplantation of MSCs and HLA-mismatched MSCs after nonmyeloablative conditioning. Twenty hematologic malignancy patients were compared to 16 historic controls who also received nonmyeloablative conditioning and HLA-mismatched HCT. MSC cotransplantation was associated with a decrease in 1-year nonrelapse mortality (HR = 0.2, 95% CI 0.04–0.9, $p = 0.03$) and 1-year overall mortality (HR = 0.4, 95% CI 0.1–0.9, $p = 0.03$). Severe, acute GVHD was low in the cotransplantation group, without an increased relapse risk, suggesting that GVHD was ameliorated without abrogating the graft-versus-tumor effect. Engraftment was prompt in both groups but there was no discernible enhancement of engraftment with MSC coinfusion; one patient receiving MSCs experienced primary graft failure. This study shows that MSC cotransplantation is feasible in the setting of nonmyeloablative HLA-mismatched HCT, where the risk of GVHD and graft rejection is higher, but no clear engraftment benefit was reported.

In summary, studies of MSC infusion at the time of HCT aimed at enhancement of engraftment, and prevention of HPC graft failure have not yielded conclusive results. While safety of MSC infusion largely has been demonstrated, the studies assessing the effect of MSC infusion on engraftment have been limited to small, nonrandomized studies that utilize historic controls. Results have been variable and several studies have failed to demonstrate engraftment enhancement with MSCs. Larger, randomized studies are needed to settle whether the use of these cells is justified.

Infusion of MSCs for Treatment of Graft Failure

Primary graft failure after HCT represents a rare but serious, life-threatening complication of the HCT procedure. Management strategies include use of recombinant hematopoietic growth factors, modifications of immunosuppressant regimens, infusion of "backup" autologous HPCs, or second allogeneic HCT. Outcomes remain dismal, even after second allogeneic HCT, as evidenced by a recent observational study from the Center for International Blood and Marrow Transplant Research (CIBMTR) [58]. Such patients with prolonged bone marrow failure are at high risk of succumbing to infection and hemorrhage.

In addition to development of strategies for early identification of those patients at high risk of engraftment failure, new therapeutic alternatives are needed for this complication. MSCs may prove of value in this setting, with their bone marrow stromal regenerative potential and the additional capacity to ameliorate graft rejection without significantly increasing risk of infection.

Fouillard and colleagues [59] reported a case of a 40-year-old woman with AML in complete remission who exhibited primary graft failure after autologous HCT. Partial recovery of her counts was achieved with administration of granulocyte colony-stimulating factor and erythropoietin therapy administered three times a week. Three years after HCT she received culture-expanded MSCs harvested from her HLA-mismatched brother. MSC dose was 2.78×10^6/kg. Rapid and sustained recovery of her neutrophil and platelet counts was observed, while no effect on hemoglobin concentration was observed. Granulocyte-macrophage colony-forming units (CFU-GM) and colony-forming unit fibroblasts (CFU-F) were increased 1 month and 1 year after MSC infusion. There were no side effects or adverse reactions to the MSC infusion and no GVHD was observed. Studies of MSC engraftment in the recipient 1 month post MSC infusion showed male DNA was detected at a frequency of 10^{-5} per cell, whereas 1 year after infusion, it was no longer detectable, results that are compatible with very low levels of MSC engraftment observed in animal studies. This report suggests that MSCs can potentially be used for treatment of engraftment failure, although the mechanism mediating their benefit is not yet elucidated.

Fang and colleagues [60] reported two pediatric patients with severe aplastic anemia (SAA) who presented with graft failure after receiving HLA-identical sibling peripheral blood HCT. The first case, an 11-year-old girl, had relapse of SAA after her second HLA-identical sibling HCT. Haploidentical culture-expanded adipose tissue MSCs harvested from the patient's mother were infused after a third infusion of HPCs from her HLA-identical sister. Neutrophil count reached 0.6×10^9/L by 16 days after HCT, while the platelet count apparently recovered by 20 days, although the details were not provided by the authors. No acute or chronic GVHD was observed. The second case was a 12-year-old boy with SAA in relapse after HLA-identical sibling HCT. A second HCT from the same donor was followed by infusion of culture-expanded adipose tissue MSCs harvested from his mother. Neutrophil recovery occurred by 15 days while self-sustaining platelet count was achieved by 19 days. Two months after transplant the patient experienced "grade alpha" (apparently grade I) acute GVHD of the skin that completely responded to corticosteroid therapy. Both patients had sustained hematopoietic function beyond 2 years at the time of publication. Several reports have documented the safety and feasibility of infusion of culture-expanded MSCs harvested from adipose tissue [61, 62]. These reports suggest that coinfusion with HPCs may improve the rates of graft failure. Although referred to as equivalent to marrow-derived MSCs, experience with this source of cells is still limited and it is possible that there are subtle biologic and immunologic differences not yet identified. These positive results warrant proceeding with larger studies of MSC infusion and coinfusion with HPCs for treatment of engraftment failure.

Meuleman and coauthors [63] recently reported the results of a pilot clinical trial of infusion of culture-expanded MSCs without coinfusion of HPCs. Patients included had received HCT and achieved full donor chimerism but with poor engraftment. The authors defined the latter as persistent posttransplant

pancytopenia, with blood neutrophils $< 1 \times 10^9$/L and platelets $< 50 \times 10^9$/L at 30 days after HCT and despite treatment with a minimum of 10 days of granulocyte colony-stimulating factor. Patients with complete engraftment failure or rejection were excluded from this study. Six patients were included, all received myeloablative, allogeneic, mobilized peripheral blood HCT; three patients received HLA-identical related donor and three haploidentical related donor hematopoietic grafts. Although full donor chimerism was observed in all cases, bone marrow examination showed hypoplasia and all patients had varying degrees of pancytopenia. MSCs were obtained from bone marrow aspiration of the original HPC donors. The MSC dose was 1×10^6 MSC/kg. No acute side effects to MSC infusion were observed. Two patients manifested hematologic recovery after MSC infusion; both had received HLA-identical sibling HCTs and were in first complete remission, in comparison to the more heavily pretreated other subjects. One patient presented early CMV infection (day 12) and subsequently died several months later from repeat CMV infection. The relationship between the viral infection and MSC infusion is unclear, as MSCs have not been shown to affect virus-specific T cell function [64, 65]. This study demonstrates the feasibility of MSC infusion without HPCs and suggests MSCs may aid in hematopoietic recovery in those patients who have residual hematopoiesis, likely through a combination of stromal reconstruction, cytokines, and modulation of rejection. Therefore, heavily pretreated patients or those with profound pancytopenia may benefit from larger doses of MSCs and possibly with coinfusion with HPCs as other investigators [52] have observed.

Conclusions

Although MSCs have been the subject of intensive laboratory and clinical research over the last 15 years, their in vivo properties and effects after administration in the clinical setting of HCT have not yet been fully established. Their biologic properties, including constitutive secretion of bioactive molecules, capacity to differentiate into bone marrow stroma, and remarkable immunomodulatory capacity, have suggested that these cells may have a role to facilitate hematopoietic engraftment and prevent development of GVHD.

Small pilot studies demonstrated the safety of infusion of HLA-compatible MSCs and suggested a possible benefit in hematopoietic engraftment rates. Subsequent studies have demonstrated the feasibility of HLA-haploidentical and HLA-unmatched MSCs in a variety of settings, including coinfusion with HLA-identical and haploidentical peripheral blood HPCs as well as with UCB grafts. These studies, however, have not conclusively demonstrated that MSCs provide an advantage in terms of hematopoietic engraftment rates. Larger, randomized studies have been performed to evaluate the effect of MSCs for treatment and prophylaxis of GVHD. In the next few years, we anticipate the design and conduct of trials to evaluate the effect of MSC infusion on hematopoietic engraftment.

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Chapter 25
MSCs for Graft-Versus-Host Disease

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Abstract Clinical graft-versus-host disease (GVHD) is a severe inflammatory condition and the main immune complication of allogeneic hematopoietic cell transplantation (HCT). While most patients respond favorably to standard treatment interventions, others do not. Thus, GVHD remains the principal limitation to the wider application of HCT. Even with dramatic increases in our understanding of the pathobiology of GVHD over the last half century, true progress in clinical care for individuals with GVHD has been limited. Recently, the unexpected ability of cultured mesenchymal stromal cells (MSCs) to modulate immune responses has captured considerable scientific and clinical interest because of their potential to limit immune injury and to repair tissues. Diverse non-hematopoietic cell types present in bone marrow, collectively termed stromal cells, provide a conceptually novel and practically elegant opportunity for anti-GVHD therapy. Here, we summarize the MSC experience most relevant to GVHD therapy and the reasons that MSCs hold the promise of fulfilling a major unmet need in the management of clinical GVHD.

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_25, © Springer Science+Business Media New York 2013

Introduction

Three preconditions for GVHD were proposed by Billingham in 1966 [1]. First, immunologically competent cells (i.e., mature T lymphocytes) need to be contained in the donor graft. Second, the recipient needs to be immunocompromised. This situation is created by the conditioning regimen of intensive chemotherapy or chemoradiotherapy delivered before HCT to make space for the new donor hematopoietic system and to reduce the likelihood for host immune rejection of the allogeneic donor hematopoietic graft. Third, the recipient must express tissue antigens that are different from the donor tissue antigens (or alternatively, express host self-antigens that are recognized inappropriately). Such human leukocyte antigens (HLA), encoded by the genes within the major histocompatibility complex (MHC) cluster, are expressed on all nucleated cells in the human body. When host cells recognize donor cells as foreign, this interaction initiates activation of allogeneic T cells by host antigen-presenting cells, such as dendritic cells [2]. Physical injury, especially to the gastrointestinal tract, as a consequence of the conditioning regimen used before HCT, causes a cytokine storm (e.g., tumor necrosis factor alpha-TNFα) and thus clonal expansion of donor T cells [3]. Donor T cell immune recognition of host antigens and activation of allogeneic T cells mediate further expression of cellular and inflammatory factors (e.g., from activated mononuclear phagocytes) that collectively amplify the local tissue injury and general inflammatory response.

Graft-Versus-Host Disease

Clinical Phenotype

While GVHD is a multi-organ, destructive disorder, the relevant target organ damage involves epithelial cell necrosis—most obvious in the skin, gastrointestinal tract, and liver. A maculopapular rash often begins on palms and soles and may become generalized erythroderma with desquamation and bullae. Lower gastrointestinal tract involvement manifests in abdominal pain and voluminous secretory and bloody diarrhea. Upper gastrointestinal disease is characterized by anorexia, dyspepsia, nausea, and vomiting. Hepatobiliary dysfunction presents typically with hyperbilirubinemia and jaundice.

Standard Therapy

Without intervention, almost all HCT recipients develop significant acute GVHD [4], accompanied by profound immunosuppression and risk of fatal bacterial, viral, and fungal infections. Therefore, multiple drugs typically used in combination

(e.g., cyclosporine A, tacrolimus, methotrexate, and mycophenylate mofetil) or strategies to deplete T cells *ex vivo* or in vivo have to be used to prevent the donor anti-host immunological complications of allogeneic HCT. Despite universally used preventative measures, development of GVHD accounts for the largest share of morbidity and mortality after HCT. Virulent cases of acute GVHD require therapy, typically with corticosteroids as a first-line treatment. If the GVHD is severe or unresponsive to steroids, second-line treatments such as anti-thymocyte globulin (a polyclonal immunoglobulin prepared by injecting rabbits or horses with lymphocytes) or other approaches such as extracorporeal photopheresis are used.

MSCs

Even though MSCs in living organisms may function as pericytes (i.e., adventitial reticular cells in the subendothelium of vascular wall) [5] and serve as a reservoir of reparative cells in parenchymal organs, their identity and physiological functions remain an enigma. Nevertheless, the MSCs obtained from bone marrow, umbilical cord blood, placenta, Wharton's jelly, and adipose tissue can be easily cultured. While MSCs isolated from different tissues differ, and even the ones isolated from the same tissue remain heterogeneous, they retain adherence to plastic and a remarkable capacity to expand rapidly in vitro, which has allowed attempts at cellular definition and experimentation [6–8].

In addition to the ability of cultured MSCs to engage in tissue repair (predominantly by paracrine mechanisms) [9–11] and their putative supportive role in the engraftment of hematopoietic cells [12–15], MSCs are anti-inflammatory, antiproliferative, anti-apoptotic, anti-fibrotic, pro-angiogenic, and immunomodulatory [16–22]. With respect to the immunomodulatory properties of MSCs, recently reviewed by us [23] and others [24], MSCs typically inhibit the proliferative response of naive T cells to allogeneic antigen-presenting cells (APCs) [17, 25–28], resulting in reduced expression of MHC class I and II antigens and costimulatory molecules [16, 29–32]. Moreover, the inhibitory effects of MSCs on monocyte maturation may incapacitate APCs such that they are unable to maximally support a T cell response [16, 29, 30, 33, 34]. MSCs can secrete immune suppressive molecules such as prostaglandin-E2 (PGE2) [16], transforming growth factor β-1 [26], and IL-10. Upregulation of intracellular pathways such as the essential amino acid catabolic pathway indoleamine 2,3 dioxygenase (IDO) by MSCs [35] results in a state of amino acid starvation (tryptophan depletion) and the accumulation of potentially toxic metabolites that suppress T cell immune responses [36]. Upregulation of stress response pathways [37–39] contributes to immune suppression. T cells exposed to MSCs fail to efficiently progress through the cell cycle that leads to the generation of immune regulatory cells such as CD4+ 25+FoxP3+ T regulatory cells (Tregs). In turn, Tregs can suppress T cell proliferation, Interferon gamma (IFNγ) secretion, and GVHD lethality [40, 41]. The ability of MSCs to modulate immune response appears to be linked to their antiproliferative effects, which are independent of tissue of origin and stage of culture, but

may be enhanced by activation of MSCs, e.g., by pretreatment with INFγ. Their immunosuppressive properties have been harnessed in a recent surge of clinical applications—several thousand patients are estimated to have been treated with MSCs to date—most of which target steroid-resistant GVHD. The canonical work on this subject has been done by Le Blanc and colleagues [20, 42, 43].

MSC Therapy in Murine Models

Despite their immune suppressive properties, MSCs are not uniformly efficacious in preventing murine GVHD. For example, allogeneic or syngeneic MSCs failed to reduce GVHD lethality in some studies. In our own studies, allogeneic MSCs did not home to secondary lymphoid organs and were unable to reduce GVHD lethality. Even upon intrasplenic injection, GVHD lethality was unimpaired [32], although some rodent GVHD studies have shown that MSCs can be efficacious in that setting [44–48]. It is likely that the location of the immune suppressive population and its persistence at sites of GVHD initiation are critical determinants of their potency. Additional factors such as timing of the MSC infusion and presence of pro-inflammatory and anti-inflammatory cytokines—especially within the microenvironment in which MSCs reside—likely influence their biological potency.

MSC Therapy in Clinic

In humans, GVHD that is resistant to standard therapy with steroids, calcineurin inhibitors, and anti-thymocyte globulin is almost always lethal. Thus, when the immunomodulatory capacity of MSCs was uncovered, they were quickly applied for this purpose. Clinical trials of MSCs for various indications have been reviewed recently [23].

First reported in 2004, MSC infusion improved gastrointestinal and hepatic manifestations of severe GVHD. Remarkably, when the signs and symptoms of GVHD returned in this single patient, subsequent MSC infusion improved the clinical status again [43]. The first patient to receive MSC treatment for GVHD was a male patient with grade IV acute GVHD of the gut and liver who had undergone allogeneic stem cell transplantation (SCT) with a matched unrelated donor [43]. The patient's GVHD was unresponsive to all types of immunosuppression. He was infused with 2×10^6 haploidentical MSC/kg recipient weight with a miraculous response including a decline in bilirubin and normalization of stools. After infusion of MSCs, a DNA analysis of the patient's bone marrow showed minimal residual disease (MRD) of his acute lymphoid leukemia (ALL). After discontinuation of cyclosporine, the acute GVHD recurred but was still responsive to a second MSC infusion. Encouraged by this proof-of-principle, eight additional patients with steroid-refractory GVHD were treated, six of whom had a favorable clinical response [49].

A larger European cooperative trial [42] followed with 55 patients, 30 of whom had a complete clinical response. These data have served as a platform for further improvements. By taking into account the biology of acute GVHD and its treatment, the potential therapeutic benefit of MSC infusions can be optimized.

First, it is reasonable to assume that an early, rather than late, intervention with MSC infusions is more beneficial to the patient. Compared to more than 3 weeks between the onset of GVHD and MSC therapy in studies before 2009, new trials aim to administer MSCs in about a third of that time.

Second, in order to circumvent the possibility of an immune response to the fetal bovine serum used to prepare the MSCs [50, 51], Le Blanc and colleagues cultured MSCs in platelet lysate [52, 53]. Therapy with such MSCs led to clinical responses in approximately one-half of the patients [52, 54].

Third, steroid-responsive GVHD may be suitable for MSC therapy. The first data on MSC therapy in de novo GVHD [55] suggest that the treatment response may be higher in that setting. Related to that, MSCs may be beneficial in the prevention of GVHD as well. Of the nearly 50 patients with leukemia who were co-infused with HSCs and MSCs from their HLA-matched sibling donors, approximately one-third developed acute GVHD and two-thirds experienced chronic GVHD [56]. When reduced intensity conditioning and third party MSCs were used in patients with leukemia, the survival was improved compared to patients who have not received MSCs, presumably due to fewer GVHD-related complications [57]. Similarly, when MSCs were co-infused with umbilical cord blood grafts, fewer cases of GVHD occurred when compared to the control patients who received no MSCs [58–60].

Although acute and chronic GVHD are linked, the first reports on patients treated for cGVHD did not indicate an apparent benefit from MSC infusion [49, 53]. Subsequently, Zhou et al. reported on four patients treated with repeated MSC infusions for sclerodermatous GVHD, with gradual improvement [61]. More recently, 19 HCT recipients with standard treatment-refractory chronic GVHD have been treated with MSC infusions [62]. The cumulative response for skin was 78%; responses in oral mucosa, liver, and gastrointestinal tract were 90–100% as graded by the NIH scoring system [63]. A helper T cell imbalance, with an increase in IL-4- and IL-10-producing Th2 cells, has been implicated in the pathogenesis of both cGVHD and other immune-mediated disorders. In accordance with this hypothesis, in the study by Zhou et al., MSC treatment decreased Th2 cells.

Mechanisms of Action of MSCs

The view of the mechanisms whereby MSCs function as immunomodulatory and reparative cells has evolved simultaneously. Initially, most studies focused on T cells, but recently it has become increasingly clear that other immune cells, such as natural killer (NK) cells, B cells, dendritic cells, and even monocytes and macrophages, are influenced by MSCs. This interaction, however, is not simple [24]:

- Both expression of soluble factors and cell-cell contact between MSCs and immune cells appear to be operational in MSC-mediated immune modulation.
- These interactions are sensitive to cell numbers and concentrations of individual soluble factors [64, 65].
- MSC behavior changes over time after initial immune injury and is dependent on histological context [26, 27].
- It remains unknown whether these effects are nonspecific and antiproliferative in nature, or whether genuine suppression of immune cells occurs [8].
- Most information has come from in vitro experimentation and animal models, and thus its relevance for meaningful clinical applications remains to be determined [66].

MSCs are likely to persist in clinically meaningful numbers only briefly after intravenous infusion. Despite that, it appears certain that MSCs regulate effector and regulatory immune cells in complex and significant ways [23]. In addition to the interactions described above, other factors—such as the microenvironment in which MSCs reside in vivo or into which they home after infusion, the timing of the MSC infusion, and the presence of pro-inflammatory and anti-inflammatory cytokines—are likely to be critical determinants of their immunomodulatory potency. In an even broader view, the biological potency of MSCs on the regulation of alloresponses is almost certainly intertwined with other functions of these multitalented cells, most prominently with their ability to engage in productive tissue repair [11].

Conclusions

Evidence that MSC therapy is safe is quickly increasing. The risk of human (unlike mouse) [67] MSCs becoming immortal and tumorogenic during *ex vivo* expansion appears extremely low. No significant infusional toxicity associated with MSC application has been described. Furthermore, engraftment of infused cells appears low, and ectopic tissue formation has not been observed in human MSC recipients. Still, there are many variables relevant to the application of MSCs in the therapy and prevention of GVHD: MSC cell dose, age and sex of the donor, tissue source of MSCs, cell expansion culture protocol used and number of population doublings before infusion, stringency of batch-release criteria, MSC purity and composition, single or serial MSC infusions, impact on GVHD in children versus adults, with myeloablative versus non-myeloablative conditioning, recipients of transplantation for malignant or nonmalignant disease, and impact on relapse [68] and on rate of infections after transplantation.

It is critical to realize, however, that the efficacy of MSC therapy is still not entirely clear. We do not know which patients with GVHD benefit the most from MSC therapy. MSCs need to be studied both in mechanistic preclinical models and in clinical trials with well-defined endpoints and controls to better understand the therapeutic potential of these multifunctional cells. This should also be matched

with clinical observations, which would help predict the response of individual patients in order to make MSC therapy patient specific and thus more efficacious.

Grant Support Acknowledgment

Jakub Tolar: Children's Cancer Research Fund, MN; Fanconi Anemia Research Fund; Progeria Research Foundation; and NIH R21 A1079755.

Katarina Le Blanc: The Swedish Cancer Society, the Children's Cancer Foundation, the Swedish Research Council, the Tobias Foundation, the Cancer Society in Stockholm, the Swedish Society of Medicine, the Stockholm County Council, the Sven and Ebba-Christina Hagbergs Foundation, and Karolinska Institutet.

Bruce R. Blazar: R01 HL56067 and R01 AI 34495.

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Chapter 26
MSCs in Pediatric Hematopoietic Stem Cell Transplantation

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Abstract Hematopoietic stem cell transplantation (HSCT) is an accepted treatment for some children with certain life-threatening conditions such as malignant diseases, immune deficiency, inborn errors of metabolism, bone marrow failure syndromes, and hemoglobinopathies. The introduction of alternative donor population such as cord blood and haploidentical transplantations has resulted in this treatment becoming more readily available. Inherent risks with alternative donor sources are mainly immune-mediated complications, with graft rejection or graft failure and graft-versus-host disease (GVHD) as main obstacles. Mesenchymal stromal cells (MSCs) through cell-to-cell interactions, production of growth factors, and secretion of matrix proteins play a vital

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_26,

role in the regulation of hematopoiesis and exhibit a wide range of immunomodulatory and anti-inflammatory properties in vitro and in vivo. Use of MSCs in phase I/II clinical studies with HSCT in children indicates that their infusion is safe and effective in preventing graft failure after T-cell-depleted allogeneic HSCT from an HLA-disparate relative as well as in rescuing patients with severe, steroid-refractory GVHD.

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is a proven treatment for selected children with malignant and nonmalignant hematological conditions, either acquired or hereditary diseases [1]. Since the first successful bone marrow (BM) transplants were reported [2, 3], significant changes and developments in transplantation medicine have been made. In this respect, one of the most relevant insights has been the use of alternative donors and sources for hematopoietic stem cells. Indeed, for many years the only type of donor used was an HLA-identical sibling. In the 1980s, the establishment of unrelated donor registries enabled the option of transplantation to a significant proportion of patients lacking a suitable HLA-compatible family donor. Registries of volunteers, currently containing more than 16 million HLA-typed potential stem cell donors, have been established worldwide to facilitate unrelated donor transplantation [4]. This large number of unrelated volunteers has resulted in an increase in the percentage of nonidentical sibling HSCT, from 32% between 1991 and 1994 to 61% between 1999 and 2002 [5]. Despite this, a significant number of children requiring urgent HSCT lack an HLA-identical donor, either related or unrelated. Moreover, substantial delays in finding a suitable donor outside the family can impair the possibility of a successful transplant either because of relapse during the donor search or due to the interval development of complications precluding transplant eligibility.

In the last decade, haploidentical mismatched family members, as well as unrelated umbilical cord blood (UCB) units, have been successfully used to allow patients lacking an HLA-identical donor to undergo HSCT [6–8]. The advantage of using either a haploidentical relative or UCB mainly refers to the ease and speed of stem cell procurement, making them exceptionally useful, where HSCT is urgently required, as well as providing alternative transplant opportunities to children otherwise without a compatible donor. Recent studies have shown that both these sources are acceptable alternatives to an HLA-matched unrelated donor HSCT [1, 9]. The choice between these options is determined by the patient's disease and condition, the urgency of transplant, the associated risk/benefit ratio to the patient, and, last but not least, the treatment center experience. However, graft failure, graft-versus-host disease (GVHD), poor immune reconstitution, and subsequent infections complicate alternative donor HSCT and reduce the overall effectiveness of this approach. As such, innovative strategies are required to reduce the burden of these effects and thereby improve outcome. Mesenchymal stromal cells (MSCs), because of their unique characteristics, have been used in clinical phase I/II studies in children as a strategy to minimize transplant-related morbidity and mortality associated with allogeneic HSCT.

Mesenchymal Stromal Cells

In addition to hematopoietic stem cells, the BM comprises a population of MSCs, which represent the precursor cells for stromal tissues and are known to support hematopoiesis [10, 11]. In particular, marrow stromal cells comprise a heterogeneous population of cells, including reticular endothelial cells, fibroblasts, adipocytes, and osteogenic precursor cells that provide growth factors, cell-to-cell interactions, and matrix proteins that play a role in the regulation of hematopoiesis [12]. Initially, MSCs were considered to function only as a hematopoietic supportive network, but recent research has shown they are integral to the development of the stem cell niche. They play a crucial role in the development and differentiation of the hematopoietic system through cell-to-cell interactions and by secreting a number of growth factors and regulatory cytokines [13–15]. Although BM serves as the primary reservoir for MSCs, their presence has been reported in a variety of other tissues [16–18]. The very low frequency of MSCs in certain tissues such as peripheral blood and umbilical cord blood has led to controversy as to whether or not MSC can be isolated from such sources [19–22]. In fetal blood the frequency has been reported to decline with gestational age, from about $1/10^6$ mononuclear cells (MNCs) in first-trimester fetal blood to $0.3/10^6$ MNC in term cord blood [18]. Recently, MSCs have been successfully isolated from human amniotic fluid [23]. At present no unique phenotype has been identified that allows the reproducible isolation of MSCs precursors with predictable developmental potential. The isolation and characterization of stromal cell fraction, therefore, still relies primarily on their ability to adhere to plastic and their expansion potential. No specific marker has been shown to specifically identify true MSCs, and *ex vivo* expanded cells are characterized by a combination of both positive (CD105, CD73, CD90, HLA class I) and negative (CD34, CD45, CD14, CD31) markers [11, 24].

Recently, the identification and prospective isolation of mesenchymal progenitors, both in murine and human adult BM, have been reported, based on the expression of specific markers [25–31]. Despite the identification of these new MSC markers, none of the available reagents are capable of identifying true mesenchymal progenitors. Whether culture-expanded MSCs differ from their progenitors *in vivo* is uncertain, as proliferation on plastic surfaces and culture conditions may induce both phenotypic and functional changes. Techniques have become available to isolate and grow mesenchymal progenitors and to manipulate their growth under defined *in vitro* culture conditions. As a result, MSCs can be rapidly expanded to numbers that are required for clinical application. This has allowed the clinical testing of culture-expanded MSCs in the context of hematopoietic stem cell transplantation.

Standard conditions for expansion of MSC's include the presence of serum, in most instances fetal bovine serum (FBS), with serum batches routinely prescreened to guarantee both the optimal growth of MSCs and the biosafety of the cellular product [32–34]. The use of FBS has raised concerns about the possible transmission of zoonoses or, especially if repeated infusions are needed, the risk of immune reactions

in the host and consequent rejection of the transplanted cells [35, 36]. Indeed, Horwitz et al. reported sensitization in a child with *osteogenesis imperfecta* treated with repeated infusions of MSCs [36]. Doucet et al. first demonstrated that the growth factors contained in platelet lysate (PL) were able to promote MSC expansion in a dose-dependent manner [37]. This observation was further corroborated by the data published by other groups, showing that a culture medium supplemented with 5% PL is superior to 10% FBS in terms of clonogenic efficiency and proliferative capacity of MSCs [38, 39]. The use of PL resulted in a more efficient expansion with significant time saving while preserving comparable *in vitro* MSC immunomodulatory functions [38, 39]. To date, clinical data on the safety and efficacy of MSCs have been obtained mainly with cells expanded in the presence of FBS. Little comparable data is available with MSCs cultured in alternative medium supplements. Extensive experimental and clinical testing is required before MSCs expanded in the presence of alternative expansion media can safely substitute FBS-prepared MSCs in clinical studies. The EBMT consortium has developed common expansion protocols for MSCs isolated from bone marrow to be used in its on-going and future clinical studies.

Immunomodulatory Properties of MSCs

Experimental models suggest that MSCs have potent immunomodulatory effects, primarily through the inhibition of effector functions, thus offering a promising option for treating immune-mediated disorders including GVHD and autoimmune diseases (AID) [40–45]. MSCs are poor antigen-presenting cells and do not express MHC class II or co-stimulatory molecules. They have been demonstrated to suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli [40] and inhibit the response of naïve and memory antigen-specific T cells to their cognate peptide [40]. Accordingly, expanded MSCs do not stimulate T cell proliferation in mixed lymphocyte reactions (MLR) and are able to downregulate alloreactive T cell responses when added to mixed lymphocyte cultures [40, 41]. However, in an immunocompetent host, MSCs have been shown to elicit an immune response (in the context of a murine model of reduced intensity allogeneic stem cell transplantation) [42]. Human MSCs altered the cytokine secretion profile of DCs and naive and effector T cells (T helper 1 [T(H)1] and T(H)2) to induce a more anti-inflammatory or tolerant phenotype [41–43]. Although various hypotheses have been proposed, the mechanisms at the basis of MSC suppression of T cell proliferation remain unclear. Most studies demonstrate that soluble factors are involved, as the separation of MSC and peripheral blood MNCs by trans-well permeable membrane does not prevent the inhibition of proliferation. Various interactions mediating suppression of T cell proliferation have been proposed, but as yet the mechanisms remain unclear. What is evident is that most studies demonstrate that soluble factors are involved, as the separation of MSC and peripheral blood MNCs by trans-well permeable membrane does not prevent the inhibition of proliferation [46, 47]. MSCs are capable of inhibiting the maturation of monocytes into dendritic cells (DCs) and of skewing mature DCs to an immature DC state [48, 49]. MSCs upregulate CD4+CD25+

Fox P3+ cells (T regulatory immunophenotype) [41], albeit their depletion has no effect on the inhibition of T cell proliferation by MSC [42]. At low NK-to-MSC ratios, MSCs alter the phenotype of natural killer (NK) cells and suppress proliferation, cytokine secretion, and cytotoxicity against HLA class I-expressing targets [43]. Some of these effects require cell-to-cell contact, whereas others are mediated by soluble factors, including transforming growth factor β1 and prostaglandin E2, suggesting the existence of diverse mechanisms for MSC-mediated NK-cell suppression. On the other hand, MSCs are susceptible to lysis by interleukin 2-activated NK cells via NKG2D [50]. The interaction between MSCs and *in vitro* B cell proliferation, differentiation, and survival is still unclear [51]. However, as T cells orchestrate B cell function, it is likely that the ultimate effect of MSCs on B cells is influenced *in vivo* by MSC inhibition on T cells [52, 53]. The pertinent *in vitro* immunomodulatory properties of MSCs have recently been summarized [45, 46].

Preclinical Results

There are abundant experimental data supporting the use of MSCs in the context of HSCT with the aim of promoting engraftment and accelerating hematopoietic recovery. Almeida-Porada et al. [54] observed that co-transplantation of human stromal cells into pre-immune fetal sheep resulted in an enhancement of long-term engraftment of human cells in the bone marrow and in higher levels of donor cells in the circulation both during gestation as well as after birth. Infusion of BM-derived osteoblasts promoted the engraftment of allogeneic hematopoietic stem cells in mice [55]. Other studies in NOD/SCID mice indicate that co-transplantation of MSCs and cord blood enhances engraftment of human hematopoietic cells in the BM of the animals, especially when low numbers of hematopoietic cells are infused [56]. Co-infusion of fetal lung-derived MSCs and umbilical cord blood-derived $CD34^+$ cells promoted the engraftment of both myeloid and B lymphoid cells in the marrow of recipient mice showing that the engraftment-promoting effect of MSCs was not lineage specific [57]. It was also found that enhancement of engraftment might be independent of the homing of MSCs to the marrow and might be mediated by the release of cytokines that promote either the homing or proliferation of hematopoietic stem cells.

Bartholomew et al. demonstrated that MSC infusions can suppress lymphocyte proliferation and prolong skin grafts in a nonhuman primate model [58]. It has been difficult to demonstrate engraftment of donor MSCs following transplantation. In some studies, gene-marked, culture-expanded MSCs were infused along with unmodified BM cells and polymerase chain reaction (PCR) evidence of marked MSCs was demonstrated in the marrow at several weeks after transplantation [54–56].

Infusion of allogeneic MSCs ameliorated lethal GVHD in mice receiving haploidentical HSCT but only when MSCs where administered early and repeatedly after transplantation [59]. Recently, favorable effects of MSCs were reported in animal models of autoimmunity, such as experimental autoimmune encephalitis; this observation is raising the possibility of MSC use in autoimmune diseases [60].

In a collagen-induced arthritis mice model, a worsening of disease was seen after administration of an allogeneic MSC cell line [61], albeit other murine studies using allogeneic MSC conversely showed demonstrable clinical improvement [62].

Pediatric Clinical Studies

Animal models may not predict the clinical situation as the immunomodulatory mechanisms between species (e.g., murine and human MSCs) may differ. Clinical application of *ex vivo* expanded MSC therapy in the pediatric HSCT setting to date has exploited their potential immunomodulatory properties as well as their abilities to support proliferation/differentiation of hematopoietic stem cells.

Inborn Errors of Metabolism

MSCs express high levels of arylsulfatase A and alpha-L-iduronidase [63]. The deficiency of these enzymes is associated with specific inborn errors of metabolism: arylsulfatase A deficiency is the cause of metachromatic leukodystrophy, whereas alpha-L-iduronidase deficiency is responsible for Hurler's disease, disorders that can be cured by allogeneic HSCT [64, 65]. Expanded MSCs were administered to patients with metachromatic leukodystrophy and Hurler's disease, who had previously undergone HSCT but had residual symptoms of their disease [66]. MSC infusion resulted to be safe, and, notably, four of five patients with metachromatic leukodystrophy showed improvement in nerve conduction velocity. MSCs have been also used to treat a bone disease, namely, *osteogenesis imperfecta* [67]. The rationale for considering the use of MSCs in this disease lies on the observation that five children with *osteogenesis imperfecta* undergoing HSCT showed donor osteoblast engraftment; this engraftment was associated with new bone formation, increase in total bone mineral content, as well as increase in growth velocity and reduced fracture frequencies [36, 68]. In a subsequent study, involving six children with severe *osteogenesis imperfecta* given HSCT and MSCs, the engraftment of *ex vivo* cultured donor MSCs was demonstrated by specific gene markers [69]; engraftment of MSCs was associated with acceleration of growth velocity.

Haploidentical Stem Cell Transplantation

T-cell-depleted HSCT from an HLA-haploidentical relative is a feasible option for children in need of an allograft but lacking a suitable either related or unrelated donor [53]. However, despite the infusion of large numbers of hematopoietic stem cells, both primary (failure to establish any hematological reconstitution or

donor chimerism) and secondary (initial engraftment not sustained) [70] graft failure, mainly mediated by host alloreactive T cells escaping the preparative regimen and favored by the profound T cell depletion of the graft, have been reported to occur in up to 15–20% of transplanted children [71, 72]. In a phase I/II pilot study, co-transplantation of BM-derived, *ex vivo* expanded MSCs of donor origin was tested in children undergoing haploidentical HSCT with granulocyte colony-stimulating factor (G-CSF)-mobilized and CD34-selected progenitor cells. The study was carried out in two centers (Pavia, Italy and Leiden, the Netherlands) participating in the EBMT-MSC consortium, using a shared protocol for *ex vivo* MSC expansion and common reagents [36]. Fourteen children were included in the pilot study. BM-derived, *ex vivo* expanded haploidentical MSCs were infused, fresh or after cryopreservation, at a target dose of $1–2 \times 10^6$/kg body weight, approximately 4 h before T cell depleted, G-CSF-mobilized positively selected CD34+ cells from the same haploidentical donor. The target number of CD34+ cells was 20×10^6 CD34+ cells/kg recipient weight. Pretransplant conditioning depended upon their underlying disease, and no pharmacological GVHD prophylaxis was given after G-CSF-mobilized peripheral blood HSCT. Results were compared with those of a historical cohort (including children transplanted in the same centers from an HLA-haploidentical relative) and were comparable in terms of age, gender, transplant indication, donor type, and number of CD34+ or CD3+ cells infused. In comparison to historical controls who had a graft failure rate of 20%, all patients given MSCs were successfully engrafted ($p = 0.03$). Hematological recovery of leukocytes was faster ($p = 0.01$) with lymphocyte (NK cells) rather than neutrophil recovery accounting for this. T and B cell reconstitution at 3 months after transplantation did not differ between controls and study patients. The incidence of viral reactivations was not significantly different between patients and controls, and in MSC-treated patients with an underlying malignant disease, no significant increase in relapse rates was noted, as compared with historical controls. Since the publication of the original cohort, we have now included a total of 30 consecutive unselected children, all of whom have been engrafted, confirming our original observations. This study and consecutive data demonstrated the safety and feasibility of MSC use in pediatric patients. Also, in the context of HLA-disparate T-cell-depleted HSCT, co-infusion of *ex vivo* expanded MSCs may modulate host alloreactivity and/or promote better engraftment of donor hematopoiesis, reducing the risk of early graft failure.

Umbilical Cord Blood

Transplantation using UCB-derived hematopoietic stem cells was first proposed in the 1980s [73] and later supported by in vitro studies [74] with human cord blood and in vivo studies with mouse models [75]. The first successful human HLA-identical UCB transplantation (UCBT), based on these preliminary findings, was carried out in 1988 in a child with Fanconi's anemia [76], and

subsequently, the first unrelated donor UCBT was performed in 1993. The use of unrelated donor UCB units, as alternative source of stem cells, offers many practical advantages [77] including:

- Relative ease of procurement compared to unrelated bone marrow [78]
- Potential HLA matches for ethnic diverse populations [79]
- Absence of risk for mother and donor
- Reduced risk of transmission of viral infections, most notable CMV
- The ability to store frozen tested material with prompt availability of cells to be transplanted [80]
- Reduce risk of development of GVHD [81]

Despite these unquestionable and relevant advantages, the success of UCBT has been limited by the lower number of cells contained in UCB units, as compared with BM grafts, resulting in a higher rate of graft dysfunction as well as a delayed immune reconstitution [81]. This delayed neutrophil and lymphocyte recovery is responsible for a higher incidence of life-threatening/fatal infections in recipients of UCBT, both during and after engraftment. The overall survival rates are similar between unrelated UCBT and unrelated BMT [82, 83]. In addition, the use of UCB as a source of hematopoietic stem cells precludes any possibility for posttransplant cellular therapy, e.g., DLI to overcome mixed chimerism or interventions aimed at treating viral reactivations using donor viral specific T cells.

Since the outcome of UCBT is dependent on the cell dose infused, attempts have been made to increase the cell content of UCB units or to optimize stem cell homing. These approaches include optimization of cord blood collection [84], *ex vivo* cord blood stem cell expansion, and direct intrabone injection of cord blood cells [85, 86]. The Minnesota group reported on their initial findings in 23 high-risk adult patients who underwent transplantation with two partially mismatched cord blood units [87]. Preliminary results have been both encouraging as to high rates of donor engraftment and low rates of GVHD. The data support that transplantation of two immunologically distinct UCB units is not associated with crossed immunological rejection, but single unit predominance. By the same rationale, MSCs also have been employed to improve engraftment rate and accelerate hematopoietic/immune recovery after UCBT. In a pediatric phase I–II clinical trial, including eight children given co-transplantation of unrelated donor UCB cells and *ex vivo* expanded third-party MSCs, infusion of MSCs proved to be safe and patients had a neutrophil recovery at a median time of 19 days after the allograft [88]. In another pediatric, phase I/II clinical study, the safety and efficacy of co-transplantation of parental MSCs was tested in 13 pediatric patients who received UCB transplantation; the results were compared with those obtained in historical controls receiving UCBT alone in the same transplant centers [33]. The study was carried out in three centers (Pavia, Italy; Stockholm, Sweden; and Leiden, the Netherlands) participating in the EBMT-MSC consortium. BM-derived *ex vivo* expanded haploidentical MSCs were infused, fresh or after cryopreservation, at a target dose of $1–2 \times 10^6$/kg body weight, approximately 4 h before UCB hematopoietic stem cells. The available cord blood unit contained $\geq 1.7 \times 10^5$/kg recipient weight CD34+ cells or a nucleated cell count

of $\geq 2.5 \times 10^7$/kg recipient weight at the time of cryopreservation. The conditioning regimen depended on the underlying disease and the GVHD prophylaxis was cyclosporine (CsA) in case of sibling donors and CsA and steroids for unrelated UCBT recipients. The number of nucleated cells infused did not differ between patients and controls. All study-patients and control-patients engrafted. In comparison to controls, the time needed to obtain neutrophil and platelet engraftment in the patient population was similar, again confirming the feasibility and safety of this approach. In contrast with preclinical results [56] and the experience reported in haploidentical transplants [32], no difference was found in either the engraftment rate or the speed of hematological recovery between the two groups, although most study patients did not receive G-CSF, posttransplant, as compared to controls. Interestingly, MSC co-infusion significantly reduced the incidence of life-threatening acute GVHD and GVHD-associated transplant-related mortality (TRM), as compared to controls [33]. This was in contrast to a study in adult patients receiving UCBT with co-infusion of third-party donor mobilized hematopoietic cells, whereby the administration of MSCs at time of transplantation had no effect on the kinetics of engraftment or GVHD prevention [89].

The difference between the results on MSC use in haploidentical and UCBT settings may be related to different mechanisms underlying graft failure. While in UCBT, graft failure may be inherent to the low numbers of stem cells infused as well as altered homing mechanisms, in the haploidentical setting graft failure may be mainly due to immune-mediated mechanisms. MSCs may also enhance engraftment of donor stem cells through non-immunological mechanisms, such as the stimulation of the functional recovery of the BM microenvironment through the secretion of paracrine mediators or, alternatively, by contributing to the rebuilding of the stem cell niche. While MSCs have been shown to engraft following systemic infusion in animal models [54, 55], in humans sustained engraftment of MSCs is probably a rare event. A number of studies have shown that marrow stroma remains host in origin following allogeneic HCT in the majority of patients [62, 90, 91]. However, limited engraftment of MSCs following HSCT in both adult and pediatric patients has been reported by other groups [36, 92–94]. Chimerism analysis of *ex vivo* expanded MSCs derived from recipient BM after co-infusion of MSCs and HSCs, both in the haploidentical and UCBT settings [32, 33], did not show evidence of donor cells in the majority of patients. This suggests that sustained engraftment of MSCs seldom occurs and therefore is unlikely to contribute to the therapeutic benefit.

Refractory Severe Acute Graft-Versus-Host Disease

Despite advances in donor HLA typing methods and donor selection, as well as in posttransplantation immune suppression, acute (a)GVHD remains a significant cause of transplant-related morbidity and mortality following allogeneic HSCT,

even in the matched HLA-identical sibling setting [95, 96]. Steroids still represent first-line treatment for established aGVHD being associated with a response rate in the order of 30–50%, while the outcome of patients with severe, steroid-refractory, aGVHD remains unsatisfactory and overall survival is poor [97–99]. Le Blanc et al. reported the successful treatment of severe steroid-refractory grade IV aGVHD of the gut and liver with haploidentical BM-derived MSCs in a 9-year-old patient who received allogeneic HSCT from an unrelated donor [100]. This seminal observation was later confirmed in a multicenter, non-randomized phase II trial of the infusion of BM-derived MSCs from HLA-identical, haploidentical family donors or unrelated donors for patients with severe steroid-refractory aGvHD [34]. A total of 55 patients were entered, of which 25 were children, aged between 0.5 and 18 years. A total of 92 MSC infusions were given with 28 patients receiving two or more infusions compared to the remaining 27 patients who received only one. Patients were treated with different regimens before administration of MSC infusions, and the timing of the infusions varied. Seven patients had received donor lymphocytes prior to the development of aGVHD. The response rates seen in this diverse cohort of severely ill patients were higher than previously described for patients with a similar degree of aGVHD, with a significant difference in survival between complete responders and partial/non-responding patients.

