Multipotent Mesenchymal Stromal Cell-Based Therapies: Regeneration Versus Repair

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Introduction

In recent years, a growing wealth of knowledge on the biology and properties of multipotent adult stem cells (ASCs) has resulted in ever-increasing expectations regarding their possible clinical uses, providing new hope for the development of novel and effective cell-based therapies for degenerative diseases, traumatic injuries, and disorders for which there are currently limited therapeutic options.

One of the most extensively studied populations of multipotent ASCs is the mesenchymal stem cells, a population of fibroblast-like, plastic adherent cells which display a defined surface marker profile (CD105, CD73, and CD90 in greater than 95 % of the culture and lack of expression of CD34, CD45, CD14 or CD11b, CD79a or CD19, and HLA class II in greater than 95 % of the culture), and is currently termed multipotent mesenchymal stromal cells (with the acronym MSCs), according to the consensus set out by The International Society for Cellular Therapy [1]. Although MSCs were first isolated from the bone marrow (BM), cells which bear MSC characteristics, and which have therefore also been termed MSCs, were subsequently derived from different sites including the adipose tissue [2–4], skeletal muscle [5], liver [6], synovial membrane [7], umbilical cord blood [8], periosteum [9, 10], and peripheral blood [11] and, more recently, from the placental tissue [12], amniotic fluid [13], and menstrual blood [14–16].

However, MSC populations from different origins display some differences in terms of their patterns of gene expression and their differentiation capacity [17, 18]. Such differences might be the consequence of at least two factors. The first of these may be considered a "operational," given that most of the information available on the phenotype and functional properties of MSCs is derived from studies performed on cells cultured in vitro; however, the culture conditions themselves may give rise to the selection of different cell populations and may also induce heritable and epigenetic cellular preconditioning, thereby altering the original cellular phenotype [17–19]. Moreover, comparison among cell populations is made more arduous due to a lack of standardization between isolation and cultivation methods applied in different laboratories [18]. A second factor, which can be considered as an "intrinsic" problem, is related to the in vivo location of MSCs in different tissues, which may differentially influence the commitment, phenotype, and functions of the cells. This aspect is further complicated by the fact that the exact locations of these cells in vivo, as well as their specific natural functions in these locations, are far from being well understood [20]. Finally, a further level of complexity is added to this scenario by the fact that MSCs isolated from specific sites still tend to be heterogeneous populations, which, when cultured, are seen to contain both undifferentiated stem/progenitor cells as well as more mature cell types, which exhibit different functional abilities [18, 20, 21].

Despite these hurdles, much attention has been dedicated to these cells because of their relative ease of isolation, their expansion ability in culture, their multipotency, their absent or low immunogenicity, their immunomodulatory properties, and their ability to home to sites of inflammation or tissue injury (reviewed in [20, 22]). Indeed, all of these characteristics support the notion that MSCs might be valuable candidates for in vivo transplantation and cell-based therapy approaches.

The initial applications for which MSCs have been used in therapy are based on their absent or low immunogenicity and their immunoregulatory functions, as well as their multilineage differentiation capacity. Indeed, on one hand, a major advantage of using human MSCs for in vivo therapies is the fact that these cells are considered to be "immunoprivileged," due to their low expression levels of human leukocyte antigen (HLA) major histocompatibility complex (MHC) class I and their negative expression of major MHC II and co-stimulatory

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molecules such as CD40, CD80, and CD86 (for a review, see [22]). Therefore, allogeneic transplantation of MSCs should not require immunosuppression of the host. In addition, several evidence also show that MSCs may play specific roles in immunomodulation, interacting with cellular components of the immune system and inducing a shift from the production of pro- to anti-inflammatory cytokines [22].

On the other hand, the fact that isolated and expanded MSCs, mostly BM-derived, have been shown to be capable of differentiating into multiple cell types in vitro suggests that these cells might also be useful in a clinical setting for tissue regeneration, with tissue engineering and regenerative purposes [23].

Interestingly, increasing evidence has recently highlighted that MSCs produce bioactive molecules (such as cytokines and growth factors), which are able to exert several types of paracrine effects (e.g., anti-scarring, anti-apoptotic, anti-inflammatory) on target cells [20]. These findings have further widened the scope of possible MSC-based therapeutic applications and forced the reinterpretation of previous results which have been obtained with these cells. Indeed, in vivo studies have revealed that although MSC transplantation improves tissue conditions in several experimental animal models of disease, as well as in human clinical trials, in many cases, such as for the treatment of myocardial infarction or fibrosis (and other cases to be described later in this chapter), the number of engrafted cells and the levels of tissue-specific differentiation of these cells within injured or diseased host tissues are often very low or undetectable and likely insufficient to account for the observed functional improvements. Therefore, in such cases, it seems that cell replacement mechanisms represent only a minor facet of the role of MSCs in tissue regeneration. Conversely, it is becoming increasingly plausible that many of the beneficial effects exerted by MSCs in vivo are related to the bioactive molecules secreted by these cells and to the reparative actions of these molecules, which act by paracrine mechanisms on surrounding host tissues.

