Cell Therapy Against Cerebral Stroke

Comprehensive Reviews for Translational Researches and Clinical Trials

Kiyohiro Houkin
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Preface

Discouragement and Hope

My senior colleagues and I have opened the door to preventing and minimizing the occurrence of stroke by developing drugs such as antiplatelet agents, antihypertension medications, and many other drugs that suppress the risk factors of stroke. Consequently, we have successfully reduced the occurrence of stroke. It may sound somewhat pessimistic, but I believe that the reduction of the occurrence of stroke by these drugs has reached a plateau. In other words, stroke is still a leading cause of death and disability and will continue to be.

Quite unfortunately, in spite of intensive basic and clinical research, few treatment options exist once stroke occurs. That is, we cannot always favorably change the clinical course of patients with stroke. Only one pivotal change of strategy has taken place recently, which is the use of t-PA and mechanical thrombectomy (MT) with endovascular techniques. However, as is well known, the number of patients who are fortunate enough to receive t-PA and MT is limited.

Many promising drugs have demonstrated superb effectiveness in vitro and in vivo in reducing the damage due to stroke. However, most clinical trials unfortunately have proved to be failures. As a clinical neurosurgeon, I have been personally involved in many of these clinical trials. As can be imagined, I was overwhelmed by despair with the poor outcome of the trials. However, I am not always driven to despair in the innovation of stroke treatment using drugs.

Possibility of a Bright Future of Cell Therapy

It goes without saying for readers of this book who have a basic interest and knowledge of cell therapy that in the late 1990s cell biology developed and became a ray of hope in the treatment of intractable disease. Because the injured neural

tissue in the central nervous system (CNS) has limited regenerative capacity, the therapeutic potential of cell transplantation has been anticipated in various pathological CNS conditions – for example, traumatic spinal cord injury, traumatic brain injury, degenerative disease, demyelinating disease, and ischemic stroke. Especially, the growing accumulation of information and induced evidence suggest that cell therapy holds great potential as stroke therapy. In this book, the authors describe recent advances and perspectives in cell therapy against stroke. First, they report on the specific issues regarding various cell sources including bone marrow-derived mononuclear cells (BMMNCs; Suda), bone marrow stromal cells (BMSCs; Kuroda), neural stem cells/neuronal progenitor cells (NSCs/NPCs; Horie), and induced pluripotent stem cells/induced neuronal cells (iPSCs/iNCs; Abe et al.). Second, they report on specific issues having to do with the protocol in pre-clinical developments and early-stage clinical trials including cell culture (Ito et al.), cell delivery (Kawabori), scaffolding in cell therapy (Osanai), bio-imaging for cell tracking (Sugiyama et al.), and functional bio-imaging (Saito). Third, they review the previous clinical trials and the development of guidelines for cell therapy (Shichinohe), and their ongoing clinical trials (Kasahara and Honmou).

Pleasure in Overcoming the Hurdles

As can be imagined, once we open new doors, another hurdle appears. For example, such effects as dose-dependence in conventional pharmacokinetics cannot always be evaluated in the effects of cell therapy. We have to develop novel methods of evaluation of cell therapy. The many ethical problems to be resolved can also be imagined. In addition, the health cost issues associated with regenerative medicine is likely to be focused on in the future. Thus, the indication of the treatment is closely related to the balance between the cost and the improvement of the quality of life (QOL) from regenerative medicine. Because stroke often causes serious sequelae such as hemiparesis, aphasia, and dementia, direct medical expenses, nursing-care costs, and the social loss by deterioration of QOL of not only the patient but also the family can arise from those sequelae. On the other hand, large expenses are also anticipated for cell therapy against stroke because it is a common disease. We must consider the cost-effectiveness and the appropriate distribution of health resources based on our present health care system, otherwise it has no future.

Moreover, as I have mentioned, it is well known that regenerative medicine has various intrinsic bioethical issues, such as the need to destroy a fertilized egg when making an embryonic stem cell, cell transplantation for Parkinson's disease using cells derived from an aborted fetus, and the possibility of human cloning. Moreover, there are specific issues in the field of cell therapy for CNS disorders. Since the early 2000s, neuroethical issues have arisen along with the development of the neurosciences such as neuromodulation by deep brain stimulation (DBS) or brain–machine interface (BMI). In the future, cell therapy will be a major target of

neuroethical considerations – for example, issues regarding enhancement due to cell transplantation or the issues of chimerism with allogeneic or xenogeneic cell transplants in the CNS. We must pay serious attention to research ethics because of the specificity of our research field.

I hope that this book will contribute to the development of cell therapy and offer a platform for further discussion of cell therapy. Above all, we authors will be very happy if this book invokes the interest of many researchers and clinicians including those who have not committed to these fields to date. We are confident that cell therapy will develop as an indispensable option for the treatment of stroke and other intractable diseases.

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Part III Clinical Trials

Part I
Optimal Cell Cources

Chapter 1
Bone Marrow-Derived Mononuclear Cells

Satoshi Suda

Abstract Stem cell-based approaches have recently attracted much attention owing to their potential therapeutic effects in patients with stroke. Bone marrow-derived mononuclear cells (MNCs), a source of stem cells, contain populations of lymphocytes, mesenchymal and hematopoietic stem cells, and hematopoietic and endothelial progenitor cells. They can be rapidly harvested from the bone marrow, separated, isolated, and then returned back into the animal or human. Experimental studies have demonstrated that the beneficial effects of MNCs may occur due to neuroprotection, modulation of inflammation and the immune response, endogenous neurogenesis, arteriogenesis, and angiogenesis. Several clinical studies have shown the safety and efficacy of MNCs in patients with ischemic stroke. Therefore, MNC treatment is a potentially attractive candidate to promote stroke recovery. Further studies are required to develop therapeutic strategies for improved protection against stroke and optimal transplantation protocols, such as cell dose, timing, delivery route, patient selection (age, gender, comorbidities, stroke subtype, and location), and combination therapy.

Keywords Bone marrow-derived mononuclear cells • Stroke • Inflammation • Angiogenesis • Neurogenesis

1.1 Introduction

Stroke is the third leading cause of death worldwide and more likely to be associated with sequelae than cardiac events. Moreover, because stroke requires long-term rehabilitation and care, this condition is associated with socioeconomic problems, such as increased family burden and medical costs.

Cell-based therapy is actively being investigated as a new potential treatment for neurological disorders, including stroke. Various types of cells, including neural stem cells (NSCs), embryonic stem cells, mesenchymal stem cells (MSCs), and

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adipose stem cells, have been found to improve neurological outcomes in animal models of stroke [1–3]. Bone marrow-derived mononuclear cells (MNCs) are a heterogeneous group of cells composed of lymphoid cells, myeloid cells, and hematopoietic and mesenchymal stem cells. A number of animal studies have suggested that MNCs are a potential treatment for limb ischemia and myocardial infarction [4, 5]. MNCs have been also shown to improve outcomes in animal models of cerebral ischemia [6–8]. Moreover, MNCs may be beneficial as a type of cell therapy, particularly during the acute stage of stroke, because they can be rapidly harvested from the bone marrow and isolated for autologous transplantation. Recently, several clinical studies have shown the safety and efficacy of MNCs in patients with ischemic stroke [9, 10].

In this chapter, I summarize current experimental data regarding the use of MNCs in the treatment of stroke.

1.2 Protective Mechanisms of MNCs Against Stroke

The experimental rationale for the use of MNCs in stroke therapy includes a number of mechanisms of action, such as differentiation into cell types relevant to repair; modulation of local and systemic inflammation; promotion of arteriogenesis, angiogenesis, and endogenous neurogenesis; and secretion of neurotrophic factors from the acute phase to chronic phase (Fig. 1.1). MNC transplantation exerts protective effect against various types of brain damage, including models of focal cerebral ischemia, transient global cerebral ischemia, chronic cerebral ischemia, and intracerebral hemorrhage (Table 1.1). First, I discuss

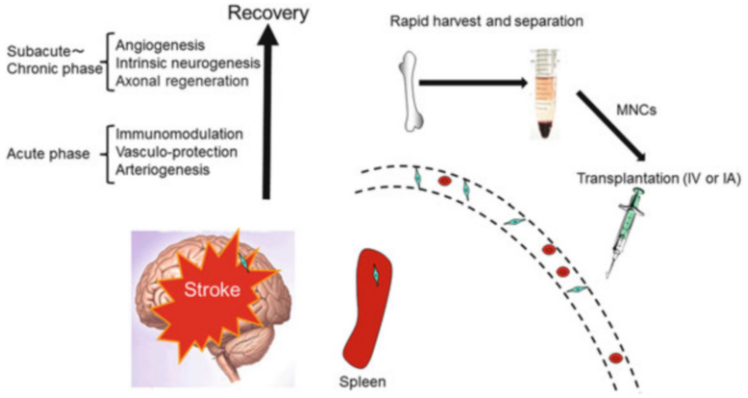

Fig. 1.1 Overview of the proposed mechanisms of bone marrow-derived mononuclear cell (MNC)-based stroke therapies. Cells transplanted by intravenous (IV) or intra-arterial (IA) injection migrate toward the ischemic boundary zone and spleen. These cells may rescue and repair the injured brain through inhibition of local and systemic inflammation and endothelial damage and the release of neuroprotective, neurotrophic, and angiogenic factors

Table 1.1 Effects of MNC transplantation in various cerebral injury models

Study	Model	Animal	Timing and route	Behavior test
Suda et al. [21]	Intracerebral hemorrhage	Male young and aged rats	24 h after onset (IV)	Staircase test, Morris water maze test
Coelho et al. [54]	Cortical ischemia	Young and middle-aged rats of both genders	24 h after onset (IV)	Cylinder test, adhesive test
Yang et al. [44]	Transient MCAO	Male young rats	24 h after onset (IV and IA)	Cylinder test, circling test, adhesive removal test
Bedi et al. [63]	Traumatic brain injury	Male young rats	72 h after onset (IV)	Morris water maze test
Brenneman et al. [14]	Transient MCAO	Male young and middle-aged rats	24 h after onset (IV)	Cylinder test, corner test
Fujita et al. [34]	Bilateral common carotid artery stenosis	Male young mice	24 h after onset (IV)	Not performed
Nakano-Doi et al. [41]	Permanent MCAO	Male young mice	48 h after onset (IV)	Not performed
Iihoshi et al. [6]	Transient MCAO	Male young rats	72 h after onset (IV)	Treadmill stress test, Morris water maze test

MCAO middle cerebral artery occlusion, *IV* intravenous, *IA* intra-arterial

the mechanism through which MNCs protect against stroke from the acute to chronic phase.