Interestingly, the results were positively influenced by the inclusion of pediatric patients. Children showed a trend towards a better response than adults, for reasons that are unclear; response rate is 80% in children compared to 60% in adults ($p=0.28$), with more complete resolution (CR) and less progressive disease in pediatric patients [34]. This better response translated into improved survival in the pediatric group: with a median follow-up of 16 months after MSC infusion, among adults, 27% (8/30) of the patients survived, as compared with 52% (13/25) of the children ($p=0.09$). Specific analysis of subsequent EBV and CMV reactivity in two patients included in this study cohort demonstrated that effector functions of virus-specific T cells were retained after MSC infusion [101]. This observation is important as infections are common in this highly immunocompromised patients and are the cause of death in a substantial number of responders [34, 102]. A more recent study of 39 pediatric patients treated in two transplant centers (Pavia, Italy and Leiden, the Netherlands), using the EBMT consortium expansion protocol, analyzed outcome according to time to 1st MSC infusion (manuscript submitted). The analysis showed that early initiation of MSC treatment (defined as within 21 days of initiating steroid therapy) in children with steroid-refractory grade III–IV aGVHD was associated with an increased complete resolution of symptoms (CR). Achieving CR translated into an overall survival advantage of 87% compared to 27% ($p\leq0.001$) in those patients who did not respond to MSC treatment. TRM was significantly lower in the group achieving CR (14%) compared to the non-CR group (60% $p\leq0.005$). Viral reactivation and death due to disseminated infection was lower in the group treated early as compared with patients treated after 21 days from GVHD onset. Early use of MSCs resulted in less exposure to successive courses of immunosuppression which probably contributed to the reduction in infections. Chronic GVHD, although not significantly reduced, showed a trend to remain as a more

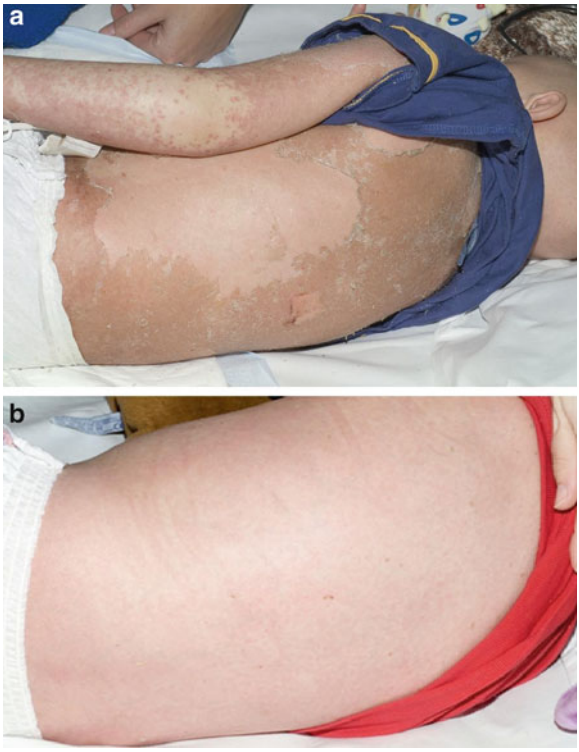

Fig. 26.1 (**a**) A 5-year-old boy post unrelated donor HSCT with progressive stage 4 acute GvHD of the skin unresponsive to treatment with methylprednisolone and cyclosporine A and subsequently tacrolimus, mycophenolate mofetil, infliximab, and daclizumab. The child also had stage 4 gastrointestinal and stage 3 hepatic GVHD at the time of entry into the study (overall grade IV) (biopsy confirmed). (**b**) Same child 10 days after the first MSC infusion. Biopsy of the skin showed no histological evidence of aGVHD. All other organs responded with complete resolution documented at 30 days postinfusion. He remains disease free with no chronic GVHD 6 years later

limited disease. This study also demonstrated that the time to complete response varied according to the organ involved with skin resolving sooner (mean 6 days, range 4–10) (see Fig. 26.1) than the gastrointestinal tract (mean 10 days, range 6–14) and hepatic symptoms taking longest to resolve (mean 13 days, range 7–18) [103]. As a result of this study we advocate administering MSCs 5–7 days after the initiation of steroids in those children failing to respond to methylprednisolone at a dose of 2 mg/kg body weight without first attempting any additional pharmacological immunosuppressive treatments.

Conclusions

The promising results of phase I/II studies of MSC transplantation now pave the way for randomized studies in the future. Although immediate feasibility and safety issues have been addressed, more information on long-term outcome of patients treated with MSC is required. The issues of dosage and timing, as well as of the efficacy of this novel therapy in comparison to other alternative strategies, need to be addressed in well-conducted, controlled studies. Biological studies should be incorporated in future clinical applications to increase the understanding of the

functional properties of MSCs relevant to their role in modulating alloreactivity and supporting hematopoiesis. In this regard, it has been recently suggested that MSCs may be used in autoimmune inflammatory bowel disease, not only because of their immunomodulatory effects but also due to their capacity for healing damaged gut epithelium [103–105]. Similarly, investigations into other autoimmune processes such as a juvenile rheumatoid arthritis, systemic lupus erythematosus (SLE), dermatomyositis, and type I diabetes mellitus are in the initial phases of development.

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Chapter 27
MSC for *Ex Vivo* Expansion of Umbilical Cord Blood Cells

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Abstract *Ex vivo* expansion of umbilical cord blood (UCB) has been proposed to increase the cell dose to enhance engraftment of UCB products used as a source of hematopoietic stem cell (HSC) transplant for hematological malignancies in adults. UCB offers several potential advantages over bone marrow from unrelated donors, including its ready availability, allowance of higher HLA disparity, and lower incidence of graft-versus-host disease which makes it an attractive source especially for minority populations. The major limitation to a wider use of this source of HSC is the relatively low number of progenitor cells in the graft. For this reason, adult UCB transplantation is usually associated with delayed engraftment and increased rates of infectious complications. UCB *ex vivo* expansion holds the promise of delivering higher cell doses and improved outcomes. Current approaches for expansion of UCB products involve initial isolation of hematopoietic stem and progenitor cells based upon expression of CD34 or CD133 prior to culture; however, this process results in variable recovery of $CD34^+$ cells and variable purity resulting in poor expansion. We have developed methods for the expansion of UCB products which eliminate the requirement for positive selection and enable the expansion of mononuclear cells by coculture on mesenchymal stromal cells. Here we discuss different methods of expansion, their shortcomings, and future directions.

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_27,

Introduction

The use of umbilical cord blood (UCB) as a hematopoietic stem cell (HSC) source has been increasing in recent years and has become an important source of HSC support following myeloablative and non-myeloablative therapies [1–6]. Unfortunately, it is not without restrictions. The major limiting factor to UCB application is the low cell dose available for transplantation. It is well documented that the total nucleated cell dose (TNC) transplanted *per* kilogram (kg) of body weight of the recipient correlates with outcomes. Patients with a total body weight of at least 45 kg who receive only a single unit of UCB have been shown to have a significant delay in time to neutrophil and platelet engraftment, as well as higher rates of engraftment failure [7–11]. For this reason, UCB transplantation remains significantly more successful in children [9, 10, 12]. However, even in small children who have received adequate cell doses, a delay is evident in engraftment of all cell lineages when compared to traditional stem cell sources [13–15] as well as delayed immune reconstitution [16, 17].

In an analysis of the effects of various demographic-, graft-, and treatment-related factors on clinical outcomes of 102 patients transplanted with a single UCB unit, the $CD34^+$ cell dose was significantly associated with the rate of engraftment, transplant-related mortality (TRM), and survival [18]. Time to neutrophil engraftment was strongly correlated with $CD34^+$ cell dose, and there was an inferior rate of engraftment and higher TRM in patients transplanted with less than 1.7×10^5 $CD34^+$ cells/kg. Based on these and other data, guidelines advocating the selection of products with higher TNC and $CD34^+$ doses emerged. Recent recommendations are that a single unit should ideally contain a minimum of $2.5–3 \times 10^7$ TNC/kg for closely matched UCB units (5/6 or 6/6 matches, considering low-resolution HLA A and B matching and high-resolution matching at HLA DRB1), with possibly greater TNC targets in the setting of a greater mismatch. Our improved understanding of optimal unit characteristics and the expansion of UCB banks have improved clinical outcomes, especially for larger pediatric and adult recipients; however, more stringent selection criteria leave more potential recipients without an UCB unit of sufficient size and/or HLA matching.

Potential Solutions

There are two general approaches aimed at overcoming the issue of low TNC associated with UCB transplantation. One approach is the infusion of more than one UCB unit in order to attain an elevated infusible cell number [19–23], and the second approach is *ex vivo* expansion of UCB units. In a recent prospective randomized trial, a double UCB transplant was compared to a transplant using one unmanipulated UCB unit combined with one unit that was expanded *ex vivo* [24].

In this trial, 71 patients with advanced hematological malignancies were randomized receiving either a myeloablative preparative regimen ($n = 41$) or non-myeloablative regimen ($n = 30$), depending on disease and clinical status. Thirty-four patients (48%) were alive at a median follow-up of 11.3 months (range, 2–49). Most of the patients on the expanded arm had some evidence of the expanded UCB chimerism posttransplant (7–82%); however, by 14 months all patients had predominance of the unmanipulated cord. These data are consistent with previous observations with *ex vivo*-expanded peripheral blood $CD34^+$ cells [25] and in a xenogeneic fetal sheep model [26] which suggest that *ex vivo* expansion may affect the durability of engraftment.

Ex vivo expansion is conducted on whole UCB units, as well as selected portions. These expanded products can then be infused concurrently with an unmanipulated UCB or sometime after infusion of the unmanipulated unit. Currently, clinical protocols aimed at proving the beneficial nature of this strategy are being conducted at a number of clinical centers [27–30] (Table 27.1). A number of approaches have been explored for *ex vivo* expansion of UCB products from liquid culture in gas permeable bags to bioreactors, and a number of groups have demonstrated that selection of $CD34^+$ cells or $CD133^+$ cells is necessary for optimal *ex vivo* expansion. In 1997 we reported that culture of UCB mononuclear cells (MNC) in a human growth factor cocktail of stem cell factor (SCF) plus granulocyte-colony stimulating factor (G-CSF) and thrombopoietin (TPO) resulted in only a 1.4-fold expansion of total cells, 0.8 fold in mature progenitor cells (granulocyte-macrophage colony-forming cells, GM-CFC), and 0.3 fold in erythroid progenitors (burst forming unit-erythroid, BFU-E) [31]. In contrast, similar culture of $CD34^+$ selected UCB cells resulted in 113-fold expansion of total cells, 73-fold expansion of GM-CFC, and 49-fold expansion of BFU-E. Based upon these results, we initiated expansion cultures in clinical trials with CD34-selected UCB cells. Processing of clinical products has led us to two conclusions: (1) Although we can significantly expand TNC and committed progenitor cells from $CD34^+$ selected cells, because of significant $CD34^+$ cell losses following the positive selection procedure, we rarely reached preselection TNC numbers. (2) The performance of clinical trials using UCB grafts in the unrelated setting requires the use of frozen UCB products. $CD34^+$ selection of frozen UCB products results in significant losses of $CD34^+$ cells (50% or greater) and often results in low purities [32]. Thus, with a 50% recovery of $CD34^+$ cells after selection, we would require at least a 400-fold cell expansion to obtain a TNC equivalent to what was started with. Again, from our experience with clinical studies, the purity of the CD34-selected product also significantly impacts the level of expansion achieved. The median-fold expansion obtained with products with a purity >50% $CD34^+$ was 139 fold, while the median-fold expansion obtained with products with starting purities <50% $CD34^+$ was only 32 fold [33]. Therefore, the use of CD34-selected products rarely results in increased cell doses of *ex vivo*-expanded cells compared to the starting unmanipulated product. Based upon these data, we have evaluated methods for expanding CB products without an initial CD34 or CD133 selection.

Table 27.1 Summary of clinical trials evaluating *ex vivo*-expanded umbilical cord blood (UCB)

Expansion method	Investigator	*N*=(adults/children)	Days in culture	TNC fold expansion	CD34+ fold expansion	# of days to ANC>500	# of days to platelets >20,000	Cytokines	Incidence of acute GVHD	Survival % (median survival)
Liquid culture	Shpall et al. [57]	37	10	56	4	28	106	SCF, TPO, G-CSF	Grade II–IV: 67% Grade III–IV: 40%	32 (17 months)
	de Lima et al. (2007) Blood 110: 3271a	35	14	23	2.3	14	34	SCF, TPO, G-CSF	Grade II–IV: 43% Grade III–IV: 7%	48 (11 months)
	de Lima et al. [24]	10	21	219	6	30	48	SCF, FL, IL-6, TPO, G-CSF	Grade II: 44% Grade III–IV: 0%	30 (25 months)
	Delaney et al. (2008) Blood 118: 3640a	5	16	660	160	14		Notch ligand Delta1, SCF, FL, IL-6, TPO, IL-3		83 (277 days)
Stromal culture	de Lima et al. (2009) Blood 114: 3394a	6	14	12	12	14.5	30	SCF, TPO, G-CSF	Grade II: 33% Grade III–IV: 0%	83 (12 months)
Bioreactor	Jaroscak et al. [28]	27 (mostly children)	12	2.4	0.5	22	71	PIXY321, FL, EPO	Grade II–IV: 36% Grade III–IV: 22%	39 (41 months)
	Pecora et al. [27]	2 (both adults)	12	2.2	1.6 (no expansion in second)	28	56	PIXY321, FL, EPO	None	100 (12 months)

The Bone Marrow Microenvironment

The bone marrow (BM) contains precursor cells that generate adherent colonies of stromal cells in vitro. This BM stroma represents the non-hematopoietic connective tissue elements that provide a system of structural support for developing hematopoietic cells. The complex cellular composition of marrow stromal tissue comprises a heterogeneous population of cells including reticular cells, adipocytes, osteogenic cells near bone surfaces, vascular endothelial cells, smooth muscle cells in vessel walls, and macrophages [34–37]. The concept that adult hematopoiesis occurs in a stromal microenvironment within the BM was first proposed by Dexter and colleagues [38], leading to the establishment of the long-term BM culture (LTMC). These studies demonstrated that an adherent stromal-like culture could support maintenance of HSC [38]. Mesenchymal stromal/stem cells (MSC) represent the major stromal cell population of the BM.

MSC were first recognized by Friedenstein who isolated cells from guinea pig BM which were adherent in culture and which differentiated into bone [39]. Surface antigens have been reported for identification and phenotyping of human MSC [40–43]. Although MSC are rare, representing approximately 0.01% of the BM mononuclear cell (MNC) fraction, they have attractive features for therapy, including the ability to expand many log-fold in vitro, and unique immune characteristics allowing their use as an allogeneic graft. They are typically isolated based upon adherence to standard tissue culture flasks. Low-density BM mononuclear cells (MNC) are placed into culture in basal media plus fetal bovine serum (FBS) (usually between 2–20%), and after 2–3 days, adherent cells can be visualized on the surface of the flask. The nonadherent cells are removed, fresh media added, and culture continued until a confluent adherent layer forms. The MSC are harvested by treatment with trypsin and further passaged expanding the number of MSC. A number of different cell populations have been isolated using different culture conditions; however, the morphology of these cells is very similar. Phenotypical characterization of MSC has been performed by many groups, and standard criteria have been proposed by the International Society for Cellular Therapy (ISCT) [44]. The minimal criteria proposed to define human MSC by the Mesenchymal and Tissue Stem Cell Committee of the ISCT consist of the following: (1) MSC must be plastic adherent when maintained in standard culture conditions; (2) MSC must express CD105, CD73, and CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR surface molecules; and (3) MSC must differentiate into osteoblasts, adipocytes, and chondrocytes in vitro.

A standard in vitro assay for MSC is the colony-forming unit fibroblast (CFU-F) assay [45]. BM MNC are plated at low density and colonies of fibroblasts develop attached to the surface of the culture dish. Based upon the results of this assay, the frequency of MSC precursor cells is one in 10^4–10^5 BM MNC. The frequency is highly variable between individuals and the number of MSC has been shown to be decreased in older people. Other studies have demonstrated that MSC precursors can be isolated based upon surface antigen

expression. Antibodies to CD271 and STRO-1 have been used to enrich MSC precursors. CD271, also known as "low-affinity nerve growth factor receptor" (LNGFR) or p75NTR, belongs to the low-affinity neurotrophin receptor and the tumor necrosis factor receptor superfamily. Selection of $CD271^+$ cells from human BM enriches CFU-F, and MSC are preferentially selected in the $CD271^+$ fraction compared to the $CD271^-$ fraction [40, 41]. Similarly, isolation of STRO-1^+ cells from BM MNC results in enrichment of CFU-F in the STRO-1^+ fraction compared to the STRO-1^- fraction [42].

Immunologic Properties of MSC

MSC are ideal candidates for allogeneic transplantation because they show minimal MHC class II and ICAM expression and lack B-7 co-stimulatory molecules necessary for T cell-mediated immune responses [41, 46]. These immunological properties are discussed in detail in other chapters of this book.

The Stem Cell Niche

The control of proliferation and differentiation of HSC occurs in the microenvironmental or "stem cell" niche. HSC have been studied in detail and shown to reside in the BM in association with stromal cells which make up the hematopoietic microenvironment [47]. The stroma consists of several cell populations including MSC, fibroblasts, and adventicular reticulocytes [34]. HSC exist in a quiescent state in close relationship with the stromal cells in the BM. These stromal cells produce cytokines and growth factors that are either secreted or expressed as membrane-bound proteins and control the differentiation and proliferation of the HSC. In vitro, MSC have been shown to support the proliferation and differentiation of HSC, generating committed hematopoietic progenitor cells over a 6-week period [38]. If the microenvironment is compromised, such as in patients who receive multiple rounds of high-dose chemotherapy regimens, normal homeostasis is disrupted, and deficiencies in blood cells occur.

Ex Vivo Expansion of Cord Blood Cells Using MSC Coculture

Based upon the ability of MSC to support hematopoietic cells, we have developed a coculture system capable of expanding UCB MNC by coculture with confluent MSC layers [33]. It has been demonstrated that MSC produce a number of hematopoietic growth factors and adhesion molecules that may stimulate the growth of hematopoietic cells. Our data reproducibly demonstrated a 10–20-fold

expansion of TNC with an 18-fold expansion of GM-CFC and 16–37-fold expansion of $CD34^+$ cells. We have also evaluated the potential of *ex vivo* expansion of frozen UCB products using MSC coculture. When cryopreserved UCB products were thawed and washed, a median of 3.3×10^8 TNC (range 1.4–3.6×10^8, $n = 5$) was achieved. For a 50-kg recipient, these CB products would provide only 0.73×10^7 TNC/kg. Therefore, none of five products would achieve the minimal target dose of 1×10^7 TNC/kg. However, when each product was expanded by culturing the MNC fraction from each product on preformed layers of MSC, a median 9-fold expansion of TNC was obtained (range of 6.5–24 fold). The median TNC post expansion was 21.6×10^8 cells (range $11–79 \times 10^8$ TNC), and a median 46-fold expansion of mature progenitor cells (GM-CFC) was achieved. For a 50-kg recipient, the expanded CB product would be equivalent to 4.3×10^7 TNC/kg (range $2.2–16 \times 10^7$), with all five expanded products now reaching the minimal target of 1×10^7 TNC/kg. In fact, all expanded products generated a dose $>1 \times 10^7$ TNC/kg based upon a 100-kg recipient. Similar preclinical *ex vivo* expansion data were obtained at The University of Texas MD Anderson Cancer Center [48] and led to the initiation of a clinical trial. This trial combines an unmanipulated UCB unit with an *ex vivo*-expanded UCB unit cocultured with MSC from a related donor member (minimum of 2/6 HLA match). Myeloablative therapy for this protocol is ATG plus fludarabine, melphalan, and thiotepa, and non-myeloablative therapy is ATG plus fludarabine, cyclophosphamide, and 200 cGy TBI. At least 1 month prior to transplant, BM is harvested from the related donor and the isolation and *ex vivo* expansion of MSC initiated. On day 14 the smaller of the two cryopreserved CB units is thawed and the *ex vivo* UCB MNC/MSC expansion coculture procedure begun. On day 0, the unmanipulated UCB unit is thawed and infused, followed by the *ex vivo*-expanded UCB cells. A median 12-fold expansion was seen in both the TNC and the $CD34^+$ subsets in the *ex vivo*-expanded CB product. For the six recipients of myeloablative therapy, the median time to neutrophil engraftment was 14.5 days (range 12–23) and platelet engraftment 30 days (range 25–51). Two of six patients developed grade II acute G*V*HD which resolved with steroids. One patient died of pneumonia in remission at day 150. Five of the six patients are alive and in complete remission at a median follow-up of 1 year with accrual continuing [49]. While the improvement in transplanted dose and neutrophil and platelet engraftment is encouraging, the flexibility of the *ex vivo* culture system (combination of growth factors and/or time in culture) will allow for future/additional modifications which may help to better craft the graft to the requirements of the patient, for example, a graft that is better "primed" to generate megakaryocytes at transplant with the goal of further improving platelet engraftment. Further, the development and availability of "off-the-shelf" third party and new, potentially more effective stromal cell lines to support HSC expansion may also prove to be beneficial [50], especially in removing the current time constraints associated with the generation of sufficient numbers of MSC from family donor-derived material, especially in cases where patients have rapidly progressing disease (Fig. 27.1).

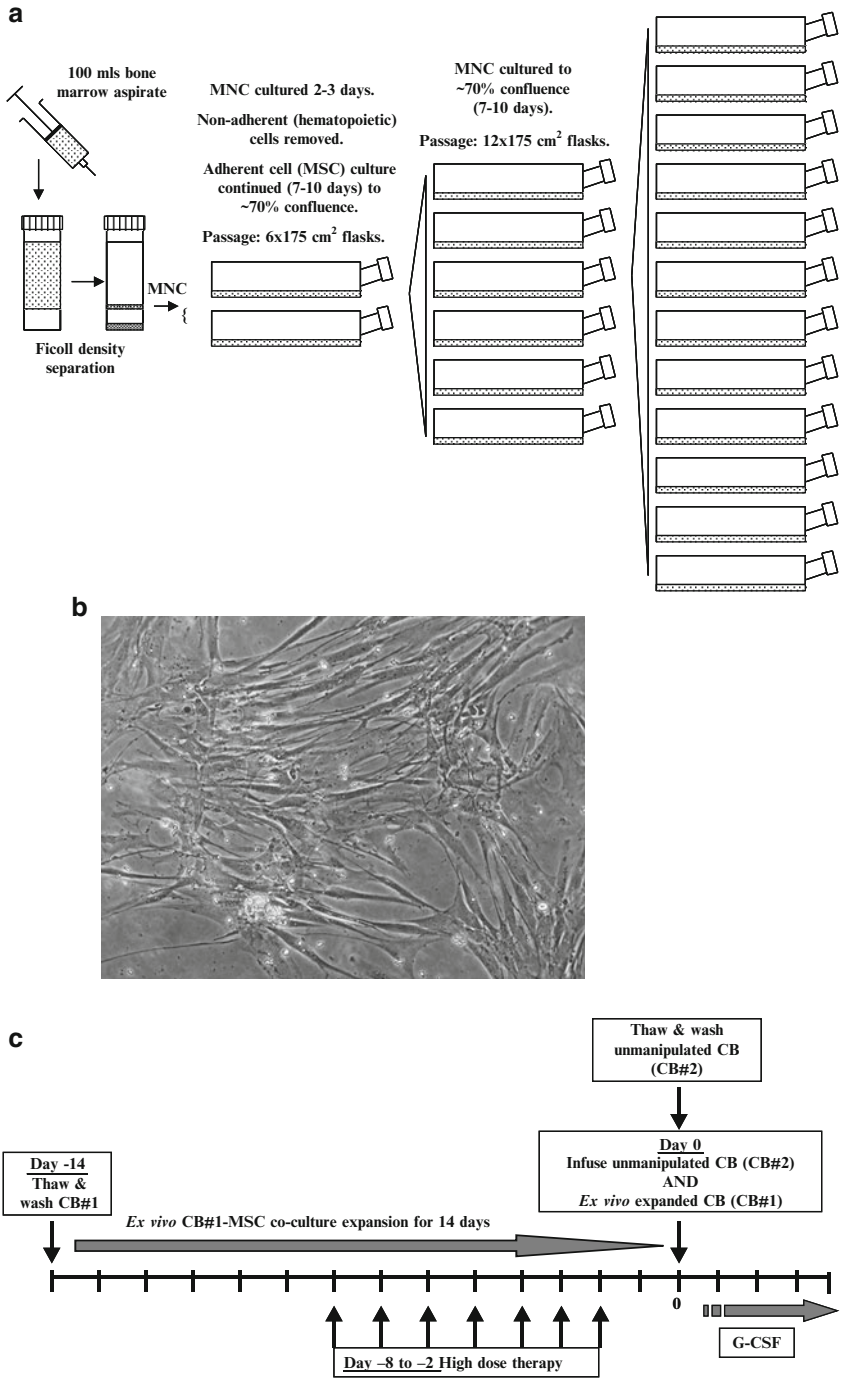

Fig. 27.1 *Ex vivo* expansion of umbilical cord blood graft using mesenchymal stromal cell (MSC) expansion technique. (**a**) Third party-derived bone marrow mononuclear cells are cultured in plastic flasks for 2–3 days to allow for 70% confluence. The nonadherent cells (hematopoietic cells) are removed and the MSC are then passaged into 12, 175 cm^2 flasks over a period of 7–10 days. (**b**) MSC show 70–80% confluence. (**c**) The UCB to be expanded is then cocultured in the separate flasks containing confluent MSC for another 14 days for maximal expansion prior to infusion

Other Expansion Methods

Liquid Culture

Cytokines: UCB cells are cultured with combinations of cytokines, growth factors, and other growth-promoting compounds in liquid culture. Prior to expansion, the isolation of relatively primitive hematopoietic progenitor cells (primarily $CD133^+$ or $CD34^+$) from UCB (as well as BM or mobilized peripheral blood) has been required [51]. The Miltenyi CliniMACS system and the Nexell Isolex device are clinical-grade devices available to perform this isolation. Once isolated, the hematopoietic progenitor cells are incubated in a culture medium supplemented with growth factors including SCF, interleukin-3, IL-6, and G-CSF [51]; SCF, TPO, and G-CSF [27, 52]; and Flt-3 ligand, SCF, IL-3, IL-6, and G-CSF [53, 54]. However, we have developed a two-step, 14-day liquid suspension UCB expansion protocol for the *ex vivo* expansion of isolated $CD34^+$ UCB cells [55], which yields more effective *ex vivo* expansion (>400-fold increase in TNC and >20-fold increase in $CD34^+$ cells) [56] than does a single-step 10-day protocol [57]. Several modifications to this particular expansion technique include (i) attempts to further optimize *ex vivo* culture conditions [58–64]; (ii) the development of serum-free culture systems [55, 57, 65]; (iii) the use of histone deacetylases, thought to promote HSC self-renewal [66]; (iv) the use of glycogen synthase kinase (GSK)-3 inhibitors reported to maintain pluripotency of stem cells [67]; and (v) the use of tetraethylenepentamine (TEPA), a copper chelator thought to modulate the proliferation and differentiation of primitive hematopoietic progenitors [68–70].

Tetraethylenepentamine: Tetraethylenepentamine (TEPA) has been shown to stimulate *ex vivo* expansion of hematopoietic progenitor cells by reducing their free copper content as well as by lowering their oxidative stress [71]. An investigation into the potential therapeutic efficacy of TEPA added in a 22-day liquid UCB expansion was conducted as a phase I/II trial by de Lima et al. [30]. In this study, ten heavily pretreated patients were allocated UCB units that were frozen into two fractions: a smaller $CD133^+$ fraction and a larger $CD133^-$ fraction. Twenty-one days before transplantation, the $CD133^+$ fraction was thawed and expanded using liquid culture technique in αMEM containing 10% FCS (HyClone) and supplemented with SCF, FL, IL-6, TPO, and TEPA. Prior to transplant, patients received myeloablative therapy, and on day 0, they received an unmanipulated UCB fraction. Infusion of the expanded fraction followed on Day +1. Nine of the ten patients engrafted at a median of 30 days (range: 16–46 days) with 100% donor chimerism despite the low TNC/kg infused in this study (mean: 1.7×10^7/kg). Platelet transfusion independence occurred at a median of 48 days (range: 35–105). Nine patients were alive at day 100, while three died during the 180-day study period due to infectious complications. No grade III or IV G*V*HD occurred. An average 219-fold expansion in TNC and a 6-fold expansion of $CD34^+$ cell numbers were achieved. Unfortunately, no correlation was demonstrable between CFU dose, $CD34^+$ cell dose, or TNC dose and engraftment due to a small sample size and heterogeneous make up of UCB units.

Further studies are therefore going to be essential in order to determine the efficacy of TEPA in improving the *ex vivo* expansion of UCB and a phase II multicenter trial with TEPA-expanded UCB is currently underway and has enrolled >40 patients (personal communication: Gamida Cell Ltd. Cell Therapy Technologies). Although liquid culture expansion is a promising technique, the optimal combination of cytokines and growth factors has yet to be defined. Liquid culture is furthermore limited by small volumes as well as the static nature of the culture system.

Notch Ligand: Delaney et al. recently used an immobilized, engineered form of the Notch ligand Delta1 in combination with recombinant cytokines (SCF, FL, IL-6, TPO, and IL-3) to stimulate *ex vivo* UCB expansion [72, 73]. $CD34^+CD38^-$ precursors were cultured with different densities of immobilized Notch ligand, Delta1ext-IgG. Lower ligand densities of immobilized Delta1 promoted maximal generation of $CD34^+$ precursor cells, including those with NOD/SCID repopulating cell activity [72]. Ten patients with high-risk acute leukemias in morphological remission, with a median age of 27.5 years and median weight of 61.5 kg, received myeloablative preparative regimen followed by infusion of one unmanipulated and one *ex vivo*-expanded cord blood graft. All units were matched to the recipient at a minimum of 4/6 six loci and at least 3/6 matched to each other, with a minimum TNC dose in the unmanipulated graft of 2.5×10^7 TNC/kg. There was a 164-fold average expansion of $CD34^+$ cells and 562-fold expansion of TNC. $CD34^+$ cell dose derived from the expanded UCB graft averaged 6×10^6 $CD34^+$ cells *per* kg (range 0.93×10^6 to 13×10^6) versus 0.24×10^6 $CD34^+$ cells *per* kg (range 0.06×10^6 to 0.54×10^6) ($P=0.0004$) from the unmanipulated UCB graft. There was no significant difference, however, in the average number of TNC *per* kilogram. The time to an absolute neutrophil count (ANC)≥500 cells/μl was shortened significantly ($P=0.002$), with a median time of 16 days as opposed to a median time of 26 days (range 16–48 days; $P=0.002$) in a concurrent cohort of 20 patients undergoing double UCB transplantation with identical conditioning and posttransplant immunosuppressive regimen.

Bioreactors: Bioreactors are also being investigated for the *ex vivo* expansion of HSC primarily using a continuous perfusion culture system rather than the use of "static" culture (culture flasks or bags) [27–29, 74–78]. These "bioreactors" are automated, continuous perfusion culture systems that have been designed to accommodate larger volumes as well as to improve gas exchange and nutrient delivery. The secreted products of mature granulocytes and macrophages are toxic to progenitors [79], and mature macrophages can directly damage cultured stroma and hematopoietic progenitors [80]. In order to eliminate these threats, a continuous perfusion of culture medium is provided that would remove these mature cells protecting the cultured cells from toxic by-products. Although the hematopoietic reconstitution of UCB $CD34^+$ cells grown in static cultures were better than stirred cultures for cell expansion, the engraftment of stirred-culture HSC was higher than static-culture HSC. Stirred-culture HSC had better multilineage reconstitution ability and colony-forming ability than static-culture HSC. Static cultures thus favor the expansion of HSC and stirred cultures are more effective in preserving functional HSC.

In a phase I trial [28], fractions of UCB were expanded *ex vivo* using Aastrom Replicell bioreactor technology and a growth factor cocktail (PIXY321, Flt-3 ligand, and erythropoietin (EPO)). The expanded cells were administered 12 days after the transplant of

unmanipulated fractions of UCB. No difference in the time to myeloid, erythroid, or platelet engraftment was observed. In a second 2-patient study, *ex vivo*-expanded UCB cells (Aastrom Replicell bioreactor) generated to augment unmanipulated UCB appeared to facilitate hematopoietic recovery [27]. A newer bioreactor that uses serum-free medium, the Dideco "Pluricell System," was used in recent preclinical and murine studies, where Astori et al. showed a MNC-fold expansion of 230.4±91.5 and $CD34^+$-fold expansion of 21.0±11.9 at 12 days, as well as improved engraftment in the NOD-SCID mouse model [81]. Other technologies such as rotating wall vessels which decrease sheer stress while maintaining consistent environment are also being evaluated [82].

Challenges Associated with *Ex Vivo* Cord Blood Expansion

Differentiation of the Progenitor Cell

Ex vivo expansion strategies are not unique to UCB and could also be applied to BM as well as mobilized peripheral blood-derived HSC [51, 52, 55], as there is evidence of functional and phenotypic heterogeneity within the HSC population [83–87]. During expansion, a major concern is selective expansion of short-term (low-quality) reconstituting HSC at the expense of long-term (higher-quality) reconstituting HSC. This potential selectivity can reduce the long-term viability of the graft while initially demonstrating early hematopoietic recovery [88]. An inherent reduction in long-term hematopoietic reconstitution potential of *ex vivo*-expanded products may be evident under certain conditions [25, 26, 88]. However, the ability to manipulate UCB to produce a short-lived, albeit rapidly reconstituting, HSC profile can be clinically useful especially when it is combined with a slowly engrafting unmanipulated UCB unit for transplantation. There is clinical data to suggest that *ex vivo*-expanded UCB products are indeed the source of the rapid, initial hematopoietic reconstitution with the unmanipulated fraction being responsible for sustained long-term hematopoietic engraftment [27]. Further, engraftment data suggests that the addition of the expanded fraction does not provide a beneficial long-term outcome [27–29]. Indeed, evidence suggests that were *ex vivo*-expanded UCB units to be used alone for transplant, the expansion of short-term reconstituting lower "quality" HSC at the expense of higher "quality" long-term reconstituting HSC following *ex vivo* expansion may lead to more rapid hematopoietic recovery, but may ultimately lead to graft failure, due to the depletion of the long-term reserve of the graft [88]. Preclinical and clinical experience suggests that this might be a valid concern with evidence of compromised long-term repopulating activity following *ex vivo* expansion in a fetal sheep model [26], loss of radioprotective and long-term engraftment potential with *ex vivo* expansion of murine BM [89], and the absence of durable engraftment from *ex vivo*-expanded $CD34^+$ cells in a clinical study [25]. However, there is also evidence that *ex vivo* expansion does not compromise the "quality" of the HSC population with evidence of self-renewal and amplification of HSC during *ex vivo* expansion [90]; the generation of *ex vivo*-expanded UCB cells capable of engraftment in primary, secondary, and tertiary xenogeneic recipients [91] and evidence of delayed engraftment in a

mouse model, suggesting that potentially more primitive, less rapidly engrafting cells, are actually preserved during *ex vivo* expansion [92]. Further studies suggest that the homing of *ex vivo*-expanded HSC to the hematopoietic microenvironment at transplant is not compromised following *ex vivo* expansion [93]. Recent evidence even suggests that the homing of UCB HSC might actually be improved following *ex vivo* expansion as the fucosylation of cell surface glycoprotein ligands becomes increased with culture. Fucosylation of specific cell surface glycoprotein ligands expressed by HSC contributes to more efficient homing and engraftment [94]. The overall durability of the graft achieved following *ex vivo* expansion is an issue that will need to be clarified as clinical expansion studies in humans progress.

Graft Contamination

Manipulation of cell products carries an intrinsic risk for infectious contamination. Therefore, strict adherence to GMP protocols is necessary. At the MD Anderson Cancer Center, in a randomized setting, we compared expanded (liquid culture) UCB units ($n=44$) to unmanipulated units ($n=48$), and no contamination was seen in either arm [95]. Similarly, no contamination was noted in another study of only expanded UCB, using bioreactor (ViaCell; $n=32$) and MSC (angioblast technique; $n=13$) for UCB expansion.

Timing of Transplantation

Another major challenge to *ex vivo* expansion is the unavoidable expansion phase which ultimately postpones transplantation by 2–3 weeks. Generally, it takes 14 days for expansion, in addition to any required testing for the transplant itself. This delay may contribute to increased risk of disease progression or relapse. In high-volume UCB transplant programs, timing of expansions can be an obstacle logistically. In order to overcome such challenges, the stem cell laboratory has to be adequately equipped with manpower and supporting technology.

Conclusions

The ultimate goal of *ex vivo* expansion is the production of an optimal number of HSC for graft transplantation as well as an appropriate number of specific progenitor cells for the purpose of rapid recovery from pancytopenia. A decrease in morbidity and mortality can be achieved if these goals can be met efficiently. A combination of expanded and unmanipulated UCB units may prove to be the most efficient method for attaining these results. *Ex vivo* expansion of umbilical cord blood cells using MSC could provide a clinically applicable methodology for this purpose.

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Chapter 28
MSCs for Autoimmune Diseases

Alan Tyndall and Chiara Bocelli

Abstract Mesenchymal stromal/stem cells (MSC), more correctly called multipotent mesenchymal stromal cells, are being examined for the treatment of autoimmune disease (AD) based on their in vitro antiproliferative properties, efficacy in animal models, apparent low acute toxicity and the early positive anecdotal outcomes in human acute graft versus host disease and AD. Small phase I/II clinical trials in Crohn's disease, multiple sclerosis and systemic lupus erythematosus have been published suggesting acceptable feasibility and safety, and some positive clinical responses. An unpublished large randomised clinical trial in Crohn's disease has failed to confirm a clear clinical advantage over placebo, although trial design may have confounded outcomes. Multidisciplinary groups are collaborating to ensure maximal use of available resources to establish the place, if any, of MSCs in the treatment of AD.

Introduction

Although often referred to as mesenchymal stem cells, the stromally derived progenitor cells referred to in this chapter as multipotent mesenchymal stromal cells (MSCs), which are being investigated for the treatment of autoimmune diseases (AD), have not yet been established as true stem cells [1]. Subpopulations of MSCs, however, demonstrate classical adult stem cell multipotency in that they are capable of differentiating in vitro and in vivo to many mesenchymal lineages, including fat, bone, cartilage and myelosupportive stroma [2–4]. MSCs can be isolated from bone marrow, skeletal muscle, adipose tissue, synovial membranes and other connective tissues of adult humans [5–8] as well as cord blood [9] and placental derivatives [10] and defined by using a combination of immunophenotypic markers and functional

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_28,

properties. Although controversy exists over the in vivo phenotype of MSCs, *ex vivo*-expanded MSCs are defined by differentiation to osteogenic, chondrogenic and adipogenic lineages, and by flow cytometry, are positive for CD73, CD90 and CD105 while not expressing the haematopoietic markers CD14, CD34, CD45 and MHC class II [11]. Importantly, MSCs exhibit antiproliferative, immunomodulatory and anti-inflammatory properties in vitro and in vivo, making them candidates for treatment of acute and chronic inflammatory AD [12, 13]. Regardless of whether or not MSCs are true stem cells, clinical benefit from MSCs may not require sustained engraftment of large numbers of cells or differentiation into specific tissues. It is possible that a therapeutic benefit can be obtained by local paracrine production of growth factors and a provision of temporary antiproliferative and immunomodulatory properties [14, 15].

MSCs as Immunomodulators

More than a decade ago, it was noticed that rodent, baboon and human MSCs, in a dose-dependent fashion, suppress T and B cell lymphocyte proliferation in mixed lymphocyte cultures (MLC) when induced by mitogens or antibodies [16–23]. This suppression is MHC independent, and indeed some degree of immune privilege was observed by many groups. In human cell cultures, the magnitude of suppression is reduced but not abolished when the MSCs are separated from the lymphocytes in transwells indicating that cell–cell contact is not always required [16, 18, 22, 24–26]. However, not all experiments have shown the same antiproliferative effects. In some studies, the influence of bone marrow MSCs on highly purified B cells resulted in the promotion of proliferation and differentiation into immunoglobulin-secreting cells [27, 28]. These controversial results are likely the result of specific experimental conditions used such as use of different lymphocyte populations. Regardless of in vitro studies, in vivo administration of MSCs leads to the inhibition of pathogenic antibodies [29, 30]. This effect was recently demonstrated to be the result of metalloproteinase processing of CCL2 produced by MSCs resulting in the suppression of STAT3 [30].

Immunomodulatory Mechanisms

It is proposed that a paracrine effect involving multiple molecules results in antiproliferative/anti-inflammatory effects. Initially, the observation that MSCs exposed to interferon gamma (IF-γ) express class II antigens but not costimulation molecules [31] suggested that inhibition of T cell responses may be related to the induction of anergy or apoptosis in cell–cell contact conditions. However, apart from one publication reporting MSC-induced apoptosis of proliferating lymphocytes [32], most publications not only excluded MSC-induced apoptosis on target cells but, in contrast, suggested that arrest of apoptosis may be a major mechanism for MSCs to impart a survival signal to immune [24, 33] as well as other cells [34]. A major

mechanism leading to inhibition of immune cell effector functions is cell cycle arrest in G0/G1, which results in inhibition of cell proliferation [22–24, 34]. Whether this effect is mediated by cell-contact mechanisms or soluble factors is still not fully understood. However, transwell experiments have demonstrated putative paracrine soluble factors including hepatocyte growth factor (HGF) and transforming growth factor-beta 1 (TGF-β1) [13], prostaglandin E2 [26, 35], indoleamine 2,3-dioxygenase [36], inducible NO synthetase resulting in STAT5 inhibition in lymphocytes [37], soluble HLA-G [38] and soluble interleukin 1 receptor [39]. Current data however demonstrate that these molecules play only a limited role, while others are yet to be discovered.

Fate of Transplanted MSC In Vivo

In rodent models, radio-labelling experiments showed localisation of MSCs after intra-arterial and intravenous infusion mostly in lungs and secondarily in liver and other organs [40]. Detailed studies in baboons (two using autologous and one allogeneic MSCs) using the green fluorescent protein retroviral construct showed that gastrointestinal tissues harboured high concentrations of transgene as measured per microgram of isolated DNA. Additional tissues including kidney, lung, liver, thymus and skin were also found to contain relatively high amounts of MSC DNA. Estimated levels of engraftment in these tissues ranged from 0.1 to 2.7%, similar in the autologous and allogeneic experiments [41].

Active homing of MSCs to bone marrow occurs largely due to the stromal-derived factor-1 (SDF-1) interacting with CXCR4 on the MSC surface [42], and similar mechanisms are operational in pancreatic islets [43] as well as in ischaemic tissue [44]. In general, mobilisation of chemokine receptors expressing MSCs and their subsequent homing to injured tissues depends on cytokines, chemokines and growth factors released during systemic and local inflammatory conditions [45] and is mediated by the coordinated interaction between integrins and selectins expressed by MSCs and endothelial cells [29, 46]. Indeed, following intravenous administration, MSCs can reach the inflamed central nervous system (CNS) where they exert a potent therapeutic effect. If active homing of MSCs to inflamed and ischaemic tissue is indeed the case, this would increase the feasibility of cellular therapy for AD, since, independent of the putative soluble factor(s) produced by MSCs, they would reach the target tissues, reducing the need for using large numbers of MSCs systemically.

A new initiative is to modify surface structures on MSCs in order to increase their penetration and integration into specific target tissues. It has been shown that recruitment of cells to bone occurs within specialised marrow vessels that constitutively express vascular E-selectin, a lectin that recognises sialofucosylated determinants on its various ligands. Sackstein et al. [47] showed that human MSCs do not express E-selectin ligands but express a CD44 glycoform bearing alpha-2,3-sialyl modifications. They converted the native CD44 glycoform on MSCs

into haematopoietic cell E-selectin/L-selectin ligand (HCELL) using an alpha-1,3-fucosyltransferase preparation and enzymatic conditions specifically designed for treating live cells, which conferred potent E-selectin binding without any detrimental effects on cell viability or multipotency. MSC homing to tumours is of theoretical concern as shown by human MSC localisation in a murine xenogenic breast cancer SCID mouse model via monocyte chemotactic protein-1 (MCP-1) [48], which, while being a potential therapeutic delivery system for cancer therapy, may pose long-term safety issues in AD treatment. MSCs also concentrate in radiation-damaged and ischaemic tissue. This on the other hand may be an important advantage when treating some acute inflammatory AD with accompanying critical ischaemia such as vasculitis or SSc. As yet, active homing mechanisms have not been demonstrated [49].

Animal Models of Autoimmune Disease and Tissue Protection

Immunomodulatory, anti-inflammatory and tissue-protective effects of MSCs are intimately related [50]. An immunosuppressive effect of MSCs in vivo was first suggested in a baboon model, where infusion of *ex vivo*-expanded donor or third-party MSCs delayed the time to rejection of histoincompatible skin grafts [17]. MSCs also downregulated bleomycin-induced lung inflammation and fibrosis in murine models, if given early (but not late) after disease induction [51]. This effect was achieved through the reduction of inflammation mediated by IL-1R antagonist secreted by MSCs and capable of antagonising IL-1α-secreting T cells and TNF-α-producing macrophages [39]. Similar results were obtained by the infusion of MSCs in an acute lung injury murine model leading to a decreased production of proinflammatory cytokines and increased levels of IL-10 [52] and in a murine hepatic fibrosis model (carbon tetrachloride induced) using an MSC line bearing the fetal liver kinase-1 (FLK1) marker [53]. In all these studies, the protective effect of MSCs on lung cells occurred despite limited levels of engraftment in the target organ or transdifferentiation. Similarly, it was shown that MSC-derived conditioned medium is enriched with many chemokines able to reverse fulminant hepatic failure through the inhibition of liver infiltration by leukocytes and subsequent death of hepatocytes [54]. Tissue-protective effects were also seen in a rat kidney model of ischaemia/reperfusion injury in which syngeneic MSCs but not fibroblasts were used. These effects were not mediated by MSC transdifferentiation but, in contrast, by bystander mechanisms including the inhibition of proinflammatory cytokines and an antiapoptotic effect on target cells [55]. In another study, it was demonstrated that the renoprotective effect of MSCs was mediated by the mitogenic and pro-survival insulin growth factor-1 (IGF-1) produced by the MSC [56]. Recently, a vasculotropic effect of infused MSC in the kidney [57] was shown, which may be relevant to SSc. It is important to remember that the final phenotype of autoimmune disease expression is mostly a combination of immune-mediated inflammation, vascular occlusion and fibrosis, all potentially modulated by MSC.