Before embarking on an in-depth discussion regarding the concepts of "regeneration" and "repair" using MSC-based therapies, clarification regarding the meaning of these two concepts is paramount. Here, MSC-based therapies for "regeneration" refers to treatments in which MSCs engraft into host tissues and "turn into" (i.e., differentiate into) specific cell type(s) required to replace defective, necrotic, or apoptotic cells and therefore rejuvenate damaged adult tissues; meanwhile, MSC-based therapies for "repair" refers to treatments in which MSCs produce bioactive factors that modulate the local host environment and induce endogenous cells or trigger a cascade of endogenous events, which lead to restoration of damaged adult tissues.

In this chapter, we will focus on those cases in which improvements in tissue function observed after MSC-based therapies do not seem to be related to the "regenerative" capacity of donor cells but, rather, are most likely due to the actions of these cells on the site of injury, thereby constituting a "reparative" activity which is associated with the regeneration of host cells. We will also discuss the concept that "regeneration" and "repair" are not mutually exclusive. Finally, we will show that reparative actions can be exerted at various levels and that identification of the mechanisms underlying the ability of MSCs to induce tissue recovery is becoming an important and challenging area of investigation, which is opening a new chapter in the therapeutic use of MSCs.

MSC-Based Therapy for Skeletal Diseases

MSCs are mainly defined simply in terms of their in vitro ability to differentiate toward the three classical mesodermal lineages (osteogenic, adipogenic, and chondrogenic) under appropriate culture conditions [18, 24–27]. On the basis of these in vitro characteristics, many attempts have been made to exploit the differentiation capabilities of MSCs in vivo to develop MSC-based approaches for the treatment of disorders affecting skeletal tissues and associated connective tissues (cartilage, tendon, and ligament) [28, 29]. Preliminary studies carried out in animal models, followed by preclinical studies and human clinical trials, have provided several evidences to support the feasibility of using MSC transplantation for this purpose, without resulting in the initiation of an immunological response (reviewed in [28]). This strategy is centered mainly on the ability of MSCs to differentiate and "turn into" cells of the specific injured tissue to be restored (regenerative approach), a process which is induced either by transplanting the cells alone or in combination with scaffolds (synthetic or natural and biodegradable), which provide mechanical and structural support, or exogenous factors (growth factors, soluble cytokines, chondrogenic, osteogenic factors), which enhance differentiation of MSC toward cells of the required tissue [28, 30]. For instance, successful results have been obtained in patients for the treatment of bone defects such as long bone nonunion fractures [31] and large bone diaphysis defects [32]. These patients have been treated with ex vivo-expanded autologous BM-derived MSCs encased in porous hydroxyapatite ceramic scaffolds, designed to match the bone deficit in terms of size and shape, and in both cases, treatment has resulted in the integration of the graft and healing of bone defects. A similar approach has also been applied with success in humans for the treatment of spinal fractures/vertebral disk injuries [33] and craniofacial defects [34, 35]. MSC-based therapy has also been suggested for the treatment of cartilaginous injuries. Indeed, numerous studies in both animal models and in humans have reported that transplantation of MSCs in combination with scaffolds results in new cartilage formation [36–38]. For instance,

Wakitani and colleagues [36] have reported treatment of patellar cartilage defects by this approach, whereby histological analyses revealed the successful repair of defects with fibrocartilaginous tissue.

Besides these approaches, which are based mainly on the local injection and implantation of MSCs, systemic transplantation of MSCs has also been shown to be a viable approach for the treatment of bone diseases such as osteogenesis imperfecta (OI). OI is a genetic disorder of bone and other tissues caused by a mutation in the genes coding for type 1 collagen, the major structural protein in bone. This disease is characterized by the occurrence of fractures, reduced bone growth, and progressive bone deformation. Horwitz and colleagues [39–41] were the first to investigate whether MSC transplantation could be used to treat patients affected by OI. In 2002, these authors reported that when children with OI were treated first with a standard allogeneic BM transplant and then with a "booster" of MSCs from the same donor (18 months post BM transplantation), clinical conditions were ameliorated, and the children began to grow again. These authors claimed that donor MSCs can engraft after transplantation and differentiate to osteoblasts as well as skin fibroblasts, thereby conferring clinical benefits attributable to the engraftment of functional mesenchymal precursors.