1.2.1 Protective Mechanisms of MNCs Against Acute-Phase Stroke

In a rodent middle cerebral artery occlusion (MCAO) model, postischemic inflammation events, such as microglial activation, neutrophil infiltration, and various pro-inflammatory cytokines, play a pivotal role in edema formation, infarct progression, and hemorrhagic transformation in the acute phase [11, 12]. Accordingly, in knockout mice or after pharmacological suppression of these inflammatory mediators, the extent of cell death and tissue damage after ischemia is decreased [13].

Bone marrow stromal cells and induced pluripotent stem cells require a period of cell culture before transplantation, whereas MNCs can be collected autologously just prior to administration. This should provide strong advantages in clinical use compared with other cell sources. Systemic transplantation of MNCs potently reduces neutrophil infiltration, microglia/macrophage activation, and inducible

nitric oxide synthase (iNOS) expression in the ischemic brain and reduces serum interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α) levels while increasing IL-10 levels after stroke [14–17]. The peripheral immune response, mediated by the spleen, is an important contributor to inflammation and enhances neurodegeneration after stroke in animals [18, 19]. Moreover, in humans, changes in spleen volume could be associated with the release of cellular components into the blood stream, which in turn may contribute to the postischemic inflammatory cascade [20]. A previous report demonstrated that systemic injection of NSCs during acute-stage hemorrhagic stroke decreases TNF-α and IL-6 mRNA levels and nuclear factor (NF)-kB protein expression in the spleen. In addition, a number of NSCs exhibit cell-to-cell contact with $CD11b^+$ spleen macrophages. Similarly, intravenous transplantation of MNCs results in accumulation of cells in the spleen, particularly in the marginal zone between the red and white pulp, partly internalized by marginal zone macrophages positive for ionized calcium-binding adapter molecule-1. Furthermore, gene expression analysis of spleen tissue has revealed significant increases in monocyte chemotactic protein-1 (MCP-1) and IL-10 [8, 21–23]. Thus, the spleen may be an important target for the development of stem cell therapies in acute stroke.

Because earlier cell transplantation may be more efficacious against spleen-induced inflammatory responses after stroke, MNCs should have advantages over other cell types. Recently, Bing et al. reported that ischemic stroke itself modulates the cytokine profile (IL-10, IL-6, MCP-1, vascular endothelial growth factor [VEGF], and TNF-α) of MNCs within the bone marrow, which may result from changes in specific cell subpopulations within MNCs. These results indicate that the application of autologous MNCs in patients with stroke may be more effective than pre-stroke or allogenic MNCs from healthy humans.

One of the most important facets of early neurovascular damage is manifested as perturbations in blood-brain barrier (BBB) function. BBB disruption leads to vasogenic edema and hemorrhagic transformation, which eventually exacerbates short and long-term disability. Protecting brain endothelial cells is critical for maintaining BBB function. MNCs suppress von Willebrand factor expression, a marker of endothelial injury, in the acute phase of ischemic stroke [16]. Moreover, some reports have shown that cell therapy protects against endothelial injury in a stroke model [24, 25]. Recently, we reported that MNCs confer protective effects against the injury of neurovascular units through inhibition of intracerebral hemorrhage-mediated upregulation of high-mobility group protein box-1, S100β, matrix metalloproteinase 9, and aquaporin 4. Thus, MNCs have protective effects against endothelial damage following stroke.

Interestingly, a recent report showed that MNCs reduce atherosclerotic plaque size and increase the collagen content of plaques through reduction of pro-inflammatory cytokines (IL-1β and TNF-α), matrix metalloproteinase 9 activity, and cleaved caspase-3 expression and upregulation of eNOS, antioxidant enzymes (glutathione peroxidase 1 and superoxide dismutase-1), insulin-like growth factor-1, and its receptor in a rabbit model of atherosclerosis [26]. Taken together, these findings suggest that MNCs modulate brain and systemic

inflammation, regulate the immune response, and protect against endovascular injury during the acute phase of ischemic brain injury.

1.2.2 Protective Mechanisms of MNCs Against Stroke from Subacute to Chronic Phase

1.2.2.1 Arteriogenesis and Angiogenesis

Many types of cell therapies do not solely target acute pathologic processes in neurological injury models. MNCs not only modulate inflammatory and immune-mediated responses but also promote repair processes in ischemic stroke from the subacute to chronic phase. Increased blood flow supply contributes to the delivery of glucose and oxygen, supporting brain tissues in order to promote recovery after stroke. MNCs increase vascular density and blood flow in various ischemic disorders, such as cardiovascular disease, peripheral arterial disease, and diabetic foot [27]. After cerebral ischemia, leptomeningeal anastomoses are the most important collateral pathways and could be a potential therapeutic target [28]. However, the spontaneous proliferation of collateral circulation cannot completely prevent the detrimental effects of vascular occlusion because arteriogenesis is slow and self-limiting [29]. Thus, stimulation of collateral growth and expansion could be another therapeutic target in the treatment of stroke [30].

Subpopulations of MNCs, such as $CD34^+$/M-$cadherin^+$ cells, can promote arteriogenesis and angiogenesis by differentiating into smooth muscle cells (SMCs) and endothelial cells (ECs) in ischemic hind limbs [31]. Some researchers have also demonstrated the neovascularization efficacy of MNCs in diabetic patients with critical limb ischemia [32]. Wang et al. reported that transplanted MNCs have the capacity to differentiate into SMCs and ECs after permanent MCAO in rats [33]. The differentiated cells exhibit enhanced arteriogenesis (particularly for leptomeningeal anastomoses) and angiogenesis by direct incorporation into the collateral vessel walls, providing powerful neuroprotective effects. These findings illustrate that MNCs have the capacity to differentiate into SMCs and ECs and are involved in the progression of arteriogenesis and angiogenesis, which may contribute to the restoration of blood flow in ischemic tissue.

In a mouse model of bilateral common carotid artery stenosis (BCAS), MNC treatment induces increase cerebral blood flow (CBF) through upregulation of endothelial nitric oxide synthase phosphorylation (Ser1177) from the early phase and the subsequent endogenous restorative response, including angiogenesis in the later phase. MNCs confer strong protection against BCAS-induced white matter damage, suggesting the potential clinical applicability of MNC treatment for subcortical ischemic vascular dementia [34]. However, in this experiment, MNC treatment did not show any evidence of direct structural incorporation of donor MNCs into ECs. Instead, donor MNCs with morphological features of pericytes were observed in the vessel walls. In another study, transdifferentiation of grafted

MNCs into cells with an endothelial phenotype was rarely observed (<1 %), as was the case in previous reports of cell transplantation by MSCs [35, 36]. These observations may indicate that MNC-induced angiogenesis largely results from the proliferation of endogenous ECs from the adjacent tissue and from circulating endothelial progenitor cells, rather than by angiogenesis derived from grafted MNCs. Although the exact mechanisms should be clarified in future studies, based on the abovementioned reports, MNCs promote arteriogenesis and angiogenesis and enhance recovery from various types of ischemic brain injury through upregulation of eNOS and VEGF, stimulation of endogenous EC proliferation, and promoting the direct differentiation into ECs and pericytes.

1.2.2.2 Endogenous and Transplanted Cell Neurogenesis

Another important aspect in brain repair is the migration of NSCs toward the damaged area. The regenerated neuroblasts may produce various factors that improve tissue integrity in the damaged brain. There may be some concordance between neurogenesis and functional improvement after brain injury [37, 38]. Moreover, NSCs residing in the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the hippocampal dentate gyrus are capable of producing new neurons in the adult brain. Increases in mitotic activity within the SVZ appear to peak between 7 and 10 days, subsequently decrease during weeks 3–5 after stroke, and thereafter continue for at least 4 months [39, 40]. Moreover, Nakano-Doi et al. found that NSCs develop in the poststroke area of the cortex in the adult murine brain [41].

Cell-based therapy induces both endogenous and grafted cell neurogenesis. Shichinohe et al. reported that approximately 20 % of transplanted MSCs express the neuronal marker NeuN in the infarct brain; however, only 1.4 % of the transplanted MNCs were found to express NeuN at 4 weeks after intracerebral transplantation in a permanent MCAO model [42]. However, MNCs stimulate the brain parenchyma cells to produce neurotrophic factors, such as fibroblast growth factor (FGF), brain-derived neurotrophic factor (BDNF), and VEGF, which activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway in NSCs to influence cell survival, proliferation, differentiation, and migration [15, 34, 43, 44]. Intravenous administration of MNCs can contribute to the proliferation of endogenous ischemia-induced NSCs through vascular niche regulation, which includes regulation of endothelial proliferation [41]. The authors speculated that enhanced endogenous neurogenesis may be attributable to increased angiogenesis and subsequent improved CBF. In another report, intravenous or intra-arterial transplantation with MNCs was shown to induce doublecortin-positive cells, a marker of neuroblasts, in the striatum and to upregulate GAP-43 expression, an important regulator of synaptic plasticity, in the ipsilateral cortex at 28 days after stroke [44, 45].

Together, these findings suggest that, although few MNCs directly differentiate into neuronal cells, which can replace injured central nervous system tissue, MNC treatment induces endogenous neurogenesis and synaptic plasticity through

production of various trophic factors, thereby activating the PI3K/Akt pathway after stroke.

1.2.3 Preclinical Studies of MNCs Efficacy Against Stroke and Future Directions

A systematic search of PubMed identified 20 preclinical studies of MNCs in the setting of experimental stroke. MNCs can be safely harvested from rodents and reinfused by intravenous or intra-arterial injection in a rodent stroke model. Moreover, the favorable effects of MNCs on functional recovery were robust across rodents, route of delivery (intravenous or intra-arterial), type of MNCs (allogenic or autologous), time of administration in relation to stroke (from 90 min to 3 days after stroke onset), and MNC dosage (from 1×10^6 to 3×10^7 cells). These findings indicate that, in animal models of stroke, MNCs exert therapeutic effects over a wide dose range, may be administered as late as 3 days after cerebral ischemia, and may be beneficial regardless of cell source or route of delivery.