Evidence supporting the paracrine hypothesis for MSCs' mediated effect on target tissues was provided also by studies demonstrating that MSCs modified with the pro-survival gene Akt1 can rescue ischaemic cardiomyocytes and restore ventricular functions [58]. Restoration of retinal function and substantial delay of retinal degeneration through inhibition of photoreceptor apoptosis was obtained through subretinal transplantation of MSCs [59]. Inhibition of apoptosis, prolonged survival and proangiogenic effects were also detected in hypoxic endothelial cells upon exposure to IL-6 and VEGF-rich MSC-derived conditioned medium [60]. Neuroprotective effects were observed also by MSCs' infusion in animal models of stroke [61]. Cerebral ischaemia is often a major issue in severe autoimmune diseases such as SLE and vasculitis. It is noteworthy that common paracrine mechanisms independent from transdifferentiation appear to support the therapeutic plasticity of MSCs for a wide range of experimental diseases.

Based on their immunomodulatory features together with tissue-protective properties and, possibly, some capacity of transdifferentiating, MSCs represented an ideal strategy to treat autoimmune disorders. Experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis (MS), was the first AD model in which the therapeutic potential of MSCs was addressed. The intravenous administration of syngeneic MSCs resulted in both clinical and histological improvement. The response was dependent on time of MSC treatment, the earlier the better, and was associated with the induction of tolerance towards the immunising myelin antigen MOG (myelin oligodendrocyte glycoprotein) [62]. In another paper, similar amelioration was obtained with human MSCs in a PLP (proteolipid protein)-induced model of EAE that showed engraftment of MSCs in mouse CNS but with limited evidence of transdifferentiation into neural cells [63]. Several other studies in EAE confirmed the beneficial effect of MSCs injected systemically [29, 64], intraperitoneally [65] or locally inside the CNS [64], suggesting that not only MSCs exert a potent inhibition of the autoimmune attack to the CNS but are also endowed with significant neuroprotective effects despite limited evidence of CNS infiltration [15]. It is noteworthy to emphasise that also neural stem cells [66] and, more recently, human embryonic stem cell-derived neuronal precursor cells [67] displayed a striking beneficial effect upon administration in EAE-affected mice through bystander mechanisms leading to immunomodulation of autoreactivity and neuroprotection.

Similar effects have been observed in experimental models of rheumatoid arthritis where MSCs alone [68, 69] or genetically modified overexpressing IL-10 [70] prevented tissue destruction and suppressed the autoimmune response against type II collagen.

In an experimental model of diabetes induced in mice by streptozotocin, it was observed that MSCs promote endogenous repair of pancreatic islets and renal glomeruli [71]. Similarly, co-infusion of MSCs and bone marrow cells, following sublethal irradiation, inhibited proliferation of pancreatic β-cell-specific T cells isolated from the pancreas of diabetic mice and restored insulin and glucose levels through the induction of regeneration of recipient-derived pancreatic β-cells in the absence of transdifferentiation [72]. The immunosuppressive effect of MSCs on T cells was exploited also in a multiorgan autoimmunity mouse model where MSCs homed in

the mesenteric lymph nodes, significantly improving the autoimmune enteropathy [73]. These results confirm the therapeutic plasticity of MSCs owing to their capacity of modulating systemic autoimmune responses and protecting target tissues.

MSCs and Human Experience

A recent survey indicated that around 1,000 humans have received MSC for various indications [74]. No adverse events during or after MSC infusion have been observed, and no ectopic tissue formation has been noted. Similar to what was observed in animal models, after infusion, MSCs are likely to remain in the circulation for a very short time, probably no more than an hour [75]. Although durable stromal cell chimerism has been difficult to establish, low levels of engrafted MSCs have been detected in several tissues [76–78], confirming preclinical studies. It is possible that sufficient therapeutic benefit is obtained by local paracrine production of cytokines and growth factors, resulting in temporary immunosuppression by the MSC infusion. However, long-term safety remains on open issue, especially regarding tumour surveillance. While encouraging results support an effect of infused MSCs in the prevention of GVHD, an increased occurrence of leukaemia relapses has been reported due to the inhibition of leukemic cell-specific T cell responses. Some human AD such as rheumatoid arthritis, Sjögren's syndrome and dermatomyositis already have a higher risk of malignancy, making follow-up a vital issue.

MSCs from Autoimmune Disease Patients

Autologous bone marrow-derived MSCs are potently antiproliferative to stimulated T cells from normal subjects and autoimmune (RA, SSc, Sjögrens, SLE) patients [79, 80]. In SSc patients, the MSCs were normal with respect to proliferation, clonogenicity and differentiation to bone and fat [80]. However, one group has shown defective differentiation into endothelial precursors in bone marrow-derived MSC from SSc patients [81], which should be considered when choosing autologous or allogeneic MSC sources for SSc treatment, since defective angiogenesis is a major feature of this AD. In other studies, MSCs isolated from MS patients [82] and Crohn's disease patients [83] exhibited the same properties as MSCs from healthy donors in terms of proliferation, phenotype, in vitro differentiation and immunosuppressive ability. Taken together, these results support the utilisation of autologous MSCs from autoimmune patients.

However, the potential cancer-related risks of MSC infusion should be considered cautiously. In fact, MSCs are being tested widely as potential agents for increasing neovascularisation in critical ischaemia settings, with the attendant risk of increasing tumour growth [84]. MSCs may also play a role in reducing tumour surveillance, as

shown in a murine melanoma model [85]. Finally, in vitro MSC manipulation may increase the risk of cytogenetic abnormalities that may result in cancer development upon in vivo administration as reported in mice [86]. It is likely that AD patients receiving MSC in experimental trials will have already been exposed to various potentially oncogenic agents such as cyclophosphamide.

MSCs in the treatment of autoimmune disease patients

Currently, few peer-reviewed publications concerning the results of using MSC in human autoimmune disease are available (Table 28.1). A small series of ten MS patients from Iran were reported using autologous intrathecal MSCs. The conclusion of this study was that intrathecal infusion of MSCs is feasible, although the clinical results were mixed and inconclusive [87]. Anecdotal report of MSC administration, intrathecally (in all) and intravenously (in some), in 15 patients with MS and 19 patients with amyotrophic lateral sclerosis (ALS) has been published providing evidence of the acceptable safety and feasibility of such treatment [97]. Although efficacy was not the primary end point, stabilisation of the clinical state was observed in some patients. In addition, migration of ferumoxide-labelled MSCs to the brain was reported as well as in vitro immunomodulation, as demonstrated by increased regulatory T cells (T-reg), decreased proliferative responses of lymphocytes and increased costimulation molecules and MHC class II on myeloid dendritic cells.

Small numbers of patients with MS have received mixtures of adipose tissue-derived MSCs, both autologous and allogeneic, given intrathecally and IV, with some clinical improvement but no MRI changes [90]. Single-case reports of MS [89], SLE alveolar haemorrhage [96] and SSc [88] patients using allogeneic MSCs appear promising as were outcomes in 14 Crohn's patients with treatment-resistant fistula giving a 71% closure rate using autologous adipose tissue-derived MSCs [91]. More recently, phase I/II studies with allogeneic bone marrow have been published in renal SLE (15 cases) [93] or umbilical cord (16 cases) [94]-derived MSCs. Most patients improved clinically and serologically, but follow-up is short and the prior therapy may have impacted outcomes [98]. Two SLE cases treated with autologous bone marrow-derived MSC failed to respond [95]. In a study of Crohn's disease (ten patients) using autologous BM-MSCs, three improved (though none achieved remission) and three progressed and required surgery [83].

Bone marrow-derived mononuclear cells containing MSCs were injected locally in ischaemic fingers and toes in two patients with SSc with an overall positive outcome and no significant toxicity [92]. This study has not used culture-expanded MSCs. In all the published small series, feasibility and safety seem acceptable, although efficacy remains an issue to be resolved by large prospective randomised trials. Two such trials, in acute GVHD and Crohn's disease, have been reported orally at meetings; both failed to reach the primary end points, attributed by the principal investigators to study design; nevertheless, both studies confirmed the feasibility and safety of MSC administration in these patients.

Table 28.1 Case reports and small series of patients with autoimmune diseases receiving MSC as therapeutic agents

Autoimmune disease	No. of patients	MSC product	Route	Outcome	Reference
Multiple sclerosis	10	Allogeneic bone marrow	Intrathecal	Mixed	[87]
Scleroderma	1	Allogeneic bone marrow	IVI	Improved	[88]
Multiple sclerosis	1	Allogeneic umbilical cord	IVI	Improved	[89]
Multiple sclerosis	3	Mixed allogeneic and autologous fat	Mixed IVI & intrathecal	Improved clinic MRI unchanged	[90]
Crohn's fistulae	14	Autologous fat	Intra-fistula	71% closure	[91]
Scleroderma digital ulcus	2	Autologous blood and marrow MNC	Local	Improved	[92]
Lupus nephritis	15	Allogeneic bone marrow	IVI	Improved SLEDAI and proteinuria	[93]
Lupus nephritis	16	Allogeneic umbilical cord	IVI	Improved SLEDAI and proteinuria	[94]
SLE	2	Autologous bone marrow	IVI	No change	[95]
SLE alveolar haemorrhage	1	Allogeneic umbilical cord	IVI	Improved	[96]
Crohn's disease	10	Autologous bone marrow	IVI	Improved	[83]
Multiple sclerosis	15	Autologous bone marrow	Intrathecal (all) plus IVI [5]	Some stabilised	[97]
Amyotrophic lateral sclerosis	19	Autologous bone marrow	Intrathecal (all) plus IVI [9]	No change	[97]

Open Issues and Future Directions

Several issues need to be evaluated for human MSC trials in AD; in some countries, cells manipulated *ex vivo* in media containing animal protein, such as fetal bovine serum, are not permitted. Human platelet lysate seems a valid alternative [99]. The use of growth factors such as FGF has been implicated in karyotypic changes in vitro, but not in vivo in one clinical trial [100]. FGF also induces the expression of functional MHC class II antigen on MSCs in vitro, which could affect their immunological behaviour in vivo [101]. This effect is negated by TGF β1, a component of platelet lysate, further supporting its use in MSC expansion protocols. Several groups are planning prospective randomised clinical trials in renal lupus, MS, type I diabetes mellitus and Crohn's disease. Important is the setting of clear therapeutic targets and harmonisation of cell products, especially MSC source and type (autologous or allogeneic), cell expansion conditions and trial protocols. In addition, long-term safety data collection across disciplines is required, and an international interdisciplinary registry of MSC-treated patients has been launched [102].

Conclusions

There are now data to suggest that the benefit/risk ratio for MSCs in human is acceptable, mostly derived from acute GVHD studies. The fact that conditioning of the patient with cytotoxic and other immunosuppressive agents is not necessary prior to MSC infusion makes this treatment attractive in critically ill patients who would be unable to undergo an autologous haematopoietic stem cell transplantation, an alternative strategy for severe AD.

Choice of patient, i.e. multisystem, inflammatory disease similar to acute GVHD or less active end-stage disease, depends on whether the study aim is efficacy or feasibility safety.

Immune privilege of MSCs is not guaranteed in an immune-competent host, but that may not be important if the MSCs home to the target organ and survive long enough to exert a therapeutic effect. An international, coordinated and interdisciplinary effort is required in order to achieve robust data on a large enough cohort of patients necessary to understand the proper role of MSC treatment among the current therapeutic options for autoimmune diseases.

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Chapter 29
MSCs for Induction of Solid Organ Allograft Acceptance

Marc Dahlke, Philipp Renner, and Elke Eggenhofer

Abstract Over the past four decades, the transplantation of solid organs (liver, kidney, heart, pancreas, and lung) has become standard clinical therapy. Despite its overall success, the shortage of donor organs and the need for lifelong immunosuppression continue to pose major challenges that need to be addressed. This chapter summarizes the preclinical efforts undertaken to investigate the potential use of mesenchymal stromal cells in the field of solid organ transplantation and presents insights into the concepts of ongoing early clinical studies.

Introduction

Previous chapters of this book have outlined the basic biology of mesenchymal stromal cells (MSCs) and the preclinical and clinical use of MSCs in proof-of-principle animal models and human diseases, respectively. Clinically, the first experience with MSCs was obtained in the field of bone marrow transplantation [1]. In this setting, the immunosuppressive and immunomodulatory effects of MSCs were successfully used to control the immune reaction of transplanted bone marrow against the host (graft-versus-host disease, GVHD) and the immune reaction of the host immune system against donor bone marrow (engraftment) [2].

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_29,

Solid Organ Transplantation: Effective Treatment with a Hitch

The success of solid organ transplantation has been the achievement of interdisciplinary medicine, bringing together basic research scientists and clinical practitioners from a wide range of specialties. Solid organ transplantation can cure patients suffering from otherwise fatal, progressive diseases. Around the globe, transplantation of cadaveric allogeneic kidneys, hearts, livers, pancreas, and lungs has become a clinical standard, while living-donor approaches (kidney, liver), split techniques (liver), islet cell transplantation (pancreas), and additional indications (small intestine) are rapidly gaining acceptance.

Yet, two problems still limit the success of solid organ transplantation, namely, the lack of enough donor organs and the complications associated with lifelong immunosuppressive therapy. The shortage of donor organs has become so serious that death on the waiting list is common, and the socio-economic cost of treating waiting-list patients is becoming a big burden. As a result, use of donor organs of suboptimal quality has become an ethically understandable approach. These so-called marginal organs present an increased risk to the recipient, and it is difficult to predict how "marginal" a graft is going to be acceptable for an individual patient. Therefore, strategies to improve marginal organs before transplantation are also needed, and cellular therapy with MSCs appears to be a promising approach.

The second main problem in solid organ transplantation is the need for lifelong immunosuppressive therapy after transplantation of an allogeneic graft. All of the drugs currently used for immunosuppression, in addition to being costly, cause acute and long-term side effects. In addition to intrinsic toxicities, such as the neurotoxicity of tacrolimus or the renal toxicity of cyclosporin, the risk for malignancies and opportunistic infections is also markedly increased in solid organ transplant recipients under chronic immunosuppressive therapy. In fact, the overall success of organ transplantation as a curative therapy is, at least in part, governed by the side effects of immunosuppressants used. Yet, pharmacological immunosuppressants continue to be indispensable to ensure long-term allograft function, and weaning of immunosuppression can be dangerous. To overcome this dilemma, strategies achieving operational transplant tolerance without (or with a significantly reduced need for) pharmacotherapy are required. One attractive option to achieve cell-based immunoregulation is the implementation of immunomodulatory MSC therapies.

MSCs in Solid Organ Transplantation: New Strategy for Old Problems?

Although solid organ transplantation in itself is clinically successful, most life-shortening events in transplantation patients are the consequence of either side effects of immunosuppressive medication or graft failure due to tissue injury, infection, or rejection. Therefore, to further improve the outcome of transplant recipients,

the next generation of immunomodulative therapies will have to go beyond simply suppressing effector immune cells while at the same time providing a beneficial safety profile. New hope to achieve these goals has been raised by investigating the application of MSCs in transplantation recipients. A series of recent data support the idea that MSCs may be beneficial through both their immunomodulatory and regenerative properties [3].

Some of the most intriguing preclinical models are discussed in detail below. Most published observations to date indicate that MSCs are a promising cell population suitable for clinical use in solid organ transplantation: First, none of the clinical trials conducted so far (for GVHD, Crohn's disease, and other disorders) have reported unwanted side effects directly related to the administration of MSCs, suggesting that MSCs have an acceptable short-term safety profile [1, 4]. Second, MSCs have been found to be immunosuppressive both in vitro and in vivo, an ability that we will discuss in greater detail below. Third, MSCs also have regenerative functions, which may positively affect organs damaged by ischemia-reperfusion injury. Fourth, MSCs can be culture expanded to large quantities and thus be multiplied to nearly unlimited numbers, which distinguishes them from most other cell populations considered for cellular therapy in transplantation medicine. Taken together, MSCs seem to be a cell population suitable for clinical use in transplantation.

Transplant Immunology and How MSCs Interfere

Transplanted organs can be rejected by a multitude of different immunological mechanisms. Although our understanding of the biology of rejection has markedly improved over recent years, the complexity of the anti-graft immune response is still not fully elucidated. Historically, the discovery of major and minor histocompatibility complexes has shown that rejection is mainly driven by antigen recognition through T cells. Alloreactive effector T cells can either attack the graft through the concerted action of helper-inductor and cytotoxic T cells or give rise to B cells producing antibodies against alloantigens [5]. Remarkably, MSCs express MCH class I and II molecules at low levels only and, as a result, do not provoke a pronounced immunological response after infusion into an immunocompetent host. This feature of MSCs has been referred to as their "immunoprivileged status," although the term is misleading because many models have shown that MSCs are in fact capable of inducing an immune response. Whether the (low) immunogenicity of MSC can be harmful in transplantation patients or beneficial in other instances is subject of ongoing research.

A series of in vitro studies have shown that MSCs can suppress T cell proliferation upon non-specific or alloantigen-driven stimulation [6, 7]. This suppressive effect is directed against both CD4 and CD8 T effector cells but is not directly dependent on MHC, because most experiments have shown that donor, recipient, and third-party MSCs can all exert a comparable suppressive function

[8]. The exact immunological mechanism of MSC-mediated T cell suppression is still unclear, but it is accepted that both soluble factors (such as nitric oxide, prostaglandin E_2, interleukin-10, indoleamine 2,3-dioxygenase, and metalloproteinases) and cell contact-driven effects are contributing factors (as reviewed by [9]). Also, recent results suggest that MSCs require a proinflammatory micromilieu to be activated and exert their immunosuppressive function [10]. In the context of organ transplantation, a "proinflammatory" state is usually present in any graft early after transplantation, providing an environment for MSC therapy to be potentially effective. Importantly, however, other conditions, such as an anti-inflammatory micromilieu, can potentially cause MSC to boost T cell responses and to promote rejection [11]. As the relationship between MSCs and T cells is bidirectional, it is important to always consider the particular micromilieu in which MSCs are infused. This is especially important for solid organs early after transplantation since all transplant patients require immunosuppressive drugs, and most immunosuppressants have been found to interfere with the interaction between MSCs and T cells [12]. Animal models have shown that immunosuppressants that abrogate T cell activation, such as calcineurin inhibitors, decrease the effectiveness of MSCs, whereas other immunosuppressive drugs do not [13]. An example of an immunosuppressive drug that does not inhibit the effect of MSCs is mycophenolic acid, which allows T cell activation and interferon-gamma secretion but still inhibits mitotic divisions [14].

In addition to mechanisms leading to rejection that involve T cells, an increasing number of studies outline the relevance of other immune cells, especially of those belonging to the systems of innate immunity. The spectrum of cells involved in both rejection and tolerance ranges from natural killer cells (NKs) to macrophages, monocytes, and dendritic cells (DCs) [15]. Among these, MSCs can inhibit NK cell proliferation and cytolytic activity under certain conditions. Furthermore, MSCs can also inhibit DC maturation, promoting a more "tolerogenic" DC type, and alter the expression of chemokine receptors on DCs [16, 17]. APC modification could resemble a central mechanism for MSC-mediated immunomodulatory effects, especially as costimulation of T cells through APCs is an important pathway for allo-reactions and target of pharmaceutical therapies [18].

From Theory to Practice: MSCs in Preclinical Models

Before any novel therapy can enter the clinical field, extensive in vitro and animal testing is mandatory. Although it is sometimes difficult to judge how much preclinical testing in animals is necessary to address both the safety and efficacy of a new therapy before starting human studies, a critical database is indispensible. We and others believe that the preclinical knowledge base for MSC therapy in solid organ transplantation is sufficient and have therefore initiated first clinical studies in humans. To understand how these clinical study decisions were made, the most important pieces of preclinical evidence are presented in brief.

MSCs in Skin Transplantation Models

Over the past decade, MSCs have been administered in several skin and solid organ transplantation animal models with promising but also sometimes conflicting results. Bartholomew and colleagues were the first to use MSCs in a baboon skin transplantation model. In their study, baboons received donor-derived MSCs intravenously on the day of transplantation, and skin graft survival was subsequently prolonged from 7.0 ± 0 to 11.3 ± 0.3 days [8]. Although graft survival in this first study was not markedly prolonged, the effect of MSCs in this acute rejection model was considered immunologically relevant and has encouraged further investigations applying MSCs in a variety of organ transplantation models: For example, skin transplantation experiments by the group of Sbano examined the effect of MSC infusion for allogeneic skin transplantation in rats. When applied without concurrent cyclosporin A immunosuppression, donor-derived MSCs led to shortened allograft survival. When applied with concurrent cyclosporin A, however, allograft survival was prolonged [19].

MSCs in Animal Models of Solid Organ Transplantation

Skin is the most antigenic tissue in the body, presenting a major obstacle for allograft survival. As a result, indefinite survival of skin allografts was not achieved with MSCs. This is in contrast to transplantation models for vascularized organs, in which MSCs can lead to operational tolerance and stable graft acceptance without continuous immunosuppression. Our own first experiments in a rat heart transplantation model indicated that MSC injections were not effective in prolonging heart allograft survival [20]. However, after modifying the protocol, we found that donor-derived MSCs induce long-term allograft acceptance (>100 days) when applied concurrently with a short course of low-dose mycophenolate mofetil (MMF). Again, when MSCs were injected without concomitant immunosuppression, all animals rejected their heart graft within 6 days, earlier than animals receiving no treatment, suggesting that allogeneic MSCs elicit an anti-donor immune response in vivo [14].

Casiraghi et al. investigated the role of MSCs in a murine semi-allogeneic heart transplant model. Infusions of donor-derived B6C3 MSCs in B6 recipients induced a profound T cell hyporesponsiveness and prolonged B6C3 cardiac allograft survival [21]. In another experimental system, Ge et al. found that treatment involving infusion of MSCs into BALB/c recipients 24 h after receiving a heart allograft from a C57BL/6 donor significantly abated rejection and doubled mean graft survival time compared to untreated recipients. Furthermore, combination therapy of MSCs and low-dose rapamycin led to long-term heart graft survival (>100 days). The treated recipients readily accepted donor skin grafts but rejected third-party skin, indicating the establishment of tolerance [22].

The Role of Concurrent Immunosuppression

Although most preclinical results so far provide arguments in favor of a beneficial effect of MSC administration in vivo, they cannot easily be transferred to the more complex setting of a human transplantation patient. In all animal experiments in which long-term acceptance was achieved, concurrent treatment with immunosuppressive drugs was also used. Most of the available data indicate that MSC therapy can indeed lead to prolonged allograft survival, provided that MSCs are accompanied by appropriate immunosuppressive pharmacotherapy. It is therefore undoubtedly reasonable to combine MSC-based cell therapy with drug-based immunosuppression in clinical trials.

Answers to Many Questions yet Many Questions to Answer

The current body of evidence suggests that the application of MSCs may well be safe and effective in prolonging graft survival. Yet, clinicians face a number of difficult questions for the design of their first clinical trials.

First, the source of MSCs is a matter of discussion among experts. For example, recipient-derived MSCs seem to be the safest choice for patients, but it is impractical to obtain and store a sufficient number for every patient on the waiting list. Donor-derived MSCs are an even less realistic option, since patients will need MSCs within a short time before or after transplantation; as most organs come from deceased donors, no donor MSCs are likely to be available. Third-party MSCs as an "off-the-shelf" product are thus the most convenient option but may be less specific and could lead to allosensitization.

Second, there is no uniform opinion about the most suitable timing, route of injection, and cell number for the injection of MSC. Nearly all published animal models have used different protocols, leaving limited possibilities of deducting substantiated data for these variables. Considering the fact that even the pharmacokinetics and pharmacodynamics of many small molecules are different between animals and humans, it can be expected that such interspecies differences are even more relevant for cellular therapeutics, such as MSCs. The appropriate route for MSC injection and the optimal number of cells to be injected are additional open questions. Third, concurrent pharmacological immunosuppression has to be chosen according to the few facts that are known about the interplay between MSCs and immunosuppressants.

Apart from the above parameters, little is known about the long-term effects of MSCs. Although most tracking studies suggest that MSCs somehow "vanish" after infusion, few but alarming studies show that MSCs can also localize to organs or even support tumor growth [23, 24].

Facing the Future: First Clinical Trials

Encouraged by the available preclinical data, several clinical transplantation trials with MSCs are in preparation, while first promising results were published recently. Consensus on standardized procedures, MSC production, and ethical issues has been developed, paving the way for comparable results grounded on protocols with a maximum of patient safety [23, 24].

In a pilot study published in 2010, Perico et al. treated two patients receiving kidneys from living-related donors with a T cell-depleting induction therapy and a single dose of autologous MSC on day 7 after transplantation. Both patients experienced graft dysfunctions during the observation period, but graft function was stable after 1 year of follow-up [25]. In a clinical trial by Tan and colleagues, 159 kidney transplantation patients were allocated to either treatment with MSC plus standard dose of calcineurin inhibitors (CNIs), MSC plus low-dose CNIs, or induction therapy with anti-IL2 receptor treatment plus standard-dose CNI. While there was no difference between these groups concerning patient and graft survival, subjects treated with MSC instead of anti-IL2 receptor induction had a lower incidence of acute rejection and a significantly decreased risk for opportunistic infections [26]. This study underscores the importance and influence of the concomitant immunosuppressive protocol used but also shows that MSC therapy has the potential to be beneficial for transplant patients.

Additional phase I/II studies are registered to test both safety and efficacy of MSCs in solid organ transplantation, involving mostly kidney transplantation but also lung or liver transplantation [27]. For the latter, our own group developed a study protocol for the assessment of safety and feasibility of multipotent adult progenitor cells, a third-party cell product with comparable abilities to MSCs [28]. The results of these studies will set the course for further evaluation of MSC-based therapies in solid organ transplantation.

Conclusions

Cellular immunotherapy is a young and rapidly changing field of transplantation medicine, with great hopes and a multitude of possible applications. The use of mesenchymal stromal cells in solid organ transplantation is attractive, as MSCs might contribute to new solutions in antirejection and regenerative therapy. First clinical experiences are promising, but future trials with carefully designed concomitant immunosuppressive protocols are needed to finally establish clinical evidence for the safety and efficacy of MSCs in this field.

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Chapter 30
MSCs for Gastrointestinal Disorders

Maria Ester Bernardo, Angela Maria Cometa, and Franco Locatelli

Abstract Recent experimental findings and clinical trials have shown the ability of mesenchymal stromal cells (MSCs) to home to damaged tissues and to produce paracrine factors with anti-inflammatory properties, resulting in reduction of inflammation and functional recovery of the damaged tissues. These properties, in the context of regenerative medicine, are being tested in chronic inflammatory disorders of the gastrointestinal (GI) tract. Phase I–II clinical trials indicate that intravenous and local injection of ex vivo-expanded MSCs are feasible and safe in luminal and fistulizing Crohn's Disease (CD) and in end-stage liver diseases. Large randomized clinical trials are warranted to properly establish the role of MSC therapy for these diseases, in comparison with conventional treatment. This chapter focuses on recent research on the anti-inflammatory/reparative properties of MSCs and discusses the potential clinical applications of MSC-based cellular therapy in GI disorders.

Introduction

Mesenchymal stromal cells (MSCs) were first described as a population of adherent cells isolated from the bone marrow (BM) which were non-phagocytic, exhibited a fibroblast-like appearance, and could differentiate in vitro into bone, cartilage,

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_30,

adipose tissue, tendon, and muscle [1]. Moreover, after transplantation under the kidney capsule, these cells gave rise to the different connective tissue lineages [2]. Based on their multilineage differentiation capacity, MSCs were first considered as a therapeutic tool for treatment of bone and cartilage diseases, in particular for bone and articular cartilage defects, as well as for the treatment of *osteogenesis imperfecta* [3–6]. Moreover, due to their immunomodulatory and engraftment-promoting properties, the clinical role of MSCs has been also explored in the context of phase I–III clinical trials, in the arena of hematopoietic stem cell transplantation, to facilitate engraftment and to treat steroid-resistant acute graft-versus-host disease; initial studies demonstrated the feasibility and safety of MSC intravenous infusion in these settings [7–9].

More recently, experimental findings and clinical trials have focused on the ability of MSCs to home to sites of injury and to promote, at this level, tissue repair through the production of trophic factors and anti-inflammatory molecules, resulting in functional recovery of the damaged tissue and reduction of inflammation [10–15]. Based on these encouraging preliminary findings, numerous potential clinical applications will be discussed including for gastrointestinal (GI) disorders and, in particular, inflammatory bowel disease (IBD).

Testing MSCs in In Vivo Animal Models of Gastrointestinal Disorders

MSCs have been tested in animal models of experimental colitis and liver failure due to their ability to home to inflamed sites and to repair injured tissues, and in view of their immunomodulatory properties (see Table 30.1) [16–32]. In preclinical models of IBD, MSCs are beneficial when administered intraperitoneally [16], intravenously [27], or directly into colonic tissue surrounding mucosal lesions [17]. In particular, topical implantation of BM-derived MSCs promotes the healing process in a rat experimental model of colitis induced by intraluminal instillation of 2,4,6-trinitrobonzene sulfonic acid (TNBS). The direct injection of MSCs into the colonic submucosa was associated with accelerated healing of the damaged tissue, as documented both macroscopically and immunohistologically [17]. In similar models of experimental colitis, MSCs of different tissue origin (isolated from adipose tissue and gingiva) alleviated the signs and symptoms of the disease by displaying immunomodulatory functions and ameliorating inflammation-related tissue destruction [16, 18].

It was recently shown that MSCs are as good as hematopoietic stem cells in populating in the injured regions of the colon after TNBS-induced damage [28] and can promote and restore epithelial barrier integrity in an experimental model of dextran sulfate sodium-induced colitis [19]. BM-derived MSCs administered by direct injection in the gastric tissue surrounding the lesion in a rat model of gastric ulcer were associated with significantly accelerated healing of the organ damage, compared with controls. The infused MSCs expressed vascular endothelial growth

Table 30.1 In vivo animal models of MSCs in gastrointestinal disorders

Context	Outcome	Refs.
Rat, TNBS-induced experimental colitis	Stimulated intestinal mucosa healing	[16–18]
Rat, dextran sulfate sodium-induced colitis	Favored and restored epithelial barrier integrity	[19]
Rat, gastric ulcer	Accelerated organ damage healing via angiogenesis	[20]
Dog, small intestine regeneration model	MSC seeded on collagen sponge grafts, promoted regeneration	[21, 22]
Immunodeficient mice, radiation injury model	Homing to radiation-injured tissues, accelerated structural recovery of small intestine	[23, 24]
Mouse, experimental injury of the anal sphincter	Surgery + MSC: stimulated tissue repair	[25]
Mouse, abdominal sepsis model	Decreased mortality, improved organic animal function	[26]

TNBS 2,4,6-trinitrobonzene sulfonic acid, *MSC* mesenchymal stromal cells

factor (VEGF) and hepatocyte growth factor (HGF) which were believed to contribute to the reparative process, possibly by induction of angiogenesis [20].

Tissue engineering based on MSC seeding on collagen sponge grafts in a canine model enabled regeneration of the small intestine although the newly formed intestine lacked a muscle layer [21]. Successful regeneration was also shown in an animal model of intestinal excision and repair with an MSC-enriched collagen-agarose three-dimensional patch on a human or swine aortic fragment [22]. Tissue regeneration after radiation injury was obtained with BM-derived MSCs intravenously infused into irradiated immunodeficient mice. The MSCs were capable of specifically homing to radiation-injured tissues, increased self-renewal of the gut epithelium, and accelerated structural recovery of small intestine [23, 24].

In a murine model of experimental injury of the anal sphincter, treatment with surgery and injection of BM-derived MSCs were effective in promoting tissue repair [25]. In a recent study, BM-derived MSCs were infused intravenously in an animal model of abdominal sepsis before and after its induction and was associated with decreased animal mortality of up to 50% and improvement in organ function [26].

MSC Potential Mechanisms of Action

Although the mechanisms through which MSCs exert their regenerative potential in tissue repair are not fully understood, it is likely that their ability to secrete soluble factors capable of stimulating functional recovery of injured cells and their capacity to home to sites of damage and modulate immune responses play a role [10–15]. Indeed, in most reported studies, the beneficial effect of MSCs did not

seem to be associated with their differentiation into the resident cell types, but appeared to be mostly related to their antiproliferative and anti-inflammatory effect, likely through paracrine mechanisms [13, 14]. It is reasonable to speculate that the therapeutic benefit of MSCs is due to the release of soluble factors and chemokines (such as HGF, insulin-like growth factor, prostaglandin E2, nitric oxide) produced by the cells and/or by the local microenvironment and that MSC survival is not necessary to produce a meaningful clinical effect [13, 14]. Moreover, current experimental and clinical data indicate that sustained engraftment of MSCs within the damaged organ is not required or it is limited to a small number of cells. In this regard, studies in baboons using a green fluorescent retroviral construct suggest engraftment of MSCs in the gastrointestinal tract and in various other tissues is in the range of 0.1–2.7% [29].

Homing of MSCs to target tissues after infusion might depend on cytokines, chemokines, and growth factors released during systemic and/or local inflammatory conditions and might be mediated by the interaction with integrins and selectins expressed on the surface of MSCs, such as CXCR4 [30–32]. Another possible mechanism of action of MSCs is displayed by their ability to stimulate the generation/differentiation of regulatory T cells. This has been shown in an experimental murine model of Crohn's disease (CD) in which MSC infusion was efficacious in both preventing and curing colitis and was associated with the induction of FoxP3+ regulatory T cells [16].

Biological and Functional Properties of MSCs Derived from Patients with Gastrointestinal Disorders

Contradictory results have been published on the biological and functional properties of ex vivo-expanded MSCs from patients affected by autoimmune and chronic inflammatory diseases [33–35]. For example, MSCs isolated from patients with systemic sclerosis were reported to be functionally impaired in vitro, while other reports document that the MSCs of systemic sclerosis patients, as well as those from patients with other autoimmune diseases, exhibit comparable phenotypical and functional properties of their healthy counterparts [33, 34].

Whether "diseased" MSCs, isolated from patients affected by IBDs or other GI inflammatory disorders, are functionally impaired or display similar characteristics as those of healthy donors needs to be properly investigated in each specific clinical context. In this regard, it has been demonstrated that MSCs isolated from BM of patients affected by refractory CD show the typical biological and functional properties of MSCs cultured from healthy donors [15, 35]. In particular, MSCs isolated from CD patients (CD-MSCs), in the presence of either fetal bovine serum or platelet lysate as culture supplements, display the typical spindle-shaped morphology and differentiation ability into osteoblasts and adipocytes. Their surface markers do not differ from those of healthy donors, with the exception of increased

expression of HLA-DR at early culture passages in CD-MSCs that was lost after subsequent passage [35]. Moreover, CD-MSCs do not show a propensity to undergo spontaneous transformation after long-term in vitro culture, as assessed by both conventional and molecular karyotyping [35]. In addition, CD-MSCs are capable of inhibiting in vitro polyclonally induced proliferation of peripheral blood mononuclear cells (PBMCs) in both autologous and allogeneic settings, as shown also for those isolated from healthy donors [15, 35]. Duijvenstein et al. demonstrated also that MSC proliferation is not affected by coculture with immunosuppressive drugs commonly employed in CD patients [15].

The data on CD-MSCs provide the experimental background for considering their use as in the management of CD patients. Indeed, in non-profoundly immunodepressed subjects, such as those affected by inflammatory and degenerative disorders, patient-derived MSCs, rather than third-party cells, are to be preferred. In this setting, allogeneic and/or third-party cells might be recognized as non-self and rejected by CD patients after infusion, without having the chance to display their beneficial tissue-healing effect.

Similar exploratory studies to establish the functionality of MSCs should be performed before considering their clinical use in various inflammatory and degenerative disorders of the GI tract.

Clinical Application of MSCs in Gastrointestinal Disorders

In recent years, there has been a significant increase in the number of clinical trials employing MSCs to treat digestive diseases, mainly for CD, in its luminal and fistulizing forms, and for liver failure (see Table 30.2).

Luminal Crohn's Disease

Despite the large number of therapeutic options available, there is a growing number of CD patients with relapsing/refractory disease [46]. In view of this consideration, and of the serious side effects of more aggressive therapies, alternative strategies are needed both to increase the proportion of CD patients achieving stable remission and to improve their quality of life. Based on the encouraging results obtained in preclinical studies [16–28], an open-label phase II clinical trial testing Prochymal® (Osiris Therapeutics, Inc. Columbia, MA) for treatment-resistant CD was carried out and showed a statistically significant reduction in the mean CD activity index (CDAI) score of 105 points by day 28 with one-third of the patients achieving clinical remission [36, 37]. Moreover, a positive correlation between dose and response was found, with the patients receiving the highest dose achieving a greater response (average CDAI reduction of 137 vs. 65).

Table 30.2 Clinical application of MSCs in gastrointestinal disorders

Context	Outcome	Refs.
Luminal CD		
Phase II trial, autologous MSC i.v.	Significant reduction of CDAI, 33% clinical remission	[36, 37]
Phase I, 10 pts, autologous BM-MSC i.v.	Feasibility and safety, clinical response in 3 pts	[15]
Fistulizing CD		
Phase I, 5 pts, autologous AT-MSC l.i.	Promoted fistula repair	[38]
Phase II randomized controlled trial, 49 pts, autologous AT-MSC l.i.	70% response in fibrin glue + MSC vs. 16% in fibrin glue alone (also fistulas of cryptoglandular origin)	[39]
1 pt, rectovaginal fistula, allogeneic AT-MSC l.i.	Partial healing of the fistula, no MSC rejection	[40]
Phase I–II trial, 10 pts, autologous BM-MSC l.i.	Feasibility and safety, CR in 7 pts, reduction of CDAI/PDAI	[41]
Other gastrointestinal disorders		
Cirrhosis, phase I trial, 4 pts, autologous BM-MSC i.v.	Safety, improvement in quality of life, in 2 pts amelioration of liver function and MELD	[42]
End-stage liver disease, phase I–II, 8 pts, autologous BM-MSC, 6 i.v., 2 via portal vein	Safety, amelioration of MELD	[43]
Ulcerative colitis, 39 pts, allogeneic BM-MSC i.v.	Improved inflammation indices, increased remission duration	[44, 45]

CD Crohn's disease, *CDAI* CD activity index, *pts* patients, *BM* bone marrow, *i.v.* intravenous infusion, *AT* adipose tissue, *l.i.* local injection, *CR* complete response, *PDAI* perianal disease activity index, *MELD* Model for End-stage Liver Disease

The results of a phase I study of autologous BM-derived MSCs (two intravenous infusions, 1 week apart) in the treatment of moderate to severe luminal CD refractory to conventional therapies were recently published [15]. Feasibility and safety of this novel approach were demonstrated; a clinical response, defined as a drop in CDAI score >70, was seen in three patients, while remission, defined as CDAI < 150, was not achieved in any of the patients treated. Two patients dropped out due to disease worsening, three required surgery, and the remainder showed only a reduction of the CDAI score <70 points. As regards mucosal healing, an improvement in inflammation, as evaluated by using the CD endoscopic index of severity, was seen in two patients, while in other five cases, no significant improvement was evident. Moreover, a trend of lower CD4+ T cells and higher CD4+ CD127+ regulatory T cells, together with a decrease of pro-inflammatory cytokines, was found in mucosal biopsies after treatment [15].

Fistulizing Crohn's Disease

External fistulas, a disabling and difficult-to-treat manifestation of CD, are associated with a high relapse rate despite a large therapeutic armamentarium [47]. During

the last decade, biological agents have become first-line treatment; however, the benefit in terms of sustained fistula healing is limited, and therefore, alternative therapeutic strategies are warranted.

In a phase I clinical trial, autologous, adipose tissue (AT)-derived MSCs were successfully employed to treat complex perianal fistulas in five patients affected by CD with promising results [38]. In 2009, the same group performed a phase II, randomized, controlled trial in which patients with complex perianal fistulas of cryptoglandular origin ($N=35$) or associated with CD ($N=14$) were randomly assigned to local treatment with fibrin glue or fibrin glue + autologous AT-derived MSCs. Fistula healing was observed in 17 of the 24 (70%) patients who received AT-derived MSCs in addition to fibrin glue versus 4 of the 25 (16%) patients who received fibrin glue alone, irrespective of the cryptoglandular or CD origin of the fistulas [39]. Histological and electron microscopy analyses of mucosal biopsies harvested from 5 CD patients treated with intrafistular injections of AT-derived MSCs showed processes indicative of mucosal healing with highly vascularized areas, abundant collagen fibers, and fibroblasts and areas of stratified perihelium, in the absence of neoplastic transformation [48].

Garcia-Olmo et al. also reported the case of a CD patient with a rectovaginal fistula who, for the first time, was treated with a local injection of allogeneic AT-derived MSCs; the administration was not associated with adverse effects and/or rejection of the cells, and partial healing of the fistula was observed [40].

In a recent phase I–II study, 10 CD patients with actively draining, complex perianal fistulas, refractory to or unsuitable for current available therapies, were treated with intrafistular injections of autologous BM-derived MSCs ($20–30 \times 10^6$ MSCs, scheduled every 4 weeks for a median of four infusions) [41]. No adverse events were recorded during the procedure and up to 12-month follow-up period. At surgical evaluation, 7/10 patients benefited from complete closure of fistula tracks, while 3/10 experienced a partial response. Moreover, all patients showed a significant reduction of both CDAI and perianal disease activity index (PDAI) scores, achieving disease remission (CDAI ≤ 150, PDAI ≤ 8) usually after the second procedure. At endoscopic evaluation, a healthy rectal mucosa with a normal vascular pattern was evident in all cases, and magnetic resonance imaging revealed the presence of regenerative tissue along the fistula tracks, without evidence of fibrosis in any patient. Moreover, the percentage of both mucosal and circulating regulatory T cells increased during the treatment and remained stable until the end of 12-month follow-up.

These data indicate that local injection of ex vivo-expanded MSC is feasible, safe, and efficacious in treating refractory fistulizing CD, thus suggesting that the possibility to cure this debilitating condition by cellular therapy may be a real prospect in the near future.

Other Gastrointestinal Disorders

MSCs have been employed to treat liver cirrhosis in a limited number of patients; preliminary results confirm the safety of this approach and suggest a trend in clinical

improvement [42, 43, 49]. In particular, in a phase I study, autologous BM-derived MSCs were infused in four patients with cirrhosis via peripheral vein without adverse events. All patients reported an improvement in quality of life, while two patients showed amelioration of liver dysfunction and specific disease score, such as the Model for End-stage Liver Disease (MELD) score [42]. In a phase I–II study, autologous BM-derived MSCs were administered to eight patients with end-stage liver failure, six via the portal vein and two via a peripheral vein. No adverse effect was registered, and MELD and prothrombin international normalized ratio (INR) improved significantly [43].

Concerning the use of MSCs in ulcerative colitis, there is only one publication in a Russian journal in which 39 ulcerative colitis patients and 11 CD patients were treated with systemic infusion of allogeneic BM-derived MSCs ($1.5–2.0 \times 10^8$ cell/patient) [44]. The authors reported that MSCs improved both clinical and morphologic indices of inflammation, increased duration of remission, and reduced the risk of relapse and hospital admissions when compared with conventional therapy (5-aminosalicylic acid and glucocorticosteroids), with a follow-up period of 4–8 months. The subsequent analysis of cost-effectiveness showed that this new therapeutic strategy is useful in saving both direct and indirect costs [45].

Studies are also planned for the treatment of other GI autoimmune and inflammatory disorders, such as autoimmune enteropathy and celiac disease.

Conclusions

The clinical trials performed for GI disorders to date suggest that MSC use is feasible and safe. No severe adverse reactions have yet been recorded in humans after MSC administration, either immediate, infusional toxicity, or later effects; however, longer follow-up is necessary to draw definitive conclusions on potential long-term adverse events (in particular, ectopic tissue and tumor formation) [50–52]. Although improvement in clinical and laboratory scores has been reported in most trials of MSCs for GI disorders, formal demonstration of the superiority of this approach is lacking. Methodological limitations related to the small numbers of subjects treated in each study, the lack of a control group in the majority of these studies, the variability of the cellular product (MSC tissue source, protocols of MSC expansion), and in the administration routes (intravenous versus local infusion) further preclude the soundness of interpretation of results. The execution of large multicenter randomized clinical trials addressing response to MSC therapy, compared with conventional treatment for different GI disorders, is essential.

Moreover, to fully exploit the potential of MSCs as a novel therapeutic strategy in GI disorders, more in vivo work is required to increase the knowledge of how MSCs mediate their protective/reparative effect and reduce inflammatory responses. Experimental and clinical data collected indicate that MSCs exert their therapeutic benefit mainly through paracrine mechanisms, in the absence of a sustained engraftment of the transplanted cells within the damaged organ in

most cases [10–15, 29, 41]. Furthermore, optimal timing of MSC administration, cell dose, and schedule of administration need to be defined in chronic inflammatory disorders. For example, in the context of IBD, MSCs may not be a "once-in-a-life treatment," but could represent a helpful tool during the active and severe phases of the disease. Whether the simultaneous administration of other immunosuppressive treatments could potentiate or abolish the therapeutic benefit of MSCs also needs to be addressed. Whether autologous or allogeneic MSCs are preferable needs also to be clearly investigated. It is reasonable to speculate that in chronic inflammatory diseases of the GI tract, in which sufficient time for MSC harvest and ex vivo expansion is available, autologous cells can be employed, provided that they are functionally active given the potential for the rejection of allogeneic cells. The risk that MSCs could contribute to fibrosis in liver diseases needs also to be precisely ascertained, given that some reports suggest a possible role of MSCs in the fibrogenic process which typically leads to worsening of the disease [53, 54].

In conclusion, once more data on the mechanism of action, safety, best source, dosage, and most efficacious route of administration of MSCs are obtained, MSC-based cellular therapy is likely to become an effective therapeutic tool to enhance tissue repair and blunt the exaggerated inflammation in chronic inflammatory GI disorders.

Acknowledgment This work has been partly supported by grants from Istituto Superiore di Sanità (National Program on Stem Cells), MIUR (Ministero dell'Istruzione, dell'Università e della Ricerca, Progetti di Rilevante Interesse Nazionale, PRIN), from Associazione Italiana per la Ricerca sul Cancro (AIRC) special grant "5 × 1,000" to FL, and from AIRC IG9062 to MEB.