Even though these findings support the notion that the beneficial effects observed are likely due to the differentiation of MSCs into the cell types needed for tissue regeneration, they still leave open the possibility that differentiation is not the only mechanism underlying the effects observed. For instance, although MSC-based therapy for cartilage regeneration was first conceived on the basis of the ability of these cells to differentiate toward the chondrogenic lineage, in some cases, doubts have been raised concerning the origin of newly formed cartilage [28, 38] and the mechanisms involved [28]. Indeed, it remains to be demonstrated as to whether new cartilage tissue is derived directly from the differentiation of transplanted MSCs, therefore representing a regenerative action of these cells, or from the ability of the transplanted cells, through paracrine mechanisms, to inhibit host inflammatory responses or stimulate the growth and/or activity of endogenous progenitors and chondrocytes [28], and therefore acting through a reparative mechanism. For instance, in the case of diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA), which are degenerative joint diseases associated with progressive and often severe inflammation, it seems that the beneficial effects of MSCs are attributable mainly to the induction of endogenous progenitor cells and their anti-inflammatory and immunosuppressive activities [42]. In addition, as is also the case when MSCs have been applied for OI treatment, some authors have reported that the levels of donor MSCs in bone, skin, and other tissues were less than 1 %. Although Horwitz and colleagues [41] claimed that these low levels of engraftment were adequate to confer clinical benefits, a reinterpretation of these results by others questioned whether the beneficial effects observed were due only to MSC differentiation into osteoblasts to form bone and suggested that most probably, bioactive factors secreted by the MSC also supported the observed growth and improvement of clinical conditions [19, 43].

MSCs-Based Therapy for Other Pathological Conditions

Besides differentiation toward mesodermal lineages, efforts have been devoted to investigate the ability of MSCs to differentiate across germinal boundaries outside of the mesenchymal lineage and to therefore also include the endodermal and ectodermal lineages, a process often referred as "trans-differentiation." Although results concerning this property of MSCs are still debated [17, 44], several studies indicate the presence of multipotent cells with MSC characteristics, which, under particular conditions, not only differentiate into cells of the mesodermal lineage but also into cells resembling neurons [17, 45–47], hepatocytes [48–52], and cardiomyocytes [53, 54], as well as endothelial [55] and pancreatic cells [56, 57].

Over the years, a wide variety of experimental conditions have been set up in an attempt to trigger and study trans-differentiation in vitro [17, 52]. Generally, these protocols are based on induction of differentiation by the addition of soluble factors to the culture medium (e.g., growth factors, cytokines, corticosteroids, hormones, chemical demethylating agents), as well as the reconstitution of cell-matrix and cell-cell interactions, with the intent of creating a microenvironment and signals to drive cell differentiation toward a specific lineage in vivo under the normal developmental/homeostatic conditions of a specific tissue/organ [17, 52, 58]. After induction, the potential resulting differentiation is monitored by evaluating cellular morphological changes (i.e., changes to neuron-like, hepatocyte-like, and cardiomyocyte-like features), the expression of various tissue-specific genes, as well as assessing any acquired abilities of the cells to exert tissue-specific functions. The literature currently includes a multitude of papers reporting successful results in this field. Nevertheless, concerns remain regarding the interpretation of the results achieved, given that there is a lack of standardization between the existing reports in terms of the methods used to induce differentiation, as well as in the criteria applied for phenotyping in vitro-generated differentiated cells. Indeed, on one hand, differentiation strategies are hampered by great variability between protocols used by different groups and also by the fact that in most cases, the signals that drive natural differentiation in vivo remain to be completely defined, therefore making it very difficult to reproduce them in vitro. On the other hand, phenotyping is

compromised by several aspects such as (*i*) the lack of specificity of differentiation markers used to evaluate the grade of differentiation achieved; (*ii*) molecular and functional heterogeneity of the starting MSC populations used, which constitutes an additional variable for consideration in efforts to ascertain the transdifferentiation ability of cells; and finally (*iii*) possible artifacts resulting from the fact that the cells used have been removed from their natural in vivo location and are subsequently grown in a nonphysiological, chemical ex vivo environment and may therefore undergo cytoskeletal and phenotypic alterations that might be misinterpreted as a "true" transdifferentiation phenomena [17, 59]. For example, several studies have described methods to direct MSCs to differentiate into specific neuronal subtypes [46, 47]; however, the positivity of the results obtained has been questioned, given that undifferentiated MSCs express a considerable repertoire of neural genes, and therefore, the expression of these genes after induction may not be the result of differentiation (reviewed in [17]). In addition, some of the neural markers, which have been analyzed, such as nestin, are not restricted to neural tissues but are also expressed in a variety of mesodermal cell types [17, 60, 61]. Similar criticisms can be applied to the interpretation of MSC transdifferentiation toward the hepatogenic lineage in vitro. Indeed, the hepatic differentiation markers often employed (such as tyrosine aminotransferase, phosphoenolpyruvate carboxykinase, and liver-enriched transcription factors) are not "true" hepatocyte markers, given that they are also expressed in other somatic cells such as cells of the lung, intestine, pancreas, and kidney or are expressed by MSCs even before induction of differentiation [52, 59, 61–64].

In spite of such limitations, in vitro transdifferentiation of MSCs has been demonstrated repeatedly, and such studies have driven scientists to investigate the potential of MSCs to "transdifferentiate" in vivo after transplantation in animal models. For instance, Kopen and co-workers [65] were one of the first groups to demonstrate that MSCs isolated from BM, when injected into the central nervous systems (CNS) of newborn mice, were able to migrate throughout the forebrain and cerebellum without causing disruption to the host brain architecture. Some of these cells were shown to differentiate into astrocytes, as well as engrafting into neuron-rich regions, suggesting that neural differentiation had occurred. These results were subsequently confirmed by other groups in vivo [44, 66–68]. Similarly, transplantation of MSCs derived mostly from BM, and adipose tissue has been shown to result in engraftment in the heart and differentiation toward the cardiomyogenic lineage [69, 70], while it has been shown that MSCs isolated from different sources may also generate hepatocyte-like cells in vivo (reviewed in [59]).