However, most studies demonstrating the positive effects of MNC treatment in stroke have used healthy young animals. The Stroke Therapy Academia Industry Roundtable (STAIR) Committee has recommended that after initial studies demonstrate positive effects in healthy young animals, additional studies in aged animals and/or animals with comorbidities, such as hypertension and diabetes, should be performed if that is the intended population for clinical trials [46]. The aged brain has faster astroglial scar formation, increased microglial reactivity, and greater pro-inflammatory cytokine release, which impairs axonal growth and neuronal tissue recovery [47, 48]. Clinical and experimental results have indicated that hypertension and hyperglycemia are related to increase infarct volume and worse functional outcomes after stroke [49–52]. Furthermore, studies haves shown that gender may be also a relevant factor affecting injury, outcomes, and the effect of therapies, including thrombolysis [53]. Therefore, the inclusion of older animals, comparisons between genders, and comorbidities should be considered during experimental design in order to assess the actual potential efficacy of therapies for stroke. Several studies examining the treatment of brain ischemia with MNCs have included these factors. For example, intravenous transplantation with MNCs (3×10^7 cells) at 24 h after stroke has been shown to result in sensorimotor recovery of thermocoagulation-induced cortical ischemia in middle-aged male and female rats [54]. In another study, intra-arterial transplantation of autologous MNCs (4×10^6 cells) at 24 h after ischemia was shown to enhance recovery in focal ischemia in middle-aged rats [14]. Intravenous transplantation with MNCs (1×10^7 cells/kg) at 24 h after intracerebral hemorrhage (ICH) was shown to improve spatial learning, alleviate memory impairment, and reduce brain edema and atrophy. On the other hand, intravenous transplantation of MNCs (8×10^6 cells/kg) at 24 h after ischemia could not reduce functional deficits or ischemic lesion volume in aged hypertensive rats, regardless of the donor's age [55]. Because MNCs offer the

particular advantage of acute and autologous transplantability, age, gender, and comorbidities may influence both the patients' susceptibility to and the functionality of the MNC graft. More studies are needed to identify the impact of these factors when studying the efficacy of MNC transplantation for stroke.

Savitz et al. reported that intravenous infusion of autologous MNCs within 24 and 72 h after stroke may be effective compared with that of control stroke patients matched for age and National Institutes of Health Stroke Scale score [9]. Taguchi et al. reported that intravenous infusion of autologous MNCs within 7–10 days of stroke onset is a safe and feasible therapy, leading to improved functional recovery and increased cerebral blood flow and metabolism in patients with severe ischemic stroke [10]. On the other hand, a phase II, multicenter, randomized clinical trial demonstrated that intravenous infusion of autologous MNCs at median of 18.5 days after stroke onset is safe, but failed to show beneficial effects on clinical outcomes [56]. These clinical results suggest that earlier transplantation of MNCs from stroke onset may have greater effects on outcomes, consistent with previous experimental results [6, 16]. However, patients can deteriorate within the first few days after stroke [51, 57]. Therefore, strategies to expand the therapeutic time window for clinical application of MNCs are also important. Previous studies have shown that the effects of cell therapy may depend on the number of engrafted cells in the injured brain [24, 58]. Most grafted cells are thought to die within a few days of systemic administration [59]. Thus, it is imperative to identify therapies that can protect donor MNCs from this hostile microenvironment after stroke. We speculate that MNCs combined with appropriate pharmacological therapy, hypothermia, and/or rehabilitation may create a suitable microenvironment for transplanted MNCs and local cellular repair in the ischemic brain [60–62].

Understanding the influence of age, gender, comorbidities, and other variables on the therapeutic effects of MNCs after stroke will be important for identifying the characteristics of patients most likely to benefit from MNCs and for improving our understanding of the fundamental limitations of MNCs in the treatment of stroke. Furthermore, we must clarify the appropriate partners for cell-based therapies against stroke.

1.3 Conclusion

In conclusion, experimental studies have strongly suggested the therapeutic potential of MNC transplantation against various types of brain damage, including models of focal cerebral ischemia, transient global cerebral ischemia, chronic cerebral ischemia, and ICH. Currently, several clinical trials are examining the efficacy of using MNCs in the treatment of stroke. It is necessary to consider the failures of neuroprotective agents for acute stroke that have occurred within the past 30 years. Further translational studies are needed to establish optimal protocols in the clinical setting.

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Conflicts of Interest None.

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Chapter 2
Cell Therapy for Ischemic Stroke with Bone Marrow Stromal Cells

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Abstract In this article, the authors review recent advancements and perspective on cell therapy for ischemic stroke with bone marrow-derived cells, including bone marrow stromal cells (BMSCs) and multilineage-differentiating stress-enduring (Muse) cells. They can be easily isolated from the patients themselves and transplanted into them without any ethical and immunological problem. Animal experiments have shown that direct transplantation of these adult stem cells significantly enhances the recovery of motor function in various types of neurological disorders, including ischemic stroke. They aggressively migrate toward the damaged tissue and proliferate in the host brain. The BMSCs may contain heterogeneous subpopulations and contribute to functional recovery through multiple mechanisms, including neuroprotection, inflammatory modulation, cell fusion, and neural differentiation. On the other hands, Muse cells may promote functional recovery after ischemic stroke by reorganizing the infarct brain.

Keywords Bone marrow stromal cell • Cell therapy • Muse cell • Ischemic stroke • Transplantation

2.1 Introduction

There are few drugs to effectively rescue the patients with ischemic stroke in spite of the huge efforts to develop them for longer than 50 years [1]. As alternative approach, cell therapy has recently been expected as one of the promising strategies to enhance functional recovery after ischemic stroke. A variety of cells have been

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studied as the candidate donor cells for this purpose. These include embryonic stem (ES) cells, neural stem cells, induced pluripotent stem (iPS) cells, and bone marrow stromal cells (BMSCs). Of these, the BMSCs may have the most enormous therapeutic potential among them, because they can be obtained from the patients themselves and easily expanded without posing any ethical and immunological problems. The BMSCs are non-hematopoietic cells in the bone marrow and regulate the proliferation and differentiation of hematopoietic cells. The transplanted BMSCs significantly enhance functional recovery after the insults in animal models of ischemic stroke. On the other hands, recent studies have shown that the adult stem cells, including BMSCs, are observed in the peripheral blood and play an important role in repairing the injured tissues [2, 3].

Based on these observations, some of preliminary clinical testing has already been conducted to evaluate the safety and therapeutic effects of BMSC transplantation for the patients with both acute and chronic neurological disorders [4–10]. However, it should be reminded that a variety of questions or problems still remain to be solved in order to establish BMSC transplantation as scientifically proven entity in clinical situation [2]. This article reviews recent knowledge on therapeutic impacts of BMSC transplantation on ischemic stroke.

2.2 Basic Aspect of BMSC Transplantation for Ischemic Stroke

Recent studies have shed light on the mechanisms through which the transplanted BMSCs enhance functional recovery after cerebral infarct. They aggressively migrate toward the damaged lesions through some chemokines. Recently, CXCR4, a specific receptor for stromal cell-derived factor (SDF)-1α, is believed to play an important role in their migration in the CNS [11]. There are few studies whether the engrafted BMSCs retain their proliferative activity in the host brain or not. Yano et al. (2005) labeled the GFP-expressing BMSCs with a superparamagnetic iron oxide (SPIO) agent and transplanted into the ipsilateral striatum of the mice infarct brain. As the results, they found that the BMSCs actively proliferate, migrate toward the lesion, and partially express the neuronal phenotype in the host brain after transplantation [12].

Nowadays, the BMSCs are known to produce some neuroprotective or neurotrophic factors and support the survival of the host neural cells [13]. This hypothesis is readily reasonable because the BMSCs per se support the homing, proliferation, and differentiation of the hematopoietic cells in the bone marrow by producing a variety of cytokines [14]. The conditioned medium of BMSCs significantly promotes neurite outgrowth from the dorsal root ganglion [15]. They also release soluble neuroprotective factors, including nerve growth factor (NGF), hepatocyte growth factor (HGF), and brain-derived neurotrophic factor (BDNF), and significantly ameliorate glutamate-induced damage of neurons [16]. The

BMSCs markedly promote the neurite extension from the neurons in the organotypic slice of the brain and spinal cord [17, 18]. The BMSCs also protect the neurovascular integrity between basement membrane and astrocyte end-feet and ameliorate brain damage in stroke-prone spontaneous hypertensive rats [19]. Recently, Shichinohe et al. (2014) demonstrated that the BMSCs serve the "nursing effect" to the damaged neurons and activate the neural stem cells in the host brain by producing BDNF [20]. Therefore, the transplanted BMSCs trigger endogenous signaling pathways of survival and repair in neurons by secreting soluble neurotrophic factors.

Both neutrophils and macrophages are well known to play an important role in the early inflammation after cerebral infarct [21]. Indeed, their inflammatory response may be an essential process to clear cellular debris and initiate the healing pathways. Simultaneously, however, these inflammatory reactions may also give rise to cytotoxic damage to the surviving neurons, astrocytes, and endothelial cells in the peri-infarct area [21]. The BMSCs have currently been investigated as donor cells for novel cell therapy to prevent and to treat clinical disease associated with aberrant immune response. In the host, the BMSCs may attenuate pro-inflammatory cytokine and chemokine induction and reduce pro-inflammatory cell migration into sites of injury and infection [22]. Therefore, the transplanted BMSCs may prevent excessive inflammatory response and prevent further tissue damage in the peri-infarct area.

The BMSCs are believed to differentiate into neural cells in the host's brain. This theory is based on the findings that BMSCs simulate neuronal morphology and express the proteins specific for neurons in vitro [23, 24] or in vivo [25, 26]. It may sound strange that the BMSCs have the ability to differentiate into the neural cells. However, the BMSCs per se express the genes related to neuronal and glial cells [27]. Recent studies also show that the BMSCs can alter their gene expression profile in response to exogenous stimuli and increase the genes related to the neural cells [27–29]. Sanchez-Ramos et al. (2000) showed that a small fraction of BMSCs cultured in epidermal growth factor (EGF) or retinoic acid/BDNF expressed nestin, NeuN, or GFAP and that the proportion of NeuN-expressing cells increased when BMSCs were cocultured with fetal mouse midbrain neurons [23]. Wislet-Gendebien et al. (2005) cocultured the BMSCs with cerebellar granule cells and assessed their fates. They found that the nestin-expressing BMSCs express other neuronal markers and that BMSC-derived neuron-like cells fire single-action potentials in response to neurotransmitters such as glutamate [30]. Hokari et al. (2008) also demonstrated that a certain subpopulation of the BMSCs morphologically simulated the neuron and expressed the neuron-specific proteins without any evidence of cell fusion, when cocultured with the neurons [16]. These findings strongly suggest that at least a certain subpopulation of the BMSCs have the potential to alter their gene expression profile and to differentiate into the neural cells in response to the surrounding environment. More importantly, the findings indicate that only the subgroup of BMSCs with potential of neural differentiation can survive in the host brain for a long time (>4 weeks). In fact, the engrafted BMSCs express γ-aminobutyric acid (GABA) receptor and improve the binding

potential for ^{125}I-iomazenil in the peri-infarct area [31]. They also improve glucose metabolism in response to sensory stimuli when transplanted into the rat cold injury model [32]. Furthermore, ^{18}F-fluorodeoxyglucose (FDG) PET study has very recently shown that the BMSCs markedly improve the recovery of glucose metabolism in the peri-infarct neocortex, when stereotactically transplanted into the infarct brain at 7 days postischemia [33]. According to recent work by Liu et al., the BMSC may enhance the axonal sprouting from the survived cortical neurons in the peri-infarct area [34]. Furthermore, Chiba et al. have recently found that the BMSCs are integrated into the neural circuits of the host spinal cord and promote functional recovery [35]. These biological properties of BMSC may play a key role to enhance functional recovery after ischemic stroke.