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Chapter 31
MSCs for Cardiac Repair

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Abstract Cardiovascular disease has reached epidemic proportions worldwide. Cell-based therapy for advanced heart and vascular disease may offer new hope for those afflicted. Although a variety of cell types are under investigation, mesenchymal stromal/stem cells (MSCs) have compelling features that offer advantages as an off-the-shelf therapy. These cells are multipotent, anti-inflammatory, and immunoprivileged and can incite regenerative growth factors via paracrine mechanisms. Animal studies and clinical trials are underway to characterize these cells and demonstrate efficacy for acute and chronic heart disease. This chapter reviews the current understanding of MSCs for heart disease, preclinical and clinical experience to date, delivery methods under investigation, and exciting new approaches to boost therapeutic efficiency.

Introduction

Cardiovascular disease is a leading cause of morbidity and mortality. Nearly 2,400 Americans die of cardiovascular disease each day. Healthcare expenditure related to cardiovascular disease exceeds $260 billion/year in the USA [1]. Myocardial infarction leading to left ventricular dysfunction and congestive heart failure is the greatest contributor to these dire statistics. A clear need exists

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_31,

to curb adverse remodeling of the ventricles and restore myocardial function. Cell-based therapy has emerged as an exciting and potential revolutionary approach to the treatment of advanced heart and vascular disease. Successful demonstration of the cardiovascular regenerative potential of unselected, bone marrow-derived mononuclear cells in small animal models led to an explosion of clinical trials testing this treatment approach in acute and chronic heart disease. Despite early enthusiasm from preclinical studies, human trials have demonstrated either negative or only modest benefits. This has prompted a reevaluation of the investigational approach. The latest generation of human cardio-regenerative trials is focusing on selected cell populations such as mesenchymal stromal cells (MSCs). Herein, we discuss the potential therapeutic mechanisms of MSCs for cardiovascular disease, review preclinical and clinical trial evidence with an emphasis on ischemic heart disease conditions, and highlight novel approaches to optimize cell delivery and therapeutic efficacy.

Potential Therapeutic Mechanisms of MSCs

MSCs are non-hematopoietic, multipotent cells with mesodermal transdifferentiation capacity [2–4]. These cells offer several advantages over other cell types for cardiovascular applications (Fig. 31.1). They can be isolated from multiple sources, including bone marrow, adipose tissue, umbilical cord blood, and cardiac tissue. They can also be expanded and banked with relative ease [4, 5]. MSCs are assumed to be immunoprivileged due to the relatively low expression of MHC class II, and other co-stimulatory molecules, and therefore, do not activate host T cells even in highly inflammatory environments [6–8]. These features permit "off-the-shelf" allogeneic cell therapy. In vitro studies have also demonstrated that MSCs are anti-inflammatory [9–12]. These cells can secrete a wide variety of pro-angiogenic and cardioprotective cytokines such as vascular endothelial growth factor, fibroblast growth factor-2, insulin-like growth factor-1, and hepatocyte growth factor in response to ischemia-reperfusion injury, hypoxia, and other stressors [13–15]. The potential for MSCs to transdifferentiate into cardiac tissue constituents remains controversial and is discussed elsewhere in this book.

Animal Studies

MSCs delivered to ischemic myocardium in small (mouse, rat, rabbit) and large (dog, pig, sheep) animal models have resulted in robust functional benefits including reduction of infarct size and improved myocardial function, perfusion, and vascular density. MSCs have successfully been tested in small animal models of myocardial infarction (MI) induced by transient or permanent coronary artery

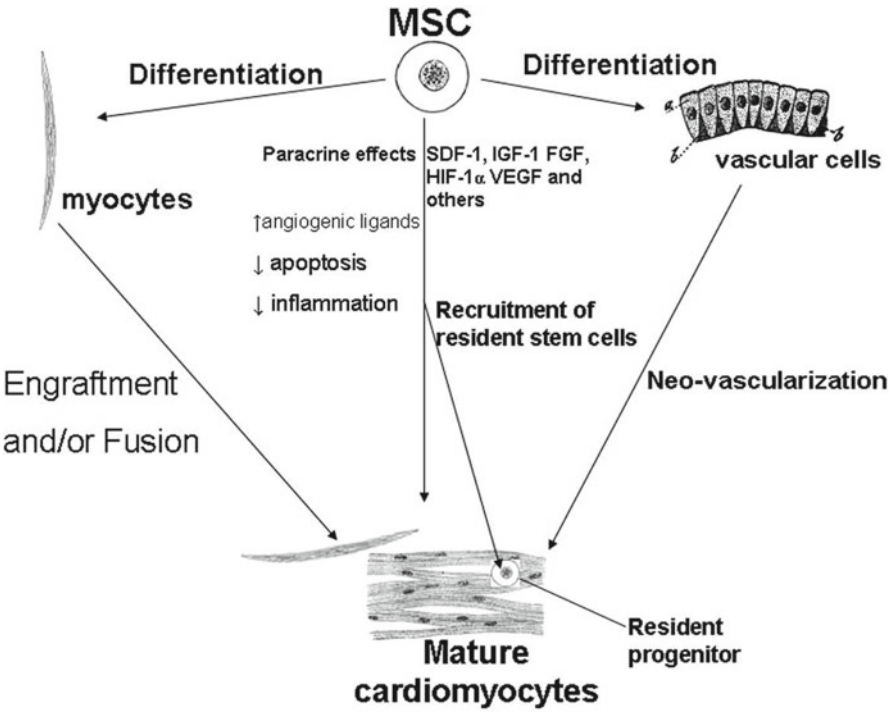

Fig. 31.1 Mechanisms of MSC therapy for cardiac disease. Paracrine factors: stromal cell-derived factor 1 (SDF-1), insulin-like growth factor (IGF-1), fibroblast growth factor (FGF), hypoxia-inducible factor 1α (HIF-1α), and vascular endothelial growth factor (VEGF)

ligation [16], in addition to chronic heart failure models [17] (see Table 31.1). Methods of cell delivery in small animals have included intramyocardial injection within the infarct and adjacent border area [21], systemic intravenous infusion [20], ultrasound bubble delivery [22], and through biocompatible patches seeded with cells [19].

Large animal models permit investigation of clinically translatable delivery methods, such as intracoronary infusion or transendocardial delivery [23]. While these delivery methods have proven successful, few studies compare delivery methods head to head. For example, comparisons between systemic intravenous, intracoronary infusion, and transendocardial injection catheter of MSCs in pigs showed no difference in infarct size, but greater acute retention with intracoronary infusion than direct intramyocardial injection or intravenous infusion (Table 31.2) [25, 28]. Similar studies in dogs demonstrated no differences in functional improvements between delivery methods but greater acute retention of cells with intramuscular injection compared to intracoronary infusion [27, 29]. Studies in other species also show an increased retention of cells with direct intramyocardial injections [21]. Finally, one study found no difference between intracoronary

Table 31.1 Small animal models of MSC therapy for cardiovascular disease

Species	Cardiac model	Delivery method/timing	Cell type/number	F/U weeks	Functional endpoint	Histologic endpoint	Reference
Mouse	Permanent coronary artery ligation	Direct intramuscular injection in peri-infarct zone/ immediate	BM MNC/5 × 10^5 BM MSC/5 × 10^5	6	Echo: improved LV function with MNC but not MSC	Some transplanted cell survival	van der Bogt et al. [18]
Mouse	Permanent coronary occlusion by cryo-injury	Bio-Patch/30 days post-op	BM MSC/5 × 10^5 on patch	2	Pressure volume loop: improved dp/dt	Increased angiogenesis, no change in infarct size	Derval et al. [19]
Mouse	Permanent coronary artery ligation	Intravenous/1 h post injury	BM MSC/1 × 10^6	4	Pressure volume loop: increased contractility, no change in end diastolic volume	No change in infarct size Cells home to peri-infarct regions	Boomsma et al. [20]
Rat	Aorta clip to create pressure overload + heart failure	Intracoronary >10 week after clip placement	BM MSC/1 × 10^6	4	Echo: improved function PV loop: improved hemodynamics	Decreased metalloproteinase expression	Molina et al. [17]
Rat	Coronary artery ligation	Direct injection into borderzone/1 h, 1 week, 2 week post injury	BM MSC/2 × 10^6	4	Echo: improved function	Reduced infarct size Increased neoangiogenesis	Hu et al. [16]
Rat	Transient coronary artery ligation - reperfusion	Direct intramuscular injection in ischemic region vs. intravenous infusion	BM MSC/2 × 10^6	1	Not assessed	15% of MSCs retained in the heart for direct intramuscular injection. None for intravenous	Hale et al. [21]
Rabbit	Coronary artery ligation	Direct intramuscular injection	BM MSC/1 × 10^7 + ultrasound bubbles	4	Echo: improved function	Increased neoangiogenesis Reduced infarct size	Xu et al. [22]

BM MSC bone marrow mesenchymal stromal/stem cells, *BM MNC* bone marrow mononuclear cells, *MRI* magnetic resonance imaging

Table 31.2 Large animal models of MSC therapy for cardiovascular disease

Species	Cardiac model	Delivery method/timing	Cell type/number	F/U weeks	Functional endpoint	Histologic endpoint	Reference
Pig	Surgical ligation or balloon occlusion of coronary artery	Transendocardial injection to ischemic zone/3 days post injury	BM MSC/ 100–200 × 10^6	8	MRI: improved LV function Pressure volume loop: normalized systolic and diastolic function	Reduced infarct size Transplanted MSCs detectable up to 8 weeks peri-infarct	Amado et al. [24]
Pig	Balloon occlusion reperfusion of coronary artery	Intravenous/immediate Intracoronary/ immediate Transendocardial/ immediate	BM MSC/ 50 × 10^6	2	n/a	No change in infarct size Retention: slightly better for intracoronary infusion vs.	Freyman et al. [25]
Pig	Balloon occlusion reperfusion of coronary artery	Transendocardial/12 week post injury	BM MSC/ 200 × 10^6	12	MRI: reduced infarct size, improved ejection fraction	Increased vascular density Evidence of engraftment	Quevedo et al. [23]
Dog	Ameroid constrictor on coronary artery	Direct intramuscular injection/4 week post injury	BM MSC/ 100 × 10^6	4	Echo: improved function	Increased vascular density	Silva et al. [26]
Dog	Surgical ligation of coronary artery	Intracoronary/7 days Transendocardial/7 days	BM MSC/ 100 × 10^6	2	Electromagnetic mapping: improved ischemic area with transendocardial injection	No differences in infarct size for either delivery mode Intracoronary infusion of MSC causes microvascular plugging	Perin et al. [27]

BM MSC bone marrow mesenchymal stromal/stem cells, *MRI* magnetic resonance imaging

infusion and direct intramuscular injection [30]. Similar engraftment has been observed when cells are delivered to the epicardium as compared to the endocardium [31]. While it would seem logical that delivery methods that increased MSC retention would confer enhanced functional benefits, there is little evidence to date to confirm this claim.

Most animal studies have been performed with bone marrow-derived MSCs, although other sources of cells such as adipose-derived MSCs have been tested. Direct comparisons of adipose verses bone marrow-derived MSCs were performed in a mouse MI model. Wu et al. showed that nearly all MSCs were dead by 5 weeks and there were no improvements in function in either group compared to control mice [18]. A second study failed to prove that bone marrow mononuclear cells transplanted to ischemic myocardium were superior to MSCs, skeletal myoblasts, and fibroblasts [32].

Age and health of the MSC donor may affect the therapeutic potential of MSC therapy. MSCs harvested from young mice improved myocardial function and reduced infarct size in older mice, whereas MSCs harvested from 18-month-old mice had no effect [33]. A similar finding occurred in rats [34]. Cell culture work also suggests that MSCs are subject to molecular genetic changes during aging that are observed during passage in culture [35]. MSCs from donors with other comorbidities aside from aging may also affect the viability of the cells and the efficacy of the treatment.

The number of cells injected ranges from 25 to 450 million cells in large animal models to 500,000 to 1 million cells in small animals. Treatment at each of these levels has shown efficacy. In dose-escalation study in swine, transendocardial injection of MSCs (8.6×10^5–1.6×10^7 cells/kg bodyweight) showed no difference in myocardial function between doses or control. A reduction in infarct size was observed at all doses compared to controls, although no differences were observed between doses [36]. In a similar study in pigs, intravenous delivery of MSCs (1×10^3 up to 1×10^6/kg bodyweight) demonstrated an improvement in myocardial function and a reduction in infarct size starting with doses over 1×10^5 MSC/kg [37]. In sheep, 25, 75, 225, or 450×10^6 MSC were injected into the infarct border zone. Improvements in ventricular function were noted at all doses, but only the lowest two doses had a reduction in infarct size [38]. From these studies, it would appear in large animals that a dose of 1×10^5–1×10^6 cells/kg is likely optimal. Higher doses delivered intravenously or via local intracoronary infusion could result in toxicity to the lungs or potentially limit blood flow in the coronary arteries [25, 27]. In addition, higher doses of cells injected may increase the risk of mutations or other safety issues such as tumor formation [37]. Lower cell dose limits to efficacy have not been established in animal models to date.

Temporal delivery of MSCs ranges from immediate to 12 weeks post-MI in animal models. Optimal delivery time was tested in rats at 1 h, 1 week, and 2 weeks post-MI. While all time points showed some benefit, the greatest benefits were observed with cells injected 1 week post-MI [16, 39]. Others have suggested that delivery of cells between 2 days and 1 week has the same benefits [40]. Most studies have shown myocardial contractility improvement and infarct size reduction

after single dose cell delivery within 2–4 weeks. Generally, progressive decreases in ventricular function continue beyond 4 weeks. Therefore, it may be beneficial to deliver cells at multiple time points particularly in light of the evidence showing positive effects with the first injections at 28 days post-MI or later [26, 41] Salient points to consider include the inflammation response from the tissue soon after infarction to the healed over and mature scar that would be present 2–3 weeks postinfarction.

Studies with a primary focus of safety in animal models have been limited to immediate effects of the cell delivery. All methods appear to be reasonably safe during and immediately after cell delivery with the exception of delivery directly in the coronary arteries. Some reports show some plugging of the arteries by the cells causing adverse events [25, 27, 42]. Longer term safety studies have not been systematically performed in animals except in the mouse where tumor formation has been noted in sites remote from the injection site. This may not translate well to larger animal models as it appears that rodent MSCs may not always be genetically stable [43–45]. Indeed, in one study in swine, the 3-month histopathology showed no evidence of rejection, calcification, teratoma formation, or myocardial infarction [46].

Clinical translation of the methods employed and favorable outcomes observed in animal studies is a reasonable prospect. Studies employing large animal models may be more predictive of human trial results, due to similar cardiac physiology as compared to rodent models. However, the lack of comorbidities in animals as compared to patients with advanced heart and vascular disease is a limitation. These comorbidities can independently alter the course of clinical outcomes, adversely affect the quality of autologous MSCs, and reduce the responsiveness of recipient heart tissue to transplanted cells.

In summary, positive results in animal studies have translated to only modest improvements in human studies. The effects of cell aging, chromosomal stability in vivo, host tissue responsiveness, and the influence of comorbidities on cell potency may partially explain the disparity. Expanded understanding of cell retention in vivo, engraftment, viability and function of cells, mechanism of action, optimization of cell type, dose, timing, long-term safety and efficacy, and delivery methods is required.

Clinical Trials

Numerous safety and efficacy trials of unselected and selected bone marrow cell populations have demonstrated feasibility and safety of cell therapy and delivery for cardiovascular indications. Strong claims of efficacy have been limited by relatively small sample sizes. The cardiovascular clinical trial experience with selected MSCs has so far been limited. We review the clinical experience using bone marrow cells and isolated MSCs, in the context of recent myocardial infarction and chronic ischemic cardiomyopathy.

Recent Myocardial Infarction

MI is usually caused by abrupt thrombotic occlusion of a coronary artery, occurs in 1 million patients annually in the USA, and is associated with 25% mortality over 3 years [47]. Options to restore flow in the infarct-related artery include primary coronary angioplasty with stents, or thrombolytic therapy. Patients, who present late or in whom therapy to restore coronary artery flow fails, suffer a poor prognosis. A proportion will develop progressive adverse ventricular remodeling leading to congestive heart failure and sudden death. The goal of cell therapy in the early post-MI period is to curb future adverse remodeling.

Strauer et al. transplanted unfractionated bone marrow mononuclear cells (BMNC) in patients who had suffered recent myocardial infarction treated emergently by primary angioplasty with stent [48]. Briefly, 5–9 days post-MI, a bone marrow aspirate was performed from the iliac crest. The mononuclear cell fraction was then infused directly into the infarct-related coronary artery using catheters that are familiar to interventional cardiologists. A coronary balloon was transiently inflated during infusion to prevent backflow of cells. At 3 months, infarct size decreased, ejection fraction increased, and cardiac perfusion was improved compared to a parallel control group. Fernandez-Aviles et al. reported improved 6-month regional and global LV function with cardiac MRI at 6 months in 20 patients using a similar cell source and delivery approach [49].

Following these initial results, several cell therapy trials for recent MI patients have been performed. The Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI) trial compared intracoronary infusion of circulating peripheral blood mononuclear cells (29 patients) compared to BMNCs (30 patients) [50]. Similar functional and viability improvements were seen in both groups, and both showed improvements over control patients. The Bone Marrow Transfer to Enhance ST-elevation Infarct Regeneration (BOOST) was the first randomized-control trial to compare intracoronary infusion of BMNCs (30 patients) against placebo (30 patients) [51]. At 6 months, cardiac ejection fraction improved in the BM-treated patients; however, this benefit was not sustained at 1 year. Janssens et al. reported results from a double-blind, randomized controlled trial of intracoronary infusion BMNCs. At 4 months, the infarct size was reduced; however, there was no significant improvement of ejection fraction, myocardial flow, or metabolism in infarcted segments using highly sensitive imaging techniques such as magnetic resonance imaging (MRI) [52]. The Autologous Stem Cell Transplantation in Acute Myocardial Infarction (ASTAMI) trial was a double-blind, randomized trial of intracoronary BMNC infusion with 50 patients in treated and control groups, respectively [53]. No improvement in infarct area, size, and function with intracoronary BMNC therapy was observed at 6 months. The Reinfusion of Enriched Progenitor Cells and Infarct Remodeling in Acute Myocardial Infarction (REPAIR-AMI) trial was a double-blind, randomized trial of nearly 100 patients per group of BMNC injected via intracoronary route versus control [54].

The BMNC-treated group had a significant 2.5% increase in LV ejection fraction at 4 months assessed by cine ventriculography. Furthermore, the treatment group had a reduction in the major cardiovascular adverse events at 1 year. The Myocardial Regeneration and Angiogenesis in Myocardial Infarction with Granulocyte Colony-Stimulating Factor (GCSF) and Intracoronary Stem Cell Infusion (MAGIC) study randomized 27 patients to GCSF-mobilized peripheral blood mononuclear cell infusion compared to GCSF alone compared to placebo following emergency stenting for MI [55]. The treated group showed improved myocardial function and perfusion.

Cytokine therapy with granulocyte colony-stimulating factor (GCSF) is effective for mobilization of bone marrow-derived progenitors into the peripheral circulation. Early clinical studies suggested that GCSF therapy after MI reduces ventricular remodeling and improves ejection fraction [56]. However, recent larger and randomized controlled trials failed to demonstrate benefit with GCSF administered to post-MI patients [57, 58]. A similar neutral effect was observed in patients with chronic ischemia and refractory angina [59]. With the evidence available, it appears that cytokine mobilization alone is not effective treatment for cardiovascular disease.

In the aftermath of the initial experiences with unselected bone marrow mononuclear cells, clinical investigators have recently explored bone marrow cell subpopulations. Notable among these are MSCs. Chen et al. randomized 69 patients to postprimary angioplasty for MI to undergo intracoronary MSC injection compared to saline at 18 days post-MI [60]. Improvements in cardiac dimension, function, and perfusion were seen in the MSC-treated group at 3 months. Katritsis et al. delivered autologous MSCs in 11 patients, 4 months following anteroseptal MI [61]. Safety in the approach and improvements in cardiac wall motion were observed in this cohort.

In the USA, Hare et al. demonstrated the safety of intravenous administration of bone marrow-derived allogeneic MSCs in patients with recent myocardial infarction [62]. Fifty-three patients were enrolled, in three dose ranges, with the upper dose at 5.0×10^6 cells/kg body weight. Key hypothesis-generating observations were a reduction in ventricular arrhythmia episodes and improved pulmonary function. Quantitative echocardiography demonstrated no significant improvement in ejection fraction at 3 and 6 months; however, an MRI sub-study showed a significant improvement in ejection fraction that persisted to 12 months. These results have provided support for a current Phase II study (ClinicalTrials.Gov NCT00877903).

Chronic Ischemic Cardiomyopathy

Congestive heart failure (CHF) is a clinical syndrome resulting from myocardial injury that impairs the heart's ability to circulate blood sufficiently to meet the metabolic needs of the body. CHF is common and can lead to frequent

hospitalization and sudden death. Initially, the principal goal of cell therapy for CHF was myocyte replacement; however, this paradigm is being reevaluated. Newer concepts such as paracrine factor-induced reverse geometric remodeling and neoangiogenesis are being proposed as potential beneficial mechanisms for therapy.

Autologous bone marrow mononuclear cells and skeletal myoblasts have been delivered in small series of CHF patients with reduced ejection fractions by catheter-directed transendocardial injections [63], intracoronary infusion [64], and direct epicardial injections during concomitant coronary artery bypass surgery [65, 66]. Intramyocardial deposits of skeletal myoblasts lead to apparent ventricular arrhythmia, perhaps due to poor electrical integration within the myocardium [66]. Nevertheless, short-term improvements in cardiac function combined with modest symptom relief were observed, forming the basis for future, larger scale clinical trials.

The Prospective Randomized Trial of Direct Endomyocardial Implantation of Bone Marrow Cells for Therapeutic Angiogenesis in Coronary Artery Diseases (PROTECT-CAD) trial randomized 28 chronic ischemia patients to catheter-directed intramyocardial BMNC transplant or control [67]. Significant improvements in exercise time, cardiac function, and perfusion were observed. Strauer et al. recently reported improvements in exercise capacity, ejection fraction, and 5-year mortality with intracoronary infusion of unfractionated bone marrow cell therapy in 191 patients with chronic ischemic cardiomyopathy [68].

Unlike unfractionated bone marrow, trial data for MSCs are currently limited. Moyheddin-Bonab et al. report observations from a small cohort of eight patients with ischemic cardiomyopathy who received autologous MSCs delivered by a surgical epicardial approach at the time of bypass or via intracoronary infusion at the time of an angioplasty procedure. Compared to a matched cohort, improvements in functional capacity, ejection fraction, and ischemic burden were seen at 1 year. Hare et al. at the University of Miami have launched an early phase trial entitled "The Transendocardial Autologous Cells (hMSC or hBMC) in Ischemic Heart Failure Trial (TAC-HFT)." This trial is enrolling patients who have ischemic heart failure and reduced ejection fraction (ClinicalTrials.Gov NCT00768066). Treatment arms include unselected autologous bone marrow mononuclear cells, autologous MSCs, and placebo. The study agents are delivered via a unique transendocardial catheter with a corkscrew-shaped tip (Helix, Biocardia Inc.). The primary outcome is safety, and key secondary outcomes are improvements in heart function measured by magnetic resonance imaging and improvements in patient overall functional capacity. A second early phase, ongoing study from the same group, entitled "The Percutaneous Stem Cell Injection Delivery Effects on Neomyogenesis in Dilated Cardiomyopathy" (POSEIDON-DCM) is comparing allogeneic versus autologous MSCs (ClinicalTrials.Gov NCT01392625). The delivery approach and endpoints are similar to the TAC-HFT study.

Summary of Clinical Trial Observations

The evidence to date suggests that autologous unfractionated bone marrow mononuclear cells delivered locally into infarcted myocardium and chronically ischemic myocardium is safe and may offer modest but detectable benefit in the prevention of post-MI adverse remodeling and restoration of ventricular function, respectively. Enriched mesenchymal stem cells delivered systemically and locally also appear to be safe; however, larger randomized trials are necessary to convincingly demonstrate efficacy.

Cell Delivery Methods

A variety of cell delivery methods to treat cardiac disorders have been utilized in preclinical and clinical trials. The optimal delivery route is still not known; however, there are several advantages and disadvantages of each approach (See Table 31.3). Systemic intravenous delivery is least invasive, but relies on intact cardiac homing signals. This may be suitable for very recent MI, where these signals may be strong, but less suitable for chronic heart failure, where these signals are barely detectable. In addition, a large proportion of cells trap in the lung vasculature, which may adversely influence the efficacy and safety of this approach [69]. Local direct epicardial intramuscular delivery via an open thoracotomy is most invasive, and risky, particularly in fragile populations with advanced heart disease. Alternatively, minimally invasive local delivery may be achieved using catheters. Infusing cells through a catheter directly into a patent coronary artery uses techniques familiar to interventional cardiologists, but acute retention is low, and large cells or "clumps" may result in worsened ischemic injury from vascular plugging [27, 70]. Intramuscular catheter delivery from "inside" the heart chamber (also called transendocardial) is another minimally invasive delivery method. X-ray fluoroscopy [24], electro-anatomic mapping [63, 71–78], real-time magnetic resonance imaging (MRI) guidance [79, 80], and 3D MRI to X-ray fluoroscopy registration and overlay [81] are imaging methods used to guide catheters and track injection locations (See Fig. 31.2). Transendocardial "intramuscular" administration has the potential to offer improved cell retention over infusional approaches and does not rely on patent coronary arteries [29].

"Boosting" MSCs to Treat Cardiovascular Disease

Methods to "boost" MSC for enhanced therapeutic potential can be broadly categorized into (1) genetic modification, (2) environmental conditioning, and (3) biologic scaffolds.

Table 31.3 Methods to deliver cells to the heart

Delivery method	Advantages	Disadvantages
Intravenous infusion	Least invasive	Relies on intact homing mechanisms to site of injury
	Does not require cath suite or operating room	Increases likelihood of cell retention in other organs (i.e., lungs, liver, spleen)
Intracoronary infusion	Uses techniques very familiar to cardiologists	May cause microvascular plugging and infarct extension (especially MSCs)
	Minimally invasive	Requires patent coronary artery
	Ability to give high cell doses locally	
Transendocardial catheter injections	Minimally invasive	Risk of cardiac perforation
	Ability to give high cell doses locally	Concern for arrhythmogenesis
	Can target key areas (i.e., infarct borders) with imaging tools	
	Potentially improved acute retention due to less washout effect	
Retrograde delivery via the coronary sinus	Minimally invasive	Washout risks low cell retention
		Risk of coronary sinus perforation
Direct intramyocardial injection	Target areas visualized	Invasive – requires major surgery
	Ability to give high cell doses locally	Concern for arrhythmogenesis
	Improved retention over infusional approaches	
Cell-based patch repair	Engineered scaffolding tailored to stem cells and application	Invasive – requires major surgery
	Large cell doses can be applied	Concern for arrhythmogenesis

Gene Modification

Targeted overexpression of growth factors has been accomplished by transfecting cells with plasmids or viral vectors bearing the gene of interest. Vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), angiopoeitin-1 (Ang-1), hepatocyte growth factor (HGF), heme oxygenase-1 (HO-1), and stromal-derived factor-1 (SDF-1) are protective growth factors that promote neovascularization. MSCs overexpressing VEGF [82, 83], FGF-2 [84], Ang-1 [85], HGF [86], HO-1 [87], SDF-1 [88], and the SDF-1 target CXCR-4 [89] have shown improved cell survival, neoangiogenesis, and improved ventricular recovery postischemic injury. These techniques offer the advantage of achieving a more specific cell modification; however, transfection efficiency, duration of effect, and toxicity related to certain viral vectors represent significant challenges.

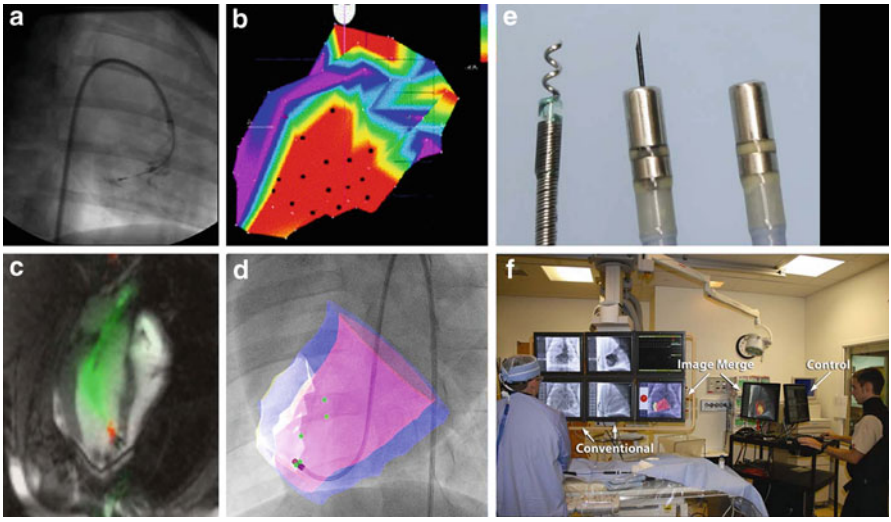

Fig. 31.2 Imaging methods used to guide transendocardial injection of cells. (**a**) X-ray fluoroscopy with Stiletto catheter (Boston Scientific). (**b**) Electro-anatomic mapping (NOGA XP, Bioscence Webster). (**c**) Real-time MRI with modified Stiletto, Boston Scientific. (**d**) MRI to X-ray "fused" overlay with detached Myostar catheter (Cordis Biologic Delivery Systems). (**e**) Transendocardial injection catheters used in clinical trials. Helix catheter, Biocardia Inc. (*left*) and Myostar catheter, Cordis Biologic Delivery Systems, with needle deployed and undeployed (*middle and right*). (**f**) MRI to X-ray "fused" overlay in use during transendocardial injection procedure in a clinical biplane catheterization laboratory (Philips FD10), University of Wisconsin-Madison

Environmental Conditioning

Genetically modifying cellular DNA can result in unpredictable consequences, and several important safety issues remain before rapid translation to clinical trials. Instead, many groups are exploring methods to expose MSCs to environmental conditions that may coax the cells to a more favorable phenotype. For example, coculturing MSCs with VEGF, SDF-1, FGF-2, insulin-like growth factor-1, bone morphogenetic protein-2, and transforming growth factor-alpha appears to improve MSC cardioprotection following acute ischemia-reperfusion injury [90–94]. Hypoxia preconditioning of MSCs upregulates Akt, eNOS, VEGF, and CXCR-4 expression and attenuates ventricular remodeling post-MI [95, 96].

Biologic Scaffolds

Poor MSC engraftment efficiency may be due to mechanical stresses imposed in the beating heart [24, 97, 98]. Several preclinical studies have demonstrated the feasibility, safety, and preliminary efficacy of mounting MSCs within a biologic scaffold, to then implant into the injured heart [99, 100].

Conclusions

Preclinical and clinical data supporting the use of MSCs to treat a variety of cardiovascular disorders are encouraging. MSCs possess interesting and useful cardio-regenerative, paracrine, and immunomodulatory properties that may translate into "off-the-shelf" therapy for acute and chronic heart disease. Although the optimal delivery method is still unknown, there have been significant advances in imaging and minimally invasive tools to target key areas of the heart. Finally, significant efforts are underway to boost the therapeutic potential of MSCs. Success in these approaches may result in a robust, tailored, cell-based treatment approach for cardiovascular disease in the future.

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Chapter 32
MSCs for Treatment of Acute Lung Injury

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Abstract Acute lung injury (ALI) is an important cause of acute respiratory failure in critically ill patients. Although there has been progress in reducing mortality with improved supportive care, there are no specific therapies to reduce mortality. This chapter summarizes some of the preclinical studies that have supported the hypothesis that allogeneic human bone marrow-derived mesenchymal stromal/stem cells (MSCs) could be effective for the treatment of clinical ALI. This chapter also considers some of the steps required to translate allogeneic human MSCs therapy for treatment of patients with ALI, including regulatory requirements and the details of designing an early phase clinical trial.

Introduction

Mesenchymal stromal/stem cells (MSCs) are multipotent stem cells that were first isolated from the bone marrow and have the capacity to differentiate into bone, cartilage, and fat. However, MSCs do not have the same degree of plasticity as

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_32,

embryonic stem cells [1, 2]. There are some practical advantages in the potential use of the bone marrow-derived MSCs that will be considered in this chapter such as a finite life span that may be relevant in any clinical trial. We now know that adult MSCs can be isolated from many tissues and organs, not only the bone marrow, but also the placenta and adipose tissue. The International Society for Cellular Therapy has defined MSCs by several criteria, which include ability for self-renewal and differentiation. The phenotype of MSCs is characterized by expression of specific cell surface markers (including CD105, CD90, and CD73) as well as being negative for other surface markers (including CD11b, CD14, CD34, and CD45) [3], and it also has the capacity to differentiate into mesenchymal cell lineages under in vitro conditions [1].

Several investigators have documented the ability of MSCs to modulate immune responses, including the function of dendritic cells, T and B cells, as well as neutrophils and macrophages. These interactions are mediated by release of both pro- and anti-inflammatory cytokines, as well as lipid mediators including prostaglandin E_2. MSCs also produce and secrete growth factors, most notably keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF). Further, MSCs produce angiopoietin-1, a molecule that can improve both endothelial and epithelial barrier permeability [1]. MSCs are capable of production (or induction in other cells types) and the release of antimicrobial peptides such as LL-37 and lipocalin-2/NGAL.

Preclinical Animal and Cell Studies of Acute Lung Injury

Previous studies have reported the potential value of allogeneic human MSCs for the treatment of clinical disorders including sepsis, acute renal failure, hepatic failure, and acute myocardial infarction [1]. This section will focus on preclinical studies that have supported the potential utility of MSC therapy for ALI in preclinical models.

In 2003, Ortiz et al. reported that MSC therapy reduced fibrosis in a bleomycin-induced lung injury model in mice and that the observed effects did not depend upon significant engraftment (<5%) [4]. A subsequent study in 2007 by Ortiz et al. reported that the release of interleukin-1 receptor antagonist (IL-1RN) by MSCs was primarily responsible for the therapeutic effect observed [5]. In addition, in a mouse model of lung injury induced by intraperitoneal endotoxin administration, intravenous delivery of MSCs prevented lung edema and inflammation [6].

Our own research group reported that the intratracheal delivery of bone marrow-derived mouse MSCs 4 h after instillation of high-dose endotoxin into the lungs resulted in significantly less acute lung injury and improved survival in mice [7]. The MSCs reduced the degree of pulmonary edema and demonstrated improvement of lung injury when compared to PBS as well as cellular controls such as mouse lung fibroblasts (3T3) and apoptotic MSCs. The beneficial effects of MSCs were associated with a decrease in the levels of proinflammatory cytokines as well

as an increase in anti-inflammatory cytokines, including IL-10 and IL-13. Our subsequent studies have demonstrated that the bone marrow-derived mouse MSCs also reduce mortality in live *E. coli*-induced lung injury in mice. Interestingly, the number of bacteria recovered from the lungs was less with MSCs therapy compared with the saline or fibroblast controls [8]. The effect of MSCs on reducing the number of bacteria in these studies with the mouse bone marrow-derived MSCs appeared to be related in part to the release and induction of lipocalin-2 (also known as neutrophil gelatinase-associated lipocalin – NGAL), a well-known antimicrobial protein.

The antimicrobial effects of the bone marrow-derived human MSCs were tested in another mouse model of *E. coli* pneumonia. In these studies, human MSCs released substantial quantities of human cathelicidin (hCAP-18/LL-37), a potent antimicrobial peptide. In these studies, *in vitro* data demonstrated significant inhibition of *E. coli*, *P. aeruginosa*, and/or *S. aureus* growth by human MSCs in part through the secretion of LL-37. The antimicrobial effects of MSC-secreted products were also demonstrated in *E. coli* pneumonia in mice using an anti-LL-37 antibody [9]. Subsequent studies from our group have demonstrated that MSCs upregulate the capacity of blood monocytes to phagocytize bacteria. These observations were made in an intraperitoneal Gram-negative sepsis model, in which the survival was improved with MSC therapy, as compared to controls treated with saline or fibroblasts. Decreased bacteremia was noted in the MSC-treated mice, which was associated with the enhanced monocyte phagocytosis [10]. There was no effect on neutrophil phagocytosis. Another group of investigators reported improved bacterial clearance, related to enhanced splenocyte-mediated phagocytosis as demonstrated in CD11b-positive cell population isolated from the spleen of MSC-treated mice in a cecal ligation and puncture peritonitis model [11]. Others reported a beneficial effect of intravenously delivered bone marrow-derived mouse MSCs in peritoneal sepsis secondary to cecal ligation [12]. In these latter studies, the therapeutic benefit was in part explained by an increased production of the anti-inflammatory cytokine, IL-10, with reprogramming of alveolar macrophages through release of PGE_2 by intravenously infused MSCs.

In addition to studies of the adult lung in preclinical animal models, there have also been some important publications in which MSCs have been used to treat hyperoxic-induced lung injury in rodent models of bronchopulmonary dysplasia. One study reported that intratracheal administration of bone marrow-derived MSCs improved the hyperoxic neonatal lung injury, based on both physiologic and structural studies [13]. In vitro studies suggested that the protective effect might have been related to paracrine products released by the MSCs. In another study [14], intravenous MSCs given shortly after birth reduced hyperoxic lung injury with less inflammation and less pulmonary hypertension. Also, the use of the MSC-conditioned media, given intravenously, replicated all of the beneficial effects of the MSCs themselves, providing evidence that paracrine factors released by the MSCs were responsible for the beneficial effects. Thus, there may be opportunities for translating MSC therapy in infants with neonatal lung injury [13–15].

Preclinical Studies in the *Ex Vivo* Perfused Human Lung Preparation

In order to further evaluate the potential therapeutic value and identify mechanistic pathways by which MSCs might be effective in ALI, we have tested MSCs in our *ex vivo* perfused human lung preparation. This preparation was originally developed to study the alveolar epithelial fluid clearance and lung fluid balance in the human lung [16]. Subsequently, we adapted this preparation to study the effects of endotoxin-induced acute lung injury in the human lung. In order to make the model physiologically relevant, approximately 100 ml of fresh human blood with viable neutrophils is added to the perfusate resulting in an average hematocrit level of the perfusate of about 4%. The lungs are inflated with 10 cm of H_2O continuous positive airway pressure using 95% oxygen and 5% carbon dioxide. In the initial experiments, allogeneic human MSCs were administered into the bronchus of the right middle lobe that had been injured 1 h earlier with a high dose of endotoxin (6 mg). Normal human lung fibroblasts served as controls. The results indicated an impressive reversal of endotoxin-induced lung injury in the MSC-treated lungs. MSCs treatment restored both the increase in lung endothelial permeability and in extravascular lung water to a closer to normal values (Fig. 32.1). Furthermore, alveolar fluid clearance was normalized, though the endotoxin administration had eliminated the capacity of the alveolar epithelium to remove the excess alveolar fluid (Fig. 32.2). Histologically, the MSC-treated lungs following endotoxin-induced injury appeared similar to control lung tissue [17]. There was also an associated reduction in the quantity of proinflammatory

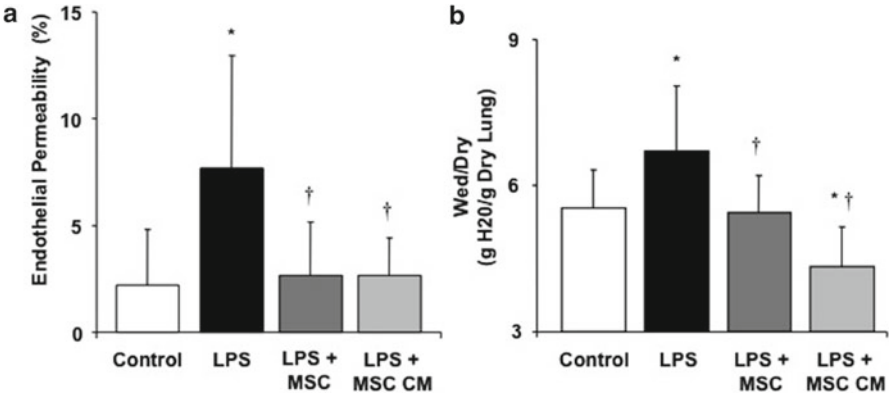

Fig. 32.1 Effect of human MSCs or MSC-conditioned media (CM) on lung endothelial permeability to protein and wet/dry ratio. Instillation of MSCs or MSC-CM into the endotoxin-injured RML or LLL 1 h later restored lung endothelial permeability to protein (**a**) and wet/dry (W/D) ratio (**b**) to control values. Data are expressed as mean % endothelial permeability or W/D ratio ± SD, $n = 4–5$ lungs; $^{*}P < 0.0001$ vs. control lobe, $^{\dagger}P < 0.0011$ vs. LPS (0.1 mg/kg) injured lobe for lung endothelial permeability and $^{*}P < 0.0014$ vs. control lobe, $^{\dagger}P < 0.005$ vs. LPS (0.1 mg/kg) injured lobe for the W/D ratio by ANOVA (Bonferroni) (Reprinted from Lee et al. [18])

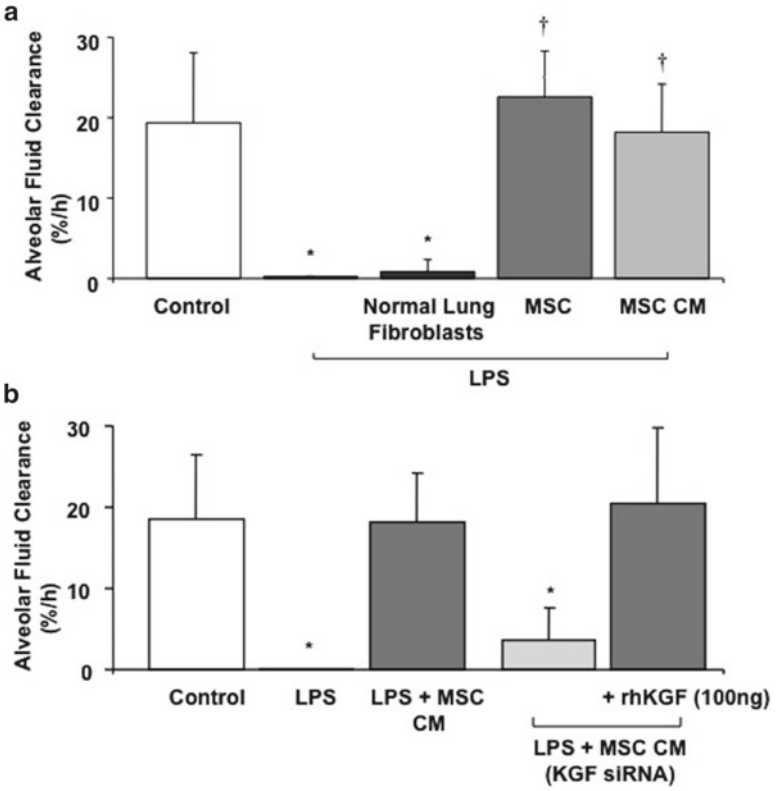

Fig. 32.2 Effect of human MSCs or MSC-CM on alveolar fluid clearance (**a**). MSCs or MSC-CM restored the decrease in alveolar fluid clearance in the lung lobe injured by endotoxin to control values at 4 h. $n=3$–4; $^{*}P<0.0006$ vs. control AFC; $^{\dagger}P<0.0001$ vs. LPS (0.1 mg/kg) AFC by ANOVA (Bonferroni). Effect of the CM of human MSCs pretreated with a KGF siRNA on alveolar fluid clearance (**b**). Administration of the CM of MSCs grown for 24 h pretreated with the KGF siRNA (#10818, Ambion) into the endotoxin-injured lung lobe after 1 h prevented the restoration of AFC with the CM alone. The addition of recombinant KGF (100 ng) to the CM pretreated with KGF siRNA restored the decrease in AFC to control values. Data are expressed as mean AFC ± SD, $n=4$–5 lungs; $^{*}P<0.0012$ vs. control lobe by ANOVA (Bonferroni) (Reprinted from Lee et al. [17]). KGK has been reported by several investigators to reduce lung injury in preclinical models of ALI [18].

cytokines in the MSC-treated lungs. Interestingly, all of these effects were replicated by administration of conditioned media from the cultured human MSCs. We were able to identify one important paracrine factor that mediated some of this effect by focusing on KGF, a molecule that has been reported previously by us and other investigators to have beneficial effects on experimental ALI [17]. MSCs produce substantial quantities of KGF, and siRNA knockdown of KGF production by MSCs resulted in a marked reduction in the beneficial effect of administering conditioned media from MSCs to the endotoxin-injured lobe in the *ex vivo* perfused human lung preparation. In a separate series of experiments, recombinant KGF increased alveolar fluid clearance in the presence of endotoxin-induced lung injury (Fig. 32.2) [18].

Additional studies were carried out in cultured alveolar epithelial human type II cells that had been injured with cytomix, a combination of tumor necrosis factor (TNF)-α, IL-1β, and interferon-γ often used as a surrogate for ALI pulmonary edema fluid, in order to simulate proinflammatory, cytokine-mediated lung injury that occurs in the acutely injured lung. Interestingly, the conditioned media from cultured human bone marrow-derived MSCs reversed the increase in alveolar epithelial permeability to protein (as measured by the trans-epithelial flux of ^{131}I-albumin) in this in vitro model. Angiopoietin-1 was the critical factor released by MSCs that restored more normal epithelial permeability [19].