Despite these promising studies, doubts have again been raised regarding interpretation of the positive differentiation results reported. Indeed, the level of engraftment observed in these studies is generally very low, and the differentiation achieved in vivo has not given rise to fully mature cells and is often poorly characterized. These limitations are likely due to problems with the cell delivery strategies adopted in these studies (i.e., local injection vs. intravenous/systemic administration), as well as to the often questionable analysis undertaken on the phenotype of the differentiated cells, and finally, to the fact that current tracking techniques for the study of engraftment and differentiation remain modest. Furthermore, some researchers suggest that the morphological and phenotypic changes observed in MSCs after transplantation are a result of fusion between donor cells and host cells, rather than true transdifferentiation [71–73].

Even so, MSC-based therapeutic approaches have been tested in a range of animal models of human diseases (followed also by testing in humans), for treatment of conditions such as myocardial infarction, brain and spinal cord traumatic injury, stroke, and fibrosis.

For instance, much effort has been dedicated to investigating whether MSC-based therapy may be beneficial for the treatment of myocardial infarction/ischemia and heart failure. Myocardial ischemia, whether acute or chronic, triggers a cascade of events leading to cellular injury or death, resulting in the sending of signals that cause the inflammatory phase which is characterized by macrophage and neutrophil infiltration, subsequently leading to scar formation, loss of structural integrity and cardiac mass, and ultimately ending, in severe cases, in congestive heart failure [70, 74]. Under these conditions, self-regeneration capacity is extremely limited [58, 70]. A large number of studies have been performed to test the feasibility of MSC-based treatments of such disorders, with the main aim of developing a "regeneration approach": i.e., whereby transplanted MSCs would engraft into host tissues and differentiate into new cells with cardiomyocyte-like features and functions, thereby correcting the heart failure through the replacement of dead resident cells. By the time the studies to test this hypothesis were conducted, MSCs were indeed thought to contribute to tissue function by means of differentiation and replacement mechanisms. Transplantation of MSCs into post-infarct animal models was shown to improve post-ischemic cardiac functions and trigger a reduction in infarct size and, in some cases, to decrease mortality [75–79]. However, one of the most intriguing observations was that the transplanted cells frequently produced functional improvement despite the small numbers of cells which were seen to be engrafted in recipient heart tissues. Furthermore, in many studies, the transplanted cells did not persist in the recipient animals in the long term, while in other reports, this factor was not even investigated. Meanwhile, some authors showed that the in situ differentiation of transplanted MSCs in the heart toward the

cardiomyocyte lineage was often incomplete, while in other cases, this was only partially characterized or not assessed at all [58, 70]. Iso and colleagues [80] reported a significant improvement in cardiac function and fibrosis after infusion of human MSCs into immunodeficient mice with acute myocardial infarction, despite the fact that no engrafted donor cells could be detected after 3 weeks postinjection. Furthermore, Dai et al. [81] found that allogeneic MSC transplantation into a rat myocardial infarction model resulted in an improvement of global left ventricular function at 4 weeks and that donor cells survived in the infarcted myocardium for up to 6 months, with expression of markers that suggest that the transplanted cells had differentiated toward muscle and endothelial phenotypes, although without fully adopting an adult cardiac phenotype, and not resulting in a visible replacement of scar tissue with sheets of muscle cells. Intriguingly, the time needed for the MSCs to differentiate toward the myogenic lineage was longer than expected, while the therapeutic effects of the injected MSCs were evident even before cells expressing cardiac-specific markers could be detected and within a time frame that was too short to reflect the occurrence of true regeneration. Therefore, the mechanisms whereby transplantation of MSCs improved cardiac function remained to be further investigated, but the authors suggested that a transient paracrine mechanism may have been at play. Strong support for paracrine actions of MSCs in cardiac repair have come from studies performed by Gnecchi and colleagues [82, 83]. In particular, these authors demonstrated that the administration of conditioned (and therefore cell-free) medium from MSCs overexpressing Akt-1 (a prosurvival gene) in a rat model of coronary occlusion resulted in a reduction in infarct size and cardiac apoptosis, possibly through the release of paracrine factors, such as VEGF (vascular endothelial growth factor), FGF-2 (fibroblast growth factor-2), HGF (hepatocyte growth factor), IGF-I (insulin-like growth factor-1), and TB4 (thymosin ß4) [83]. Since some of these factors could also have proangiogenic activities, their paracrine functions may have been responsible for inducing neovascularization in the injured heart [83, 84].