Based on these observations, the exogenous transplantation of BMSCs is now believed to enhance functional recovery through multiple mechanisms, including nursing effect, anti-inflammatory action, and neural cell differentiation, in patients with ischemic stroke.

2.3 Translational Aspect of BMSC Transplantation for Ischemic Stroke

As described above, the observations in basic experiments are quite encouraging, and some clinical trials of BMSC transplantation have already been started for patients with ischemic stroke. Bang et al. intravenously injected the autologous BMSC into five patients with severe neurological deficits due to ischemic stroke at 5–9 weeks after the onset and concluded that autologous BMSC infusion is a feasible and safe therapy that may improve functional recovery [4]. Honmou et al. intravenously transplanted the BMSC into 12 patients with ischemic stroke 36–133 days post-stroke [36]. Very recently, Lee et al. performed an open-label, observer-blinded clinical trial of 52 patients with ischemic stroke and followed up them for up to 5 years. As the results, they concluded that intravenous transplantation of autologous BMSC could be safe and effective strategy for ischemic stroke [9]. These studies indicate that BMSC transplantation may be at least safe and feasible for patients with ischemic stroke.

However, there are many variables that may affect the efficacy of BMSC transplantation in clinical setting. Thus, they include donor cell factors (safety, autologous, or allogeneic, ex vivo cell expansion), patient factors (age, stroke type), treatment factors (interval since onset, delivery route, cell dose), and validation factors (neurological assessment, imaging) [37, 38].

First, allogeneic cells would permit "off-the-shelf" use even within 24 h after the onset, but force a long-term medication of immunosuppressant. Autologous BMSC from patients themselves would be ideal as the donor cells for restorative medicine, but require several weeks for ex vivo expansion. Therefore, it should be scientifically proven that the BMSC can enhance functional recovery after ischemic stroke

even when transplanted several weeks after the onset. More importantly, it would be critical to establish the feasible protocol to "safely and rapidly" expand the BMSC. Thus, the BMSCs have been cultured in the medium including fetal calf serum (FCS) in the majority of animal experiments and even clinical trials [4]. However, the FCS carries the potential risk of prion, viral, or zoonoses contamination. Alternatively, autologous serum is employed to expand the BMSC, but may require a large amount of serum [36]. Very recently, human platelet lysate (PL) is proven useful to expand the BMSC as the alternative substitute. The human BMSCs expanded with the FCS-free, PL-containing medium retain their capacity of migration, survival, and differentiation and significantly promote functional recovery when stereotactically transplanted into the infarct brain. Therefore, PL may be a clinically valuable and safe substitute for FCS in expanding the hBMSC to regenerate the infarct brain [39–41].

Second, the BMSCs are transplanted *within 24 h or 7 days* after the insults in the majority of animal studies, whereas they are usually transplanted several weeks (or even several months) after stroke onset in previous clinical trials [4, 9, 36]. In order to resolve this problem, pharmacological modulation may be useful to promote in vitro proliferation of the cultured BMSC to shorten the interval between stroke onset and cell therapy. For example, granulocyte colony-stimulating factor (G-CSF) significantly enhances the proliferation and growth factor production of the cultured BMSC and accelerates functional recovery by BMSC transplantation into the infarct brain [42]. Such pharmacological modulation would be essential in considering autologous stem cell therapy for older patients with ischemic stroke, because adult stem cells, including BMSC, suffer the effect of aging and reduce their self-renewal and differentiation capacity [43]. Very recent study has also demonstrated that G-CSF significantly promotes the proliferative capacity of BMSC harvested from the aged rats [44]. These observations should be taken into considerations when establishing the treatment protocol in clinical situation.

Third, the BMSC can be transplanted directly, intravenously, intra-arterially, or intrathecally. Although direct, intracerebral, or stereotactic injection permits most efficient delivery of the donor cells to the damaged tissue, less invasive procedure would be optimal. Intravenous or intrathecal transplantation is attractive because of its noninvasive, safe technique for the host CNS, but has been reported to result in less pronounced cell migration and functional recovery than direct cell transplantation [45]. Alternatively, the intra-arterial injection of BMSC may be valuable to noninvasively deliver them to the damaged CNS [46, 47]. Therefore, optimal transplantation technique should be developed to serve maximally safe and efficient results. However, there are limited numbers of studies that directly compare the therapeutic effects of these delivery routes under the same conditions. It is urgent issue that tests the effects of each delivery route on functional recovery after cerebral infarct. Recently, Kawabori et al. transplanted the BMSC into the infarct brain directly or intravenously at 7 days after the insult, that is, clinically relevant timing. As the results, they concluded that intravenous administration of BMSC has limited effectiveness at clinically relevant timing and intracerebral administration should be chosen for patients with ischemic stroke [48]. Furthermore, they directly

transplanted the BMSC (1×10^5 or 1×10^6) into the infarct brain at 1 or 4 weeks after the insult and found that earlier transplantation requires a smaller number of donor cells for beneficial effects [49]. These observations strongly suggest the importance of timing, delivery route, and cell dose to yield therapeutic effects of BMSC transplantation for ischemic stroke. Similar translational research should thoroughly be conducted to establish the scientifically proven protocol prior to the start of clinical testing.

Finally, it would be essential to develop the techniques to serially and noninvasively track the fate of the transplanted cells in the host CNS. Cell tracking technique would also be important as a "*biologically relevant end point*" [1]. Magnetic resonance (MR) imaging, nuclear imaging, and optical imaging are the candidate modalities. The donor cells can be identified on MR imaging by labeling with a superparamagnetic iron oxide (SPIO) agent [41, 50]. On the other hands, optical imaging technique may also serve future technology to visualize the BMSC engrafted in the damaged CNS. Quantum dot (QD) emits near-infrared (NIR) fluorescence with longer wavelength (800 nm) that can easily penetrate the living tissue. Very recent study has shown that the QD-labeled BMSC can be clearly visualized under in vivo fluorescence imaging through the skull and scalp for at least 8 weeks when transplanted into the infarct brain of rats [47, 51]. In addition, imaging technology would be valuable to assess the effects of BMSC transplantation on the function of host brain. ^{18}F-fluorodeoxyglucose (FDG) PET may be a useful tool to visualize the beneficial effects of BMSC transplantation for ischemic brain in clinical situation [32, 33]. Miyamoto et al. reported that direct BMSC transplantation improved glucose metabolism in the infarct brain, using micro-PET/CT apparatus. ^{123}I-iomazenil is a radioactive ligand selective for the central type of benzodiazepine receptor and is known useful to visualize the neuronal integrity on single photon emission computed tomography (SPECT), which is a more widely available apparatus in clinical situation than PET. Using ^{23}I-iomazenil SPECT, Saito et al. also reported that the BMSCs enhanced functional recovery by improving the neuronal integrity in the peri-infarct area, when directly transplanted into the infarct brain at clinically relevant timing [52].

Based on these 15-year observations in our laboratory, we are going to start a novel clinical trial of stereotactic BMSC transplantation for patients with ischemic stroke (RAINBOW trial). In this trial, we will harvest the bone marrow from the patients themselves and expand the *autologous* BMSCs within 1 month, using allogeneic PL without any animal proteins. Then, we will label the BMSC with SPIO agent and stereotactically inject the BMSCs into the brain adjacent to cerebral infarct. After BMSC transplantation, we will not only monitor neurologic function but also serially track the engrafted SPIO-labeled BMSCs, using MR imaging. ^{18}F-FDG will also be employed to serially visualize the effect of BMSC transplantation on glucose metabolism in the infarct brain. Preliminary data would be reported within 2 years.

2.4 Muse Cell Transplantation for Ischemic Stroke

Very recently, Dezawa and co-workers successfully isolated stress-tolerant adult human stem cells from cultured skin fibroblasts and BMSCs. These cells can self-renew, express a set of genes associated with pluripotency, and differentiate into endodermal, ectodermal, and mesodermal cells both in vitro and in vivo. When transplanted into immunodeficient mice by local or intravenous injection, they were integrated into damaged skin, muscle, or liver and differentiated into cytokeratin 14-, dystrophin-, or albumin-positive cells in the respective tissues. Furthermore, they can be efficiently isolated as SSEA-3-positive cells. Unlike authentic ES cells, their proliferation activity is not very high, and they do not form teratoma in immunodeficient mouse testes. The findings are quite attractive, because non-tumorigenic stem cells with the ability to generate the multiple cell types of the three germ layers can be obtained through easily accessible adult human mesenchymal cells without introducing exogenous genes [53]. These cells were named as multilineage-differentiating stress-enduring (Muse) cells. Furthermore, they have proven that Muse cells are a primary source of induced pluripotent stem (iPS) cells in human fibroblasts [54]. The results strongly suggest that a certain subpopulation of BMSCs may have the biological properties of neural differentiation and contribute to regenerate the infarct brain.

Recent studies strongly suggest the possibility of Muse cells as biologically powerful stem cells for patients with ischemic stroke. Thus, they can survive in the infarct brain, differentiate into the neurons, and promote the recovery of motor function when directly engrafted into the murine model of ischemic stroke at clinically relevant timing (7 days) after the insult. A majority of engrafted Muse cells express neuronal markers in the infarct brain at the peri-infarct area [55]. Interestingly, motor function starts to improve at 5 weeks after Muse cell transplantation, which indicate that Muse cell require about 1 month for their migration, differentiation, and integration into the host brain. Furthermore, Muse cells promptly committed to neural/neuronal lineage cells when cocultured with stroke brain slices in vitro and significantly improve motor function when directly injected into the rat brain subjected to middle cerebral artery occlusion at 2 days after the insult [56].