Thus, several paracrine factors have been identified that can reduce injury and enhance repair, including IL-1RN, IL-10, KGF, prostaglandin E_2, and angiopoietin-1 (Fig. 32.3). In addition, release of antimicrobial peptides and enhancement of monocyte phagocytosis appear to be important pathways for inhibiting bacteria-induced lung injury.

In subsequent unpublished studies, we tested clinical grade human, bone marrow-derived allogeneic mesenchymal stem cells supplied by the University of Minnesota Production Assistance for Cellular Therapy (PACT) group, an organization funded by the NHLBI-NIH for the production of GMP grade cellular products for potential clinical trials. These cells have been administered to the perfusate (equivalent to intravenous administration) in both endotoxin- and live bacteria-induced models in a perfused *ex vivo* human lung preparation. The results indicate that intravenous administration is as effective as intrabronchial administration in reducing lung injury and restoring alveolar fluid clearance in the setting of live *E. coli* infection in the human lung. For these experiments, human MSCs were cryopreserved by the University of Minnesota PACT and thawed and administered in the perfusate similar to clinical administration via the intravenous route.

In addition, we tested the same cryopreserved human allogeneic MSCs in a rat model of acid-induced lung injury for safety using doses of 1, 5, or 10×10^6 cells/kg body weight. In these unpublished rat studies, these three different doses of human MSCs were compared to vehicle, Plasmalyte A, alone. The results showed that the administration of even the higher doses of MSCs over a 5-min time period 1 h following acid-induced lung injury had no effect on central venous pressure, systemic blood pressure, airway pressures, or arterial blood gases. Further, additional safety studies have been carried out in a sheep model of severe ALI in which the sheep were injured by inhalation of hot cottonwood smoke and instillation of *Pseudomonas aeruginosa* into both lower lobes. In these unpublished studies, a single dose intravenous administration of 5 or 10×10^6 cells/kg body weight over 60 min one hour after the development of acute lung injury did not result in any adverse hemodynamic or respiratory effects. There were no changes in heart rate, systemic blood pressure, pulmonary arterial pressure, pulmonary vascular resistance, airway pressure, or arterial blood gases over the first 1–2 h following infusion. Further, by 24 h, there was evidence of improvement in oxygenation with a significant increase in the PaO_2/FiO_2 ratio in the MSC-treated sheep.

Thus, the preclinical work has provided encouraging data for proceeding to test allogeneic human MSCs for the treatment of ALI in patients. Allogeneic human

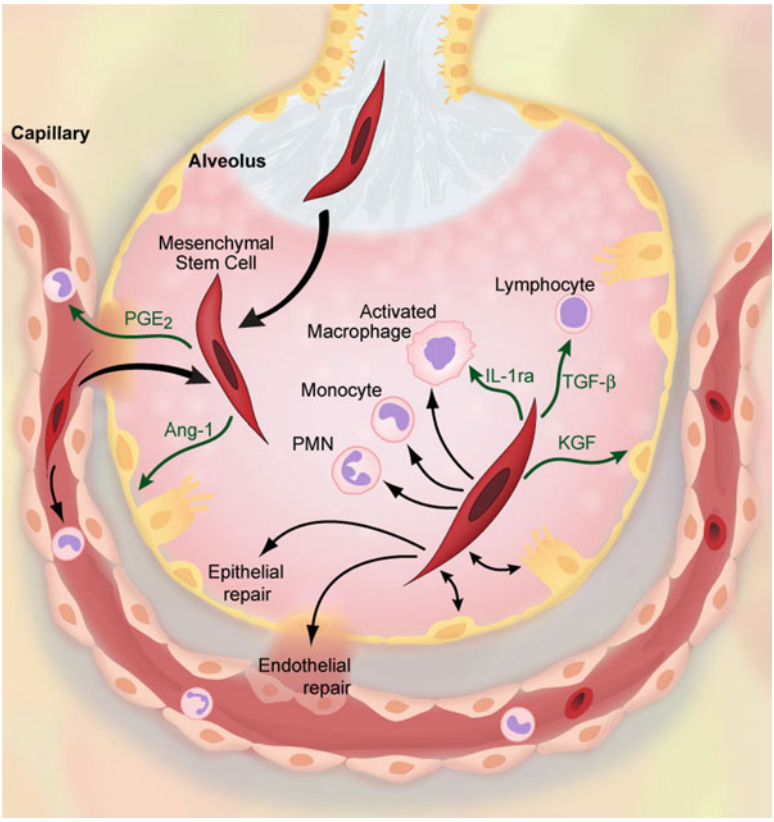

Fig. 32.3 In acute lung injury (*ALI*), the therapeutic properties of mesenchymal stem cells (MSCs) rely on paracrine mechanisms and interaction with other cells. Multiple mechanisms have been identified through which MSC therapy may repair the alveolar epithelium and endothelium during ALI, such as (**a**) secretion of paracrine soluble factor, which restores alveolar fluid clearance, lung permeability, and inhibits bacterial growth and (**b**) immunomodulation of innate and adaptive immune cells, which reduces alveolar inflammation. Although not fully characterized, the potential for engraftment by in vivo-modified MSCs and the presence of endogenous adult stem cells with characteristics similar to MSCs may also contribute to this therapeutic effect. *Abbreviations*: *Ang-1* angiopoietin-1, *IL-10* interleukin-10, *KGF* keratinocyte growth factor, *MSC* mesenchymal stem cell, *PGE_2* prostaglandin E_2, *PMN* polymorphonuclear neutrophils (Reprinted with permission from Lee et al. [1])

MSCs have been administered by Osiris, Inc. for a variety of clinical disorders, including acute myocardial infarction, graft-versus-host disease, chronic obstructive pulmonary disease, and inflammatory bowel disease [20]. To date, there have been no significant adverse events reported and it is important to recognize that allogeneic human MSCs do not elicit a strong immunologic response, in part because of markedly reduced expression of MHC class type I and type II cell antigens [1]. A recent article reviewed clinical trials with MSCs and found no serious adverse events [21]. However, more trials focused on safety are needed.

Steps Required to Test MSCs for Treatment of Clinical Lung Injury in Adults

In order to test allogeneic human MSCs for clinical lung injury, appropriate FDA approval for an investigational new drug (IND) must be obtained. This process is currently ongoing. We have submitted a pre-IND packet to the FDA and they have responded by providing us with several guidelines, including additional preclinical animal studies and criteria for toxicology and standard requirements for cell-based therapeutics. Our plan is to use the clinical grade allogeneic human MSCs provided by the PACT group from the University of Minnesota. These are the same MSCs that we have been using in our recent mouse, rat, sheep, and *ex vivo* perfused human lung studies. Since cell-based therapy is a new therapeutic initiative in lung diseases, special attention needs to be given to issues of consent and exclusions. As a general guideline, we believe that MSC therapy should be tested in patients with more severe lung injury, specifically patients with acute respiratory distress syndrome (ARDS) who have severe hypoxemia. Also, although allogeneic human MSCs have been administered to many hundreds of patients with a variety of diseases including inflammatory bowel disease, acute myocardial infarction, and graft-versus-host disease [20], we intend to conduct a dose escalation phase I study to test for safety of allogeneic MSCs in patients with acute lung injury. After a successful phase I study, specifically no major safety issues in terms of acute hemodynamic or respiratory issues, we will proceed with a phase II clinical trial.

The randomized, placebo-controlled, double-blind phase II trial will be focused on enrolling patients with moderate-to-severe ALI. An important endpoint will be the severity of acute lung injury as defined by the four-point ALI score or the oxygenation index [22]. Several other clinically relevant endpoints will be monitored, including oxygenation index, pulmonary dead space, ventilator-free days, non-pulmonary organ dysfunction, and mortality. The current plan is to study approximately 60 patients with a 2:1 randomization of MSC treatment vs. controls. Because of the small size of this initial trial, it will not be powered to identify a significant difference in major endpoints such as ventilator-free days and mortality. Further, several studies will be carried out on bronchoalveolar lavage samples, as well as plasma samples done to study the biologic effects of MSCs and identify potential mechanisms that account for the effect of MSCs in the clinical setting of ALI [23]. For example, we will measure pro- and anti-inflammatory cytokines, biochemical markers of endothelial and alveolar epithelial injury, and growth factors such as keratinocyte growth factor and vascular endothelial growth factor. In the bronchoalveolar lavage samples, we will measure total protein as an index of lung permeability as well as the number of neutrophils and monocytes. There will also be careful monitoring of patients for adverse effects on hemodynamics or respiratory status.

Conclusions

MSCs are effective in multiple preclinical models of ALI, in part, through the release of paracrine soluble factors and their beneficial effects on inflammation, lung protein permeability, alveolar fluid clearance, and total bacterial load. More recently, these results have been obtained using clinical GMP-manufactured, cryopreserved allogeneic human bone marrow-derived MSCs. In both small and large animal models of ALI, MSC treatment with a single dose has not resulted in adverse hemodynamic or respiratory complications at doses of 5 or 10×10^6 cells/kg of body weight. Based on the preclinical studies, we are currently in the process of designing and initiating a clinical trial for safety and limited efficacy endpoints in patients with ALI. There is also evidence that mitochondrial transfer from MSCs to injured alveolar epithelium in endotoxin-induced ALI is an additional mechanism of benefit [24]. Despite the significant progress described in the literature, we still have much to learn to understand how MCSs may be of therapeutic benefit in multiple disorders.

Acknowledgments The authors thank Andrew Manies for his assistance in the preparation of this manuscript. Dr. Matthay's effort were supported by NHLBI R37 HL51856 and HL51854, Dr. Lee by HL093026, Dr. Krasnodembskaya by HL51854, and Dr. Eldridge by NHLBI PACT Contract: HHSN268201000010C. Dr. Serikov was supported by the CHORI Jordan Family Fund.

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Chapter 33
MSCs for Diabetes

Luca Inverardi, Giacomo Lanzoni, Juan Dominguez-Bendala, and Camillo Ricordi

Abstract Mesenchymal stromal cells (also often referred to as mesenchymal stem cells, MSCs) hold promise as a powerful tool in the treatment of human diseases. Because of their potent immunomodulatory properties, they are likely to impact in a positive fashion diseases in which inflammation and/or autoimmunity play a key role. Furthermore, they have been shown to possess plasticity as precursors of cells of various types. Their differentiation potential could span cell differentiation products that do not normally derive from the mesoderm, including cells of endodermal origin. These two remarkable features make them potentially ideal candidates for regenerative approaches. This chapter will briefly discuss the current state of the art in the emerging field of their use for autoimmune type 1 diabetes.

Introduction

Type 1 diabetes mellitus (DM) occurs, in most instances, as a consequence of the autoimmune destruction of pancreatic insulin-producing beta cells [1]. Ill-defined environmental factors trigger disease onset in individuals with a predisposing genetic background [2]. The resulting insulinopenic diabetes leads to hyperglycemia that requires hormonal substitutive therapy. It is not uncommon that type 1 DM is recognized when patients present with diabetic ketoacidosis, a medical emergency that unfortunately can still result in death [3]. Conventional treatment of type 1 diabetes is based on frequent monitoring of blood glucose levels and exogenous administration of insulin via multiple daily injections or through a microinfusor (pump). While effective in achieving reasonable blood glucose control, these therapeutic approaches often fail to prevent complications linked to

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_33,

hypo- or hyperglycemia [4]. In the former case, the outcome can be acute and potentially catastrophic (hypoglycemic coma); in the latter, the outcome is usually chronic and debilitating (e.g., macro- and microangiopathy, neuropathy, kidney failure, and blindness).

Therefore, there is great interest in defining alternative approaches whereby a better and safer control of blood glucose levels can be achieved, resulting in the prevention/reversal of complications. One such approach is represented by islet transplantation [5], which, when successful, results in remarkable improvement of blood glucose control and normalization of hemoglobin A1C in the absence of hypoglycemic episodes [6]. This, in turn, leads to marked amelioration of the patients' quality of life [7]. Recent data indeed suggest that complications may be significantly slowed or prevented in islet transplant recipients [8].

For islet transplantation to become an elective treatment for patients with type 1 diabetes, however, at least two fundamental hurdles need to be overcome. First, current immunosuppressive regimens utilized to prevent islet transplant rejection and recurrence of autoimmunity are often characterized by side effects, some of which can have a serious impact on the recipient's health status [9]. Therefore, it is urgent that we refine treatments to maintain efficacy while reducing/eliminating unwanted complications while we work to accomplish the ultimate goal of transplantation immunology, the induction of tolerance through safe, clinically applicable protocols [10].

Second, we need to identify an inexhaustible source of transplantable, insulin-producing cells, so that we can overcome the issues linked to the use of human deceased donors, including the scarce number of pancreata available and the intrinsic variability of organs and differences in yield and quality, to name just a few [11].

Mesenchymal stromal cells (MSCs, also often referred to as mesenchymal stem cells) may represent a valuable tool to address both these outstanding issues, since they possess immunomodulatory properties (relevant to the improvement of current immunosuppression protocols and possibly to tolerance-inducing strategies) and may represent a valid source of precursor cells that could differentiate into glucose-responsive, insulin-producing cells. This chapter will briefly review our current knowledge of MSC for diabetes and help define future challenges as we move ahead to assess their clinical uses.

MSCs were initially described as the stromal component of the bone marrow hematopoietic niche [12, 13]; they have now been successfully isolated from numerous additional anatomical sources that include the adipose tissue, cord blood, Wharton's jelly, periodontal ligament tissue, synovium, skeletal muscle and dermis, tendons, blood vessels, and many others in different animal species [14–26]. MSCs are defined by the simultaneous fulfillment of selected criteria that include adherence to plastic in culture, expression of selected surface markers in the absence of lineage-specific marker expression (e.g., immune and hematopoietic cells) and differentiation potential toward bone, cartilage, and fat [27, 28].

While these criteria suffice to define MSCs, it is now apparent that MSCs from different sources may have substantial differences that encompass expansion potential, expression of markers of uncommitted progenitors, and may differ in their immunomodulatory properties, an observation of relevance to their clinical use [29–34].

While it is widely accepted that MSCs have anti-inflammatory and immunosuppressive properties, it is not entirely clear how these effects are mediated. The concept that directs contact with effector cells, the production of soluble mediators, and the activation of regulatory cell subtypes all may contribute to the MSC effect is now gaining acceptance [25, 35–44].

Given that autoimmunity is the determining factor in initiating type 1 diabetes while intervention at onset may halt the process, transplantation of insulin-producing cells is required for treating established diabetes. Hence, it is likely that issues of allorejection and recurrence of autoimmunity need to be addressed. An autologous source of insulin-producing cells could overcome the former, leaving autoimmunity recurrence as the single remaining hurdle.

MSCs and Autoimmune Diabetes

The natural course of autoimmune diabetes often includes a "honeymoon period" during which the patients' blood glucose control and metabolic parameters improve, shortly after diagnosis, to the point that it is not uncommon for the insulin dose requirements to drop significantly. The honeymoon period is unfortunately short-lived, and eventually patients progress in the disease course, and insulin requirement increases as the beta-cell mass in the pancreas decreases. This observation clearly suggests that interventions aimed at altering the course of the disease (slowing/halting beta-cell mass attrition) have better odds of success if implemented early after diagnosis. Furthermore, the identification of populations at high risk of developing type 1 diabetes (e.g., first-degree relatives with positive autoantibodies) would extend the target for intervention to individuals with a beta-cell mass that is likely larger than that of patients at disease onset.

Most studies that have examined the role of MSCs in models of autoimmune diabetes, i.e., nonobese diabetic (NOD) mice and streptozotocin (STZ)-induced diabetes mellitus (DM), conclude that a protective effect is observed. For example, a single administration of MSCs in prediabetic NOD female mice (at 4 weeks of age) conferred significant protection, with about 60% of the treated animals developing diabetes, compared to 100% in the control group. In the same report, coadministration of MSCs with diabetogenic T cells (from diabetic NOD mice) to irradiated NOD male recipients revealed a dose-dependent protective effect of MSCs on diabetes development, compared with controls. Analysis of the mechanisms of action of MSCs in this model suggests that regulatory T cells may be a key component of the observed protective effect [45].

In an independent study, administration of congenic MSC (from NOR) to NOD mice shortly after disease onset led to reversal of hyperglycemia in most treated animals, an effect that persisted for 12 weeks, well beyond the time frame of administration of MSC (given for 4 weeks after disease onset). In-depth mechanistic analysis of the observed protective effect revealed key roles of the modulation of cytokine profile, of effector T-cell number (reduced by MSC administration), and of plasmacytoid DC frequency (increased by the treatment) [46].

Interestingly, the same group previously reported that the use of NOD-derived MSC in syngeneic NOD recipients had no preventive effect on disease progression, while the use of allogeneic MSC did; furthermore, the administration of NOD MSCs to NOD recipients led to the formation of tumors in a large number of the animals. This raises important questions about the validity of preclinical models assessing safety of MSCs and highlights the possible inherent flaws of the NOD animal model, the most widely used for the study of autoimmune diabetes occurrence and manipulation [47].

Human bone marrow-derived MSCs were found to have a protective effect on both pancreatic beta cells and renal glomeruli when transplanted in streptozotocin-treated NOD SCID mice. MSCs did not release detectable human insulin, but mediated endogenous repair – or regeneration – of mouse beta cells and thus ameliorated blood glucose control [48]. While very encouraging in demonstrating the efficacy of xenogeneic source(s) of MSCs, this report did not address whether profound immune incompetence (as in the NOD SCID mice) is a key prerequisite for the xenogeneic MSCs to function, a reasonable assumption, and one that needs to be formally addressed.

A more recent study [49] confirmed a positive effect of human bone marrow (BM)-derived MSCs on the glycemic profile of STZ-treated NOD/SCID mice and unveiled interesting differences in the mechanism of action of cultured MSC when compared to BM-derived mixed progenitor cells.

Using the same model of diabetes induction (low-dose STZ) thought to lead to autoimmune destruction of beta cells, Ezquer et al. showed that administration of bone marrow-derived, autologous MSCs can revert hyperglycemia and protect mice from diabetes-induced nephropathy [50]. The same authors reported that insulin treatment of the animals did not impair the positive effect of MSCs and that the administration of an additional dose of MSCs did not increase efficacy [51].

Similar data were reported in an experimental rat model; autologous, BM-derived MSC was used to promote regeneration/repair of beta cells in a model of STZ-induced diabetes, and the positive effect was linked both to MSC homing to the pancreas, with the establishment of a microenvironment supportive of beta-cell function/survival, and to a systemic shift of the immune profile toward an anti-inflammatory phenotype with enhanced interleukin (IL)-10/IL-13 production and a higher frequency of regulatory T cells (T-reg) [52].

Are MSCs the only key players in the mediation of the observed effects? Or does coadministration of additional cell sources play an important role in promoting/enhancing efficacy? While most studies suggest that MSCs are the only critical effectors, at least one report concludes that they may not suffice to consistently achieve reversal of diabetes after STZ treatment and that coadministration of bone marrow cells is instrumental in therapeutic success in the mouse model [53].

Studies with human cells support a positive role of MSCs in down-modulating autoimmunity in patients with diabetes, as suggested by the observation that allogeneic MSCs of bone marrow origin abrogate the Th1-type response seen in patients with type 1 DM when their peripheral blood mononuclear cells are challenged in vitro with the disease-relevant antigen, GAD65. Coculture with MSCs results in

a reduction of interferon-gamma (IFN-γ) production and in an increase in IL-4. The addition of prostaglandin (PG)E2 inhibitors reverses the MSC immunomodulatory effect, suggesting a key role for this pathway [54].

Quite intriguing is the observation that MSCs harvested from diabetic mice have a profoundly altered phenotypic profile, are senescent, and undergo apoptosis, suggesting the possibility that at least some of the physiological roles of MSCs may be impaired in pathological conditions such as diabetes, resulting in a shift to a proinflammatory, non-tolerogenic milieu [55]. These data are not seen in all models, since MSCs from rats with hyperglycemia were shown to be comparable in their efficacy to those of rats with normal blood glucose levels [56].

In addition, MSCs appear to have angiogenic and trophic potential that improve, in a co-transplant setting, the ability of pancreatic islets to survive the first few days after transplantation. Coating islets with endothelial cells and MSCs could indeed limit the immediate blood-mediated inflammatory reaction that leads to early loss of a significant portion of the transplanted tissue. Moreover, it could warrant a rapid anastomosis with the existing vascular bed resulting in an improved islet perfusion [57, 58].

It has also been reported that MSCs can act as trophic mediators on different cell types, creating an environment supportive to cell survival. Anti-inflammatory, antiapoptotic, and proangiogenic factors are primarily implicated. Soluble factors such as interleukin-6 (IL-6), interleukin-8 (IL-8), vascular endothelial growth factor-A (VEGF-A), hepatocyte growth factor (HGF), and transforming growth factor beta (TGF-β) are actively released by MSCs and likely mediate a trophic effect on pancreatic islets, enhancing their survival and function after transplantation [59, 60].

Data of an ongoing clinical trial of MSC (Prochymal™) administration in patients with type 1 diabetes at onset will soon be reported. The trial is based on the administration of allogeneic MSC in the absence of immunosuppression, a choice that appears justified based on patient safety [61]. Additional trials are currently enrolling patients, including one that utilizes autologous MSCs at Uppsala University [62].

MSCs and Islet Transplantation

Several models of islet transplantation can be used to address the effect of MSCs in animals, and preclinical data indicate positive effects of MSCs in promoting engraftment and increasing survival and function of beta cells. In islet transplantation, the mass of viable beta cells administered to the recipient correlates with function, and a marginal (suboptimal) mass leads to increased delay to function and to a lower success rate. Interventions that lead to an increase in the success rate and/or a decrease in the time to function in marginal mass transplant models are therefore considered protective of islet mass/function. MSCs from recipient rats mediated such an effect when co-transplanted with allogeneic islets, resulting in long-term survival and sustained normoglycemia [63]. The effect of MSCs in this model could

be due either to an anti-inflammatory effect or an immunomodulatory effect or to a combination of both. Interestingly, in the study the authors also report beneficial effects of MSC administration when a marginal mass of syngeneic islets is implanted in the recipients, strongly suggesting that the anti-inflammatory effect plays an important role in mediating the observed positive outcomes [63]. Similar data were reported in a model of syngeneic marginal mass in rats co-transplanted with autologous BM-derived MSCs, with co-administration resulting in better function. The positive effect observed in this study was paralleled by increased neoangiogenesis at the implant site, a key mechanistic observation that highlights the multiple mechanisms of action and MSC effects [64]. These "trophic" effects result in a better maintained islet structure/morphology, increased function, and better vascularization, also in mice that received co-transplants of syngeneic islets and MSCs versus controls receiving only islets [65, 66].

In stringent models of transplantation in fully allogeneic recipients, the coadministration of MSCs with islets led to highly significant prolongation of graft survival in rodent models [67, 68]; analysis of the MSC immunosuppressive mechanisms in one study revealed its dependence on the production by MSCs of matrix metalloproteinases (MMP)-2 and 9. In vivo inhibition of MMP-2 and MMP-9 completely reversed the protective effect on graft survival [69]. Additional mechanisms that MSCs utilize to mediate their immunomodulatory effects include the modulation of dendritic cell function and additional T-cell subsets (e.g., regulatory T cells) as well as alterations in cytokine production (e.g., IL-10) and secretion of other compounds (galectins, prostaglandins, IDO, etc.) [44, 45, 70–80].

Timing of infusions appears to be an important factor when applying MSCs as trophic mediators or immunoregulators. Cytokines related to the inflammatory process during rejection may act as activators of MSC function and thus enhance MSC efficacy. In a preclinical model of islet graft rejection in nonhuman primates, Berman and colleagues found that intravenously administered MSCs derived from the BM of either islet donors or third-party marrow donors were able to reverse rejection episodes. MSCs infused several days after graft destabilization were ineffective [81].

Different sources of MSCs appear, at least preliminarily, to share comparable immunomodulatory effects; for example, Ohmura and colleagues recently reported that adipose tissue-derived stem cells (ADSC) co-transplanted with islets enhanced survival and function of islet grafts in diabetic mice. ADSC were isolated with an automated method (developed by Cytori Therapeutics, Inc.) and were not fully characterized as MSCs. These cells prolonged survival of a marginal islet mass. Promotion of revascularization and inhibition of immune aggression were suggested as possible mechanisms of action. The authors claimed that ADSC committed to endothelial fates, but the data reported were not sufficient to definitively draw such a conclusion [82]. Similarly, when co-transplanted with islet in streptozotocin-induced diabetic mice, kidney-derived MSCs were found to improve the capacity of islet grafts to reverse hyperglycemia. They supported the maintenance of the natural organization of islet cells and of the rich islet vascularization. Of note, kidney-derived MSCs transplanted alone did not reverse hyperglycemia nor enhanced the regeneration of pancreatic beta cells [65].

BM-derived cells also contribute to the pancreatic mesenchymal populations, raising attention to the role of trafficking marrow-derived cells. In these experiments the differentiation potential of pancreatic MSCs toward endocrine fates was found to be negligible, but an important supportive effect was found in co-transplantation experiments with islets, possibly related to the expression of trophic and proangiogenic factors [83]. We believe there is sufficient evidence that MSCs have the ability to modulate autoimmunity in type 1 DM and promote beta-cell function/survival/regeneration. MSCs can positively influence allorecognition and rejection but several outstanding issues need to be addressed before they can be widely used clinically.

1. *The Definition of the Ideal Source of MSCs*: *Autologous*, *Allogeneic*, *or Xenogeneic*. While an easy assumption is that an autologous source is preferable to avoid rejection or the need for immunosuppression, the observation of tumor growth in some experimental settings of autologous MSCs administration (NOD) deserves in-depth scrutiny to understand whether this is strictly an occurrence seen in murine models or may be a more common event. Also, the reported (albeit somewhat controversial) negative impact of disease conditions such as diabetes on MSC function, viability, or expansion potential may critically influence the choice of the appropriate cell source. Definitive data in humans are needed to make rigorous choices.
   The use of allogeneic MSCs will require an understanding of their fate, i.e., how long they persist in the host in the absence of immunosuppression, and whether in fact their life span is important. The use of xenogeneic MSCs may currently seem unjustified (after all, MSCs can be obtained quite easily from the prospective recipient), but may have theoretical advantages, including the genetic manipulation of the donor and the generation of large clinical-grade batches.
2. *The Site of MSC Harvest*. It is now well accepted that MSCs can be harvested from multiple anatomical locations, and it has been widely assumed that MSCs derived from different sources are largely equivalent, at least in terms of surface marker expression and differentiation potential. Both characteristics are in fact a prerequisite for their definition as MSCs. On the other hand, analysis of marker/gene expression profiles reveals differences that may have a profound impact on MSC function. For example, MSCs derived from the periodontal ligament have markers of neural crest derivation (shared by MSCs derived from the eyelid fat) that are not seen in MSCs derived from the BM or the adipose tissue [84]; and MSCs derived from the cord blood express markers of pluripotency such as OCT 4 that are not seen in MSCs from other sources such as the adipose tissue or the BM [85]. Whether these differences have an impact on the immunomodulatory effects mediated by MSCs is still not fully understood, but the concept that immunomodulatory functions of MSCs may depend on origin (and likely other variables) is gaining momentum.
3. *Expansion and Culture*. We know very little about ideal culture conditions for MSC growth/expansion. Furthermore, we have modest knowledge concerning the effect of culture/expansion on the preservation of function(s) of MSCs. This

may turn out to be critically important in the successful utilization of MSCs as a therapeutic tool. A relevant example of the impact of culture conditions on MSC characteristics (including the expression of markers of "stemness" and senescence) is the observation that selection and culture of BM-derived MSCs in low oxygen lead to the expansion of cells with unique features, as reported by Schiller and d'Ippolito [86].

4. *Single Agent or Composite Strategies*? The use of MSCs as a single tool may fall short of expectations, as suggested by the work of Urban and others who postulate that the composite use of multiple cell sources (e.g., BM, Islets of Langerhans) may lead to much improved outcomes [53]. Many intriguing hypotheses can be formulated, and many experiments are warranted to explore these possibilities.
5. *Is There a Dark Side to MSCs*? Our desire to categorize cell subsets, cytokines, and hormones in a simple and uncontroversial manner (proinflammatory vs. anti-inflammatory, immunogenic vs. tolerogenic) is often met with frustration, as we discover time and again more complicated scenarios. Many cytokines and immune cell subsets can have opposite effects that are dependent on numerous interrelated variables, such as dose, time, and site of production/administration, interaction with the environment, and others. MSCs, as a relatively new player in the immunomodulation/inflammation field, have been linked mostly to effects that are desirable, dampening autoimmunity and delaying rejection of allogeneic transplants. Recently, however, this assumption has been challenged by the observation that priming of MSCs under different conditions may result in their shift to a phenotype that may lead to chronic inflammation and fibrosis [30, 32, 33, 87]. The definition of the key variables that shift the MSC function will be of critical importance to their safe and effective use in vivo.

MSCs as a Source of Insulin-Producing Cells

Almost a century ago, the discovery of insulin revolutionized the treatment of diabetes mellitus. Nowadays, hopes for a cure are coming from stem cells. Exciting findings are originating from attempts to differentiate stem cells toward functional pancreatic endocrine fates, but we may still be many steps away from a cure for diabetes. MSCs are among the most actively investigated cells in the diabetes research field: not only have they proven their worth as immune regulators (see section above), but they exhibit an everexpanding differentiation range that may even encompass the pancreatic endocrine beta-cell fate.

MSCs possess a widely recognized ability to differentiate into a variety of therapeutically valuable tissues, distinctly those of the connective tissue family [88]. Recently, MSC populations have been consistently shown to commit to pancreatic-like fates under certain experimental conditions, even though doubts remain about their ability to become *bona fide* beta cells. Diverging claims and interpretations may be attributed to the fuzzy definition of MSCs, the intrinsic variability expected from cells derived from many different sources, the heteroge-

neity of cultured populations and the variety of culture conditions that have been used for their expansion and differentiation. Attempts at harmonizing the criteria for their definition resulted in the unification of cells of disparate origins sharing mesenchymal multipotency under the all-encompassing MSC acronym [27, 28]. However, this categorization still fails to establish whether MSCs are one and the same. The answer to this question will dictate whether different clinical applications will require the use of MSCs from specific sources [89–94].

A "gold standard" protocol for the differentiation of MSCs along the beta-cell lineage has not been defined yet. Indeed, such enterprise could be hampered by the possibility that MSCs of different origins may not be the same entity. Furthermore, the notion that MSCs can differentiate into beta cells challenges the embryological dogma that mesoderm cannot give rise to endoderm-derived beta cells. However, cells of different origins are known to behave in noncanonical ways when subjected to nonphysiological conditions. From a therapeutic perspective, perceived violations of the norm may not be of great concern as long as MSC-derived cells are shown to safely restore normoglycemia in patients [95].

Strategies to Induce β-Cell Differentiation

The general aim of beta-cell differentiation protocols is to obtain clinically relevant numbers of insulin-producing, glucose-responsive cells that could be used to restore beta-cell mass in diabetic patients. The major strategies applied to induce in vitro MSC commitment in this direction make use of a combination of endocrine-promoting factors and culture conditions found to have a beneficial effect on islets or beta-cell mass. After the tentativeness of initial approaches, which yielded questionable results, current efforts are focused on refining culture conditions to more accurately recapitulate pancreatic development. It is noteworthy that the mechanisms of action of many inductive agents remain obscure (Table 33.1).

A significant breakthrough in the field came with the definition of stage-specific inducers of pancreatic beta (β)-cell specification in human embryonic stem cells (ESCs) [96–98]. Although successful at yielding true β-cells in an in vivo setting, in order to be clinically applicable, this method still needs to overcome problems in the efficiency of differentiation and the control of teratoma formation [99, 100]. Nevertheless, research on ESCs (and their more recently described reprogrammed counterparts, induced pluripotent stem cells) [101] is paving the way for the development of more efficient MSC differentiation protocols. Indeed, methods similar to those developed for ESC were recently proven effective in MSCs derived from the umbilical cord blood [85].

As mentioned before, MSCs derived from different sources may need specific inductive cues for pancreatic endocrine commitment, due to the diverse microenvironments and ontogenetic history of the specific tissue into which they were integrated. As an example, bone marrow-derived MSCs undergo osteogenesis after BMP6 exposure and chondrogenesis after TGF-β stimulation, whereas adipose

Table 33.1 Agents commonly used for the induction of MSCs toward the pancreatic beta (β)-cell phenotype

Inductive agent	Effect and mechanism of action
Nicotinamide	Poly(ADP-ribose) polymerase (PARP) inhibitor. Activates cyclic AMP signaling. Induces hepatic and pancreatic progenitors to insulin-producing cells. Increases the rate of proinsulin biosynthesis, obscure mechanism of action
Exendin A	Glucagon-like peptide 1 (GLP-1) agonist, potent inducer of pancreatic islet differentiation. Promotes beta-cell replication and neogenesis from ductal precursors. Inhibits beta-cell apoptosis and stimulates functional activity
β-cellulin	Regulates growth and differentiation of pancreatic endocrine precursors
Activin A	Member of the TGF-β superfamily, regulates beta-cell neogenesis in vivo
Glucose	At high concentrations (20–30 mM) stimulates beta-cell replication; induces insulin expression
β-mercaptoethanol	Obscure mechanism of action
Pyruvic acid	Precursor in the biosynthesis of Ala, Tyr, Phe, and Tpt. Obscure mechanism of action
Epidermal growth factor (EGF)	Expands undifferentiated pancreatic embryonic cells. Involved in several pathways
Basic fibroblast growth factor (bFGF)	bFGF and FGF receptors are overexpressed in several pancreatic malignancies and may be related to progenitor expansion. Involved in several pathways
Hepatocyte growth factor (HGF)	Stimulates proliferation and migration. Morphogenetic and antiapoptotic. Binds c-Met and stimulates replication by activating PI3K and AKT
Retinoic acid (RA)	Defines intestinal morphogenesis from the endoderm. Acts in the dorsal bud before the expression of Pdx1 but after pancreatic specification. Affects the choice between exocrine and endocrine fates. High concentrations of retinoic acid stimulate endocrine versus exocrine commitment
Vascular endothelial growth factor-A (VEGF-A)	Involved in modeling the islet vasculature. By reciprocity, endothelial cells induce islet development
Connective tissue growth factor (CTGF)	Involved in several pathways. It is expressed during embryonic life in beta cells, ductal cells, and endothelial cells. Regulates embryonic beta-cell proliferation and localization
Transforming growth factor beta (TGF-β)	Conflicting results regarding the effect on the beta-cell mass and exocrine cell mass during development

tissue-derived MSCs respond to the supplementation with BMP6 by adopting a chondrogenic fate [102]. Variations in inductive requirements must be taken into account in the process of designing differentiation protocols and add a grade of complexity when comparing the behavior of different MSC populations. On the

other hand, in the same way that there is no search for individualized protocols for different ESC lines (despite their well-known variability in developmental potential), the definition of a universally robust method for MSC regardless of their origin still remains a legitimate pursuit.

Sources of MSCs with β-Cell Potential

The demand of beta cells for transplantation in diabetic patients has triggered a widespread quest for cells with beta-cell potential. MSCs from various sources have been investigated, and several findings support their ability to commit to the pancreatic endocrine fates.

Pancreatic Islet-Derived MSC

For obvious ontogenic reasons, pancreatic tissues could be considered among the most promising sources of stem cells with islet-specific differentiation potential. The hypothesis that putative pancreatic endocrine progenitor cells are responsible for islet regeneration suffered a significant setback in the mid-2000s when elegant lineage-tracing experiments in a transgenic mouse model seemingly demonstrated that normal turnover and regeneration of beta cells occurs by replication of existing beta cells [103, 104]. Nevertheless, the adult pancreas appears to harbor several types of progenitor cells that may contribute to beta-cell replenishment, and some progenitors may give rise to MSC-like cultures with organ-specific potential.

Thus, Zulewski and colleagues reported the isolation from rat pancreatic islets of nestin-positive islet-derived progenitor (NIP) cells. These fibroblast-like cells were induced in vitro toward pancreatic endocrine phenotypes. Despite the limitations of a less than optimal induction protocol and a rather incomplete analysis of the differentiation outcomes, this work opened the path for a search of multipotent MSC in pancreatic islets [105]. These progenitors were able to reverse hyperglycaemia in diabetic mice [106]. Human nestin-positive MSC-like cells were isolated from fetal pancreas: these cells showed some potential to commit along the beta-cell lineage [107].

The hypothesis that a reversible process termed epithelial-to-mesenchymal transition could occur in insulin-expressing cells has also been presented. Cultured insulin-positive cells "transitioned" to a population exhibiting a mesenchymal phenotype. These cells were then induced to differentiate into glucagon- or insulin-positive cells. However, the content of insulin of these putative "redifferentiated" β-cells was two orders of magnitude lower than in real ones, which cast doubts on the validity of the model [108]. In similar experiments, islet cells acquired mesenchymal markers during culture expansion and were partially redifferentiated to a glucose-regulatable insulin-releasing phenotype by betacellulin exposure [109]. A flaw in these experiments was the absence of lineage tracing to determine the in vivo

counterpart or the nature of the colony-initiating cells. In a mouse model of committed pancreatic endoderm-traced cells (Pdx1 and insulin lineage-traced cells), the isolation of fibroblast-like cells from pancreatic islets showed that they did not derive from an endoderm undergoing epithelial-to-mesenchymal transition [110]. Somewhat in contrast with these findings, lineage-tracing experiments performed on human islets supported beta-cell dedifferentiation toward a mesenchymal phenotype [111, 112]. The investigators followed the progeny of beta-cells using insulin as a marker of terminally differentiated cells, but this may not be necessarily true, given that a rare population of multipotent islet cells was recently shown to express insulin [113].

After immortalizing a human islet-derived single-cell expanded MSC clone, Eberhardt and colleagues elegantly showed their multidifferentiation potential toward mesenchymal, pancreatic, and hepatic commitments. The immortalization step introduced a bias for the description of stemness as a natural characteristic but allowed for significant expansion [114]. Gallo and colleagues reported that islet-derived MSCs appeared immunophenotypically similar to BM-derived MSCs and shared a mesenchymal differentiation pattern. After an induction based on serum deprivation, the cells gained the ability to secrete limited amounts of insulin [115]. Similar experiments performed in rodent models pointed to a link between the MSC populations derived from islets and an in vivo counterpart of pericytic nature [116].

Also starting from human islets, Davani and colleagues derived cell populations similar to MSCs. These cells showed mesodermal multidifferentiation potential and were found to commit in vitro in hormone-expressing cells and to partially mature in vivo into glucose-responsive insulin-secreting cells [117]. Notably, the putative culture-initiating cell population appeared to basally express low levels of insulin [117]. These cells were reported to cycle between an epithelial phenotype in clusters and a mesenchymal phenotype in conventional culture conditions [118].

Epigenetic traits suggestive of an endocrine-committed progenitor state were described in islet-derived mesenchymal precursor cells [119]. It was observed that, after extensive expansion, these cells underwent silencing of the insulin gene, but not of glucagon. Even after forcing the expression of the transcription factors Pdx1, NeuroD1, and MafA, the epigenetic marking inhibited beta-cell maturation. The α-cell commitment was instead readily activated [120]. From a transcriptional point of view, islet-derived MSCs might theoretically be primed toward the pancreatic endocrine lineage. Islet MSC lacks some mesenchymal differentiation attributes characteristic of BM-MSC, but can go further along the endocrine pathway [121]. Islet-derived MSCs may derive from intraislet CD90+/CD105+ progenitors, cells of pericytic nature [122, 123].

Taken together, these findings seem to consistently suggest that pancreatic islet-derived MSCs may have the peculiar ability to enter the pancreatic endocrine differentiation path, although the level of transcriptional and functional maturation is still far from that expected of true beta cells. Functional differentiation in vivo has been reported, but additional research is necessary to unequivocally establish the soundness of these claims.

Exocrine Pancreas-Derived MSCs

Events occurring during both organogenesis and regeneration suggest that pancreatic progenitor cells may reside within the pancreatic ducts [124]. Waves of differentiation and migration, described as ductal budding, start from ductal progenitors and ultimately give rise to neoislets. Populations of cells isolated from human exocrine pancreas and reported as mesenchymal in nature may be related to these progenitors. Results similar to those reported with islets were obtained by culturing adult human pancreatic ductal tissue. Thus, cell cultures established from ductal tissue were found to express transcription factors of the endocrine commitment (Pdx1, Isl1, Nkx2.2, Nkx6.1, nestin, Ngn3, and NeuroD) and a panel of MSC markers. It was also observed that MSCs from the ductal epithelium were capable of differentiating in vitro into osteocytes, adipocytes, and chondrocytes, thus sharing characteristics with MSCs from other anatomical sources. Moreover, they were found to adopt endodermal fates, such as hepatocytes and beta cells, although the expression of endocrine hormones was faint at best. Functional cell types were not consistently obtained [125–127]

Sordi and colleagues raised important concerns regarding the differentiation potential of pancreatic MSCs toward endocrine fates, documenting it as negligible in their culture conditions. The authors pointed out that contaminating cells of endodermal derivation could be responsible for the earlier findings. Primary cultures are still contaminated by endodermal cells, mainly short-lived CD133+ ductal epithelial cells, bearing a transcriptional profile that is consistently one of pancreatic endoderm, although these cells rapidly senesce and are negatively selected by culture conditions designed for MSCs [83].

Bone Marrow-Derived MSCs

For historical and practical reasons, the bone marrow (BM) has been extensively investigated as a source of stem cells. MSCs represent a rare and relatively variable population in the BM. Chen and collaborators found that culture conditions based on high glucose concentration, nicotinamide, and beta-mercaptoethanol were able to stimulate the expression of insulin in rat BM-MSC, but in vivo data did not support the conclusion that beta-cell differentiation had been achieved [128]. Similarly, Oh and colleagues reported that BM-MSCs cultured in high glucose and dimethyl sulfoxide (DMSO) differentiated into insulin-producing cells. Surprisingly, xenotransplantation of differentiated cells in diabetic mice gave rise to islet-like clusters and supported long-term normalization of glycemia. Unfortunately, these results could not be independently replicated [129].

Extracts from the rat regenerating pancreas after partial pancreatectomy were used to stimulate the commitment of rat BM-MSCs toward pancreatic phenotypes. After this treatment, BM-MSCs gave rise to islet-like clusters and expressed pancreatic endocrine hormones and released insulin in response to glucose challenge.

Despite the fact that the exocrine tissue would be the main contributor to these extracts, it is noteworthy that the regenerated tissue after partial pancreatectomy is highly enriched in endocrine tissue. Thus, the induction toward endocrine fates may be more effective than previously thought [130].

Wu and colleagues observed commitment toward the endocrine phenotype after exposing rat BM-MSCs to high glucose, nicotinamide, and exendin-4, but functional and in vivo data were not supportive of competent differentiation [131]. More recently, Paz and colleagues found that betacellulin overexpression in rat BM-MSCs stimulates the production of insulin in vitro and reverts the hyperglycemic state in STZ-diabetic rats [132]. Given the limited specificity of these induction protocols, one may argue that the transcriptional blueprint responsible for the adoption of the pancreatic endocrine phenotypes may somehow be more accessible in rat BM-MSC.

Moriscot and coworkers differentiated human BM-MSCs by manipulating the extracellular microenvironment and introducing exogenous transcription factors involved in early pancreatic development: Pdx1, Hlxb9, and FoxA2 [133]. Li and colleagues observed that the Pdx1 transcription factor is sufficient to convert human BM-MSCs into insulin-producing cells. The overexpression of the Pdx1 gene gave rise to cells with some signs of functional activity, as determined by glucose-regulated insulin release in vitro. Their transplantation in diabetic mice ultimately resulted in functional differentiation [134]. Similar approaches confirmed that human BM-MSCs undergo an incomplete commitment in vitro commitment, but after transplantation in diabetic mice, the cells progressed toward maturation into endocrine phenotypes and decreased the hyperglycemia. The diabetic environment may indeed represent a strong stimulus to push maturation forward [135]. Sun and colleagues found that human BM-MSCs from type I diabetic patients could be turned into insulin-producing cells after induction with a multistage differentiation protocol. Despite the somehow preliminary nature of the data supporting differentiation, the investigation of the potential of MSC from diabetic patients is an intriguing avenue of research [136]. Others confirmed the differentiation potential of MSCs from diabetic patients, highlighting dissimilarities between human BM-MSCs derived from diabetic versus healthy donors [137]. Xie and co-workers observed that a multistep differentiation protocol yielded functional insulin-producing cells. Induced BM-MSCs gave rise to cells expressing multiple genes involved in pancreatic beta-cell development, including Nkx6.1, Isl1, β2/NeuroD, Glut2, Pax6, nestin, Pdx1, Ngn3, insulin, and glucagon. Moreover, these cells were able to release insulin in a glucose-dependent manner and ameliorated the diabetic conditions of STZ-treated nude mice [138].

Limbert and colleagues recently reported the reprogramming of telomerase-immortalized BM-MSCs toward pancreatic endocrine lineages. Two transcription factors acting as master regulators in the beta-cell differentiation cascade, namely, Pdx1 and Ngn3, were overexpressed as transgenes. The differentiation cascade was partially activated and insulin-producing cells were generated. The induced insulin biosynthesis and secretion were found to be glucose insensitive, evidencing the need for additional maturation factors [139].

In summary, research on BM-MSCs is rich in reports that describe cells of mixed phenotypes and at variable degrees of commitment along the pancreatic lineage. However, claims to the effect that these cells may represent an efficient source of new beta cells are not yet fully backed by the data and need to be more thoroughly substantiated.