Our group has also recently hypothesized paracrine mechanisms to explain the observation that application of amniotic membrane fragments (known to also contain MSCs) onto infarcted rat hearts significantly reduces post-ischemic cardiac dimensional alterations and improves myocardial function for up to at least 60 days after ischemia [85]. Interestingly, in this study, no engraftment of amniotic cells was detected in host cardiac tissues, suggesting that the benefits observed may not have been related to engraftment of amniotic cells into the ischemic rat hearts, but more likely, due to release of soluble factors that may have modulated the ischemic inflammatory process, resulting in prolonged survival of host tissue cells.

However, it is important to keep in mind that "regeneration" and "repair" do not necessarily mutually exclude each other. For instance, Amado and colleagues [76] claimed that the cardiac improvements exerted by BM-derived MSCs in pigs with myocardial infarction might be the result of both mechanisms: transdifferentiation of transplanted MSCs toward the cardiomyocyte lineage (regeneration) and increased endogenous reparative mechanisms (repair), potentially through the release of factors such as VEGF, which is linked to both neoangiogenesis [86] and stem cell homing and migration [87].

MSC-based therapy has also been investigated for the treatment of several models of CNS diseases, affecting both the brain [traumatic brain injury and cerebral infarct (ischemic stroke)] and the spinal cord (traumatic spinal cord injury) [88]. Although it has been suggested, as reported above, that differentiation of MSCs into cells of neural lineage may occur both in vitro and in vivo, in most of these studies, regeneration through MSC transdifferentiation is, once again, unlikely to be the major mechanism underlying the observed functional recovery. Indeed, these studies reported that in general, few of the transplanted MSCs expressed astrocytic or neural markers, and these were far too few in number to provide cellular replacement. In particular, Mahmood and colleagues [89] found that MSC transplantation into a rat model of traumatic brain injury resulted in increased endogenous cell proliferation and improved functional recovery, with only few MSCs observed to express neural markers. Conversely, in more than one case, functional improvement was observed in association with an increase (either locally or in the cerebrospinal fluid) in the levels of soluble factors, such as the neurotrophic factors NGF (nerve growth factor), BDNF (brain-derived neurotrophic factor) [90, 91], GDNF (glial cell line-derived neurotrophic factor), activin A, TGFβ-1 (transforming growth factor-1), and TGFβ-2 [92]. Similarly, intravenous administration of MSCs into a rat model of stroke was shown to improve functional recovery, increase FGF-2 expression, reduce apoptosis, and promote endogenous cellular proliferation [93]. Although results regarding the use of MSCs for the treatment of spinal cord injury remain controversial, many studies have provided evidence that administration of these cells may also induce functional recovery in this scenario, even when only a low level of neural differentiation is documented [88].

MSC-based approaches have also been explored for liver disorders. Although much effort has been dedicated to testing whether cell therapy using MSCs could be used as a potential alternative to hepatocyte transplantation in order to cure metabolic and acute liver diseases, with debatable results obtained to date [59, 94], other authors have been prompted to investigate whether MSCs, mainly BM derived, could be used for the treatment of chronic liver disorders such as liver fibrosis. The results which have been obtained so far are controversial (i.e., reduction versus enhancement of fibrosis),

and open questions also remain regarding the mechanisms involved [95, 96]; however, at least two possible mechanisms have been proposed for the potential therapeutic function on liver fibrosis exerted by MSCs: one implies their ability to engraft into the liver and differentiate toward the hepatogenic lineage, therefore participating in the regeneration of the endogenous parenchyma; the other possible mechanism is related to the ability of MSCs to produce or activate paracrine mediators, such as IL-10 (interleukin-10), TNF-α (tumor necrosis factor-α), and HGF, which lead to reduction/modulation of fibrosis (for a review, see [96] and [97]). For instance, Parekkadan et al. [98] have shown in vitro that MSCs produce IL-10 and TNF-α, which may have inhibitory effects on proliferation of hepatic stellate cells (HSC) (one of the main sources of ECM-producing myofibroblasts) and collagen synthesis, while MSC-derived HGF was seen to be responsible for a marked induction of HSC apoptosis. In addition, Chang et al. [99] reported that after the injection of human BM-derived MSCs labeled with GFP into a rat model of liver fibrosis [carbon tetrachloride (CCl4) induced], liver fibrosis was significantly decreased, and the degree of fibrosis reduction paralleled the number of donor cells observed in liver sections. Although these authors reported dubious results regarding differentiation of MSCs into hepatocytes, they also suggested that the observed decrease in fibrosis could have been due to production by MSCs of matrix metalloproteinases (MMP) with anti-scarring activity, as well as HGF, which could have exerted anti-apoptotic effects, thereby increasing hepatocyte proliferation. Paracrine effects have also been hypothesized by Tsai et al. [100] to explain an observed reduction in liver fibrosis in the absence of differentiation of engrafted Wharton's jelly-derived cells, which had been transplanted into rats with (CCl4)-induced liver fibrosis, probably by inducing a reduction in the expression of profibrogenic TGF-β1 by biliary epithelial cells.