Therefore, Muse cells, the unique and promising adult stem cells, are expected available for application into clinical situation for ischemic stroke through further studies.

2.5 Conclusion

Direct transplantation of BMSCs/Muse cells may be one of promising strategies to promote functional recovery in patients with ischemic stroke in very near future. Further translational approaches would accelerate clinical application of cell therapy for ischemic stroke, using these bone marrow-derived cells.

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Chapter 3
Neural Stem Cells/Neuronal Progenitor Cells

Nobutaka Horie

Abstract Neural stem/progenitor cells (NSCs) are defined as cells with the potential for self-renewal and differentiation into neurons, astrocytes, and oligodendrocytes. These cells can be derived from several sources, including embryonic stem cells and fetal tissue. NSCs have been found to exist not only in the developing brain but also in the mature mammalian brain. NSCs were initially cultured as floating neurospheres in the presence of epidermal growth factor from adult and embryonic murine forebrain. Cell transplantation using these cells has evolved as a promising experimental treatment approach for stroke. Additionally, the activation of endogenous neural stem/progenitor cells has recently been employed for stroke treatment. This review provides an introduction to neural stem/progenitor cells and briefly describes some advances in neural stem cell transplantation for stroke.

Keywords Neural stem/progenitor cells • Neurosphere • Subventricular zone • Subgranular zone • Transplantation

3.1 Cell Biology

As initially observed by the pioneering neuroscientist Santiago Ramon y Cajal, the mature central nervous system (CNS) was thought to be distinguished from the developing nervous system by the lack of growth and cellular regeneration; it was believed that nerve paths were something fixed, ended, and immutable and had no regeneration potential in the adult CNS [1]. However, recent advances in neuroscience have revealed the falsehoods in this myth. In 1992, Reynolds, Weiss, and colleagues for the first time isolated neural stem cells (NSCs) and propagated them in the presence of epidermal growth factor (EGF) to give rise to large cellular spheres that they termed "neurospheres" [2, 3]. Neurons and glial cells are derived from common immature NSCs, which are defined as self-renewing and multipotential cells (Fig. 3.1). NSCs have been found to exist not only in the developing

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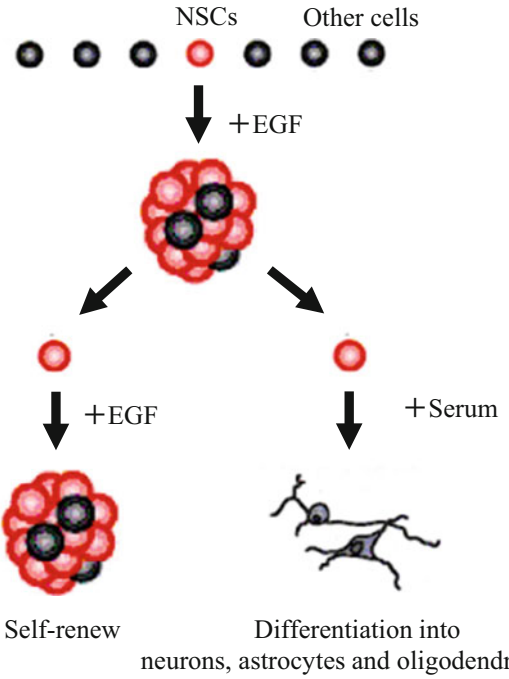

Fig. 3.1 NSC and neurosphere method

brain but also in the mature mammalian brain. Cultured NSCs derived from murine embryonic brains can be propagated by incubation in serum-free medium containing EGF and subsequently differentiated into neurons and astrocytes by incubating in low-serum medium (e.g., 1 % fetal bovine serum-containing medium without EGF) [4].

NSCs exist in at least two regions of the adult brain – the subventricular zone of the lateral ventricle and the subgranular zone of the hippocampus. Newborn neurons are incorporated into existing functional networks and are thought to have important innate and adaptive roles in cognition, behavior, and tissue repair [5]. Notch signaling, which is highly active in quiescent NSCs in these areas, plays a pivotal role in maintaining the undifferentiated and quiescent state of NSCs [6–8]. Interestingly, NSCs give rise to their own niche cells through asymmetric segregation of Notch ligand Delta-like 1 during mitosis, a process that may contribute to initialization of activated NSCs to return to a basal NSC state (undifferentiated and quiescent) [9]. Conversely, transcription factors including basic helix-loop-helix (bHLH) transcription factors regulate NSC proliferation and differentiation of each cell type [10]. Proneural bHLH genes, such as Ascl 1 (as Mash 1) and Neurogenin 2, promote neuronal fate determination and suppress astrocytic gene expression [11, 12]. The bHLH gene Olig 2 regulates oligodendrocyte specification, whereas the bHLH genes Hes 1 and Hes 5 maintain NSCs by repressing proneural gene expression [13, 14]. In addition, Ascl 1 and Olig 2 regulate oligodendrocyte and motor neuron development, respectively [13, 14]. A

recent report showed that oscillatory control of these factors determines NSC multipotency and fate [15].

NSCs first expand by rapid cell division to generate a large number of different types of neurons during the early stage of brain development. After this neurogenic period, NSCs mostly lose their neurogenic potential and begin to preferentially generate glial cells during postnatal stages (astrogenic phase). Early stage NSCs have a greater capacity to proliferate and self-renew than late-stage NSCs [16]. This suggests that NSCs lose their neurogenic potential during development, which might be a disadvantage for neuronal repair in adult CNS. Kishi et al. found that neocortical NSC chromatin becomes globally condensed in a stage-dependent manner and that high-mobility group A (HMGA) proteins, which are chromatin architectural proteins, are necessary for the open chromatin state in early stage NSCs [17]. They also found that reduced HMGA protein levels and resultant global chromatin condensation are involved in restriction of the NSC differentiation potential during neocortical development [17]. Thus, HMGA proteins are capable of reprogramming late-stage NSCs into cells with early stage-specific capacities.

Developmental studies and experimental data have enabled us to determine that the terminal cell differentiation state is reversible and that altering the balance of specific transcription factors could be a powerful strategy for inducing pluripotency [18]. It has recently been demonstrated that induced neural stem cells (iNSCs) can be obtained from rodent and human somatic cells, such as fibroblasts, through forced expression of defined transcription factors [Sox2, Klf4, and Myc (also known as c-Myc) and Pou3f4 (also known as Brn4)] [19]. To date, two different approaches have been successfully used to obtain iNSCs: a direct method and an indirect method that involves an intermediate destabilized state. The possibility to induce characterized iNSCs from human cells, e.g., fibroblasts, has opened new horizons for research in human disease modeling and cellular therapeutic applications in the neurological field [20].

3.2 Ischemia-Induced NSC Activation

In vitro studies have shown that hypoxia enhances proliferation of cultured NSCs and modifies the ability of the cells to differentiate [21–24]. Conversely, reduced glucose has been shown to suppress proliferation and increase differentiation of murine neural stem cells [25]. It is now well known that endogenous neurogenesis occurs in certain brain areas after cerebral ischemia, such as the subgranular zone of the dentate gyrus in the hippocampus [26], subventricular zone of the lateral ventricle in the striatum [27], and cortical layer [28]. Some evidence indicates that these neurons reestablish connections and contribute to functional recovery [29, 30]. These new neurons migrate into the impaired lesion, where they express markers of projection neurons. However, the majority of new neurons die during the first weeks after stroke and are only capable of replacing a small fraction of necrotic mature neurons [31]. Recently, electrical stimulation has been reported to

elicit NSC activation and strengthen intrinsic neurogenesis as well as chemical stimulation, which could be suitable for the clinical application to stroke, because it is well established and its potential complications are manageable [32].

3.3 NSC Transplantation for Stroke

3.3.1 Interaction Between Transplanted NSC and Host Brain

Transplantation of NSCs has been proposed as a promising therapeutic strategy in almost all neurological disorders, including Parkinson's disease [33], Huntington's disease [34], Alzheimer's disease [35], multiple sclerosis [36], amyotrophic lateral sclerosis [37], spinal cord injury [38], and ischemic stroke [39], which are characterized by the failure of CNS endogenous repair mechanisms to restore damaged tissue and rescue lost functions [40]. If the use of NSC transplantation is to be translated to clinical use, it is important to understand the mechanisms of action for improved recovery. The initial hypothesis assumed that NSCs would replace lost neurons and circuits. However, evidence for widespread afferent and efferent neuronal projections is lacking. NSCs prevent neuronal-programmed cell death and glial scar formation mainly via paracrine secretion of nerve growth factor, brain-derived neurotrophic factor, ciliary neurotrophic factor, and glial cell-derived neurotrophic factor. Recent preclinical data confirmed that transplanted NSCs may exert a "bystander" neuroprotective effect. Results also identified a series of molecules – immunomodulatory substances, neurotrophic growth factors, stem cell regulators, and guidance molecules secreted from NSCs, which are temporally and spatially orchestrated by environmental cues [41]. The bystander effect is a multistep process that depends on the timing of cell injection and route of cell transplantation [42]. Once injected, NSCs migrate and home to injured sites [43, 44], likely due to constitutively expressed chemokine receptors, such as CXCR4, cell adhesion molecules, and integrins, which allows the NSCs to follow chemoattractant gradients and reach damaged lesion sites [45]. Following migration to the injured areas, transplanted NSCs survive in close proximity to blood vessels (Fig. 3.2), where they interact with inflammatory cells, endothelial cells, astrocytes, and microglia. If the NSCs are transplanted into a non-injured brain, NSC migration does not occur [43, 44]. Conversely, NSCs have the potential to integrate into the injured brain after differentiation into appropriate cells. However, this remains undetermined and it is unclear whether this contributes to functional recovery. The major concern in utilizing these cells is the capacity of NSCs to form tumors, although tumorigenicity is less for fetal-derived NSCs than for embryonic-derived NSCs [46].