Adipose Tissue-Derived MSCs

Adipose tissue is a high-yield source of MSC [140]. Given its ready availability and ease of collection, it offers important advantages as a source of MSCs when compared to bone marrow. Adipose tissue-derived MSC (AT-MSC) can easily be banked for prospective use in autologous or allogeneic settings. AT-MSC and BM-MSC gene expression and differentiation patterns are widely overlapping [141]. Work by Timper and colleagues suggested that AT-MSCs could undergo differentiation into insulin-producing cells. Their protocol stimulated the formation of cells expressing endocrine transcription factors, insulin, glucagon, and somatostatin. However, they failed to demonstrate that the resulting insulin-expressing cells can secrete insulin in response to glucose challenge [142]. Adipose tissue-derived stromal cells (ADSC), a heterogeneous population sharing many similarities with AT-MSC, were investigated by Okura and colleagues. Unfortunately, the initial population was not characterized, thus limiting subsequent comparisons. Nonetheless, human ADSC responded to a multistep differentiation protocol reaching the stage of insulin-, glucagon-, and somatostatin-producing cells and showed signs of functionality in vitro [143]. Kajiyama and colleagues primed the commitment of mouse ADSC with the ectopic expression of Pdx1: this did not consistently drive maturation in vitro, but the in vivo diabetic microenvironment stimulated differentiation. The cells reached a degree of functional maturation, ameliorated hyperglycemia in the long term, and increased the survival rate of diabetic mice [144].

Trivedi and colleagues reported the initiation of a clinical trial involving the use of adipose tissue-derived cells and bone marrow-derived cells in patients with insulin-dependent diabetes mellitus [145]. An update of this trial was recently presented, with data from 11 patients [146]. Adipose tissue-derived cells were not thoroughly characterized as MSC. Pancreatic endocrine differentiation was stimulated in vitro, but inconsistent data were reported. Adding a variable and confusion in data interpretation, induced adipose tissue-derived cells were coinfused in human patients with culture-expanded BM cells. Bone marrow and adipose tissue donors were blood group-matched family relatives. Both adipose tissue-derived and bone marrow-derived cells were poorly characterized before the procedure. No adverse side effects related to the infusion were observed, and transplantation was reported to result in reduced insulin requirements in treated patients. Unfortunately, this work was defective in several major ways: as mentioned above, the nature of transplanted cell was not fully defined, the cell dose was not reported, and the mechanisms remain obscure [146]. These concerns raise doubts about the interpretation of the outcomes.

Cord-Blood-, Cord-, and Placenta-Derived MSCs

The term fetal adnexal tissues, commonly referred as placental tissues, are available in large quantities and can be easily procured. Their use raises no ethical concerns. The nature and age of these tissues provide even more striking advantages. Placenta, cord, and cord-blood tissues may retain a higher developmental plasticity [85] and potential for replication than other adult tissues. In addition to the obvious benefit for autologous transplantation, the possibility of banking fetal adnexal stem cells may enable us to match most if not all prospective recipients. Cell populations similar to MSCs have been derived from umbilical cord blood (UCB) and from several fetal adnexal tissues, including the umbilical cord matrix (Wharton's jelly, WJ), chorion, and amniotic membrane [147, 148]. Gao and colleagues found that UCB-derived MSCs (UCB-MSC) can be turned into insulin-producing cells, but the differentiation protocol applied did not result in functional maturation [149, 150]. Hu and colleagues expanded these findings and detected human insulin upon transplantation of these cells in diabetic animals, although hyperglycemia was not corrected [151]. More recently, Prabakar and colleagues investigated the differentiation potential of human UCB-MSCs toward beta cells by applying a stepwise differentiation protocol based on known pancreatic developmental cues. A relatively large proportion of undifferentiated UCB-MSCs constitutively express the key transcription factors Pdx1, Ngn3, NeuroD1, Nkx6.1, and Isl1, all critically involved in pancreatic endocrine development. Upon differentiation, the resulting cells were shown to secrete insulin in a glucose-responsive manner both in vitro and in vivo [85].

Solid tissues from the placenta have also been investigated as sources of insulin-producing cells. Several such populations have characteristics consistent with MSC. Chang and colleagues observed that placenta-derived MSCs (PD-MSC) can undergo pancreatic endocrine commitment after exposure to a differentiation protocol based on EGF, bFGF, and HGF. The cells improved hyperglycemia in STZ-treated SCID mice, but the differentiation stage was not fully investigated [152]. Kadam and collaborators observed that multipotent placenta (chorionic villi)-derived MSC are able to secrete insulin in a glucose-responsive fashion. At variance with findings by others, the cells appeared to secrete insulin even in the undifferentiated state [153]. Chiou and colleagues overexpressed MafA in PD-MSC and observed upregulation of the expression of pancreatic development-related genes and endocrine hormones. Transplantation in STZ-induced diabetic mice improved blood glucose levels [154]. Considered together, these findings suggest that placenta-derived MSC has the potential to enter the differentiation pathway toward insulin-producing cells, but more steps need to be taken to demonstrate a functional beta-cell phenotype and enhance differentiation efficiency.

The umbilical cord matrix, termed Wharton's jelly (WJ), is another rich and uncontroversial source of MSCs. Immersed in the Wharton's jelly, and difficult to separate from the rest of the matrix, are the omphalomesenteric duct remnants, leftovers of the yolk sac stalk. Intriguingly enough, the yolk sac is one of the three tissues that expresses insulin during the fetal life, together with the fetal liver and

the developing pancreas [155, 156]. Chao and colleagues derived WJ-MSCs and induced pancreatic endocrine differentiation by means of a protocol based on neural-conditioned medium. The cells activated genes involved in the appropriate differentiation cascade and expressed insulin in response to physiological glucose levels [26]. Kadam and Bhonde extended the findings describing multipotent nestin-positive WJ-MSCs able to differentiate into β-like cells [153, 157], and Wang and colleagues reported promising in vivo results in NOD mice [158]. The Wharton's Jelly thus appears as a promising source of MSCs with the potential to undergo beta-cell commitment.

Outstanding Issues Regarding MSC Differentiation

Can MSCs fully mature into functional beta cells? Current differentiation methods need to be refined. Even if functional activity has been documented by several groups, robust, rigorous demonstration is still needed.

What is the best induction strategy? Considerable advances are coming from attempts to recapitulate pancreatic organogenesis. However, many of the differentiation approaches reported so far do not take into account natural islet physiology and organogenesis and most simply disregard the importance of cell-to-cell and cell-to-matrix interactions. Envisageable strategies will probably integrate fundamental cues in relation to the natural organ development and structure [159, 160]. The yields of current differentiation protocols are rarely reported, and functionality is even more rarely compared with islets as gold standards. Much effort will probably be needed to obtain clinically relevant numbers of functional insulin-producing cells. Most studies have focused on deriving beta cells alone, but probably an islet-like organoid could act as a system and thus be preferable for the systemic management of blood sugar levels.

What is the best tissue source for retrieving MSCs with beta-cell differentiation potential? And does MSC from different sources need different differentiation cues? As MSCs from different tissues were presumably exposed to different inductive milieus during their development and reside in altogether disparate physiological niches during adulthood, it would be reasonable to expect that they will have different capabilities in vitro as well.

Autologous, allogeneic, or xenogeneic cells? The immune recognition of transplanted cells, especially into an immunologically primed diabetic environment, represents a big question mark. Undoubtedly, the ability to bank stem cells would offer the possibility of matching allogeneic recipients. The use of autologous cells would instead bypass the limitation of immune rejection based on alloimmunity, but the differentiation potential of autologous MSCs may be profoundly affected by the pathology of the disease [55]. Moreover, the same pathogenetic processes that acted on the original beta cells may ultimately act against transplanted insulin-producing cells.

On the other hand, having a potentially unlimited source of insulin-producing cells may provide enough regenerative units for the long-term management of

diabetes. But how do culture expansion and differentiation affect the integrity of the cells? Ex vivo manipulation may come at a price, with a rise in the risk of genomic instability, tumorigenicity, and progressive exhaustion of differentiation potential.

Should we transplant terminally differentiated cells, endocrine-committed progenitors, or stem cells? The function is needed as soon as possible, but the stability of the phenotype and the duration of survival of the beta-like cells are unclear. It is conceivable that progenitors and stem may dynamically contribute to the differentiated pool and sustain function in the long term.

Will the diabetic environment stimulate or derange the differentiation? And in any case, what is the risk of undesired, ectopic differentiation or tumor formation? If cells were placed into a retrievable device, it would be possible to excise or interchange the graft in case of need. A device could even protect cells against immune attack. Nevertheless, the integration in the recipient tissue and appropriate vascularization appear to be fundamental in dictating fate.

What is the best anatomical site for transplantation and how should the cells be delivered? Intrapancreatic injections may expose the cells at the best environment possible, but this approach may be associated with important iatrogenic risks. Extracellular matrix components could help with engraftment, survival, and differentiation.

How specific is the transcriptional fingerprint currently associated with the pancreatic endodermal progenitor cells and their differentiation cascade? Finding similar expression patterns in MSC derived from several tissues may have opposite significance. They may represent shared mechanisms governing access to the endocrine commitment, indicating that endocrine progenitors may be recruited (and thus isolated) from distant sites. Or they may have different roles in other morphogenetic contexts, hence pointing to a flaw in our initial assumption. The design of future research will have to carefully consider all these concerns [161].

Conclusion

Accumulating evidence supports the ability of MSCs to positively influence allorecognition and rejection: these protective effects could be exploited in co-transplant settings with pancreatic islets. MSCs have the potential to modulate autoimmunity and could directly target the autoimmune aggression in type 1 diabetic patients. Moreover, MSCs can promote β-cell function, survival, and regeneration, attractive capacities that could address the loss of functional β-cell mass in both type 1 and type 2 DM. Furthermore, MSCs could represent a source of insulin-producing cells, but current differentiation strategies need to be evolved in order to obtain clinically relevant numbers of β-like cells. Outstanding issues regarding safety, efficacy, and mechanisms of action need to be addressed before MSCs can be widely used in clinical settings. Nonetheless, the noted advantages of this plentiful cell type make them a very promising potential tool for the treatment of diabetes.

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Chapter 34
MSCs for Renal Repair

Anna M. Gooch and Christof Westenfelder

Abstract Acute kidney injury (AKI), the abrupt loss of renal function that results from ischemia, sepsis, or nephrotoxin-induced damage to vascular and tubular structures, is characterized by inflammatory processes, cellular apoptosis, and necrosis. Patient mortality remains high and treatment is largely supportive. Even mild AKI can lead to chronic kidney disease (CKD), further underscoring the urgent need for therapeutic innovations. Mesenchymal stromal cells' (MSCs) potent anti-inflammatory, immunomodulatory, organ protective, and reparative properties make them excellent potential candidates for prevention and treatment of AKI. Multiple preclinical studies using various AKI models have confirmed that, via paracrine mechanisms, MSC treatment improves survival, ameliorates and reverses injury, and prevents progression to CKD. Preliminary results from the first Phase I clinical trial (safety, preliminary efficacy), in which we infused allogeneic MSCs into subjects at high risk for AKI following on-pump cardiac surgery, paralleled preclinical observations, suggesting that MSC therapy is safe and effective in preventing both postoperative AKI and progression to CKD.

Introduction

Acute kidney injury (AKI), or the sudden loss or cessation of renal function, is a common complication in at-risk populations and manifests as a sudden increase in serum creatinine and/or reduction in urine output. While the outward manifestations

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_34,

are simple, AKI is the result of a complex array of damage to vascular and tubular structures and processes in the kidney. Due to AKI's complex pathophysiology, therapies designed to target a single or only a few of the involved pathways are unlikely to be effective, as has been observed in several clinical trials with targeting drugs. Consequently, treatment has remained largely supportive. It is now recognized that even mild acute renal injury often leads to progressive, chronic loss of renal function and other adverse outcomes, underscoring the urgent need for innovations in therapy. Mesenchymal stromal cells' (MSCs) known anti-inflammatory, immunomodulatory, organ-protective, and reparative properties make them excellent potential candidates for prevention and/or treatment of AKI. Indeed, multiple preclinical studies using either ischemia/reperfusion or nephrotoxic models of AKI have confirmed that treatment with MSCs improves survival, amelioration and reversal of injury, and clinical progression to CKD. Although underpowered, preliminary results from the first phase I clinical trial (dose escalation, safety), employing infusion of allogeneic MSCs in subjects who underwent on-pump cardiac surgery and who were at risk for development of postoperative AKI parallel preclinical results, suggesting that MSC therapy appears both safe and effective in preventing postoperative AKI, as assessed by the RIFLE and AKIN criteria, as well as preventing progression to CKD up to 2 years postoperatively.

At-Risk Populations and Frequency of Acute Kidney Injury

Acute kidney injury (AKI) is a common complication caused by ischemia/reperfusion injury to the kidney or by exposure to renal toxins [1]. It is characterized by an abrupt and sustained partial or complete loss of renal function resulting in disturbed volume, electrolyte and acid base balance, and "uremic" multisystem complications due to the retention of toxic waste products, a heightened inflammatory state, and the generation of reactive oxygen species [2]. Depending upon the degree of injury, such derangements can result in life-threatening multi-organ complications. Indeed, the mortality rates from AKI range from 15% in the general population to 80% for patients with multi-organ failure and sepsis or for those who develop it postoperatively [3].

AKI is most frequently seen in patients with shock, sepsis, trauma, and after major surgery. Patients undergoing cardiac surgery are at high risk with up to 30% of all cardiac surgery patients developing AKI [4, 5]. Many studies of cardiac patients have consistently found certain factors to be associated with increased risk of developing AKI following on-pump surgery. These risks include but are not limited to the type of procedure performed, with valve procedures being of particularly high risk; patient age greater than 65; female patient gender; underlying renal disease; diabetes mellitus, type I or II; congestive heart failure; combined surgeries; and cardiopulmonary bypass surgery time greater than 2 h [4–7].

Even when renal function appears to fully recover following AKI, it is now recognized that a significant proportion of patients will go on to lose renal function gradually, developing chronic kidney disease (CKD) or end-stage renal disease (ESRD) and eventually requiring chronic hemodialysis or a renal transplant [8–10]. Such progression may be a consequence of either undiagnosed or incompletely resolved AKI and is characterized by continued inflammatory and fibrotic processes and microvascular rarefaction within the kidney [8, 11, 12]. Recently, it has been observed that even minor changes in serum creatinine (SCr), indicating only a mild renal insult, can result in increased mortality risk and other adverse outcomes [10, 13–16]. The treatment-resistant nature of AKI, its high morbidity and mortality rates, as well as the now recognized frequent progression of AKI to CKD underscore the urgent need for advances in treatment modalities [1, 10, 14, 17, 18].

Definition and Classification of Acute Kidney Injury

The clinical signs of AKI are an acute and sustained increase in serum creatinine (SCr) and/or decrease or loss of urine output, reflective of an acute decline in renal function resulting from a renal injury that has caused structural and functional changes in the affected kidney. Prior to 2004, there was no uniformly accepted definition of AKI. In order to allow for consistent classification, staging and diagnosis, as well as to facilitate comparison of results of studies and trials in a standardized, defined fashion, AKI staging or classification systems were developed [2, 16]. The first such commonly used system is referred to as the RIFLE classification system, RIFLE being an acronym for the stages: risk, injury, failure, loss of function, and end-stage renal disease. Each stage represents either the degrees or clinical outcomes of AKI as clinically manifested through defined, incremental, percentage increases in SCr (mg/dL) relative to baseline and/or decrease or cessation of urine output for defined lengths of time [2]. A second commonly used system, the acute kidney injury network (AKIN) classification system, is a simplified version of the RIFLE system, with clinical outcomes L(oss of function) and E(nd stage renal disease) removed, and R(isk), I(njury) and F(ailure) replaced with stages 1, 2, and 3, respectively. Due to emergent data indicating that even small increases in SCr were associated with adverse outcomes such as increased mortality risk or progression to CKD, the AKIN system introduced a minimum, acute, sustained change in SCr of 0.3 mg/dL above baseline as constituting injury [13, 16].

Pathophysiology of Acute Kidney Injury

As has been determined from extensive preclinical studies, an acute renal insult, whether ischemic or toxic, damages the kidneys at both the vascular and tubular levels, and inflammatory processes contribute to further injury [19–24]. Decreased

renal blood flow, oxidative stress, increased intracellular calcium concentrations, and ATP depletion lead to tubular cell apoptosis and necrosis which, in turn, lead to tubular obstruction and leakiness. Tubular cells that survive such damage undergo cytoskeletal changes and loss of cell polarity, further impairing tubular transport functions [25]. In addition, tubular cells respond to ischemic stress by inducing multiple proinflammatory genes such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, IL-8, transforming growth factor-beta (TGF-β), and others, thus further augmenting destructive processes. Damage to the renal vasculature results in loss of vascular tone and regulatory function, which leads to congestion, leakiness, edema, and accumulation of inflammatory cells in the microvasculature. Generated reactive oxygen species and induction of proinflammatory genes and consequent inflammatory processes in tubular cells result in further renal damage [19–21, 23, 24, 26–28].

Treatment of Acute Kidney Injury

Despite a number of promising preclinical studies of novel therapies in animal models, including those with atrial natriuretic peptide (ANP), erythropoietin, and insulin-like growth factor-1 (IGF-1), such therapies have largely proven ineffective when tested in human clinical trials [29–31]. AKI has remained frustratingly resistant to treatment, with treatments being mainly supportive and involving renal replacement therapy when injury is severe or prolonged.

Possibilities for the failures of promising preclinical therapies to translate to human patients were examined recently by Jo et al. [32]. One possibility cited for failures of translation is the presence of complicating comorbidities in human patients, such as diabetes and underlying renal disease, but unaccounted for in animal models, where AKI is induced in otherwise healthy animals. A second possibility is the complex pathophysiology of the disease and the single target nature of many pharmacological therapies. Jo et al. suggest, and we concur, that given the number of pathways that contribute to AKI, and the likely compounding effects of comorbidities, multiple pathways must be targeted simultaneously or successively to be effective in protecting and repairing the injured kidney [32].

Preclinical Studies on the Use of MSCs in Treating Acute Kidney Injury

Rationale

As discussed above, the apparently simple clinical manifestations of AKI belie the highly complex cascade of vascular, inflammatory, and tubular injuries within

the kidney itself. The work of many laboratories has indicated that prevention or repair of those injuries requires modulation of the inflammatory response and stimulation of organ-protective and regenerative pathways.

As established elsewhere in this book, mesenchymal stromal cells (MSCs) offer the advantage of acting through multiple mechanisms in disorders such as AKI with its highly complex pathophysiology. In addition to their ability to differentiate into multiple lineages, MSCs have been shown to have immunomodulatory capabilities and to express growth factors known to be renoprotective in experimental AKI [20, 33, 34], thus previously leading us and others to postulate that MSCs may have therapeutic potential for prevention and treatment of AKI.

Preclinical Data with MSCs

Our and others' preclinical studies in animals with experimental AKI have demonstrated that MSCs are renoprotective against different forms of injury including from nephrotoxins such as cisplatin, myoglobin and hemoglobin [35–37], and ischemia/reperfusion [38, 39]. Such preclinical studies have confirmed that administration of MSCs to animals with experimental AKI protects kidneys from further damage, hastens renal repair, facilitates regeneration of damaged renal tissues, and, importantly, prevents progression to CKD [35–42].

That such protective and reparative effects are primarily the result of secretion of soluble factors and thus paracrine actions, rather than engraftment, is now apparent. MSCs are known to secrete growth and other factors such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and IGF-1 that have been shown to be renoprotective; and treatment of AKI with MSCs is associated with downregulation of proinflammatory genes such as TNF-α, interferon-gamma (INF-γ), and IL-1β and upregulation of anti-inflammatory genes such as IL-10 [39, 41, 43, 44]. Tracking studies by many groups, including our own, have observed no significant engraftment of MSCs infused for treatment of AKI, arguing against engraftment as an important protective mechanism [39, 45–47].

Importantly, factors secreted by MSCs in the absence of the MSCs themselves have been shown to provide renoprotection. Bi et al. in a study employing cisplatin-induced AKI in mice found that intraperitoneal administration of conditioned medium from cultured MSCs alone was sufficient to enhance tubular cell proliferation and limit apoptosis [35]. Similarly, studies have shown that injection of MSC-derived microvesicles provides renoprotection from glycerol-induced and ischemia/reperfusion-induced AKI [48, 49]. Thus, despite some observations that MSCs might have the ability to differentiate into renal cells [37], the common observation of lack of engraftment coupled with the ability of MSC-derived factors alone to provide renoprotection argues in favor of paracrine mechanisms of protection.

Clinical Trials of MSCs for Acute Kidney Injury

Study Design and Methods

Because of our and others' promising preclinical data demonstrating the efficacy of MSCs in preventing or reducing the renal damage associated with AKI and its long-term sequelae of progression to CKD, as well as clinical evidence that MSCs are effective in the treatment or prevention of other conditions such as osteogenesis imperfecta and graft-versus-host disease [50–53] (see also www.ClinicalTrials.gov), we undertook a phase I clinical trial to test the safety, feasibility, and preliminary efficacy of administered allogeneic MSCs in preventing or reducing the incidence of AKI in at-risk patients undergoing on-pump (cardiopulmonary bypass machine) cardiovascular surgery [20, 24, 54]. In this trial, allogeneic MSCs, derived from the marrow of healthy, screened donors, using FDA-approved protocols, were culture expanded under current good manufacturing practice (cGMP) conditions at the University of Utah Cell Therapy Facility, Salt Lake City, Utah. MSCs were administered in a dose escalation protocol and infused via a femoral catheter into the suprarenal aorta immediately after the subject came off pump and was hemodynamically stable.

The trial was approved by the FDA and the institutional review boards (IRB) of the two centers where the trial was conducted, St. Mark's Hospital and Intermountain Medical Center, both in Salt Lake City, Utah. The clinical data on the study subjects are reported with their consent and with approval of the respective IRBs. An independent Data and Safety Monitoring Board (DSMB) consisting of a general nephrologist, a nephrologist/medical epidemiologist, and a cardiovascular surgeon reviewed the trial protocol, approved the trial, and continues to monitor the clinical data from all enrolled and treated subjects.

Clinical Setting and Enrollment Criteria

Eligible subjects for this trial were patients scheduled for on-pump cardiac surgery for various causes (valve replacement and/or coronary artery bypass surgery) that were at high risk for developing AKI as defined by comorbid conditions. On-pump cardiovascular surgery patients were chosen as study subjects for several reasons. First, as discussed in section ("Introduction"), such patients are vulnerable to developing AKI postoperatively, and the risk of developing AKI becomes even greater when such patients have other underlying risk factors or comorbidities such as older age, diabetes, or CKD [4, 6, 7]. Second, the time and cause of potential renal injury, the on-pump surgery, is defined. Third, the time of intervention (administration of MSCs) in relation to the time of injury is defined. Fourth, baseline and postoperative data can be collected at defined times relative to the time of injury, thus allowing for meaningful analysis

and interpretation of collected data, assessment of renal function, safety, and potential efficacy of the intervention.

Objectives

The primary objective was safety: to test whether infusion of allogeneic MSC into the suprarenal aorta of patients who have undergone on-pump cardiac surgery, coronary artery bypass grafting (CABG), and/or valve surgery and who were at high risk for AKI following surgery is safe. This was assessed by monitoring subjects postoperatively for the occurrence of adverse events (AEs) and serious adverse events (SAEs) that may be attributable to the MSC therapy. Specifically, subjects were monitored for AEs and SAEs in the immediate postoperative period through discharge from the hospital, once monthly for 6 months and at three annual visits [20, 24, 54]. Additionally, as the protocol involved the use of allogeneic MSCs, subjects' sera were tested for the presence of antibodies to the MSCs they had been infused with at approximately 1 month post infusion.

The secondary objective of this trial was assessment of preliminary efficacy of MSC administration for the potential prevention and treatment of postoperative AKI. Although a priori underpowered, preliminary efficacy in this trial was assessed by determining the comparative frequency and severity of postoperative AKI using standard markers of AKI (serum creatinine, BUN, urine output, creatinine clearance, electrolyte and acid–base balance), need for temporary or permanent dialysis, length of hospital stay, need for readmission within 30 days of surgery, associated 30-day mortality, and progression to CKD within 6 months or longer after surgery.

Subjects

Sixteen subjects were enrolled in the trial and were divided equally among three dosing groups, i.e., low, medium, and high MSC dose.

Basic demographic and preoperative risk factors for each subject can be found in Table 34.1. To summarize, all subjects were Caucasian and ranged in age from 59 to 86 years, with a mean age of 71.8 years. Eleven subjects were enrolled at Intermountain Medical Center and five at Saint Mark's Hospital. Five subjects were female; eleven were male. As of October 2010, all still followed subjects were at least 1 year after surgery.

All enrolled subjects had at least one preoperative risk factor for development of postoperative AKI. The preoperative risk factors for AKI among the subjects are listed in Table 34.1 and summarized as follows:

- Diabetes mellitus (DM): 11 subjects had DM; one with DM-I, ten with DM-II.
- CKD: 12 subjects had CKD broken down as follows, CKD-I: one subject, CKD-II: six subjects, CKD-III: five subjects.

Table 34.1 Subject Demographics

Cohort	Subject	Age (years)	Gender	Preop SCr (mg/dL)	Type of surgery	Pump time (min)
Cohort 1 (low dose)	1	59	M	1.04	CABGx3	116
	2	79	F	0.68	Mitral valve	88
	3	74	M	1.4	CABGx1	123
	4	66	F	0.63	Aortic valve, aortic graft	155
	5	70	F	1.01	CABGx4	135
	6	82	F	1.3	Aortic valve, ascending aorta replacement, CABGx1, balloon pump	245
Cohort 2 (middle dose)	7	65	M	0.94	CABGx5	127
	8	78	M	1.3	CABGx2	59
	9	70	M	1.6	CABGx4, aortic valve	171
	10	67	M	1.52	Aortic valve	98
	11	62	F	1.1	Aortic valve	100
Cohort 3 (high dose)	12	66	M	1.79	CABGx5	179
	13	75	M	1.42	Aortic root	127
	14	75	M	1.8	CABGx4	127
	15	86	M	1.2	Mitral valve	120
	16	81	M	1.8	CABGx4 plus balloon pump	207

- Hypertension (HT): 13 subjects.
- Congestive heart failure (CHF): eight subjects.
- Chronic obstructive pulmonary disease (COPD): two subjects.
- Pump time ≥ 2 h: 11 subjects.

The types of surgeries for each subject are listed in Table 34.1. To summarize, eight subjects underwent CABG alone, six had valve surgery alone, and two had both CABG and valve surgery.

Case-Matched Historical Controls

For assessment of preliminary efficacy, study data were compared to historical data that were collected and available for analysis from groups of historical, case-matched control patients. These matched controls were selected from a database of 9,163 cardiovascular surgical patients at Intermountain Medical Center who were followed longitudinally from surgery through death. Data on these patients were collected between August 1992 and December 2007. Phase I subjects were matched to controls by age, gender, type of surgery (CABG + graft number/valve replacement or repair), and preoperative underlying comorbidities (CKD and stage, diabetes, etc.). Of the patients in the database, 195 matched the Phase I subjects using these criteria. For each subject, a minimum of four historical control group patients matched. Studied endpoint data from the control groups were given as averages. These averages were then compared to the subjects' endpoints [20, 24, 54].

Results

Safety

Safety was assessed by monitoring the subjects for the development of AEs and SAEs postoperatively. Relatedness of any AE or SAE to administration of MSCs was assessed by the primary investigators (PIs) and reviewed at each dose level by the DSMB.

In addition, subjects' sera were tested for the presence of anti-MSC antibodies. As discussed in this book and elsewhere, human MSCs do not express HLA-DR and blood group antigens and are negative for co-stimulatory CD-40, CD-80, and CD-86, which implies that the infusion of allogeneic MSCs to humans should not elicit an antibody/immune response [55]. However, if human MSCs are cultured in fetal calf serum (FCS), as is routinely done, retained bovine antigens may induce alloantibody production, as has been reported [56]. To avoid the possibility of eliciting an immune response in Phase I study subjects, administered human MSCs were cultured without FCS for the Phase I clinical trial.

Adverse Events

There were 72 AEs (SAEs included) recorded over the first 6 months of the study. All 16 enrolled subjects experienced at least one AE. The most common AE was pain, which was most frequently associated with sternotomy. The most commonly affected body system was the cardiovascular. Of the cardiovascular AEs, edema was the most common. In the judgment of the PIs, sixty-one of the AEs were deemed unlikely to have been attributable to MSC treatment, and thirteen were deemed not related to MSC administration. In addition to monitoring for AEs, representative sera from all three dosing cohorts were tested at approximately 1-month postoperative period for the presence of anti-MSC antibodies. None of the subjects developed anti-allogeneic MSC antibodies, as previously reported by other investigators [56].

Serious Adverse Events

Of the 72 recorded AEs, 6 classified as SAEs. These SAEs occurred in four clinical trial subjects. No SAE was attributed by the PIs to be the result of MSC administration. One subject experienced sternal nonunion, requiring two readmissions to the hospital, one within 30 days of surgery. The remaining SAEs all involved the cardiovascular system. The most serious resulted in the death of one subject, who died suddenly and unexpectedly at home 25 days postoperatively, most likely from a fatal cardiac arrhythmia. After careful review of this subject's data, the PI concluded that the event was unlikely to have been related to MSC administration. The subject's death was fully adjudicated by the FDA and reviewed by the DSMB, which concluded that there was "no plausible connection between the study drug and the patient's death" [24, 54].

Safety Conclusions

Four factors indicate that allogeneic MSCs are safe for administration to at-risk cardiovascular surgical subjects at the low, intermediate, and high doses tested. First, no SAEs or AEs to date have been attributed to MSC therapy. Second, the DSMB has had no safety concerns after reviewing all of the subjects' data. Third, no adverse effect on renal function or other organ systems was seen in any of the 16 treated subjects. Fourth, no subject developed an antibody response to MSCs within a month of infusion.

Preliminary Efficacy

Preliminary efficacy of MSC therapy in preventing or ameliorating postoperative AKI and its associated long-term sequelae of progression to CKD was determined by

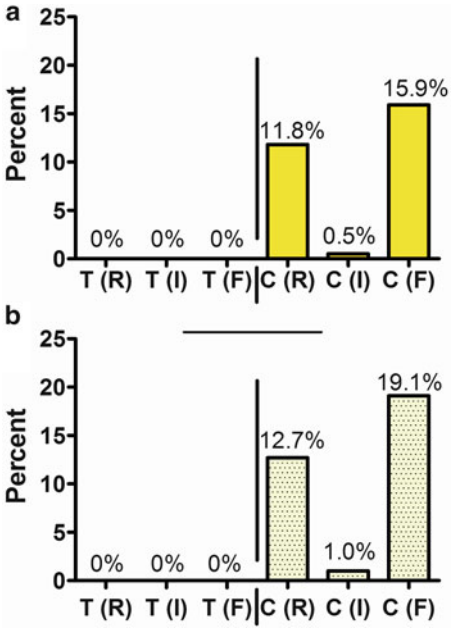

Fig. 34.1 Renal outcome at discharge in all Phase I MSC-treated subjects and their historical controls (*panel* **a**, *top*) and those with underlying CKD (*panel* **b**, *bottom*) (RIFLE criteria). (**a**) Renal outcome (RIFLE criteria) at discharge in MSC-treated subjects (*left*; $n = 16$) and historical controls (*right*; $n = 64$). (**b**) Renal outcome (RIFLE criteria) at discharge in MSC-treated subjects with underlying CKD (*left*; $n = 12$) and historical controls (*right*; $n = 48$)

assessment of renal outcomes per se and in comparison with those of historically matched case controls. These outcomes were development of postoperative AKI as defined by the RIFLE or AKIN criteria in the immediate postoperative period (postoperative day 1 through hospital discharge) and development or progression of CKD within 6 months or more of surgery. Length of hospital stay, need for readmission for any reason within 30 days of surgery, and death for any reason within 30 days of surgery were also assessed. These variables are discussed below.

Development of Postoperative Acute Kidney Injury

Development of postoperative AKI, as defined by the both the RIFLE and AKIN criteria, was assessed in the study subjects and matched historical controls using a comparison of their preoperative (baseline) and hospital stay SCr values measured on postoperative days 1, 2, 3, and the day of discharge. The results of such assessment are shown in Fig. 34.1. Two subjects experienced transient elevations in SCr levels, one on postoperative day 2, and the other on postoperative day 3 that were >0.3 mg/dL above baseline, but neither elevation constituted a 50% increase above baseline, neither was sustained, and both were completely resolved at the time of discharge (data not shown). As shown in Fig. 34.1, none of the study subjects met any of the RIFLE criteria by the time of discharge, while approximately 16% of historical matched control patients met the criteria

for failure. When comparing just those subjects with underlying CKD (12 of the 16 subjects), who may thus be at greater risk of developing postoperative AKI, to their historical matched control cohorts, approximately 19% of the matched controls met the criteria for failure by discharge. Though underpowered, these results are statistically significant. Upon examination of other indicators of renal injury (reduced urine output, elevated BUN, need for dialysis, etc.), no MSC-treated subject exhibited any such signs, while notably, approximately 6% of historical controls required temporary dialysis.

Progression to Chronic Kidney Disease

To assess the long-term efficacy of MSC treatment, SCr measurements were taken for each treated subject monthly for 6 months postoperative and compared with values at those time point from historical matched controls. Aggregate results of the preoperative, discharge, and 6-month measurements are shown in Fig. 34.2. As illustrated in Fig. 34.2, SCr values are an average of 0.3 mg/dL higher than baseline at 6 months postop in historical control groups. In contrast, SCr values remain essentially unchanged from baseline at 6 months postoperative in treated subjects, particularly in those with underlying CKD. Individual SCr values from baseline to 2 years postop further indicate that this stable level of renal function continues long term in MSC-treated subjects.

Length of Hospital Stay, Need for Readmission Within 30 Days, and 30-Day Mortality

A comparison of length of hospital stay between the MSC-treated subjects and the matched historical controls is shown in Fig. 34.3. MSC-treated subjects' length of hospital stay was approximately 7 days, while that of the matched historical controls was approximately 11 days, or approximately 4 days longer. While 6% of all treated subjects (and none of those with underlying CKD) required hospital readmission within 30 days of surgery, approximately 18% of matched historical control patients did. Mortality rates within 30 days of surgery between the treated and historical control groups were comparable.

Preliminary Efficacy Conclusions

The above preliminary efficacy data demonstrate that none of the study subjects developed significant AKI in the immediate postoperative time in the hospital nor after discharge. Significantly, no patient required dialysis immediately or late after surgery. Compared with historical controls, MSC-treated subjects fared better in the long term, showing no development or progression of CKD within 2 years.

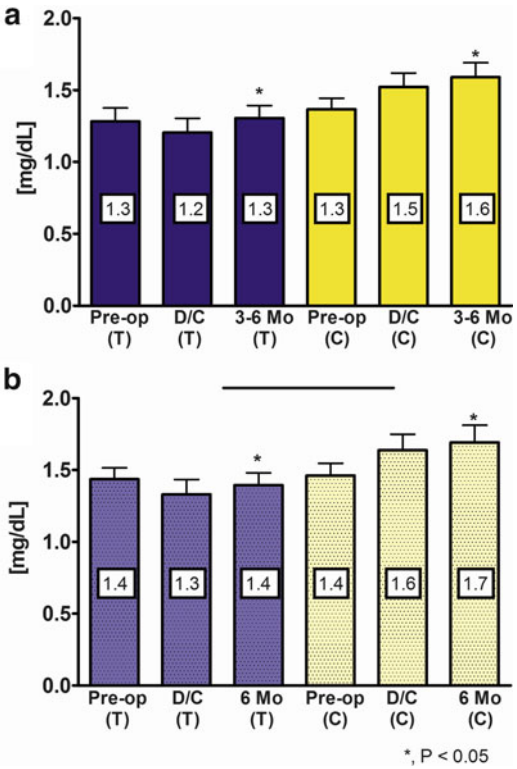

Fig. 34.2 Average SCr (± SEM): preoperative (pre-op), upon discharge (*D*/*C*) and 6 months postoperative (6 months) in treated (*T*; *blue bars*) vs. historical controls (*C*; *yellow bars*). (**a**) All Phase I subjects and their matched historical controls ($n=16$, treatment; $n=64$, control) (*top*). (**b**) Just those Phase I subjects with underlying CKD and their matched historical controls ($n=12$, treatment; $n=48$, control) (*bottom*). *Indicates a significant difference between the 6-month time point for the MSC-treated group vs. the 6-month time point for the historical control group. For the historical control group, the difference between the preoperative SCr values and the 6-month values is also significantly different ($p<0.05$), while they are not significantly different for the MSC-treated group

Conclusions

The safety and preliminary efficacy results from this first Phase I clinical trial employing allogeneic MSC infusion in order to prevent AKI following cardiac surgery mirror those of our preclinical studies. The Phase I results indicate that MSCs are safe, with no AEs or SAEs directly attributable to MSC administration, and that this therapy is potentially renoprotective. This efficacy extends not just to the short-term prevention of AKI commonly experienced following cardiac surgery but to the long-term prevention of CKD, a currently well-recognized consequence of even small elevations in SCr subsequent to cardiac surgery or other insults. Definitive confirmation of the efficacy of this MSC-based therapy of AKI awaits the conduct of adequately powered, randomized, placebo-controlled, multicenter Phase II trials.

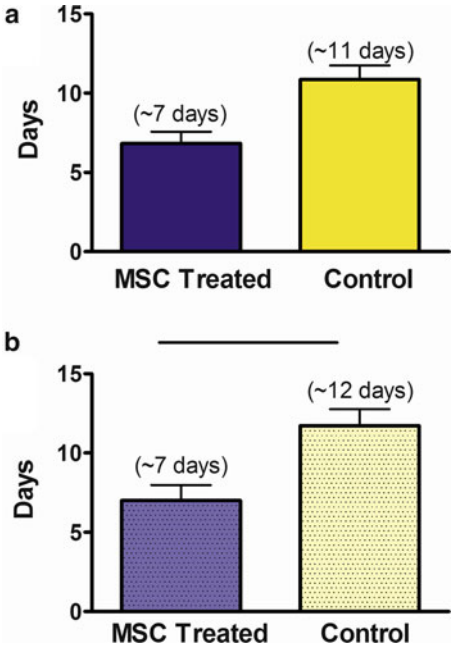

Fig. 34.3 Mean (± SEM) length of hospital stay (*LOS*) for MSC-treated subjects (*blue bars*) and matched historical controls (*yellow bars*). (**a**) All Phase I subjects and their matched historical controls ($n=16$, treatment; $n=64$, control) (*top*). (**b**) Just those Phase I subjects with underlying CKD and their matched historical controls ($n=12$, treatment; $n=48$, control) (*bottom*)

Acknowledgments At the time of the Phase I clinical trial, both Christof Westenfelder and Anna Gooch were either consultants to or recipients of research support from AlloCure, Inc., the trial's sponsor. Currently, both authors declare they have no competing interests. The authors' research was also in part supported by funds from the Merit Review program of the Veterans Administration, Washington, DC, the National Institutes of Health, the American Heart Association, the National Kidney Foundation, the Western Institute for Biomedical Research, and AlloCure, Inc. The human MSCs that were administered to study subjects in the Phase I clinical trial were prepared in the cGMP Cell Therapy Facility of the University of Utah. The outstanding skills of the PIs (Dr. John Doty, Intermountain Medical Center, Murray, Utah; Dr. David Affleck, St. Mark's Hospital, Salt Lake City, Utah), Co-PIs (Drs. Benjamin Horne and Brent Muhlestein, Intermountain Medical Center; Drs. Shreekanth Karwande and Gilbert Schorlemmer, St. Mark's Hospital), and study nurses (Jean Flores, Intermountain Medical Center; Analee Creer, St. Mark's Hospital) are gratefully acknowledged. The contributions of the members of the Data and Safety Monitoring Board for this trial, Drs. C. Kablitz, George R. Reiss, and Srinivasan Beddhu, are also greatly appreciated. Finally, the excellent bench and regulatory work of Zhuma Hu and Drs. Ping Zhang and Florian Tögel was invaluable for the successful conduct of the preclinical studies and the clinical trial.

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Chapter 35
MSCs for the Treatment of Stroke, Spinal Cord Injury, and Traumatic Brain Injury: From Bench Work to Clinical Trials

Bing Yang, Ramy El Khoury, and Sean I. Savitz

Abstract Stroke, spinal cord injury, and traumatic brain injury are the major three causes of central nervous system injury. After the acute phase, most patients are left with significant motor, cognitive, and social impairments. Few treatments exist and there are no current therapeutic interventions altering their underlying pathological processes via tissue salvage, support, repair, or replacement at the cellular or subcellular level. Recent evidence suggests that the cell-based therapy exerts therapeutic benefits in relevant preclinical animal models. Furthermore, some cell types, like MSCs, have advanced into clinical trials. Here, we present the current status of MSCs in stroke, SCI, and TBI therapy from preclinical studies to clinical trials, with an emphasis on dosage, timing, and routes of delivery. We also discuss possible cellular and molecular mechanisms of action that mediate the effects of MSCs in these different disease models. Finally, we end with a discussion of important issues that require future study.

Introduction

Stroke is a leading cause of death and long-term adult disability in industrialized countries [1], but there is only one approved FDA therapy—recombinant tissue plasminogen activator (tPA) [2]. The use of tPA is limited mainly because of delay in presentation resulting in fewer than 3% of stroke patients receiving treatment [3]. The majority of stroke patients therefore have no proven therapies to enhance recovery. Neuroprotective therapies in animals have shown much promise; however, their

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_35,

successful translation from bench to bedside has still not occurred over 20 years. The focus of stroke research has now shifted from studies on acute neuroprotection toward combined neuroprotective and neuroregenerative approaches with a special emphasis on cell-based therapies [4].

Traumatic brain injury (TBI) accounts for 50% of all trauma-related death [5], and the survivors often suffer from a range of deficits in motor, cognitive, and behavioral function. Yearly, around 1.5 million people die, and more than ten million people are hospitalized or killed due to TBI [6]. TBI poses heavy economic burdens with a cost estimated at about two billion dollars per year [7]. Multiple investigational treatments have been tested, but current therapies are limited to controlling intracranial pressure and optimizing cerebral perfusion to prevent expanding edema and damage.

The annual incidence of spinal cord injury (SCI) is estimated at approximately 12,000 new cases each year. The number of people in the USA who are alive in 2009 and have SCI is around 262,000. According to the National Institutes of Health, "among neurological disorders, the cost to society of automotive SCI is exceeded only by the cost of mental retardation" [8]. Due to the limited capacity of axonal regeneration and the presence of local inhibitory factors, recovery is minimal. Limited treatment options include methylprednisolone [9], which has many critical side effects and early surgical decompression [10, 11]. A potential method for functional restoration will likely need to incorporate neuroprotective strategies along with the reestablishment of neuronal tracts and synapses.

After the acute phase of these three neurological disorders, patients are left with significant motor, cognitive, and social impairments. Rehabilitation is essential to their recovery but provides limited options to return patients to their former lives. Despite their respective etiological differences, stroke, TBI, and SCI share many common features: (1) loss of neural tissue and neural cell death, (2) post-injury inflammation, (3) progressive deterioration subsequent to the initial injury, (4) neural dysfunction, and (5) glia scar formation in chronic stages. Few treatments exist for any of them, and there are no current therapeutic interventions altering their underlying pathological processes via tissue salvage, support, repair, or replacement at the cellular or subcellular level. This suggests that an ideal therapeutic strategy could be targeted at promoting neurogenesis and angiogenesis while suppressing excessive glia reactions and dual-modulating the pro- and anti-inflammatory responses after injury.

Mesenchymal Stromal Cells

Mesenchymal stromal cells (MSCs) as a potential therapeutic strategy for acute neurological disorders have thus been investigated intensively in the past 10 years. No matter the source from which MSCs are derived, such as bone marrow or umbilical cord, they have catapulted to center stage in regenerative medicine research for

two main reasons. First, MSCs can be harvested from a patient's own body tissue, which permits autologous transplantation. Such an approach can reduce the likelihood of immune rejection and avoid some possible complications associated with allogeneic cell transplantation. Second, MSCs from the adult circumvent the moral dilemma of using fetal tissue and the ethical and political issues of using human embryonic tissues or human eggs for stem cell generation.

MSCs can be generated from a range of tissues. Bone marrow was the first source of MSCs identified by Friedenstein and colleagues in 1970. However, because the volume of bone marrow and the capacity of MSCs to differentiate decrease with age, alternative sources have been investigated. To date, human MSCs have been isolated from amniotic fluid and placenta [12], umbilical cord [13] and vein [14], Wharton's jelly, [15] lymphoid organs such as spleen and thymus [16], adipose tissue [17], periodontal ligament [18], scalp tissue [19], menstrual blood [20], and peripheral blood [21]. MSCs from most of these other tissues are currently being explored in a range of animal models of neurological disease.

Preclinical Studies of MSCs in Animal Models

The development of new therapies for acute neurological disorders typically begins with testing in preclinical animal models. Various clinically relevant animal models exist for stroke, TBI, and SCI. Ischemic stroke models usually involve the occlusion of a specific cerebral blood vessel to mimic an embolic or thrombotic occlusion that occurs in human stroke. Traumatic brain injury models involve direct contact (direct impact) versus noncontact paradigms (acceleration/deceleration). SCI models involve the severing of the spinal cord at specific levels, balloon compression, hemisection, and stretching of the cord to produce a consistent partial or complete injury. Other models involve irradiation and ethidium bromide to create a focal demyelinated lesion. While no model perfectly simulates all the events of these illnesses, they have served as a starting point to test the safety and efficacy of novel therapies. Although many animal species could and should be employed in MSC research, rodent models, especially rats, are still the most commonly used for (1) resemblance to humans in CNS anatomy and physiology, (2) genetic homogeneity within strains, (3) low cost, and (4) greater ethical acceptability. Using standard rodent models, several laboratories have shown that MSCs improve neurological recovery after stroke [22, 23], TBI [24, 25], and SCI [26, 27]. Scientists have also studied MSCs in large animal models although there are very few reports. The canine model has been used to test MSCs in stroke [28] and SCI [29], and a recent report from China demonstrated that human MSCs improve outcome in a nonhuman primate stroke model [30]. Nearly all of the published work has reported positive results. Very few studies have been published showing that MSCs do not enhance recovery [25] in models of acute neurological disorders.