MSC-Based Therapy for Immune-Related Diseases

Numerous studies have demonstrated that MSCs are able to modulate the function of different immune cells in vitro, in particular T lymphocytes and antigen-presenting dendritic cells (DCs), which play a key role in the induction of immunity and tolerance. Indeed, MSCs are able to suppress T lymphocyte activation and proliferation in vitro. This inhibition affects the proliferation of T cells after stimulation by alloantigens [101–103], mitogens [104], as well as activation of T cells by CD3 and CD28 antibodies [103, 105]. Most studies in this regard have reported that MSCs exert their suppressive function by means of soluble factors such as TGF-β and HGF [104], prostaglandin E2 (PGE2) [103], and the tryptophan catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) [106]. MSCs may also modulate immune responses through the induction of regulatory T cells [107]. Moreover, MSCs have been demonstrated to interfere with differentiation, maturation, and function of antigen-presenting DCs, likely by means of soluble factors such as IL-6 (interleukin-6) and PGE2 [108–110]. Furthermore, MSCs may also modulate B-cell functions [111, 112] and affect the cytotoxic activity of natural killer (NK) cells by the inhibition of proliferation and cytokine secretion [110, 113].

Intriguingly, several studies suggest that MSCs may exert their immunoregulatory functions specifically at sites of inflammation. Indeed, it has been shown that when MSCs are delivered intravenously in animal models, they home preferentially to sites of inflammation, where they respond to signals from the surrounding microenvironment and perform local immunoregulatory actions (reviewed in [114]). This "homing" ability has been attributed to the expression of receptors for growth factors, chemokines, and extracellular matrix on the surface of MSCs, which mediate the migration of these cells to the injured site [115, 116].

The immunosuppressive properties of MSCs have been examined in a variety of animal models, as well as in clinical studies. Although the mechanisms involved are only partially known and still under study, they very likely involve both contact-dependent mechanisms and production of soluble factors. To date, MSC-based approaches have been investigated for the treatment of alloreactive immunity (to reduce or prevent graft rejection after cell and organ transplantation), autoimmunity (to ameliorate experimental autoimmune conditions, such as multiple sclerosis (MS) and Crohn's disease), and also tumor immunity.

In particular, significant studies in this field have been performed for the treatment of the graft-versus-host disease (GVHD), a life-threatening complication arising after allogeneic BM transplantation. In this condition, cells of the immune system, which are present in allogeneic donor BM, recognize the recipient's cells as foreign and attack them, with a high risk of mortality. Interestingly, Le Blanc and co-workers [117] demonstrated that the infusion of haploidentical MSCs into a patient with severe GVHD of the gut and liver resulted in rapid recovery from acute GVHD in the gastrointestinal tract and the liver. Furthermore, other clinical studies have also applied MSC-based treatments for patients with steroid-resistant, severe acute GVHD in a multicenter, phase II experimental study [118], whereby transplantation was performed using MSCs derived from the European Group for Blood and Marrow Transplantation's ex vivo expansion procedure. More than half of the enrolled patients with steroid-refractory acute GVHD responded to treatment with MSCs, and no patients showed any side effects either during or immediately after infusions of MSCs. Two years later, just over half of those patients with a complete response were still alive. Despite these promising results, little is known about the mechanisms

exploited by MSCs to induce such beneficial effects, partly due to the fact that few data are available concerning cell survival after transplantation. Indeed, most data derived from animals indicate short survival of MSCs after injection in vivo. Le Blanc and colleagues [117] suggested that the observed clinical benefits might not require sustained engraftment of many cells but could instead result from production of growth factors or temporary immunosuppression.

MSC-based approaches have also been tested with success in rodent models of diseases such as MS and diabetes, where immunomodulation is thought to be the main operative mechanism [119, 120]. For instance, transplantation of MSCs has been shown to ameliorate the conditions of mice affected by experimental autoimmune encephalomyelitis (EAE), a murine model of human MS, which is a chronic inflammatory multifocal demyelinating disease of the CNS that predominantly affects young adults [121–123]. Zappia and colleagues [121] demonstrated that injection of MSCs in EAE mice significantly reduced the clinical severity of EAE, with a decrease in CNS inflammation (suppression of effector T cells and induction of peripheral tolerance, decreased infiltration of the CNS by T cells, B cells, and macrophages), induction of T-cell anergy at the level of lymphoid organs where MSCs seemed to engraft, and reduction of demyelination both in the brain and spinal cord of treated mice. Recently, an MSC-based therapy (intrathecal injection of autologous MSCs) has been investigated for treating patients affected by MS based on the notion that MSCs can migrate locally into the areas of lesions, where they have the potential to support local neurogenesis and rebuilding of the affected myelin [124, 125].

MSCs are also very attractive candidates for the treatment of the amyotrophic lateral sclerosis (ALS), which represents another devastating and incurable neurodegenerative disease targeting motor neurons and their connections to muscle. Human MSC transplantation has been shown to extend survival, improve motor performance, and decrease neuroinflammation in the SOD1(G93A) mouse, a murine model of ALS [126]. MSC transplantation has also been tested in patients with ALS in two phase I clinical trials, demonstrating that this procedure was safe and well tolerated and might be applicable in future cell-based clinical trials for ALS; however, the lack of postmortem data prevents any definitive conclusions regarding the presence of the MSCs after transplantation to be drawn [127].