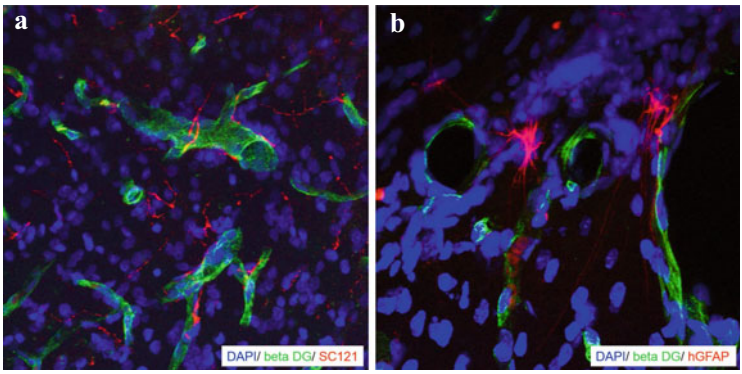

Fig. 3.2 NSCs survive in close proximity to blood vessels. Human NSCs (**a** *red*, SCS212) and NSC-derived astrocytes (**b** *red*, hGFAP) attached to vessels (*green*)

3.3.2 Endogenous Brain Repair After NSC Transplantation

3.3.2.1 Angiogenesis/Neovascularization

Transplanted NSCs migrate toward infarct lesions along existing vessels. Chemoattractants, such as stromal cell-derived factor-1 [45] and monocyte chemoattractant protein 1 [47], are reported to be critical factors associated with cell migration and homing to lesions, although the interaction between transplanted NSCs and existing vessels has not been fully elucidated. Nevertheless, the increased vascularization in the peri-infarct area after stroke is associated with functional recovery [48, 49]. Subacute NSC transplantation enhances neovascularization, and stem cell-induced vascular endothelial growth factor (VEGF) plays a critical role, as well as an anti-inflammatory effect [42]. Moreover, these vascular events correspond with two patterns of functional recovery: an early mode of recovery independent of neovascularization and delayed recovery that is NSC secreted and VEGF dependent and coincides with increased vascularization [42].

Transplanted NSCs upregulate expression of tight junction proteins, such as occludin, claudin 5, and Zo-1, and contribute to blood-barrier integrity by reducing leakage [42]. Although the functional role for neovessels has not been fully established, in addition to tissue perfusion, neovessels express trophic factors that remodel damaged tissues in the brain after ischemia, form new synapses, and attract endogenous neuroblasts originating in the subventricular zone [50].

3.3.2.2 Immunomodulation

Inflammation also plays an important role in ischemic stroke. Experimentally and clinically, the brain responds to ischemic injury with an acute and prolonged inflammatory process characterized by rapid activation of resident microglia,

production of proinflammatory mediators, and infiltration of various types of inflammatory cells into the ischemic brain tissue. However, these cellular events collaboratively contribute to secondary brain injury.

Interestingly, experimental stroke leads to splenic atrophy and spleen-derived, proinflammatory, monocyte, and macrophage mobilization into the circulation, as well as subsequent accumulation in the ischemic brain. The decreased splenic size inversely correlates with the extent of infarct volume [51, 52]. Therefore, removal of the spleen might be effective for reducing infarct volume after stroke.

Transplanted NSCs have an anti-inflammatory effect even after 2–3 weeks poststroke, and interestingly, this effect is associated with the development of neovessels [42]. Similarly to other stem cell types, NSCs exert immunomodulatory effects outside the brain upon systemic transplantation, occurring within secondary lymphoid organs [53]. NSC-secreted leukemia inhibitory factor inhibits differentiation of pathogenic Th17 cells through the extracellular signal-regulated MAP kinase suppression of the cytokine signaling 3 inhibitory signaling cascade that, in turn, antagonizes interleukin 6-mediated phosphorylation of signal transducer and activator of transcription 3, both of which are required for Th17 cell differentiation in peripheral lymphoid organs [54].

3.3.2.3 Axonal Sprouting, Dendritic Branching, and Synaptogenesis

Following ischemia, enhanced axonal sprouting takes place in the vicinity of the lesion, which extends from the intact cortex toward the deafferented cortical area [55, 56]. In rats, NSC grafts demonstrated increased corticocortical, corticostriatal, corticothalamic, and corticospinal axonal rewiring from the contralesional hemisphere, with transcallosal and corticospinal axonal sprouting correlating with functional recovery [57, 58]. Functional imaging has also shown similar remapping of the brain after stroke, indicating recruitment of both ipsi- and contralesional brain areas at least during the first few weeks following injury [59, 60].

Chronic changes in dendritic structural plasticity after stroke have also been reported with increased contralesional layer V dendritic branching peaking at 18 days poststroke, while ipsilesional layer III branching decreases at 9 weeks poststroke [61, 62]. NSCs enhance dendritic branching, length, and arborization at 3 weeks poststroke in layer V cortical neurons in both the ipsi- and contralesional cortex [57]. In vitro and in vivo studies have demonstrated that VEGF, thrombospondins 1 and 2, and slit act as mediators and are partially responsible for the NSC-induced effects on dendritic sprouting, axonal plasticity, and axonal transport [57, 63].

Some studies have shown that NSC transplantation enhances synaptophysin immunoreactivity in the ischemic boundary area after transplantation, suggesting that NSC transplantation enhances synaptogenesis [64–66]. Satisfactory functional recovery as a result of transplantation has been associated with increased expression of synaptogenesis markers [65]. Daadi et al. showed that NSCs increase expression of synaptic markers and enhance axonal reorganization in injured

areas at 4 weeks after transplantation [67]. This was also confirmed with initial patch-clamp recording [67] and electron microscopy [66].

3.3.3 Modification of NSC Grafts for Transplantation

One of the main problems with NSC transplantation is the massive graft cell death, which is possibly due to a hostile host brain environment and reduced the effectiveness of this approach. It has been reported that only 1–3 % of grafted cells survive in the ischemic brain after grafting [68, 69], mainly due to inflammatory responses in the host brain after ischemia. To address these issues, approaches to modify NSCs for longer survival have been proposed. Minocycline-preconditioned NSCs have been reported to tolerate oxidative stress after ischemic reperfusion injury and express higher levels of paracrine factors [70]. Genetic manipulation of NSCs to overexpress copper/zinc-superoxide dismutase (SOD1) was also reported to enhance graft survival in an animal model with intracerebral hemorrhage [71]. This strategy could be a highly effective approach, although its safety should be validated.

3.4 Activation of Endogenous Neural Stem/Progenitor Cells

Animal studies have demonstrated that stem cell transplantation reduces ischemic brain injury by increasing endogenous neurogenesis and angiogenesis [50, 72, 73], even in the aging brain. Functional recovery has also been achieved using cell transplantation therapy, and results show that transplanted NSCs influence the host brain by increasing endogenous striatal neurogenesis [50]. It is important to note that graft-evoked neurogenesis varies depending on graft location and stroke type [74]. Nevertheless, it remains unclear how much stroke-induced or transplanted NSC-induced neurogenesis contributes to recovery or endogenous angiogenesis, axonal sprouting, dendritic branching, and synaptogenesis.

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Chapter 4
iPS Cells and iN Cells

Toru Yamashita and Koji Abe

Abstract The discovery of iPS indicated that overexpression of master transcriptional factors might change cell fate. Recent developments in reprogramming methods have shown that somatic cells can be directly reprogrammed to various kinds of neuronal cells directly. Moreover, overexpression of a neuron-specific transcriptional factor with a viral vector can change the fate of endogenous glial cells to neuronal cells in vivo. In this chapter, we discuss the advantages, issues, and possibility for clinical application of these reprogramming methods for cell transplantation/replacement therapy.

Keywords Stroke • Cerebral ischemia • iPSCs • iNCs • iNSCs • In vivo direct reprogramming

4.1 Introduction

The number of elderly people is continuously increasing in the industrialized nations of the world, causing an increase in the number of patients that suffer from ischemic stroke. Stroke is the second leading cause of death in the world and results in a drastic reduction in the quality of life. On the other hand, effective therapeutic methods are currently very limited, especially in the chronic phase of a stroke; therefore, a novel therapeutic strategy for the chronic phase of a stroke is now urgently required. Recently, the discovery of ES and iPS seems to have opened the gate for stroke regenerative therapy. In addition, novel ways of inducing neuronal cells with direct reprogramming methods, such as induced neuronal stem cells (iNSCs) and induced neuronal cells (iNCs), have been reported (Fig. 4.1).

In this chapter, we briefly review recent progress of cell transplantation/replacement therapy with iPSCs/iNSCs/iNCs alongside our recent findings.

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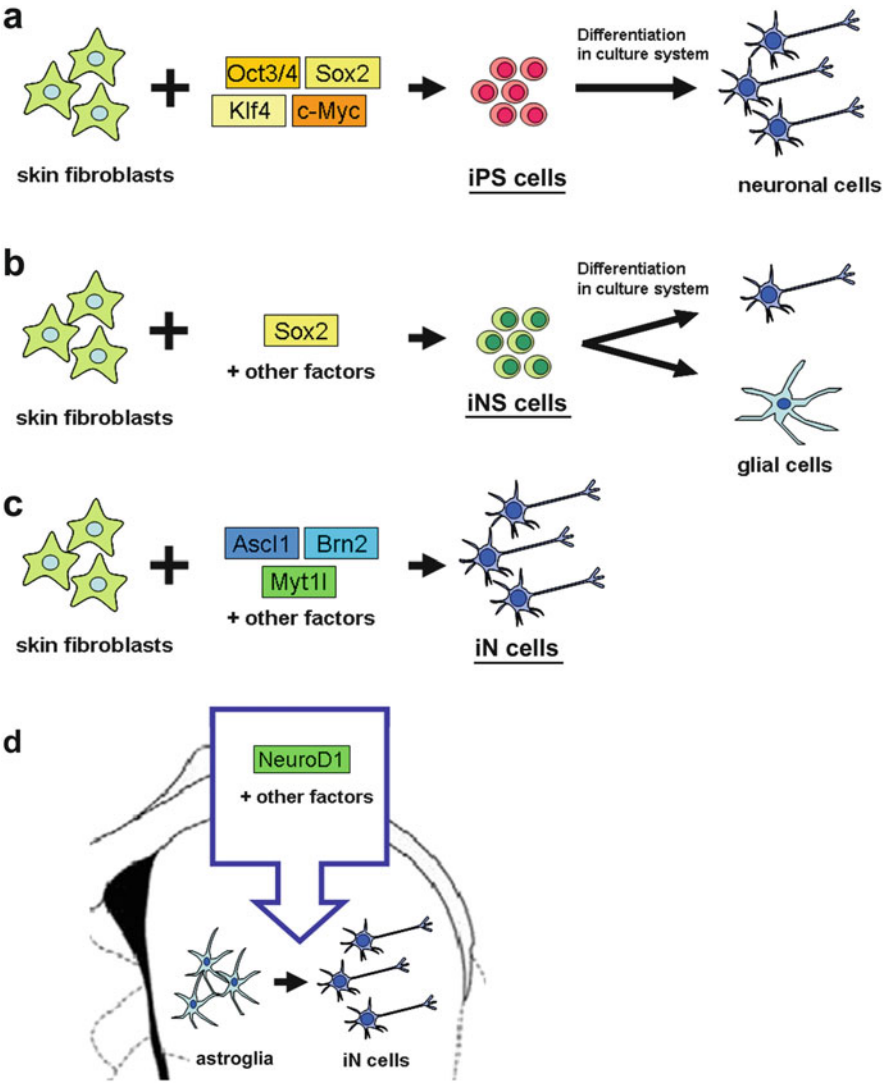