Routes of Delivery

Multiple routes of cell infusion have been investigated: intravenous (IV), intra-arterial (IA), intracerebral(IC), intracisternal (ICV), and intrathecal (IT) injection. Although most studies report positive therapeutic effects, no matter what the delivery route, the important point to consider is that every route has its limitations. Local intracerebral administration of cells provides a precise way of targeting MSCs to the injured brain, and an ICV or IT route provides an opportunity to access an even large surface of brain or spinal tissue. However, local delivery routes are invasive and carry potential complications such as seizures and bleeding at the surgical site. Whether these routes would also yield uniform biodistribution of cells throughout the brain or spinal cord is still a matter of debate [31]. Systemic administration is far more clinically feasible and least invasive. It also allows wide systemic cell distribution in which cells may selectively accumulate in the target of injury within the brain due to the release of local chemoattractants. However, it is now well established that IV cell injection results in poor engraftment at the target lesion because MSCs are trapped by filtering organs such as lungs, spleen, liver, and kidneys. IA cell delivery bypasses the peripheral filtering organs and increases MSC engraftment with uniform distribution in the ischemic brain, compared to IV delivery (21% via IA [24] vs. 0.75–18.5% via IV [32]). But IA injection is invasive and has the potential to cause microvascular occlusions and lower cerebral blood flow [33]. In one report [34], a direct comparison was made between human MSC injection via IV and IT with lumbar puncture in a rodent SCI model. At 4 days and 21 days, the engrafted MSCs were increased to 4.1 and 3.4% with lumbar puncture and intrathecal cell delivery, compared to 2.3 and 1.6% with intravenous cell delivery, respectively. However, as discussed below, an increasing number of studies have called into question whether MSCs even need to enter the brain to exert a therapeutic effect [35–39], considering the fact that only a few MSCs can migrate into the brain and survive in a short time frame but still show benefits in recovery. Engraftment of cells within the brain may therefore not be necessary for neurological recovery in such disorders as stroke or TBI. MSCs, unlike pharmacologic agents, have multiple mechanisms and do not follow the pharmacokinetics of drugs. Based on the above discussion, the optimal route of MSCs has not been determined yet, and further studies are needed to demonstrate the superiority of one approach over another.

Time Window

There have been studies investigating the possible therapeutic time window of MSCs from minutes to months after injury. Most of the studies reported promising results in the acute (less than 3 days after damage) and subacute phase (3–14 days after damage). Very fewer studies have been done in the chronic phase. Shen et al. [40] administered MSCs to female aged animals at 1 month after stroke and found improved functional outcomes. Katsuya et al. reported similar results

using a more severe injury with the same gender of MSCs [41]. But a recent report from Brazil [42] did not find benefit when MSCs were administered at 1 month after stroke. Unfortunately, there are no reports in such delayed time windows for MSCs in TBI and SCI.

It is difficult to compare the published data to identify an optimum time for MSC therapy as these studies used different animal models, delivery routes and behavioral tests, and most of these studies did not provide immunophenotypic characterization of the MSCs used. An acute delivery may be the most ideal if inflammatory signals are needed to direct MSCs to areas of injury or exert effects on the pathophysiological mechanisms at play during the acute period of injury: neuroprotection, edema formation, or immunomodulation.

Dosing

The dosing choice depends on the cell delivery route and timing. Our review of the literature seems to indicate that around 1×10^5 /kg cells are the minimum needed for cerebral local administration and 1×10^6 for systemic administration. However, increasing the number of cells does not always lead to better recovery in stroke [43, 44] and TBI [44, 45] models. Another paradigm that deserves further study is to perform multiple injections with lower dosages. As reported by Omori et al. in 2008, a single relatively high dose of MSCs proved more effective than dispersed administrations [46] of lower doses. But Kranz et al. reported a contrary result with placenta-derived MSCs that two infusions were superior to a single dosage in achieving more functional improvement [47].

Overall, MSCs have been extensively studied in animal models of acute neurological disorders, and they have the advantage of exerting multiple biological effects. However, the benefits differ between diseases and the phases of pathological conditions.

Potential Mechanism of Action of MSCs in Neurological Disorders

The mechanisms of MSCs' effects in animal models of neurological disorders are multiple and are based on cell delivery route, dosing, timing, and the type of disorder. Despite the fact that they were not designed to circulate within the bloodstream, MSCs do have the capacity to migrate to the injured region after stroke [22], TBI [48], and SCI [49]. This capacity is probably via interaction between stromal cell-derived factor (SDF)-1, expressed in neurons and endothelial cells, and its chemokine receptor-4 (CXCR-4) expressed on MSCs [40, 50]. Furthermore, some engrafted MSCs develop a phenotypic expression similar to mature neurons

and glia cells, which suggest the potential for MSCs to replace lost or damaged neural cells. Although MSCs are reported to have the capacity to differentiate into many cell linages in vitro, there are no conclusive data on trans-differentiation of MSCs to functioning neurons, either by ultrastructural or electrophysiological analysis in vivo. Moreover, neuronal differentiation rates of MSCs are low and therefore are unlikely to be substantially responsible for observed improvements in recovery in so many animal models reported to date [51, 52]. However, the pathological conditions are different in SCI. Unlike other regions in the central nervous system, the spinal cord does not possess a neurogenic niche [53, 54]. In addition, SCI is viewed as not only a predominantly "white matter problem" but also a potential regenerative therapy for SCI may need replenishment from neuronal populations within the gray matter for the purposes of intraspinal circuitry repair [55]. Nevertheless, there are conflicting results between animal models [49] and clinical trials [56] on SCI over the issue whether trans-differentiation of MSCs to neurons can occur at a sufficient level to contribute toward cell replacement. However, there are efforts investigating techniques to introduce transcription factors to MSCs to promote their differentiation into neural cells, for the purposes of SCI transplantation [57].

The question then immediately arises what are the mechanisms to account for the therapeutic effects observed in animal models. Chopp et al. have provided the most extensive data on the mechanisms of transplanted MSCs and have concluded that these cells behave as "small molecule factories" by secreting neurotrophins glial cell-derived neurotropic factor (GDNF) and brain-derived neurotropic factor (BDNF); growth factors vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), and nerve growth factor (NGF); and other supportive substances after stroke and TBI. The end result is stimulation of the brain's endogenous repair responses and facilitation of the microenviroment around local areas of injury: inhibiting apoptosis, reducing glia scar formation, and upregulating neurogenesis and angiogenesis [58, 59]. It has also been reported that MSCs express and secrete brain natriuretic peptides (BNPs) in vitro which might help reduce edema following acute brain injuries such as stroke and TBI [60].

There is also increasing evidence indicating that MSC transplantation leads to immunomodulation after CNS injury. MSCs possess immunomodulatory properties [61–63]. MSC transplantation induces the upregulation of genes that express anti-inflammatory cytokines and induces the downregulation of pro-inflammatory and immune-related genes in the injured brain, the end result of which shifts the cytokine profile from pro-inflammatory to anti-inflammatory [64]. MSCs also alter the brain's local immune cell phenotypes. MSCs reduce the number of leukocytes and modify the proliferation of microglia in the injured brain [65–67]. Furthermore, MSCs may interact with immune organs such as the spleen to generate an alteration in systemic inflammatory and immunologic responses [65]. The combination of these events may attenuate inflammation in the acute phase of brain injury. Cell-induced immunomodulation might even be the main mechanism underlying how these cells might promote recovery from SCI [68].

Tables 35.1, 35.2, and 35.3 shows current preclinical MSCs studies in animal models of acute neurological injury, which mostly involve the rat. As preclinical data continues to mount on the effects and mechanisms of MSCs in animal models of neurological disorders, clinical trials have already commenced.

Clinical Trials Testing MSCs in Acute Neurological Disorders

MSCs for Stroke

One of the first research groups to study MSCs in stroke patients began their investigations in Korea about 10 years ago. The first study, published in 2005 [99], examined the feasibility and safety of culture-expanded autologous MSCs in patients with middle cerebral artery (MCA) infarcts and neurological deficits (defined as an NIH stroke scale (NIHSS) score ≥ 7) after 7 days of symptom onset. The MSC group ($n=5$) received an intravenous infusion of 1×10^8 autologous MSCs, and the control group ($n=25$) received standard of care. In the treated patients, bone marrow was aspirated under local anesthesia from the posterior iliac crest. MSCs were isolated in culture from the aspirates and expanded for at least 30–60 days. The cells were then administered IV at 4–5 and then 7–9 weeks after symptom onset (5×10^7 cells per patient per time) were infused into a peripheral catheter for each injection. Changes in neurological deficits and improvements in function were compared between the groups for 1 year after the onset of symptoms. The Barthel index measures a person's activities of daily living and mobility, consisting of ten items and a scoring system from 0 to 100 where the higher score reflects more independence. The modified Rankin Scale (mRS) is another commonly used scale for measuring the degree of disability or dependence with a scale that runs from 0 to 6 where zero is a cure and six is death. In the groups treated with MSCs, both scoring systems improved to a greater extent compared to the control group at 3, 6, and 12 months afterward. Follow-up evaluations showed no adverse cell-related, serological, or imaging effects. Intravenous infusion of autologous MSCs appeared to be feasible and safe.

A larger study was then more recently published by the same study team [100]. They performed an open-label, observer-blinded clinical trial of 52 patients with severe middle cerebral artery territory infarcts. Patients were randomly allocated to one of two groups, those who received IV autologous *ex vivo* cultured MSCs ($N=16$) or those who did not receive cells ($n=36$), and followed for up to 5 years. Four (25%) patients in the MSC group and 21 (58.3%) in the control group died during the follow-up period, and the cumulative surviving portion at 260 weeks was 0.72 in the MSC group and 0.34 in the control group (log-rank; $p=.058$). No significant side effects were observed following MSC delivery. There was no difference in recurrent strokes or seizures between both groups. Follow-up modified Rankin Scale (mRS) score was decreased, and the number of patients with a mRS of 0–3 increased in the MSC group ($p=.046$). Interestingly, clinical improvement in the MSC group was associated with serum levels of stromal cell-derived factor-1.

Table 35.1 The preclinical studies with human MSCs in animal stroke models

Authors and year	Cell source	Species	Cell dosage	Delivery route	Delivery timing post-injury	Model type	Functional recovery
Andrews et al. (2008) [69]	hB-MSC	Rat	1.5×10^6	IC	7 days	p-MCAo	Yes
Chen et al. (2004) [70]	hB-MSC	Rat	1×10^6	IV	24 h	t-MCAo	Yes
Chung et al. (2009) [28]	hUC-MSC	Dog	1×10^6	IA	24 h	Embolic MCAo	Yes
Ding et al. (2007) [71]	hUC-MSC	Rat	1×10^6	IC	7 days	t-MCAo	Yes
Honma et al. (2006) [72]	hB-MSC hTERT-MSC	Rat	1×10^6	IV	12 h	t-MCAo	Yes
Horita et al. (2006) [73]	hB-MSC	Rat	1×10^6	IV	3 h	p-MCAo	Yes
Horita et al. (2006) [73]	hB-MSC-GDNF	Rat	1×10^6	IV	3 h	p-MCAo	Yes
Kang et al. (2003) [74]	hAT-MSC	Rat	1×10^6	IC	24 h	t-MCAo	Yes
Kim et al. (2008) [75]	hB-MSC-ngn1	Rat	1×10^6	IC	3 h	t-MCAo	Yes
Kim et al. (2008) [75]	hB-MSC	Rat	1×10^6	IC	3 h	t-MCAo	Yes
Koh et al. (2008) [76]	hUC-MSC	Rat	6×10^5	IC	2 weeks	t-MCAo	Yes
Kranz et al. (2010) [47]	hP-MSC	Rat	1×10^7	IV	8–24 h	p-MCAo	Yes
Kurozumi et al. (2003) [77]	hB-MSC	Rat	5×10^5	IC	24 h	t-MCAo	Yes
Kurozumi et al. (2003) [77]	hB-MSC-BDNF	Rat	5×10^5	IC	24 h	t-MCAo	Yes
Kurozumi et al. (2005) [78]	hB-MSC-BDNF	Rat	5×10^5	IC	24 h	t-MCAo	Yes
Kurozumi et al. (2005) [78]	hB-MSC-CNTF	Rat	5×10^5	IC	24 h	t-MCAo	No
Kurozumi et al. (2005) [78]	hB-MSC-GDNF	Rat	5×10^5	IC	24 h	t-MCAo	Yes
Kurozumi et al. (2005) [78]	hB-MSC-NT3	Rat	5×10^5	IC	24 h	t-MCAo	No
Kurozumi et al. (2005) [78]	hB-MSC	Rat	5×10^5	IC	24 h	t-MCAo	Yes
Lee and Yoon (2008) [79]	hAT-MSC	Rat	1×10^5	IC	24 h	t-MCAo	Yes
Li et al. (2010) [30]	hB-MSC	Monkey	$1, 5\times10^6$	IC	1 week	Focal MCAo	Yes
Li et al. (2008) [80]	hB-MSC	Rat	1×10^6	IV	24 h	t-MCAo	Yes

Li et al. (2002) [81]	hB-MSC	Rat	3×10^6	IV	24 h	t-MCAo	Yes
Liao et al. (2009) [82]	hUC-MSC	Rat	2×10^5	IC	24 h	IAH	Yes
Liao et al. (2009) [83]	hUC-MSC	Rat	2×10^5	IC	24 h	p-MCAo	Yes
Liu et al. (2006) [32]	hB-MSC-PIGF	Rat	1×10^7	IV	3 h	p-MCAo	Yes
Liu et al. (2006) [32]	hB-MSC	Rat	1×10^7	IV	3 h	p-MCAo	Yes
Liu et al. (2009) [84]	hE-MSC	Rat	2×10^7	IV	24 h	t-MCAo	Yes
Nomura et al. (2005) [85]	hB-MSC	Rat	1×10^7	IV	6 h	p-MCAo	Yes
Nomura et al. (2005) [85]	hB-MSC-BDNF	Rat	1×10^7	IV	6 h	p-MCAo	Yes
Omori et al. (2008) [46]	hB-MSC	Rat	$1–3\times10^6$	IV	6 h–1 week	p-MCAo	Yes
Onda et al. (2008) [86]	hB-MSC	Rat	1×10^7	IV	6 h	p-MCAo	Yes
Onda et al. [86] (2008)	hB-MSC-Ang	Rat	1×10^7	IV	6 h	p-MCAo	Yes
Skvortsova et al. (2008) [87]	hB-MSC	Rat	6×10^6	IV	24 h	p-MCAo	Yes
Toyama et al. (2009) [88]	hB-MSC	Rat	1×10^7	IV	6 h	p-MCAo	Yes
Toyama et al. (2009) [88]	hB-MSC-VEGF	Rat	1×10^7	IV	6 h	p-MCAo	Yes
Toyama et al. (2009) [88]	hB-MSC-Ang-VEGF	Rat	1×10^7	IV	6 h	p-MCAo	Yes
Toyama et al. (2009) [88]	hB-MSC-Ang	Rat	1×10^7	IV	6 h	p-MCAo	Yes
Xiao et al. (2005) [89]	hUC-MSC	Rat	$75{,}000–1\times10^6$	IV	48 h	t-MCAo	Yes
Yoo et al. (2008) [90]	hB-MSC	Rat	5×10^5	IC	3 days	t-MCAo	Yes
Zhang et al. (2004) [91]	hB-MSC	Rat	3×10^6	IV	24 h	p-MCAo	Yes

Table 35.2 The preclinical studies with human MSCs on animal TBI models

Authors and year	Cells source	Species	Cell dosage	Delivery route	Delivery timing post-injury (h)	Type of model	Functional recovery
Chopp et al. (2003) [45]	hB-MSC	Rat	$1–2 \times 10^6$	IV	24	CCI	Yes
Chopp et al. (2005) [92]	hB-MSC	Rat	$2, 4, \text{or } 8 \times 10^6$	IV	24	CCI	Yes

Table 35.3 The preclinical studies with human MSCs on animal SCI models

Authors and year	Cells source	Species	Cell dosage	Delivery route	Delivery timing post-injury	Type of model	Functional recovery
Courtney et al. (2009) [93]	hB-MSC	Rat	1×10^6	LP or IV, or local	Immediately	Cervical	Yes
Lee et al. (2009) [29]	hUC-MSC	Dog	1×10^6	Local	7 days	Lumbar	Yes
Fang et al. (2010) [94]	hB-MSC	Rat	2×10^5	Local	7 days	Thoracic	Yes
Hu et al. (2010) [95]	hUC-MSC	Rat	1×10^5	Local	24 h	Thoracic	Yes
Samdani et al. (2009) [96]	hB-MSC	Rat	1.5×10^5	Local	Immediately	Cervical	Yes
Himes et al. (2006) [97]	hB-MSC	Rat	5×10^5	Local	7 days	Thoracic	Yes
Cizkova et al. (2006) [98]	hB-MSC	Rat	1×10^6	IV	7 days	Thoracic	Yes

Based on these studies, the data thus far suggest that intravenous autologous MSCs are safe in stroke patients. However, these studies also point out that autologous MSCs using current culture techniques are not likely to be a practical option for patients with acute and subacute stroke. Whether MSCs can still improve outcome when given in the chronic setting of stroke remains an open question.

Abdullah et al. from Malaysia has a registered trial entitled "*Ex Vivo* Cultured Adult Allogenic MSCs in Ischemic Cerebral Stroke." This phase I/II trial is evaluating the safety and efficacy of intravenous *ex vivo* cultured adult allogenic MSCs in 78 patients with ischemic stroke. Patients are given an intravenous dose of allogenic MSCs at a dosage of two million cells/kg versus placebo within 10 days of stroke onset. They will then be followed for 1 year. Safety will be assessed by adverse events monitoring. Efficacy will be evaluated by both imaging (MRI) and clinical parameters using traditional endpoint analysis.

Detante et al. from France are currently enrolling patients with anterior circulation ischemic strokes in another study entitled "Intravenous Stem Cells After Ischemic Stroke (ISIS)." This study consists of three arms, enrolling 30 stroke patients within 6 weeks of symptom onset: the first group is receiving standard of care, the second group is randomized to IV injection of autologous MSCs, and the third group receives two IV injections of autologous MSCs.

Although the first trials of autologous MSCs in stroke patients show promising results supporting safety, the optimal approach regarding dosing, mode of delivery, and therapeutic window has yet to be determined. Allogeneic MSCs are just beginning to undergo testing in stroke patients, and we anticipate many more clinical trials emerging around the world.

MSCs for Spinal Cord Injury

Ra et al. investigated the safety of "autologous adipose-derived MSC transplantation in patients with spinal cord injury." Eight male patients who had suffered a spinal cord injury in the prior 12 months were intravenously administered a single injection of autologous adipose-derived MSCs (4×10^8) and had no serious adverse events during a 3-month follow-up [101].

Two other clinical trials had been identified in www.clinicaltrials.gov. Sabaawy et al. attempted to assess in 80 patients the safety of "autologous bone marrow-derived cell transplant in chronic spinal cord injury." Lasala et al. are currently enrolling patients into a study entitled "Transfer of Bone Marrow Derived Stem Cells for the Treatment of Spinal Cord Injury," which is a phase I, single-center trial to assess the safety and tolerability of an intrathecal infusion (lumbar puncture) of autologous, *ex vivo*-expanded bone marrow-derived mesenchymal stem cells in a well-defined population of spinal cord injury patients. However, no further published data can be found.

Kishk et al. [102] reported a case control sample of 64 patients, at a mean of 3.6 years after SCI. Forty-four subjects received monthly intrathecal autologous

MSCs for 6 months, and 20 subjects, who would not agree to the procedures, served as controls. All subjects received rehabilitation therapies three times weekly. After 1 year, no significant between-group improvements were found in clinical measures. Adverse effects included spasticity and 24 developed neuropathic pain. One subject with a history of post-infectious myelitis developed encephalomyelitis after her third injection. The authors suggested that autologous MSCs might have side effects and might be contraindicated in patients with a history of myelitis. The application of MSCs for chronic traumatic SCI needs further study in preclinical models.

MSCs for Traumatic Brain Injury

There are currently no registered trials of MSCs in traumatic brain injury which likely results from the fact that far less animal work has been completed in TBI. We believe it remains only a matter of time before the first studies emerge to test MSCs in this patient population given the animal data acquired thus far and the ongoing work in this field.

Summary of Clinical Trials

The above discussion illustrates that the testing of MSCs in acute neurological disorders is only in its infancy. Most studies to date indicate that the MSCs which have been administered to patients in these trials are safe. Several studies, however, began despite a more complete understanding of basic translational questions regarding timing, patient population, and delivery route.

Future Directions

As clinical trials have already commenced on MSCs for acute neurological disorders, there remain many unresolved issues.

1. Lack of Standard Immunophenotype Definition of MSCs

   There is not a single cell marker to clearly define MSCs. Different investigators use cells from different sources and employ different extraction and culture methods. Whether these differences cause differences in the generation of certain populations of MSCs or cause differences in the phenotypes of MSCs is not clear [103]. Even though MSCs share common features regardless of their source, there are still some differences in their immunophenotype and in vitro differentiation [104–107]. These issues may make it difficult to compare clinical trials testing MSCs in the same patient population, especially if the MSCs are derived from different sources of tissue. Furthermore, few published studies involving animal modeling of acute neurological disorders

include immunophenotyping which raises some concern that not all MSCs are the same in each published study.

2. Cell Expansion

   Ex vivo culture expansion is critical for generating sufficient cell amounts for clinical needs. Following good manufacture practice (GMP), all processes must be defined from cell isolation to cell culture. The cell sizes and other features of cells will change with culture passages. After 3 weeks or 12–15 population doubling, human MSCs will decrease their proliferation rate and progressively lose their multipotency [108]. Li et al. reported more pronounced results with earlier-passaged (passage two) cells than later-passaged (passage six) human MSCs in a rat stroke model [80]. However, there are more hematopoietic precursors in the initial 2–3 passage, which affects the purity of MSCs [109]. Long-term cultured MSCs show progressive arrest and enter senescence [110]. But, more importantly, using MSCs from different passages may also prevent adequate comparisons between clinical trials and between animal studies.

   Another issue is that there is no serum-free medium for clinical use of MSC cultures. The enriched fetal bovine serum (FBS) medium brings the theoretical risks of prion or zoonotic infection and graft-host immune reaction. The use of human serum with fibroblast growth factor 2 [111] or use of human platelet lysate [112] instead of FBS supplemented medium might increase the safety by excluding xenogeneic proteins.

3. Standard Translational Questions

   The optimal route of delivery, dosing, and time window still need to be better defined for a number of these neurological disorders. Noninvasive imagining techniques will help us to better answer these issues and provide a deeper understanding of the interaction between MSCs and brain and between MSCs other organs as well.

4. Clinical Variables

   To date, MSCs have been shown to be safe and effective in animal models of ischemic stroke, TBI, and SCI, but clinical trials testing MSCs for these conditions have only just begun. From the clinical perspective, many variables, including types of MSCs, cell dosing, timing of treatment, route of cell delivery, and characteristics of the patients, will likely influence the safety and efficacy of MSCs.

Conclusions

Developing effective therapies for acute neurological disorders remains a major challenge for biomedical research. Despite intensive efforts, the ability to promote functional recovery after brain and spinal cord injury remains frighteningly very limited, while the need for such therapies is increasing with an aging population. It is hoped that MSCs and other types of cell-based therapies will prove to be safe and beneficial for this patient population.

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Chapter 36
MSCs in Reconstructive Surgery

Summer E. Hanson and Michael L. Bentz

Abstract Soft tissue defects remain a major challenge in modern medicine and represent a significant burden, affecting not only physical and mental health but also productivity, healthcare expenditure, and long-term morbidity. Even under optimal conditions, the healing process leads to some element of fibrosis or scarring. Cell-based treatments involve the transplantation of progenitor/stem cells to patients through local or systemic delivery and offer a novel approach to many diseases. Mesenchymal stromal cells (MSCs) are multipotent adult progenitor cells of great interest as cellular therapeutics because numerous studies have shown that MSCs, derived from bone marrow and other tissues, possess the ability to suppress activation and proliferation of immune cells, differentiate along multiple tissue lineages, and participate in the tissue healing process through a variety of other paracrine mechanisms. Better understanding of these potential interactions could translate to the development of clinically relevant, novel cell-based therapies for soft tissue reconstruction. The clinical role of tissue-derived MSCs, though not well understood, holds promise for many applications in novel cell therapies and regenerative medicine.

Introduction

Normal healing is a complex, coordinated sequence of events proceeding from hemostasis through inflammation to organized tissue regeneration [1]. Following tissue injury, platelet aggregation initiates the clotting cascade, followed by infiltration of the wound bed with pro-inflammatory cytokine-producing leukocytes, including neutrophils and macrophages (Fig. 36.1). In later stages of healing,

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_36,

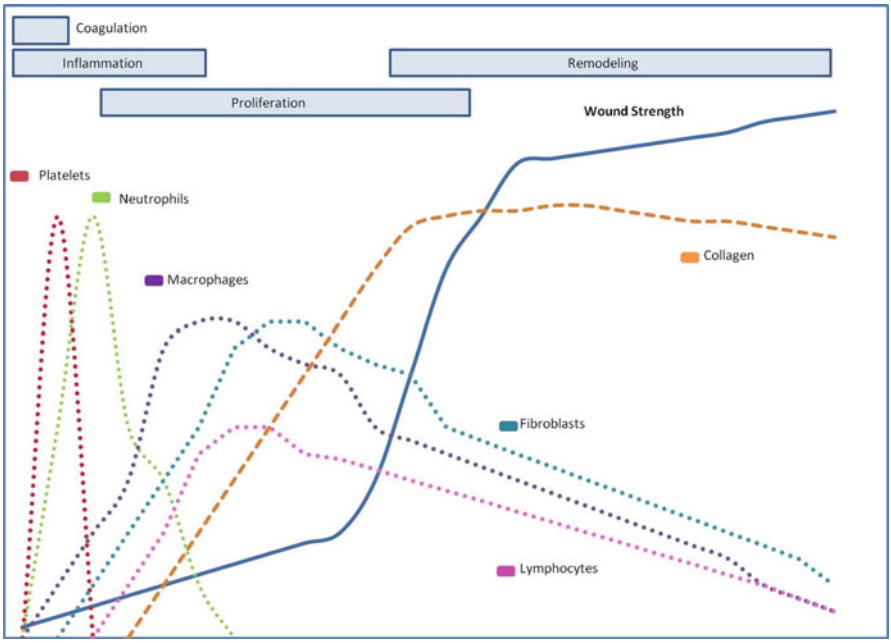

Fig. 36.1 Wound healing. Normal healing consists of multiple phases including coagulation, inflammation, proliferation, and remodeling. Early in the process, platelets and circulating neutrophils are first to arrive at the area of injury to initiate healing. Macrophages are recruited to the site of tissue injury and are predominantly responsible for the inflammatory cascade. During this same time, resident fibroblasts proliferate in the wound bed and begin to produce new extracellular matrix proteins such as collagen and hyaluronic acid. As the healing process transitions from proliferation to remodeling, collagen reorganizes, inflammation begins to decline, and the wound gains strength. The process is a complex, coordinated sequence of physiologic events and, even under optimal circumstances, leads to some element of fibrosis and scarring (Modified with permission from Witte and Barbul [55])

fibroblasts are recruited to the wound, depositing extracellular matrix (ECM) proteins such as collagen, fibronectin, and hyaluronic acid, leading to new tissue regeneration [2]. Even under the best circumstances, the healing process typically results in fibrosis or scarring. There are a number of factors that contribute to a loss of tissue integrity, whether from trauma, surgery, or underlying pathology such as a congenital anomaly, vascular insufficiency, hypertension, or metabolic derangement. Therefore, there are many aspects of the healing process that may affect decision-making and clinical outcome.

Millions of reconstructive procedures are performed each year to address a variety of defects [3]. The reconstructive ladder is a treatment algorithm historically used by clinicians as an organized approach to a wound and offers increasingly complex solutions based on the tissues lost (Fig. 36.2). Within this armamentarium are techniques ranging from primary closure and secondary intention or granulation to complex, composite tissue transplantation moving skin, muscle, soft tissue, and

Fig. 36.2 The reconstructive ladder. The reconstructive ladder is a treatment algorithm historically used by clinicians as an organized approach to a wound and offers increasingly complex solutions based on the tissues lost

bone from one part of the body to another based on microsurgical anastamosis and revascularization of the blood supply of the transferred tissues. Adjunctive treatment modalities in clinical practice include debridement, pressure offloading, dressing regimens, hyperbaric oxygen, antibiotics, and topical growth factors. However, even with most current therapies, greater than 50% of chronic wounds remain refractory to treatment [4]. New treatment strategies in wound healing and reconstruction, such as bioengineered scaffolds and cellular applications, aim to replace senescent resident cells and reestablish the normal cycle [5, 6]. In recent years, numerous biomaterials and several cell-based products have emerged on the market with Food and Drug Administration (FDA) approval, including those containing living cells. Tissue-engineered dressings such as Dermagraft ® and Apligraf ® are currently available with FDA approval for treatment of diabetic foot ulcers and venous leg ulcers, respectively, although both have shown only limited clinical success [7]. During the last decade, adult tissue-derived mesenchymal stromal cells (MSCs) have rapidly moved from in vitro and animal studies into human trials as a therapeutic modality for a diverse group of clinical applications [8].

Mesenchymal Stromal Cells

It is now evident that MSCs reside within most adult connective tissues and organs [9]. Of particular interest to plastic surgeons is the isolation of cells with characteristics similar to bone marrow (BM)-derived MSCs from the stromal vascular fraction (SVF) of adipose tissue (AT) [10]. It is well established that there are a variety of cytokines and growth factors found in adipose tissue which regulate endocrine and metabolic homeostasis, as well as precursor differentiation throughout one's lifetime [11]. Plastic surgeons have been using fat as a soft tissue filler for over a century, and during this time, techniques for harvest and administration have been modified to address the viability of cells within the lipoaspirate and SVF [12–14]. Although the primary cell type found in the SVF is rich in MSCs, there are populations of vascular endothelial cells, pericytes, and monocytes identified as well [15, 16].

Studies suggest that MSCs isolated from these diverse tissues possess similar biological characteristics, differentiation potential, and immunological properties [17–19]. Enthusiasm about MSCs for use in reconstruction and regenerative medicine has been fueled by evidence that these cells possess the ability to participate in the tissue repair process through a variety of paracrine mechanisms affecting tissue regeneration and inflammation [20–25].

Mesenchymal stromal cells possess a variety of functional characteristics which make them a desirable cell type for reconstructive applications as illustrated in Fig. 36.3. Their ability to migrate to the site of injury or inflammation, participate in regeneration of damaged tissues, stimulate proliferation and differentiation of resident progenitor cells, promote recovery of injured cells through growth factor secretion and matrix remodeling, and exert unique immunomodulatory

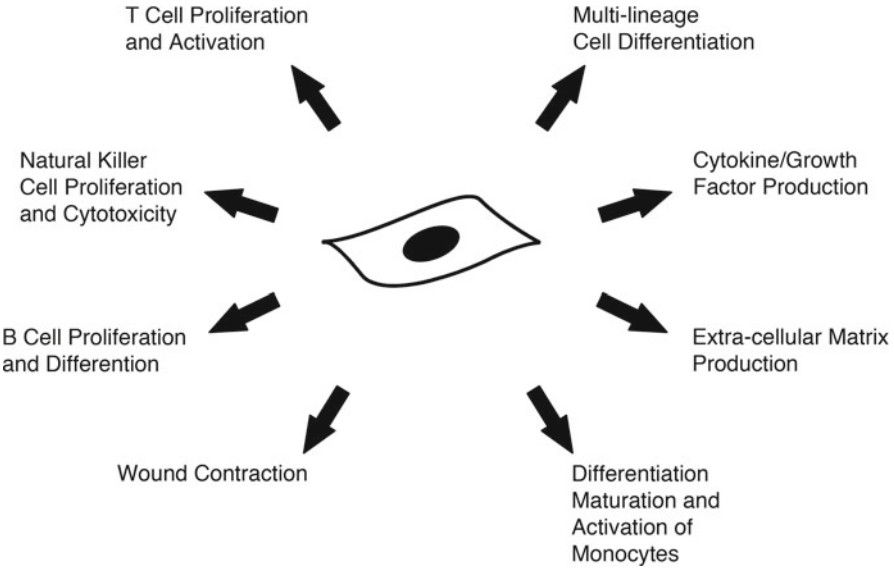

Fig. 36.3 Functional mechanisms of mesenchymal stromal cells in tissue regeneration. There are several functional mechanisms identified with culture-expanded mesenchymal stromal cells that are favorable when developing cell-based therapies for reconstructive surgery and regenerative medicine. These affect both the inflammatory and reparative pathways of the healing process (Illustration by C. Hanson)

and anti-inflammatory effects make them a potential key component in wound healing [20–25]. In fact, one of the most intriguing properties of *ex vivo*-expanded MSCs is their ability to affect the immune response through interaction with a broad range of immune cells including T lymphocytes, B lymphocytes, natural killer and dendritic cells, and macrophages [26–29]. Thus, in contrast to most pharmacological agents targeting single pathophysiological pathways, MSCs are advantageous as they could affect tissue healing and regeneration through many different routes.

Although the majority of the published literature concerns BM-derived MSCs, adipose tissue-derived MSCs (AT-MSCs) could be considered a desirable cell population due to their availability, ease of harvest, and ability to be expanded in culture for clinical use like their marrow counterparts [30]. However, to date, BM-MSCs have remained as the most commonly used preparation of MSCs for clinical applications. There have been several reports of MSCs administered in the clinical setting, most commonly systemic infusions for inflammatory conditions such as graft versus host disease or myocardial ischemia. Applications in reconstructive surgery are gaining momentum; there are more than ten clinical trials investigating MSCs in some form of reconstruction (predominantly wound healing) registered in the USA at this time (http://clinicaltrials.gov/). The current published case reports or series illustrate the potential clinical use of MSCs in reconstruction/augmentation, wound healing, tissue engineering, and composite tissue reconstruction [31–35].

Soft Tissue Augmentation and Scar

Fat grafting for cosmetic soft tissue augmentation has received much attention over the past few decades. Due to the renewed interest in fat grafting, the American Society of Plastic Surgeons commissioned a task force to evaluate the limited literature regarding such procedures and potentially develop evidence-based practice recommendations [36]. However, there is a paucity of clinical studies indicating the long-term safety and efficacy of autologous fat grafts. While the fate of the grafted fat has not been fully elucidated, there has been no concrete evidence to suggest it is less safe than biomaterial alternatives [12]. Nevertheless, much attention has been focused on enhancing the results of autologous fat grafting through the potential utilization of adipose-derived stem cells to improve graft survival.

Since the amount of fat transferred as a graft has a variable survival rate, only about 25–60% of the volume of the transferred fat remains after a few months [12]. In an effort to improve "graft take" and therefore predictablility and efficacy of autologous fat grafting, Matsumoto and colleagues developed a novel method of concurrent transfer of lipoaspirated fat with adipose-derived progenitor cells termed cell-assisted lipotransfer (CAL) [15]. In this technique, a portion of the lipoaspirated fat is processed to isolate the heterogeneous mixture of cells of the SVF; the remaining lipoaspirate is processed for fat grafting, serving as a biological scaffold for the cells. The foundation of this technology is that the additional cells will improve graft survival and reduce postoperative atrophy or resorption through enhanced angiogenesis and cell self-renewal.

Yoshiumura et al. described the outcomes of this technique for cosmetic breast enhancement in 40 healthy patients [37] using cell-assisted lipotransfer. The mean volume of injected fat was around 270 ml per breast. The authors note some resorption of the adipose tissue within the first 2 months; however, the final breast volume was augmented by 100–200 ml. Unfortunately, the authors did not offer any control patients who had fat grafts placed without using cell-assisted transfer, so it is impossible to suggest that this technique offers a significant improvement in outcome. Microcalcification was detected postoperatively via mammography at 24 months time in two patients, cyst formation was detected by magnetic resonance imaging in two patients (<12 mm), and fibrous breast tissue was observed by computed tomography at 6 months in two patients with physical findings of firm breast tissue. A similar case series by this group describes successful use of progenitor-rich fat transfer in 15 patients following breast implant removal secondary to capsular contracture or other postsurgical complications. While long-term results have yet to be established, these studies illustrate the safety and utility of combining adipose-derived cells with autologous fat grafts for cosmetic breast augmentation. Tissue processing was completed in 90 min during the operative procedure, though there was no indication as to how much time this added to the total surgical case.

Cell-assisted lipotransfer has also been used for other indications for fat grafting such as facial contouring [31]. In a small study, groups of patients with facial lipoatrophy from lupus erythematosus profundus or Parry–Romberg syndrome

(idiopathic hemifacial lipoatrophy) were treated with fat injections, with or without additional cells or CAL ($n = 3$ per group). The average volume of lipoinjection was 100 ml with cell processing taking 90 min. The CAL-treatment group had a better clinical improvement score; however, this was not statistically significant given the small study size. One patient in the non-CAL group was treated for fat necrosis. Here again, the authors established safety of the technique in soft tissue augmentation, though larger and structured clinical trials are necessary to make further conclusions.

Local Injection of Cells for Skin Rejuvenation

Much of the interest in adipose-derived stem cells in plastic surgery focuses on wound healing and replacement of tissue defects. However, the paracrine effects of these cells, including stimulation of ECM deposition and resident cell recruitment have also recently been applied to skin rejuvenation. Conventional treatments for aging skin, such as topical retinoids or laser therapy, seek to induce fibroblast activation and collagen synthesis in a manner similar to in vitro and preclinical findings of adipose-derived stem cells. A high concentration of autologous purified lipoaspirate cells in saline was injected directly into the photoaged periorbital dermis of one patient to test this theory in a clinical case report [38]. The patient underwent two injections with a 2 week interval between treatments. The report shows follow-up at 2 months after the last injection. While there is subjective improvement in the periorbital skin seen in photographs shown before and after treatments, the authors noted an increase in dermal thickness measured by ultrasonography (2.054 mm pretreatment versus 2.3217 mm post treatment). This finding, coupled with preclinical work showing culture-expanded MSCs, increases Type I collagen and a variety of growth factors in normal skin, offers promise for adipose MSCs in skin rejuvenation, and sets the foundation for clinical studies being conducted by the authors.

Wound Healing

The few clinical series focused on the application of *ex vivo* culture-expanded MSCs in human wounds have shown promising potential. When evaluating the literature, particular attention should be paid to nomenclature and methodology. Reports on the use of MSCs range from BM- or lipoaspirate without manipulation to culture-expanded MSCs (with or without skin graft or other dressing material). One of the early proof-of-principle reports of novel cell therapies of this nature in cutaneous wounds included the direct application of autologous bone marrow aspirate to wounds present for more than a year that were recalcitrant to standard therapeutics [39]. Remaining aspirate was cultured with or without hydrocortisone in the media to maximize the progenitor cells in culture. At the time of subsequent

administration, cells from the steroid containing media were mixed with cells from the steroid-free media. Ultimately, the authors report healing of all of the wounds ($n=3$) within three consecutive treatments. Two wounds closed with cell treatment alone, while one was closed with a bioengineered skin substitute (Apligraft ®). However, it is not clear if the cells cultured by these investigators were MSCs or other types of cells present in the BM, as there was no information on the characterization of the cells cultured.

A unique delivery system using fibrin glue was investigated in both acute ($n=4$) and chronic ($n=6$) wounds by Falanga and colleagues [40]. Autologous BM-MSCs were expanded in culture to passages 2–10 and characterized as MSCs by flow cytometry. These cells were then combined with fibrin spray for topical application. The acute wounds studied were surgical defects following excision of nonmelanoma skin cancers. The authors assessed that these wounds were likely to heal secondarily, but not ideal for primary closure. In this group, all wounds were healed within 8 weeks time, suggesting MSCs contributed to accelerate resurfacing. To study MSCs in the setting of chronic wounds, the authors chose lower extremity wounds present for greater than 1 year and refractory to standard treatments including topical growth factors and bioengineered skin substitutes. Wounds were significantly decreased or healed completely by 16–20 weeks. Autologous culture-expanded MSCs were used with a fibrin glue system for up to three applications. Given the variation in the baseline size of the wound bed, the study found a strong correlation between the number of MSCs applied per cm^2 surface area and the reduction in ulcer size. Biopsies of both the acute and chronic wounds treated with topical MSCs and fibrin glue show higher concentration of CD29+ cells, one of the surface markers found on MSCs. These results indicate fibrin glue potentially provides a delivery system to maintain MSCs in the acute wound bed, but allows for migration out of the fibrin matrix as healing progresses. Again, MSCs were used in a nonrandomized, multimodality treatment regimen.

Yoshikawa and colleagues [32] reported 20 patients with various non-healing wounds (i.e., burns, lower extremitiy ulcers, and decubiti) treated with autologous BM-MSCs expanded in culture and a dermal replacement (Pelnac), with or without autologous skin graft. The authors report that 18 of the 20 wounds appeared healed completely with the cell-composite graft transfer and the addition of MSCs facilitated regeneration of the native tissue by histologic examination. Moreover, the process of cell harvest, culture expansion, and application with a dermal replacement or skin graft can be repeated and performed under local anesthesia if indicated. However, these authors only used a relatively low number of cells that were available at the end of passage 0 and did not report on the characterization of cultured cells. This is especially important since passage 0, when the culture flasks are confluent after initial plating, potentially contains many other types of cells, including macrophages, which would affect wound healing as well.

The largest series so far included 24 patients with non-healing lower extremity ulcers (18 from Buerger disease while 6 were diabetic foot ulcers) randomized to receive standard wound dressings with or without autologous BM-MSCs [41]. For the MSC-treated groups, cells were culture-expanded for several days and injected

both directly into the ulcer edges as well as intramuscularly throughout the lower extremity. The authors found a significant difference in overall ulcer size, total reduction in ulcer size, and pain-free walking distance between the two groups, with more favorable outcomes seen in the MSC-treated patients. Furthermore, there was no difference in biochemical parameters studied including liver function tests, fasting blood glucose, or renal function in patients treated locally with MSCs compared to control subjects.

While the majority of wounds treated clinically with cell-based therapies have been chronic in nature, there are reports of severe radiation burn injury successfully treated with a combination of serial debridements, split-thickness skin graft, and MSC injection [42]. The cells were cultured from autologous BM aspirate and injected directly into the wound following a two-step expansion process. The cells administered were positive for surface markers characteristic of MSCs and pluritpotency confirmed with differentiation assays. Complete healing was observed within 6 months with no functional impairments noted. Although encouraging, the single case report nature of this study and combined use of other modalities to treat this case should be considered.

An additional case of severe radiation burn was salvaged with reconstructive surgery and autologous BM-MSCs in a series of applications [43]. The case report was of a male accidentally exposed to a radioactive source producing, in addition to acute radiation syndrome, a desquamating wound along the entire posterior surface of his left arm, from shoulder to elbow with limited range of motion. Once recovered from the acute insult, the patient was first treated with debridement, allogeneic dermal replacement (Integra ®) and an autologous skin graft (which ultimately failed). Subsequently, a regimen of serial debridements, complex reconstructions including latissimus dorsi and radial forearm rotational flaps, and several local injections of autologous BM-MSCs was carried out (five injections total, each >100 $\times 10^6$ cells). The authors report limitation in motion at the shoulder and elbow, but good soft tissue coverage and resolution of pain in an injury that would more commonly have lead to amputation at the shoulder.

Currently, the use of autologous adipose-derived MSCs published in wound healing is limited to complex perianal fistulas. The phase I clinical trial by Garcia-Olmo et al. was the first to show safety and feasibility of culture-expanded MSCs derived from lipoaspirate [44]. In a follow-up multicenter phase II trial by the same investigators, a group of patients with similar fistulas (related to Crohn's disease and other pathology) were treated with fibrin glue, with or without AT-MSCs, in a prospective, randomized, and controlled fashion [45]. The treatment group achieved healing in 71% of patients with a recurrence rate of 17.6%. While the underlying pathology associated with fistula-in-ano is different from that of chronic cutaneous wounds, these studies establish the foundation for similar safety and feasibility studies using MSCs derived from bone marrow, or adipose tissue, for non-healing wounds.

Purified lipoaspirate has also been used to treat wounds in 20 patients resulting from radiation therapy to the chest wall or supraclavicular region [46]. In this study, lipoaspirate was centrifuged, the oil/liquid layer discarded, and the remaining cell-augmented adipose tissue was injected into the wounded tissue; patients received

from one to six injections, based on the severity of their wound. Outcomes measured included clinical healing, symptom improvement, and recurrence. In only one case was there no sign of improvement.

Taken together, this literature shows that the addition of MSCs to non-healing wounds is associated with dermal rebuilding (in addition to remodeling), an increase in wound vascularity, and reduced fibrosis or scarring. Tissue hypoxia, inflammation, repetitive ischemia-reperfusion injury, and aging or cellular senescence are factors leading to dysfunctional wound healing [4, 47] and are all potential pathways for MSCs to exert effect. While these reports demonstrate the heterogeneity of the type of wounds treated with MSCs, they also illustrate the variations in culture and application techniques that limit the current body of evidence in support of MSC therapy.