Potential Paracrine Effects of MSCs and the Biologically Active Molecules Involved

At this point in our discussion, it is perhaps worth summarizing the putative paracrine effects of MSCs and the corresponding molecules involved in these effects. To this end, we can refer to classification of the group of Caplan [20, 128] which proposes that the paracrine effects of MSCs should be divided into (*i*) anti-apoptotic effects, through reduction/inhibition of apoptosis of resident cells, therefore limiting the area of injury; (*ii*) anti-fibrotic and anti-scarring effects, by suppression of the inflammatory response, modulating protease activity, and production of extracellular matrix; (*iii*) angiogenic effects, through promotion of angiogenesis and restoration of blood flow around the damaged area; (*iv*) supportive effects, by stimulating proliferation and differentiation of endogenous stem/progenitor cells; and, finally, (*v*) immunomodulatory effects, through inhibition of the proliferation of CD8+ and CD4+ T lymphocytes and NK cells, suppression of immunoglobulin production by plasma cells, inhibition of maturation of DCs, and stimulation of regulatory T-cell proliferation.

Table 1.1 reports some of the molecules involved in these processes and relative examples, even though the roles of many of the bioactive molecules implicated remain to be validated. These molecules may have pleiotropic effects, and their collocation in one of the abovementioned groups of paracrine effects rather than another is not restrictive. Moreover, it has recently been proposed that, besides soluble factors, cell-derived microvesicles, consisting of proteins and lipids that may also contain nucleic acids (mRNA, miRNA, and DNA), might also represent a new mechanism of cell-to-cell communication through which paracrine effects may be exerted, with the transfer of signals and molecules from one cell to another even over long distances [143, 144].

Although classification of paracrine effects and molecules could help in our understanding of this complex area of investigation, we are still far from understanding all of the mechanisms and molecules involved. Moreover, it is important to underline the fact that the molecules and mechanisms proposed should not be seen as separate actors in the paracrine actions exerted by MSCs, but rather, these actions should be viewed as the results of a combination of factors and mechanisms that work in concert to modulate the molecular composition of the local tissue environment to evoke responses from resident cells.

MSC-Based Therapy and Clinical Trials

Interestingly, in the last decade, the number of clinical trials using MSCs to treat a wide range of damaged, diseased, or inflamed tissues has been rapidly increasing. Indeed, a quick search of the site www.clinicaltrials.gov using “mesenchymal stem cells” or “mesenchymal stromal cells” as a search query and selecting only “interventional studies” (studies where individuals are assigned to receive specific interventions) and returns more than 400 trials, all of which are aimed at

Table 1.1 Potential paracrine effects of MSCs and the biological active molecules involved

Paracrine effect	Molecule	Properties	Examples
Anti-apoptotic	VEGF	Member of the platelet-derived growth factor family	MSC-secreted VEGF decreases apoptosis of endothelial and tubular cells [129]
			Adipose tissue-derived MSCs secrete VEGF and prevents cardiomyocyte apoptosis [130]
	HGF	Multifunctional factor: mitogenic, motogenic, morphogenic, and anti-apoptotic	MSC-secreted HGF decreases apoptosis of endothelial and tubular cells [129]
			Adipose tissue-derived MSCs secrete HGF [131]
			MSC-secreted HGF may decrease apoptosis and increase hepatocyte proliferation [99]
	IGF-1	Insulin-like hormone	Adipose tissue-derived MSCs secrete IGF-1 and prevent cardiomyocyte apoptosis [130, 131]
Anti-fibrotic and anti-scarring	HGF	Multifunctional factor	HGF antagonizes the pro-fibrotic actions of TGF-β by intercepting Smad signal transduction [132]
			Adipose tissue-derived MSCs secrete HGF contributing to suppression of fibrogenesis [133]
	IL-10	Cytokine	MSC-secreted IL-10 may inhibit HSC proliferation and collagen synthesis [98]
	TNF-α	Cytokine	MSC-secreted TNF-α may modulate HSC proliferation and collagen synthesis [98]
	MMP	Zn(++)-endopeptidases able to degrade ECM	BM-MSCs express MMP resulting in a significant reduction in liver fibrosis [99]
Supportive	HGF	Multifunctional factor	MSCs-secreted HGF stimulates proliferation of surviving cells [129]
	LIF	Interleukin-6 class cytokine	BM-derived MSCs express LIF and support hematopoiesis in vitro [134]
	SCF	Cytokine that binds c-Kit	BM-derived MSCs express SCF and support hematopoiesis in vitro [134]
	IL-6	Cytokine	BM-derived MSCs express IL-6 and support hematopoiesis in vitro [134]
	M-CSF	Cytokine	BM-derived MSCs express M-CSF and support hematopoiesis in vitro [134]
	FGF-2	Member of the fibroblast growth factor family	MSCs increase FGF-2 expression and promote endogenous cellular proliferation after stroke [93]
Angiogenic	FGF-2	Member of the fibroblast growth factor family	FGF-2 promotes angiogenesis directly or indirectly, by upregulating VEGF [135]
			MSC-secreted FGF-2 enhances proliferation of endothelial and smooth muscle cells [136]
	VEGF	Member of the platelet-derived growth factor family	MSC-secreted VEGF enhances proliferation of endothelial cells [136]
			MSC transplantation induces VEGF and neovascularization in ischemic myocardium [137]
	MCP-1	Small cytokine of the CC chemokine family	MSCs secrete MCP-1 [138]
			Chemoattractant protein that helps the migration of endogenous stem cells to injured sites [135]
	IL-6	Cytokine	MSCs secrete IL-6 [138]
			IL-6 induces the expression of VEGF [139]
	Angiogenin	Heparin binding protein of the RNAse superfamily	Induces new blood vessel formation [140]
			Conditioned medium from MSCs contains angiogenin [138]
	PIGF	Member of the VEGF subfamily	PIGF promotes prenatal and postnatal angiogenesis [135]
			BM-MSCs secrete PIGF [136]
Immunomodulatory	PGE-2	Lipid compound of the prostanoid class of fatty acid derivatives	MSCs constitutively produce PGE2 [103]
			PGE-2 modulates the MSC effects on T cells and NK and DCs [110, 141]
	TGF-β	Cytokine	Mediator for suppression by MSCs of T-cell proliferation [104]
			Responsible for MSC-mediated inhibition of NK proliferation [141]
	HGF	Multifunctional factor	HGF mediates antiproliferative effects of MSCs on T cells [104]
	IDO	Immunomodulatory enzyme	IDO mediates suppression of T-cell proliferation by MSCs [106]
	iNOS	Member of nitric oxide synthases family	iNOS mediates suppression of T-cell proliferation by MSCs [142]