Fig. 4.1 Summary of induction of iPSCs, iNSCs, and iNCs and direct in vivo reprogramming (Modified from Yamashita et al. [28]). (**a**) Overexpression of Oct3/4, Sox2, Klf4, and c-Myc can convert somatic cells such as skin fibroblasts into iPSCs. Neuronal cells can be obtained after differentiation in the cell culture system. **(b)** Overexpression of Sox2 with other factors can convert skin fibroblasts into iNSCs. Both neuronal and glial lineages can be obtained from iNSCs. (**c**) The combination of Asc1, Brn2, and Myt1l with other factors can directly convert skin fibroblasts into iNCs (direct reprogramming methods). (**d**) Overexpression of NeuroD1 with other factors can convert endogenous glial cells into neuronal cells in vivo (in vivo direct reprogramming methods)

4.2 Therapeutic Effect of Transplantation of Human IPS Cells in an Animal Model

In 2006, Prof. Yamanaka first established murine iPSCs by overexpressing four transcriptional factors (Oct3/4, Sox2, c-Myc, and Klf4) in mouse fibroblasts. Of note, they found that these key transcription factors (TFs) from 20 candidates were strongly expressed in embryonic stem cells (ESCs) [1]. iPSCs can retain high replication competence and pluripotency and can differentiate into various kinds of cells, similar to ESCs, indicating that overexpression of key TFs can change cell fate. Since iPSCs can be produced from a patient's skin fibroblasts, there are no immunoreactive and/or ethical issues associated with ESCs. Therefore, iPSCs are believed to be a promising cell resource for cell transplantation/replacement therapy. Several scientific papers have demonstrated that human iPS-derived neuronal stem cells/neuronal progenitors, when transplanted into the stroke murine model brain, showed a therapeutic effect such as the recovery of motor function (Table 4.1). Notably, Oki et al. generated long-term self-renewing neuroepithelial-like stem cells from adult human fibroblast-derived iPSCs and transplanted them into the stroke mouse model. They found that motor function had already recovered by the first week after transplantation. They also confirmed that transplanted cells survived without forming tumors for at least 4 months. In their experiment, functional recovery was observed soon after cell transplantation, and the observed therapeutic effect was regarded to be derived from a neurotrophic effect caused by the release of transplanted cells [2].

4.3 Discovery of iN Cells

Some Japanese research groups have started or plan to conduct clinical transplantation therapy trials using iPS cells for age-related macular degeneration, spinal cord injury, and Parkinson disease [3]. However, iPS cells can form tumors, especially in pathological conditions such as poststroke [4]. In addition, it is likely to be difficult to monitor tumor formation for more than 2 years, even if iPS cells are transplanted into a mouse model. Therefore, a new technology and strategy to induce neuronal cells in damaged brains is required. Research findings using iPS suggest that master TFs regulating the overexpression of ES cells could convert fibroblasts to ES cell-like iPS cells. From this finding, many researchers have overexpressed neuron-specific TFs in skin/lung fibroblasts and tried to convert these fibroblasts into neuronal cells. In 2010, Wernig et al. first established murine-induced neuronal cells (iNCs) by introducing three neuron-specific TFs (Ascl1, Brn2, and Myt1l) into mouse fibroblasts. They found that these iNCs showed a glutamatergic neuronal phenotype with synapses and action potential, as recorded by electric patch-clump analysis [5]. Various kinds of iNCs, including dopaminergic neurons and motor neurons, have been reported (Table 4.2).

Table 4.1 Therapeutic effect of transplantation of iPS-derived neuronal cells in the ischemic stroke model

Original cells	Induced cells	Main findings	References
Human skin fibroblasts	Neuroepithelial-like stem cells	iPS-derived neuroepithelial-like stem cells were transplanted into poststroke striatum of MCAO mice 1 week after the induction of cerebral ischemia. Motor functional recovery was observed 1 week after cell transplantation. Authors found that part of transplanted cells survived for at least 4 months, showing that grafted cells exhibited electrophysiological properties of mature neurons and received synaptic input from host neurons	Oki et al. [2]
Human skin fibroblasts	Neuronal progenitor cells	iPS-derived neuronal progenitor cells were transplanted into poststroke striatum of MCAO mice 1 week after the induction of cerebral ischemia. Motor functional recovery was observed 6 weeks after cell transplantation. At this time, part of the grafted cells survived, expressing some neuronal markers	Gomi et al. [15]
Human skin fibroblasts	Neuroepithelial-like stem cells	iPS-derived neuroepithelial-like stem cells were transplanted into the poststroke cortex of MCAO rats 48 h after the induction of cerebral ischemia. Motor functional recovery was observed 5 months after cell transplantation. Authors confirmed that grafted cells exhibited electrophysiological properties of mature neurons and received synaptic input from host neurons	Tornero et al. [16]
Human skin fibroblasts	Neuronal progenitor cells	iPS-derived neuronal progenitor cells were transplanted into the poststroke striatum of MCAO mice 1 week after the induction of cerebral ischemia. Motor functional recovery was observed 2–3 weeks after cell transplantation. Authors found that part of transplanted cells survived at least for 1 month, showing that grafted cells express neuronal markers such as NeuN. At 6 and 12 months after cell transplantation, tumor formation was not detected	Mohamad et al. [17]

Interestingly, Ascl1 appears to be a key factor in the induction of iN cells, and the specific combination of Ascl1 plus other factors can convert somatic cells to specific neuronal cells. In cell transplantation therapy, it has already been reported that induced dopaminergic neurons showed a therapeutic effect against 6-hydroxydopamine (6-OHDA)-treated rats by attenuating the level of striatal dopamine [6]. iNCs can be produced without passing through the multipotent stem cell linage as iPS cells can be regarded as safer and easier to induce within a relatively short time frame, compared with iPS cells. However, the cell cycle of iN cells stops during cell conversion, making it difficult to prepare sufficient quantities of iNCs for cell transplantation therapy. To overcome this problem, induced

Table 4.2 Scientific reports showing direct reprogramming from fibroblasts to neuronal cells

Target cells	Original cells	Combination of transcriptional factors for reprogramming	References
Glutamatergic neurons	Mice fibroblasts	Ascl1, Brn2, Myt1	Vierbuchen et al. [5]
	Mice hepatocytes	Ascl1, Brn2, Myt1	Marro et al. [18]
	Human fibroblasts	Ascl1, Brn2, Myt1, NeuroD	Pang et al. [19]
	Human fibroblasts	Ascl1, Brn2, Myt1, Olig2, Zic1	Qiang et al. [20]
	Human fibroblasts	Ascl1, Myt1, NeuroD2, miR-9/9* and miR-124	Yoo et al. [21]
	Human fibroblasts	Brn2, Myt1, miR-124	Ambasudhan et al. [22]
Dopaminergic neurons	Mice/human fibroblasts	Ascl1, Lmx1a, Nurr1	Caiazzo et al. [23]
	Mice fibroblasts	Ascl1, Lmx1a, Nurr1, Pitx3, Foxa2, EN1	Kim et al. [6]
	Human fibroblasts	Ascl1, Brn2, Myt1, Lmx1a, FoxA2	Pfisterer et al. [24]
Motor neurons	Mice/human fibroblasts	Ascl1, Brn2, Myt1, NeuroD1, Lhx3, Hb9, Isl1, Ngn2	Son et al. [25]
Neural stem cells	Mice fibroblasts	Sox2, Brn2, FoxG1	Lujan et al. [26]
	Mice fibroblasts	Sox2, Brn4/Pou3f4, Klf4, c-Myc, E47/Tcf3	Han et al. [7]
	Mice/human fibroblasts	Sox2	Ring et al. [27]
Target cells	**Original cells**	**Combination of chemical compound for reprogramming**	**References**
Glutamatergic neurons	Mice fibroblasts	CHIR99021, forskolin, I-BET151, ISX9	Li et al. [14]
	Human fibroblasts	CHIR99021, forskolin, VPA, RepSox, SP600125, GO6983, Y-27632	Hu et al. [13]

Modified from Yamashita et al. [28]

neuronal stem cells (iNSCs) were developed. In 2012, Han et al. demonstrated that a combination of TFs (Sox2, Brn4, Klf4, c-Myc) successfully induced mouse fibroblasts directly to iNSCs [7]. Han and collaborators evaluated the therapeutic effect of cell transplantation using iNSCs in the spinal cord injury rat model. They found that engrafted iNSCs could differentiate into neuronal lineages forming synapses and enhancing the recovery of locomotor function [8]. iNSCs can thus be regarded as a promising cell resource for cell transplantation/replacement therapy.

4.4 Development of iN Cell Technology

Recently, novel technologies and new findings in the field of iNCs are reported every year. In particular, in vivo direct conversion technology and chemical-induced neuronal cells are attracting the most attention. In a clinical setting, the culture medium, including bovine/calf serum, can be problematic as they may be infectious materials in the human body. Thus, if endogenous non-neuronal cells such as astroglia can be converted to required neurons, in vivo direct conversion technology could be a new, simple, and straightforward way of supplying required new neuronal cells to the human brain. Thus far, astroglia as well as pericytes have been reported to be directly reprogrammed into neuronal cells in cell culture systems [9, 10]. In 2013, Torper et al. showed that endogenous mouse astroglia could be converted into NeuN-positive neuronal cells in vivo [11]. In 2014, Guo et al. reported that reactive glial cells in the cortex of the stab-injured mice model could be directly reprogrammed into functional neurons in vivo by overexpressing a single neural TF, NeuroD1 [12]. These findings suggested that in vivo direct reprogramming technology is a hopeful method of supplying required neurons for the human central nervous system.