Tissue Engineering and Composite Reconstruction

Too often, reconstructive surgeons are faced with the additional challenge of complex tissue composition and unique functional requirements often associated with large defects. Tissue engineering strategies have been explored more recently in select clinical scenarios. Previous reports have illustrated successful tissue-engineered human organs including bladder [48] and skin [49]. The first report of a clinically successful complex tissue construct was a tissue-engineered patch made from autologous cells and a collagen matrix [50]. The patient had recurrent non-small cell lung cancer, initially treated with lung resection and radiation and later completion carinal pneumonectomy. However, he returned with breakdown of his tracheal anastamosis. Autologous dermal fibroblasts were isolated and expanded to three passages. These cells were then seeded on a collagen matrix derived from decellularized porcine jejunem and cultured in a bioreactor for an additional 3 weeks while functional tissue was formed. The engineered segment was implanted over the dehisced tracheal segment and the pleural space was filled with omentum and subscapular flaps. According to the authors, postoperative endoscopy shows an airtight graft and respiratory endothelium on the surface. Moreover, the patient's vocal quality returned, and he was able to perform physical activity at 24 weeks postoperatively.

More recently, Macchiarini and colleagues published their results with the transplantation of a tissue-engineered tracheal segment, the first of its kind [35] in a young woman with bronchomalacia and expiratory collapse refractory to standard treatment and reconstruction. Autologous bronchial epithelial cells were isolated from biopsies and expanded in supplemented media, while autologous chondrocytes were derived from bone marrow MSCs treated with chondrogenic media. The scaffold was a decellularized allograft from a cadaveric trachea. It was seeded with epithelial cells on the inner surface, chondrocytes on the outer surface, and then maintained in a bioreactor to introduce biomechanical cues that would need to be tolerated by the engineered construct. This *ex vivo* generated graft was then implanted end-to-end to

normal tracheal tissue to replace the stenotic segment. According to the authors, the lung immediately ventilated well. Subsequent evaluations showed no indication of inflammation related to the donor trachea, normal pulmonary function tests, and an immeasurable improvement in quality of life.

Engineered bone constructs are being widely investigated in the orthopedic literature [20, 51] and gaining support in select maxillofacial applications, possibly as an alternative to autologous bone grafting. The use of dental implants in edentulous patients is limited by the volume of viable bone in the posterior maxillary floor, at which site such devices are implanted. A procedure commonly associated with dental implantation is maxillary sinus floor elevation, in which autologous bone graft or synthetic bone mineral is used to augment the maxillary ridge for future implantation. One study compared 12 consecutive patients undergoing sinus floor augmentation randomized in a "split-mouth" fashion, with each patient serving as their own control [52]. Both sides of the patients' mouth were augmented with bovine bone mineral or hydroxyapatite (BioOss ®) with one side receiving standard therapy of the BioOss combined with autologous bone graft, while the other received BioOss combined with autologous BM MSCs (P0). Biopsies were subsequently taken approximately 3 months later at the time that dental implants were placed. The authors demonstrate significantly more new bone formation in the area treated with MSCs compared to standard bone graft. There were no differences in overall healing, complication rate, or ability to place dental implants between either groups.

Shayesteh and colleagues studied six consecutive patients undergoing maxillary sinus augmentation in preparation for dental implantation that were treated with hydroxyapatite–β-tricalcium phosphate ceramic loaded with culture-expanded autologous BM-MSCs (P2-3) [53]. The authors cite a 93% success rate of dental device implantation and a mean bone regenerate of 41.4% seen on panorex. A similar technique was used to provide graft material for alveolar clefts in a report of two patients with unilateral complete cleft palates. Both underwent primary repairs in infancy and presented for autogenous bone graft repair of the remaining alveolar clefts defects between ages 10 and 14. The defects were repaired using constructs composed of demineralized bone matrix (Osteoset ®) and autologous BM-MSCs (P1). There were no acute complications in the two patients reported. At 4 months follow-up, there were no fistulas or other adverse outcomes. Overall, the remaining postoperative defects were 25.6 and 34.5%, respectively, on computed tomography (CT). While this may not be an optimal outcome, it is important to note that there was no histologic assessment of the regenerated tissue, and perhaps, the new bone had not yet consolidated enough to provide a dense signal on CT.

Meijer and colleagues investigated tissue-engineered bone constructs for regeneration of bone in maxillary or mandibular defects [54]. Six patients were included in the study. Bone marrow-derived MSCs were harvested via bone marrow aspirate from the iliac crest, expanded to P3, seeded onto hydroxyapatite particles, and cultured for an additional 7 days in osteogenic culture media. The bone substitutes were placed directly into the defect site and covered with a local mucoperiosteal flap. Four months post-implantation, the patients had dental implants placed and biopsies taken. There was bone formation observed in three

of the six patients. The authors make note that in only one patient was there bone formed in the scaffold distant from the native bone defect edge, implying *de novo* bone growth; the others only showed bone formation in the scaffold at the site of the native bone edge, likely due to the osteoinductive effect of hydroxyapatite. The other three patients failed to show new bone formation. At the time of the original surgery, constructs from each of the patients were implanted in a subcutaneous pocket on the backs of athymic mice. At 6 weeks, all of the constructs exhibited new bone formation on histological analysis, although this was not observed in each of the clinical correlates. This study illustrates the unpredictable nature of translational research when developing complex tissue-engineered constructs.

Conclusions

The wealth of clinical data regarding the safety of MSCs warrants considering the use of these cells in a wider range of applications. Indeed, most of the current literature regarding the therapeutic use of MSCs in reconstructive surgery is based on small, nonrandomized clinical trials or case reports. One major confounding factor in these trials is the lack of a standardized procedure for isolation and characterization of the cells used. Some studies have used bone marrow or fat aspirates, with the assumption that these tissues are rich in MSCs, while others have used highly enriched populations of MSCs through culture expansion. While the outcomes were varied among the clinical applications, these reports are promising, offering novel solutions for challenging clinical disorders, ushering in a new era in regenerative medicine.

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Chapter 37
MSCs in Orthopedic Surgery

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Abstract Orthopedic injuries and pathology, both acute and chronic, result in debilitating problems afflicting millions of patients each year. Mainstays of treatment include physical therapy, inflammation and pain control, as well as surgical debridement, fixation, or replacement, depending on the disease process. Given the complex functional requirements of these musculoskeletal tissues, there is increasing interest in developing cell-based therapies for tissue regeneration rather than just replacement with scar, fibrosis, or alloplastic device. The case reports and series reviewed here offer unique and potentially superior approaches to ubiquitous and debilitating orthopedic diseases. Though in its infancy for regenerative applications such as those discussed here, cell-based therapy using mesenchymal stromal/stem cells (MSCs) could potentially affect the healing of musculoskeletal tissues.

Introduction

When it comes to healing and regeneration of damaged tissue, the unique mechanical environment of musculoskeletal tissue, namely, articular cartilage, ligament, tendon, and bone, currently places limits on effective treatment for their respective pathologies. Bone is, by far, the most common among these tissues to undergo reconstruction. In the USA alone, the number of bone-grafting procedures is estimated at 1 million per year [1]. Grafting is performed for a variety of defects

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_37,

related to trauma, arthritis or arthroplasty, infection, tumor resection, or congenital anomaly. The gold standard for any of these bone defects is to replace "like with like"; using autologous bone or alternatively, cadaveric bone graft as the primary construct for reconstruction. While generally reliable, autologous bone graft or vascularized flaps are highly associated with donor site morbidity. So much so that patients will often complain that the pain at the donor site is worse than the pain associated with the primary surgical site. Allografts, while solving the donor site problem, have their own drawbacks including increased chances for infection and immune response, such as hypersensitivity or foreign-body response.

Tendon, ligament, and articular cartilage pose even more difficult challenges to healing and reconstruction as they lack the ability to return to their respective pre-injury characteristics. Each tissue brings its own complications and frustrations. The gold standard for tendon and ligament healing remains primary repair whenever possible. When not possible, tendon or ligament autograft or allograft [i.e., anterior cruciate ligament (ACL) reconstruction] or synthetic sheaths are often used. Unfortunately, in all cases, the reparative tissue never regains the full characteristics of uninjured tissue. Strength of reparative tendon can be dependent on the suture used for repair or the amount of motion allowed after repair. Quality of ligamentous reconstruction can hinge on the method of reconstruction and anchorage as well the biochemical environment of the healing tissue. The majority of solutions remain mechanical in nature and lack a good biological or functional approach to connective tissue repair.

Cartilage is likely the most difficult tissue to heal and repair. Articular cartilage is an avascular, hypocellular, alymphatic tissue with minimal reparative potential. Chronic degeneration can lead to arthritis, which affects over 50 million Americans yearly [1]. Acute cartilaginous injury, while less frequent, likewise is debilitating and can subsequently lead to chronic degeneration. To date, no adequate substitute has been developed nor surgical procedure performed that successfully replicates articular cartilage. Repair stimulation via microfracture surgery or drilling leads to the stimulation of healing with type I cartilage rather than the desired type II cartilage. Osteochondral plugs or allografts, while allowing for type I cartilage repair, are technically difficult to perform and include donor site morbidity.

Functional Mechanisms of MSCs Relevant to Orthopedic Applications

It is thought that the primary mechanism of MSCs in contributing to tissue healing is through paracrine effects on resident cells or activation of the intrinsic pathway. The recruitment of cells to injured or inflamed tissue of the area of injury may have some role. While all these factors are likely to improve the environment for healing tissue, they are indirect and difficult to localize to a specific tissue. In addition to their paracrine effects, MSCs also have demonstrated a potentially more direct method of contributing to healing. MSCs have been differentiated to osteogenic and chondrogenic lineages suitable for direct implantation through a variety of scaffold materials.

Tissue engineering strategies allow for the manipulation or guidance of MSCs to clinically relevant constructs that can be used in regeneration of musculoskeletal injuries. The translation of these findings to in vivo models, or clinical applications, is currently the focus of extensive research by many groups.

Bone

There is evidence to suggest that biomaterial scaffolds, clinically used for their osteoconductive properties, such as hydroxyapatite or ceramics, can further be enhanced by adding MSCs. The role of the scaffold is to provide a microenvironment that allows for nutrient diffusion as well as biochemical, structural, and cellular stimuli that promotes survival, proliferation, migration, and differentiation of implanted or tissue resident cells. Clinically, bone marrow aspirate has been added to fracture sites for decades to promote healing or treat fracture nonunion [2, 3]. Additionally, autologous bone marrow has been combined with a variety of scaffolds to address bony defects such as cysts or segmental defects [4, 5].

In a small case series, culture expanded BM-MSCs seeded on hydroxyapatite scaffolds were used to treat large segment defects (4–7 cm) in the lower extremities stabilized with external fixation [6]. The three patients reported on had complete bone fusion at approximately 6 months and stable durability at an average follow-up of 6 years. Morishita et al. reported the use of autologous, *ex vivo* expanded MSCs cultured on hydroxyapatite pellets to address defects resulting from benign tumors of the extremities [7]. Osseointegration was evident at more than 2 years follow-up and functional measures, and quality of life were satisfactory at follow-up.

A novel method of progenitor cells (likely MSCs) derived from periosteum implanted with a 3D piece of porous coral was described for replacement of the distal phalanx of the thumb following traumatic avulsion [8]. Follow-up biopsy showed both lamellar bone and ossified tissue; perhaps more importantly, the patient had good function of his hand. As expected, the stable nature of bone and its unique ability to regenerate to full functionality allow for improved, if not impressive, augmentation with MSCs.

Ligament and Tendon

Unlike bone, ligament, and tendon healing is complicated by an acellular, avascular, tissue environment, subject to mechanical stress and motion. Current advances in tendon healing focus on the use of bioactive molecules such as hyaluronic acid (HA), transforming growth factor-β (TGF-β), and platelet-rich plasma (PRP). The goal is to find biological mechanisms to influence healing via the intrinsic pathway which leads to better organization of collagen as well as fewer complications (i.e., adhesions or excessive scar tissue).

Recently, MSCs have been investigated for use in ACL repair in several in vivo studies as an animal preclinical model of ligament and tendon healing [9].

Hankemeier et al. utilized a mixture of human B-MSC and fibrin glue and injected the mixture into rat patellar tendon defects. Follow-up analysis demonstrated more mature and organized cell structure when compared to controls [10]. Furthermore, MSC injection is used in veterinary applications to repair ligamentous injuries or inflammation. Such reports have shown an increase in vascularity and tissue formation as well as improved biomechanical functions [11]. An additional application of MSCs in ligament and tendon reconstruction includes tissue-engineered constructs which could be mechanically stressed in a bioreactor prior to implantation [12, 13]. To our knowledge, there are no clinical reports of MSCs in such injury although one can anticipate this is not far off in the spectrum of cell-based therapies in orthopedic applications.

Cartilage

Articular cartilage is a unique functional tissue allowing for both shock absorption and minimal friction for joint movement. The components of hyaline cartilage include predominantly collagen II, hyaluronic acid, proteoglycans, and glycoproteins surrounded by a peripheral rim of perichondrium. The relatively acellular and poorly vascularized nature of this tissue makes it not only susceptible to injury but difficult to heal adequately.

For decades, surgical procedures have been aimed at repairing or replacing articular cartilage, at some level, to restore motion or reduce pain. With more than 50 million Americans carrying a diagnosis of cartilage degeneration or arthritis, there is considerable interest in developing cell-based therapies for cartilage regeneration. The first reported cases using culture-expanded, autologous MSCs to repair cartilage defects were reported by Wakitani and colleagues [14]. The group treated two patients, each with patellar chondral defects, with MSCs seeded on a hydrogel of type I collagen. These constructs were implanted into the cartilage defects and covered with a periosteal flap. Within 6 months, both patients noted an improvement in their pain and activity. Biopsies at 1 and 2 years show that the tissues were repaired with fibrocartilage indicating that this approach is equivalent but not superior to current techniques. The authors now have over 10-year follow-up of these original cases which show no evidence of symptom recurrence, clinical problem, infection, or abnormal pathology.

Additional cases of this MSC-collagen-periosteal flap methodology have been reported in a handful of osteochondral defects. A young, otherwise healthy athlete presented with a defect of the femoral condyle following injury and underwent debridement with implantation of an MSC-collagen gel construct covered with a local periosteal flap. Up to 1 year after surgery, the defect was filled in with hyaline cartilage that stained weakly positive for glycosaminoglycans. The patient's symptoms improved significantly, and he resumed high level activity. A series of articular cartilage defects in the patella-femoral joint were treated in three patients (a total of nine defects) [15]. Again, autologous, culture-expanded MSCs were

embedded in a collagen gel, implanted into full thickness defects, and covered with local periosteum. At 1-year follow-up, clinical symptoms resolved, all patients were pain-free, and imaging demonstrated tissue repaired with fibrocartilage in the previous defect site. Three adolescent male athletes treated for elbow pain and associated restricted throwing motion were found to have bony fragments in the capitellum [16]. Each underwent autologous BM-MSC construct transplantation. Clinical symptoms improved significantly. These are the only cases of cell transplantation in children, and there were no clinical complications or problems at long-term follow-up.

Nejadnik and colleagues compared 72 age- and lesion-matched patients undergoing first-generation autologous chondrocyte implantation (ACI) to those treated with autologous bone marrow MSC implantation [17]. Both groups were treated with cell sheet implantation, covered with a periosteal flap and sealed with fibrin glue. In general, there was no difference between the two treatments in clinical outcome or assessment. Interestingly, patients younger than 45 years old scored significantly better than older patients in the ACI-treated group; however, there was no difference among the age groups in the BM-MSC cohort. This may be due to cellular injury or senescence of terminally differentiated cells such as chondrocytes in orthopedic diseases, while BM-MSCs may be less susceptible to local inflammation, injury, or aging.

In the first randomized, prospective controlled study, this technique was applied to osteoarthritis in patients undergoing high tibial osteotomies (HTO). Generally reserved for young, active patients, HTOs are used to change the alignment of the knee to offload damaged cartilage in an attempt to increase its life span and reduce wear [18]. Twenty-four patients undergoing HTO for medial compartment osteoarthritis of the knee were randomly assigned to receive autologous MSC transplantation or cell-free controls. Bone marrow aspirate was harvested from the iliac crest, and cells were expanded for approximately 30 days. MSCs were then embedded in a type I collagen gel and maintained in culture an additional night. At the time of surgery, patients randomized to receive cell transplantation had a segment of disease articular surface abraded and treated with the collagen gel – MSC construct followed by a periosteal cover (average 14 × 35 mm). The control group underwent a similar procedure with a cell-free collagen sponge implanted and covered with local periosteum. The mean follow-up was 16 months. The authors reported a significant improvement in scores assessing pain, function, and muscle strength before and after the procedure in both groups. There was no difference in clinical outcomes between the cell transplantation and control groups, indicating that either treatment is appropriate. Second-look arthroscopic procedures were performed an average of 42 weeks after surgery on nine of the cell-transplanted patients and showed firm, regular white cartilage in the area of sponge transplantation. Similar arthroscopy was performed in six of the control patients showing softer, yellowish, irregular cartilage in the area of collagen sponge implantation. Additionally, tissue biopsies were obtained and showed hyaline-like cartilage in the cell-treated group and fibrocartilage in the control group, suggesting a superior biological outcome. The authors observed that the arthoscopic and histological grading scores were better in the cell transplantation group versus controls.

A large defect produced by methicillin-resistant *Staphylococcus aureus* septic arthritis following trauma was treated in a similar manner by Adachi and colleagues [19]. The patient initially presented with severe knee pain and limited range of motion, imaging illustrated an osteochondral defect of the medial femoral condyle measuring 20 × 25 × 25 mm causing pain and an audible click in the knee joint. First, bone marrow aspirate was taken from the tibia under local anesthetic, and MSCs were expanded to passage three. A porous calcium hydroxyapatite sheet was suspended in the defect, and the autologous MSCs were infiltrated directly in to the matrix scaffold. Partial weight bearing was initiated at 3 weeks, at which time the patient's range of motion improved, his pain improved, and the audible click had resolved. Arthoscopy and tissue biopsy taken 1 year after the procedure demonstrated fibrous tissue with a deep layer extracellular matrix expressing glycosaminoglycans with integration into the underlying bone.

There is one case reported of direct injection of culture-expanded, autologous MSCs into the knee of a patient with osteoarthritis [20]. The patient presented with a several-year history of chronic knee pain and limited range of motion despite therapies including nonsteroidal anti-inflammatory drugs, physical therapy, and arthroscopic microfracture. He underwent autologous MSC injection, passage five, into the intra-articular space. In addition to a single injection of 22 million MSCs, the treatment protocol included injection with fresh BM nucleated cells at the same time, followed by two subsequent injections of dexamethasone and platelet lysate at 1 and 2 weeks post-procedure, all of which could potentially have an anti-inflammatory, reparative effect on the arthritic joint space. At 24 weeks follow-up, the patient had significantly improved pain scores, range of motion, and increased volume of cartilage and meniscus observed on MRI. While encouraging, these results are complicated by the multifactorial nature of the treatment protocol and the lack of definitive tissue evaluation; it is likely that the increased volume seen on MRI was fibrocartilage rather than true regenerated hyaline cartilage.

Conclusions

The overall effectiveness of repair and regeneration of cartilage, bone, or tendon in situ is limited by the size of the defect, the nature of the injury, the mechanical requirements of the tissues, and the surrounding soft tissue envelope. Current accepted techniques have focused more on mechanical requirements of the tissue than on the biology. This has led to solutions that, while effective, are limited and inferior to the original tissue. Mesenchymal stromal cell therapy offers an alternative in which cells with both osteogenic and chondrogenic differentiation capabilities are transferred to the site of injury, with a goal of more closely replicating the original tissue mechanically and biologically. The clinical cases and series reported here offer promising outcomes and applications of MSC-based therapy in a spectrum of orthopedic diseases. Well-designed randomized clinical trials are needed next to evaluate the true potential of this area of regenerative medicine. Further

work is necessary to determine the fate of the transplanted cells and constructs in terms of engraftment, inflammation, mechanical function, and durability given these unique tissues of interest.

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Chapter 38
Adipose Tissue-Derived MSCs: Moving to the Clinic

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Abstract Adipose tissue-derived stromal/stem cells (ASCs) are an abundant source of adult tissue stem cells for tissue engineering and regenerative medicine therapeutic applications. Unlike other sources of mesenchymal stromal/stem cells (MSCs), ASCs can be retrieved from liposuction aspirates or subcutaneous adipose tissue fragments in high numbers with minimal ethical considerations and can be easily expanded in vitro. The ASC have the ability to differentiate in vitro along endodermal, ectodermal, and mesodermal lineage pathways. Similar to other MSCs, ASCs display immunomodulatory and immunosuppressive properties, making them suitable for both autologous and allogeneic approaches. Furthermore, ASCs are genetically stable in short-term, although possibly not long term, culture. Due to these characteristics, ASCs are poised for clinical trials treating a broad range of conditions. Despite their tremendous promise, adipose tissue stem cell-based regenerative strategies may require additional preclinical evidence to support advancement to clinical trials. This chapter summarizes current preclinical and clinical data on the use of ASCs and discusses the future regenerative medical applications.

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_38,

Introduction

With obesity, adipose tissue can account for 50% or more of the individual's total volume, making it the body's largest organ. Subcutaneous adipose depots are accessible for harvest using tumescent liposuction techniques, which are among the most common plastic surgical surgeries performed worldwide. Multiple independent studies have described the isolation and characterization of multipotent stromal/stem cells from lipoaspirates, and this literature has increased exponentially over the past decade. These findings suggest that adipose tissue may provide an alternative to bone marrow as a source of stromal/stem cells for regenerative medical and tissue engineering applications. This chapter provides a concise review exploring the characteristics and function of these adipose-derived cells.

Basic Biology of Adipose Stromal/Stem Cells

Isolation: Stromal-Vascular Fraction Cells Versus Adipose Stromal/Stem Cells

Adipose tissue is an alternative to bone marrow as a source of mesenchymal stromal/stem cells (MSCs). The majority of adipose tissue derives from the embryonic mesenchyme, contains a large number of stromal/stem cells, and is relatively easy to obtain in large quantities. It can be found throughout the human body and classified according to either its location and/or function, as white (WAT) or brown (BAT) adipose tissue [1]. Stromal/stem cells isolated and culture expanded from adipose tissues demonstrate characteristics in common with those from other mesodermal tissues, including adherence to plastic, formation of fibroblastic-like colonies, extensive proliferative capacity, ability to differentiate into several mesodermal lineages (including bone, cartilage, muscle, and fat), and expression of common cell surface antigens [2, 3]. Several different terms have been used to describe these cells, isolated or culture expanded from adipose tissues, including preadipocytes [4–6], stromal cells [7], vascular endothelial cells [8], multipotent stem cells [136], processed lipoaspirate (PLA) cells [9], and adipose-derived stromal or stem (ASCs) cells [10–12]. Additional evidence suggests that under certain experimental in vitro conditions, non-mesodermal tissues (neuron-like cells, hepatocytes, epithelial cells) can also be formed by these cells [13–20].

Over the past decade, methods to isolate stromal/stem cells from waste tissue generated by abdominoplasty, cosmetic surgery, and tumescent liposuction procedures have been developed and their reproducibility validated by independent laboratories [21]. The isolation process goes through sequential steps including the washing and mincing of the tissue, digestion with collagenase or related lytic enzymes, and a subsequent differential centrifugation step that separates the mature adipocytes (floating supernatant) from a heterogeneous stromal-vascular fraction

(SVF) pellet that includes the ASC cells as well as circulating blood cells, fibroblasts, pericytes, and endothelial cells [21, 22]. The stromal-vascular fraction is then suspended in growth media, plated, and washed free of any nonadherent cells after overnight incubation [21, 22]. Cells are then ready for in vitro culture where they can be expanded, cryopreserved, differentiated in specific cell types, and used for tissue engineering and regenerative medicine approaches. Routinely, a milliliter of liposuction aspirates yields ~375,000 ASCs following a 4-day expansion period [23].

Various independent groups have reported the cell surface immunophenotype of ASC from humans and other species based on CD (cluster of differentiation) markers determined with fluorescent-activated cell sorting [7, 24–27]. The reported immunophenotypes have been relatively consistent, regardless of differences in isolation, culture procedures, and time in passage; furthermore, the surface marker expression profile of the ASCs have been comparable to those of bone marrow-derived MSCs [28, 29].

Nevertheless, as observed with MSCs from other tissue sources, no single definitive cell surface marker for ASCs has been identified. An increasing number of studies have shown that many types of MSC, including ASCs, reside in a perivascular location, and evidence shows that both MSC and ASCs may in fact be vascular stem cells [30–35]. In tissues, these pericytic cells differentiate into smooth muscle and endothelial cells that participate in angiogenesis and neovasculogenesis. MSCs or ASCs are recruited to injury sites where they participate in the repair/regeneration of the injured tissue [36]. These stem cells exist within tissues at various stages and on different paths of differentiation and have been associated with the dynamic capacity for growth of the vascular system [36–38].

Likewise, the preadipocytes within adipose tissue depots exhibit close relationships to endothelial cells in vivo. Cells isolated and cultured within the heterogeneous SVF express markers related to an endothelial phenotype, including CD31, CD144 (VE-cadherin), and von Willebrand factor [27]. In vivo studies further support the endothelial differentiation potential of SVF cells. Several paracrine pathways may contribute to the ability of ASCs to integrate in vivo as fully functional and differentiated endothelial cells [38]. The ASC's secretome contains angiogenic cytokines such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF), and these may underlie the ASC's angiogenic and vasculogenic properties [33].

A subpopulation of SVF cells have been characterized based on the immunophenotype CD34+CD31- [33]. In adipose tissue, CD34 is localized to the intima and adventitia of blood vessels as opposed to the media, where cells expressing alpha-smooth muscle actin (SMA) exist. By excluding the intima, which contains the CD34+CD31+ endothelial cells, and the media, which contains the CD34−CD31− smooth muscle cells, it leaves the adventitia as the putative location for the CD34+ ASCs [30, 33]. CD34 and CD140b (a pericyte marker) are expressed together exclusively in the capillary, leading some to conclude that pericytes are not the source of CD34+ ASCs [30, 33]. Several other cellular markers for vascular cells, stem cells, and stem cell niche have also been investigated as possible ASC

markers [31]. The well-known MSC marker STRO-1 is expressed in cultured ASCs [24, 29]. In adipose tissue, STRO-1 appears to be expressed exclusively in the endothelium of certain but not all blood vessels, and thus is not associated with the CD34+ ASCs [30]. In conclusion, nonexpanded adipose stem cells exist as CD34+CD31-CD104b-SMA- cells in the capillary and in the adventitia of larger vessels. In the capillary, these cells coexist with pericytes and endothelial cells; both of which could be possible progenies of ASCs. In the larger vessels, these ASC have been reported as specialized fibroblasts (having stem cell properties) in the adventitia [30].

Differentiation

Under the appropriate environmental signals, ASC can differentiate along selective lineage pathways as summarized in Table 38.1 [3, 22]. In vitro, the ASC has the ability to renew itself, consistent with the definition of an adult stem cell [72]. Despite common characteristics among stem cells from different tissues, there are epigenetic mechanisms related to the tissue of origin which may distinguish the characteristics of ASCs from those of MSCs isolated from other tissues [68, 73]. Similar to bone marrow MSC, human ASC cells exhibit substantial plasticity [22] and differentiate into several lineage pathways relevant to musculoskeletal and soft tissue repair, including chondrogenic, osteogenic, myogenic, and adipogenic cell types [3, 22, 61]. Clonogenic studies have confirmed the multipotency of individual clones of ASC, consistent with a stem cell identity, rather than that of a mixed population of unipotent progenitors [24, 74]. Manipulation of the physical and biochemical environment can be used to induce the differentiation of the cells (Table 38.1). The presence and time of exposure to different exogenous growth factors, hormones, vitamins, and other molecules in combination with the control of cell shape has a profound effect on the phenotype of ASCs cells [75]. While in vitro studies suggests that ASCs have the potential to differentiate into non-mesodermal lineages including neuronal-like cells, endothelial cells, epithelial cells, hepatocytes, pancreatic cells, and hematopoietic supporting cells [22, 48, 58, 63, 76–79], this remains highly controversial since there is currently no in vivo evidence indicating that such transdifferentiation is physiological [80].

Paracrine Function

Adipose tissue, as a source of adipokines and cytokines, plays both local and systemic roles in health and disease [81, 82]. The angiogenic factors released by ASCs may stimulate the vasculogenesis and blood supply required for the expansion of adipose tissue depots during the development of obesity. Elevated serum levels of proinflammatory and chemotactic cytokines have been attributed, in part, to the

Table 38.1 In vitro differentiation of ASCs

Cell lineage	Phenotype/behavior	References
Adipocyte	0.5% oil red-O staining (lipid accumulation)	[9, 24, 39]
Cardiomyocyte	Cardiac myosin heavy chain (MHC), troponin I, α-sarcomeric actin expression, spontaneous contraction	[40–46]
Chondrocyte	Toluidine blue (sulfated proteoglycan-rich matrix), collagen II and X production	[9, 10, 47]
Endothelial	CD31 and von Willebrand factor expression, tubular formation in Matrigel™, incorporation into microvasculature	[48–51]
Hematopoietic support	Bone marrow and platelet reconstitution, engraftment in hematopoietic organs, CD34 and CD45 expression, Sca-1/Lin^{lo}, secretion of hematopoietic support factors (G-CSF, M-CSF, GM-CSF, and IL-7)	[52–57]
Hepatocyte	Urea synthesis, maintain glycogen stores, liver-specific mRNA	[13, 14, 58–60]
Myocyte	Multinucleation, skeletal muscle myosin heavy-chain 2, myosin- and MyoD1-specific, MyoD1 expression	[9, 24, 61, 62]
Neuronal-like	Nestin, NeuN, intermediate filament, MAP2, b-III tubulin, glutamate receptor subunits NR1 and NR2 expression, electrophysiological properties	[19, 63–65]
Osteoblast	Alizarin red (mineralization), von Kossa staining, collagen I, alkaline phosphatase, osteopontin, osteonectin, osteocalcin	[9, 12, 24, 66, 67]
Pancreatic	Secretion of insulin, glucagon, somatostatin	[68–71]

increased number of resident macrophages within adipose tissue of obese individuals. Studies suggest that undifferentiated and/or adipocyte differentiated ASCs within intact adipose tissues also may contribute to the elevated proinflammatory cytokines levels found in obese individuals [52, 83].

Investigators have exploited the ASC's paracrine properties as "regenerative cells," delivering them to injured or diseased tissue where cytokines can stimulate recovery. Presumably, the ASCs modulate the "stem cell niche" of the host by stimulating the recruitment of endogenous stem cells to the injury site and promoting their differentiation along the required lineage pathway [84].

In a related manner, at an ischemic site, ASCs might provide antioxidant molecules, free radical scavengers, and chaperone/heat shock proteins. As a result, toxic substances released into the local environment at the injured/ischemic site would be neutralized, thereby promoting recovery of the surviving cells. Exciting studies have suggested that transplanted bone marrow-derived MSCs can deliver new mitochondria to damaged cells, thereby rescuing aerobic metabolism [85, 86]. It remains to be determined if ASCs exhibit a comparable ability to contribute mitochondria following ischemic injuries such as myocardial infarction.

ASCs secrete multiple growth factors, including vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), fibroblast growth factor 2 (FGF-2), and insulin-like growth factor 1 (IGF-1) [38, 49, 52]. In addition, exposure of ASCs to hypoxia, growth factors, differentiation factors, or tumor necrosis factor-α will

induce their secretion of VEGF and HGF. In general, the cytokine profile of ASCs is similar to that reported for human bone marrow-derived MSCs [52]. Both cell types secrete angiogenic, proinflammatory, and hematopoietic-supportive cytokines following exposure to common inductive factors. This suggests that ASCs, like BMSCs [87], may have clinical utility for patients undergoing hematopoietic stem cell (HSC) transplantation following high-dose chemotherapy. It may be possible to co-infuse ASCs with HSC to enhance and accelerate the recovery of normal blood cell production and restoration of immune competence while protecting against graft versus host disease [53, 88].

Immunomodulatory Properties

Tissue engineering and regenerative medical applications will require either autologous or allogeneic stem cells, depending on the clinical circumstances; each population presents unique advantages and challenges [89–91]. While autologous ASCs present limited risk to the recipient, they are best suited for elective procedures where adipose tissue can be harvested in advance to allow for cell expansion. In contrast, while allogeneic ASC present greater potential infectious risks to the recipient, they can be harvested from tissue donors and cryopreserved in bulk well in advance of any surgical procedure. Allogeneic ASC can then be used at the point of care under emergency circumstances for tissue repair. Like BMSC, it is feasible to transplant ASC across immunological barriers [25, 88, 92–94].

It has been demonstrated that cell surface receptors, such as histocompatible locus antigen-DR (HLA-DR) and hematopoietic-associated markers, which elicit a cell-mediated immune response in mixed lymphocyte reactions (MLR), are present on freshly isolated SVF cells, but decreased as human ASC were successively passaged [25]. Subsequent exposure to interferon-γ (IFNγ) has been found to induce HLA-DR expression on passaged ASCs [95]. In vivo experiments comparing syngeneic and allogeneic implants in a rat spinal fusion model determined that there was no T-cell response; however, allogeneic ASC elicited a low-titered, noncytotoxic humoral response associated with the presentation of bovine serum proteins [96]. Multiple independent studies have reported that passaged ASCs do not stimulate proliferation of activated lymphocytes [94, 95, 97]. Surprisingly, a recent study found that ASC activated proliferation of nonactivated resting T-lymphocytes by up to 25-fold that was associated with increased ASC expression of IL6, IL8, TNFα, VEGF, and bFGF [95]. Both $CD4^+$ and $CD25^+$ T cell numbers increased, and the latter population displayed immunosuppressive function. Consistent with this, the presence of both IFNγ treated and non-treated ASCs inhibited phytohemagglutinin (PHA)-stimulated lymphocyte proliferation [95]. This immunosuppressive effect was not cell-to-cell contact dependent and has been attributed to soluble factors produced by ASCs [95]. A cytokine profile determined that secreted transforming growth factor-β (TGF-β), HGF, and prostaglandin E2 (PGE2) were present in the ASC conditioned medium [98]. Independent studies have determined that PGE2

alone can suppress MLR proliferation [94, 98]. These and related studies provide important cues regarding the molecular mechanisms underlying the immunomodulatory character of ASCs [94, 95, 97–99].

Inflammatory stimuli alter the soluble factors produced by human ASCs. When incubated with TNF-α for 24 h, ASC supernatants contained significantly increased levels of vascular endothelial growth factor (VEGF), HGF, and insulin-like growth factor 1 (IGF-1) [100]. Studies have found that the presence of adherent rat ASCs modulated the immune response to a polyurethane implant, reducing the foreign body response in the host animal [101]. Interestingly, when seeded in either polystyrene or polyurethane implants, the ASC modulated their secretion of IL-6, VEGF, and TNF-α dependent on the surface upon which they were seeded [101]. Thus, the scaffold/environment plays a role in regulating the immunomodulatory character of ASCs.

Clinical Applications

There is substantial interest worldwide in the clinical use of SVF cells and ASCs for multiple disorders. Most preclinical studies using ASCs and SVF cells have used mice or rats for reasons of cost, the wide range of species-specific antibody reagents, and the availability of inbred, transgenic, and/or knockout strains. Additional work has employed large animal models (dog, goat, horse, pig, rabbit, or sheep); however, there are far fewer species-specific monoclonal antibodies suitable for cell analysis or tracking in these models compared to rodents or humans. Data regarding the safety and efficacy of SVF cells and ASCs have led to proposals for clinical trials [22, 89–91, 102]. Regulatory authorities, primarily in Asia and Europe, have allowed a limited number of clinical trials involving SVF cells and ASCs to move forward. A search of the National Institutes of Health Clinical Trials database in late 2010 with the search terms “adipose stem cells” identified >30 registered studies (Table 38.2). In contrast, this compares with the identification of >1,400 studies or >140 with the search terms “bone marrow stem cells” or “mesenchymal stem cells,” respectively.

Soft Tissue Reconstruction

Clinical trials using ASCs and SVF cells for soft tissue cosmetic and reconstructive procedures have been approved. Plastic surgeons have combined SVF cells with lipoaspirate tissue to improve outcomes in autologous fat graft procedures for breast augmentation and reconstruction [103, 104]. Fat grafting can be complicated by fibrotic changes, resorption, and necrosis, and preliminary findings suggest that SVF cells, possibly due to paracrine actions, reduce the incidence of these events [103–105]. Similar approaches have used SVF cells to treat craniofacial defects

Table 38.2 Registered therapeutic clinical trials using SVF cells or ASCs

Name of the trial	Location	Phase	Expected outcomes
Safety and efficacy of autologous adipose-derived stem cell transplantation in patients with type 1 diabetes	Philippines	Phase I, Phase II	Lowering of insulin-dependence and anti-hyperglycemic medication dosages
Efficacy and safety of adipose stem cells to treat complex perianal fistulas not associated to Crohn's disease (FATT1)	Spain	Phase III	Closure of fistulas and re-epithelization
Autologous adipose-derived stem cell transplantation in patients with lipodystrophy (AADSCTPL)	Brazil	Phase I	Presence of neovascularization, adipose transplantation reabsorption, tissue viability
Treatment of fistulous Crohn's disease by implant of autologous mesenchymal stem cells derived from adipose tissue	Spain	Phase I, Phase II	Evaluating the ASCs therapeutic effect
Autologous mesenchymal stem cells from adipose tissue in patients with secondary progressive multiple sclerosis (CMM/EM/2008)	Spain	Phase I, Phase II	To evaluate safety and tolerability related to the intravenous infusion of autologous mesenchymal stem cells
Safety and efficacy of autologous adipose-derived stem cell transplantation in type 2 diabetics	Philippines	Phase I, Phase II	Lowering of blood glucose be it fasting, random, or postprandial
Allogenic stem cells derived from lipoaspirates for the treatment of recto-vaginal fistulas associated to Crohn's disease (ALOREVA)	Spain	Phase I, Phase II	Healing of rectovaginal fistula
Safety study of autologous cultured adipose-derived stem cells for the fecal incontinence	Korea	Phase I	Improvement of anorectal and endorectal functions
Safety and efficacy study of autologous cultured adipose-derived stem cells for the Crohn's fistula	Korea	Phase I	Closure of fistula
Randomized clinical trial of adipose-derived stem cells in the treatment of Pts with ST-elevation myocardial infarction	Netherlands; Spain	Phase I	Improvement in cardiac function
Long-term safety and efficacy of adipose-derived stem cells to treat complex perianal fistulas in patients participating in the FATT-1 randomized controlled trial (LTE)	Spain	Extension of prior Phase III	Closure of the fistula; complete re-epithelization
Safety and efficacy study of autologous cultured adipose-derived stem cells for the Crohn's fistula	Korea	Phase II	Complete closure of fistula

A randomized clinical trial of adipose-derived stem cells in treatment of non revascularizable ischemic myocardium	Spain	Phase I	Improvement in cardiac function
Abdominal obesity and cardiovascular risk factors in women who survived cancer or a related illness following total body irradiation and stem cell transplant	USA	–	Compare visceral adipose tissue in irradiated women plus stem cell transplant and women who were not
Autologous stem cells derived from lipoaspirates for the non-surgical treatment of complex perianal fistula	Spain	Phase II	Complete closure at week 8 and re-epithelization. No fistula recurrence after 1 year follow-up
Study of autologous fat enhanced w/ regenerative cells transplanted to reconstruct breast deformities after lumpectomy (RESTORE-2)	UK	Phase IV	Change in breast volume and shape and improvement in skin pigmentation abnormalities at 6 and 12 months compared to baseline
Safety and efficacy of autologous cultured adipocytes in patient with depressed scar	Korea	Phase II, Phase III	Assess injection site and overall scar tissue improvement
The role of lipoaspirate injection in the treatment of diabetic lower extremity wounds and venous stasis ulcers	USA	–	Wound measurements and healing
ACELLDream for adipose CELL derived regenerative endothelial angiogenic medicine	France	–	Evaluate safety and tolerability of intramuscular injection of autologous adipose derived stroma/stem cells
Multicenter clinical trial for the evaluation of mesenchymal stem cells from adipose tissue in patients with chronic graft versus host disease	Spain	–	Reduction or suspension of immunosuppressive treatment
Development of bone grafts using adipose derived stem cells and different scaffolds	Switzerland	–	Pre-engineer large synthetic bone grafts and study the vascularization process in vivo

Source: http://clinicaltrials.gov

secondary to lipodystrophy or trauma [106]. Fat grafts without SVF cells have been used successfully to treat fibrotic changes in the skin of breast cancer patients after receiving radiation therapy [107]. These patients can exhibit reduced range of motion and poorly healing wounds [107, 108]. While fat grafting and SVF cells have been used in multiple patients, few of the published reports describe randomized controlled trials. The majority of the literature describes large case reports, and some complications (cysts, microcalcifications, fat necrosis) have been recorded following breast augmentation [109, 110]. Furthermore, recent studies indicate that ASCs promote the proliferation of metastatic, but not resting, primary breast cancer cells [111, 112]. Thus, safety concerns remain regarding the use of SVF cells and ASC for reconstruction in breast cancer survivors.

Bone Reconstruction

Orthopedic applications for SVF cells and ASCs are a realistic opportunity due to the close developmental links between adipocytes and osteoblast [113, 114], and this has been substantiated in a case report from Finland [115]. A regenerative medical team successfully used autologous ASC to repair a hard palate defect in a 68-year-old subject disfigured by the removal of a keratocyst. The ASCs, obtained from 200 ml of the patient's subcutaneous fat and cultured for 14 days, were combined with a scaffold and bone morphogenetic protein two, implanted into the patient's muscle to allow for bone formation, and then transplanted into the defect site 8 months later. With additional surgeries and prosthetic tooth implants, the patient regained the ability to drink and eat without assistance [115]. Similar outcomes have been achieved in >90% of the nearly 30 additional patients receiving this therapy (personal communication, Susanna Miettinen and Bettina Lindstrom Mannerstrom) as well as four patients undergoing cranioplasty [116]. Further work is necessary to determine if ASCs or SVF cells are equally effective in orthopedic procedures involving weight-bearing bones.

Inflammatory Disorders

Autologous SVF cell and ASC therapies have been used to repair fistulae in Crohn's disease patients, and these are the most advanced peer-reviewed clinical trials published to date [117–122]. Phase I trials with up to five patients each have documented healing in 75% of chronic fistulae using culture-expanded ASCs [119]. In a single trial with four patients, however, only 25% of fistulas healed using freshly isolated and nonexpanded autologous SVF cells delivered in combination with allogeneic CD34+ cells and MSCs via intrathecal and intravenous injections [121]. The difference between the immunogenic properties of

ASCs and SVF cells is postulated to account for these disparate outcomes [121]. While SVF cells do not display immunosuppressive features and stimulate activated T-cell proliferation in vitro, culture expanded ASCs are immunosuppressive and express low levels of immunoreactive surface proteins [25]. In light of the autoimmune etiology of Crohn's disease, these immunomodulatory features could account for the relative success or failure of the two adipose-derived cell populations.

Murine experimental autoimmune encephalitis is a preclinical model of multiple sclerosis (MS). After injection with myelin basic protein, proteolipid protein, or oligodendrocyte glycoprotein, mice display symptoms comparable to the human disease [123]. A limited number of MS patients have received intravenous infusions of autologous SVF cells [124]. While these treatments were tolerated without complications and symptoms improved in some patients, this work remains inconclusive, and further study is necessary at both preclinical and clinical levels. Additional preclinical studies indicate a potential use for ASC in the treatment of rheumatoid arthritis, but, again, further work is needed before this can proceed to the clinic [125, 126].

Ischemic Disorders

Preclinical studies by multiple international groups have demonstrated the protective effects of both SVF cells and ASCs against acute vascular injuries [48–51, 127–129]. Intravenous infusion or intramuscular injection of ASCs or SVF cells enhanced blood supply and prevented necrosis in rodent hind limb ischemic injuries. The production of the angiogenic/vasculogenic cytokines HGF and VEGF has been postulated to be involved in the mechanism of ASC and/or SVF cell actions. Theoretically, ASCs and SVF cells could be used to treat vascular insufficiency in the extremities, secondary to diabetes or small-vessel diseases. While clinical protocols have been submitted to regulatory authorities, no phase I safety results have appeared yet in peer-reviewed publications.

Acute cardiovascular ischemic events underlie multiple disorders including myocardial infarction, stroke, and related conditions associated with trauma to the brain, spinal cord, and extremities. Since the ASC secretome contains angiogenic and vasculogenic cytokines with regenerative and neurotrophic properties, these conditions represent potential targets for ASC and SVF cell therapy [40]. Preclinical results using ASC in myocardial infarction and stroke models have improved ventricular [130–132] and motor function recovery post injury [133–136]. Similar studies have explored the regenerative function of ASCs in animal spinal cord injury models [136]. Phase I trials using ASCs or SVF cells have been registered for myocardial infarction treatment; however, outcome data has been presented primarily in oral presentations at international meetings, and there are few, if any current, published peer-reviewed manuscripts [137, 138].

Conclusion

Preclinical animal models and ongoing clinical trials support the use of stromal/stem cells of bone marrow origin for the treatment of diabetes mellitus, acute myocardial ischemia, chronic heart failure, and neurodegenerative diseases due to inborn errors of metabolism, among other pathologies. Since these diseases account for a substantial portion of healthcare costs internationally, there is interest in further developing ASCs and SVF cells as alternative cell therapeutic options; however, the existing data on ASC and SVF cells do not match that available for bone marrow-derived MSC. As a next step, it will be necessary for independent research groups to document the reproducibility of preclinical and clinical findings for adipose-derived cells in peer-reviewed publications and in presentations before regulatory agencies. This work can then be leveraged by randomized, controlled clinical safety and efficacy testing of ASCs and SVF cells in the context of specific disorders. To preempt any regulatory oversight questions in the future, academic clinicians and the biotech industry should initiate voluntary long-term follow-up of ASC and SVF cell recipients. In light of data suggesting the ability ASC to transform in vitro, proactive documentation for adverse events such as tumor incidence as well as efficacy is of particular importance as these stromal/stem cells move to the clinic [90, 139].

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4,

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4, © Springer Science+Business Media New York 2013