Abbreviations: *VEGF* vascular endothelial growth factor, *HGF* hepatocyte growth factor, *IGF-1* insulin-like growth factor-1, *IL-10* interleukin-10, *IL-6* interleukin-6, *TNF-α* tumor necrosis factor-α, *MMP* matrix metalloproteinases, *LIF* leukemia inhibitory factor, *SCF* stem cell factor, *M-CSF* macrophage colony-stimulating factor, *FGF-2* fibroblast growth factor-2, *MCP-1* monocyte chemoattractant protein-1, *PIGF* placenta growth factor, *PGE-2* prostaglandin E2, *TGF-β* transforming growth factor-β, *IDO* tryptophan catabolizing enzyme indoleamine 2,3-dioxygen, *iNOS* inducible nitric oxide synthase, *HSC* hepatic stellate cells, *ECM* extra cellular matrix

curing different types of conditions, from cardiovascular diseases to those affecting the kidneys, liver, and pancreas. Even more intriguing is the fact that, while some trials implicate the importance of MSC differentiation (i.e., for skeletal diseases), most of the trials seem to prevalently rely on the paracrine effects of MSCs rather than on their differentiation abilities, therefore highlighting this new possible repertoire for therapy (for an update, see [145]).

Little is known regarding the in vivo survival of MSCs after transplantation or their possible long-term adverse effects, such as ectopic tissue formation, malignant transformation, and immunogenicity. In this regard, Breitbach and co-workers have observed calcifications in the infarcted hearts of mice that had received local MSC treatment [146]. Meanwhile, although no in vivo transformation or tumor formation has been observed in MSC-treated patients, considering that most in vivo applications using MSCs are performed using in vitro cultured and expanded cells, we cannot exclude the possibility that such in vitro manipulation may negatively alter the characteristics of these cells and induce a malignant transformation in vivo [147–149]. Finally, although MSCs are considered to have absent or low immunogenicity, recent evidence indicate that, under appropriate conditions, these cells can function as antigen-presenting cells and activate immune responses, thereby eliciting their rejection [150–152]. Therefore, further controlled studies are required to address these concerns regarding the safety of MSC for development of cell therapy approaches.

Conclusions

From this *excursus* of the current literature in the field, it is evident that the range of potential applications of MSCs in cell-based therapeutic approaches has evolved and broadened to include not only their ability to replace cells through differentiation but also on their ability to secrete biologically active molecules that exert beneficial effects on other cells and on the microenvironment which they occupy [20, 153].

Although many of the observations from preclinical models that support the beneficial effects of MSC-based approaches represent something of a "jigsaw puzzle," with many pieces still to be put together, and despite the many gaps remaining in our understanding of the mechanisms involved and possible long-term consequences of MSC transplantation, scientists continue to pursue clinical experiences and to test innovative approaches using these cells.

Meanwhile, although the original optimism for application of MSCs for tissue regeneration (regenerative medicine) has decreased in recent years, we are now beginning to appreciate a new facet regarding the potential of these cells in the clinical arena, with the concept of reparative medicine versus regenerative medicine. While the application of MSCs along either of these two lines entails the employment of differing logics and the design of different therapeutic protocols, future studies will no doubt show the importance, to differing degrees, of both of these aspects in the development of MSC-based cell therapies for treating a wide range of human conditions.

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