In 2015, two different research teams published that chemical-induced neuronal cells could be established using a cocktail of chemical compounds including forskolin (a cyclic AMP agonist) and CHIR99021 (a glycogen synthase kinase 3 beta inhibitor) [13, 14]. In this method, mouse/human skin fibroblasts were successfully converted to neuronal cells without virus vectors overexpressing TFs, suggesting that the chemical cocktail can replace previously reported reprogramming TFs, leading to easier and more stable reprogramming methods that supply neuronal cells.

4.5 Concluding Remarks

This chapter briefly highlights recent progress in the development of iPSCs, iNCs, and iNSCs for cell transplantation therapy of damaged brains following an ischemic stroke. Clinical trials using iPSCs are ongoing, but it is important to combine these technologies or to choose appropriate strategies depending on the target disease.

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Part II
Translational Researches

Chapter 5
Cell Culture

Masaki Ito and Kiyohiro Houkin

Abstract Cell production under Good Manufacturing Practice (GMP) protocol is mandatory for the proper application of therapeutic cells in clinical settings. If cells are produced under GMP conditions, chemically defined conditions and a controlled environment would be ensured. However, such practices do not specify the use of animal-derived or xenogeneic recombinant supplements, which might raise some concern for clinical-grade cell preparations. At the very least, information of these materials should be provided to the patients treated with cell therapy to ensure proper understanding and informed assent. Therefore, in this chapter, the conventional cell culture methods employed for cell preparation (isolation, expansion, and/or derivation) are discussed, with a particular focus on each of the cell types employed in clinical trials of cell therapy against cerebral stroke.

Keywords Clinical application • Cell culture • Cerebral stroke • Cell transplantation

5.1 Introduction

Cell therapy shows potential to enhance functional recovery in patients with cerebral stroke; however, there is currently no pharmacological therapy to restore lost neurological functions, especially for patients in the subacute to chronic stages after the onset [1, 2]. Although a growing number of basic research studies have demonstrated cell therapies as attractive candidates for the treatment of stroke, several questions and problems remain to be addressed in order to scientifically

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translate the discoveries obtained from basic research into clinical settings. In fact, several leaders in the field in the USA, from both academia and industry, have organized a meeting of the Stem Cell Therapies as an Emerging Paradigm in Stroke (STEPS) and have repeatedly pointed out that design considerations for basic studies of cell therapy against cerebral stroke are important to most effectively and accurately translate the research into clinical practice [3–5]. In particular, these considerations include the following important elements of preclinical testing; cell characterization, appropriate species, type of stroke models, treatment protocols, imaging of cell tracking, host response, outcome measures, requirements for safety indices, and investigating the mechanisms of action. Among these factors, safety should be a priority to facilitate extensive use of cell therapy for patients with cerebral stroke, since cerebral stroke typically affects elderly patients with significant comorbidities, including atherosclerosis and histories of malignancy. In the STEPS guidelines, safety refers to tumorigenicity, immune sensitization, biodistribution, persistence of transplanted cells, and cell fate [4]. In addition, the quality control of cell production, including clinically relevant standards of cell culture methods, might also be an essential gateway to ensure the safety of stroke patients treated with cell therapy. Most of the cell types employed in recent clinical trials, including bone marrow stromal cells (BMSCs) and neural stem/progenitor cells (NSPCs), need to be isolated, expanded, and/or trans-differentiated ex vivo before transplantation [1, 6]. The growth of any type of mammalian cell in vitro requires growth media, extracellular matrices, and environmental factors. As Chen et al. recently reported, cell production under Good Manufacturing Practice (GMP) protocol is essential for the proper application of therapeutic cells in clinical settings for cell therapy [7]. Production of large numbers of functionally mature cells with high purity at a reasonable cost within a reasonable time period is also an essential component toward clinical application [7]. Without proper quality control and clinical-grade relevance for the cell preparations, the cell resources may contain impurities, which could result in reduced efficacy of the cell therapy, potential tumorigenicity of the cells during the course of cell engineering, as well as contamination of undesired components into the transplants. For example, undesired contamination of animal-derived components or chemicals in the medium or vehicle solutions should be avoided [6, 7]. In addition, even when cells are produced under a GMP condition, i.e., under chemically defined conditions in a controlled environment, this does not necessarily ensure control over animal-derived or xenogeneic recombinant products contained in the materials used for cell preparation, which may raise some concern in clinical-grade cell preparations. Ideally, animal-derived and xenogeneic recombinant product-free cell engineering would be the best. At the very least, information of such materials should be provided to the patients treated with cell therapy for proper understanding and informed assent.

In this chapter, the use of cell culture for the cell therapy against cerebral stroke will be addressed from a translational viewpoint. Among the cell resources discussed in Part I, mesenchymal stem cells (MSCs), NSPCs, and induced pluripotent stem cells and induced neurons (iPSCs/iN) must be expanded or trans-

differentiated ex vivo. On the other hand, bone marrow-derived mononuclear cells (BMMNCs) do not need to be cultivated. To organize this chapter from the viewpoint of translational research, recent clinical trials of cell therapy against cerebral stroke, including those already published as well as ongoing trials, will be discussed first with a particular focus on cell resources and cell culture methods. Then, the common cell culture methods employed for cell preparation (isolation, expansion, and/or derivation) will be discussed.

5.2 Cell Culture Methods Adopted in Clinical Trials of Cell Therapy Against Cerebral Stroke

To provide an overview of the current status regarding cell therapy against cerebral stroke in clinical settings, review articles that comprehensively addressed this theme were searched using the PubMed database. Then, all of the completed and published clinical trials regarding cell therapy against cerebral stroke conducted worldwide were collected from the most recent review articles published in 2014 or 2015 [1, 6, 8]. In addition, some online resources were also searched including "ClinicalTrials.gov: a service of the U.S. National Institutes of Health (https://clinicaltrials.gov)" and "UMIN Clinical Trials Registry: a service of a cooperative organization for national medical schools in Japan (http://www.umin.ac.jp/ctr/)," both of which are certified registries to the International Committee of Medical Journal Editors (ICMJE). Based on these registries, information of any unpublished but currently ongoing clinical trials (including those in the recruiting, not yet recruiting but starting soon, or closed recruiting but still active stages) was collected at the end of October 2015. One ongoing and currently recruiting trial being conducted in Japan was added subsequently, although this study is not listed in either of the abovementioned registries.

As shown in Table 5.1, up to the end of October 2015, there were 25 publications that reported the results of completed clinical trials regarding cell therapy against cerebral stroke [9–33]. The first such clinical trial was conducted by Kondziolka et al. from the University of Pittsburgh and was published in 2000 [10]. Five trials were conducted and published from India, which ranked highest in number of completed and published clinical trials worldwide [18, 23, 24, 29, 32]. Four trials were from Brazil [16, 17, 21, 25] and the USA [10, 12, 14, 20], respectively. Three were from China [26–28], two were from Japan [19, 33] and Korea [9, 11], and one study each was from Cuba [15], Russia [13], Spain [22], and Taiwan [31]. The conditions studied were ischemic stroke in the acute stage (approximately 1–2 weeks after the onset), subacute stage (within approximately 3 months), or chronic stage (approximately 3 months to several years), except for one study that evaluated the effects of cell therapy for acute intracerebral hemorrhage. The study subjects were all adults older than 20 years of age, including both sexes. Some authors reported that the cell dose was determined based on previous animal studies

Table 5.1 Published clinical trials regarding "Cell Therapy against Cerebral Stroke" by the end of October 2015

Author	Year	Conditions being studied		Study type		Cell transplantation procedures			
		Stroke type	Timing of delivery after stroke	Phase category	Location	Cell type	Cell source		Delivery route
Kondziolka et al.	2000	Ischemic	7–55 months	Phase1, NR/SB	USA	Predifferentiated neuronal cells, LBS neurons®	Allogeneic	NT2/D1 human precursor cell line	IC
Kondziolka et al.	2005	Ischemic	1–6 years	Phase 2, R/SB	USA	Predifferentiated neuronal cells, LBS neurons®	Allogeneic	NT2/D1 human precursor cell line	IC
Bang et al.	2005	Ischemic	5–9 and 7–11 weeks	Phase1/2, R	Korea	MSC	Autologous	Bone marrow	IV
Savitz et al.	2005	Ischemic	1.5–10 years	Phase 1, NR/OL	USA	Fetal lateral ganglionic eminence cells, LGE cells ®	Xenogeneic	Primordial porcine striatum	IC
Rabinovich et al.	2005	Ischemic or hemorrhagic	4–24 months	NR/OL	Russia	Nerve and hematopoietic hepatic cells	Allogeneic	Human fetus immature nervous and hematopoietic tissues	IT
Suárez-Monteagudo et al.	2009	Ischemic or hemorrhagic	3–8 years	Phase 1, NR/OL	Cuba	MNC	Autologous	Bone marrow	IC
Lee et al.	2010	Ischemic	Approximately within 7 and 9 weeks	Phase 2, R/SB	Korea	MSC	Autologous	Bone marrow	IV
Barbosa da Fonseca et al.	2010	Ischemic	62–82 days	Phase 1, NR/OL	Brazil	MNC	Autologous	Bone marrow	IA
Savitz et al.	2011	Ischemic	24–72 h	Phase 1, NR/OL	USA	MNC	Autologous	Bone marrow	IV

Honmou et al.	2011	Ischemic	36–133 days	Phase 1, NR/OL	Japan	MSC	Autologous	Bone marrow	IV
Battistella et al.	2011	Ischemic	59–82 days	Phase 1, NR/SB	Brazil	MNC	Autologous	Bone marrow	IA
Bhasin et al.	2011	Stroke	3 months to 1 year	NR	India	MSC	Autologous	Bone marrow	IV
Friedrich et al.	2012	Ischemic	3–10 days	Phase 1, NR/OL	Brazil	MNC	Autologous	Bone marrow	IA
Moniche et al.	2012	Ischemic	5–9 days	Phase1/2, NR/SB	Spain	MNC	Autologous	Bone marrow	IA
Prasad et al.	2012	Ischemic	8–29 days	Phase 1, NR/OL	India	MNC	Autologous	Bone marrow	IV
Rosado-de-Castro et al.	2013	Ischemic	19–89 days	Phase 1, NR/OL	Brazil	MNC	Autologous	Bone marrow	IA or IV
Bhasin et al.	2013	Ischemic or hemorrhagic	3 months to 2 years	Phase 1/2, NR/OL	India	MNC	Autologous	Bone marrow	IV
						MSC			
Jiang et al.	2013	Ischemic or hemorrhagic	11–50 days	Phase 1, NR/OL	China	MSC	Allogeneic	Umbilical cord	IA
Li et al.	2013	Hemorrhagic	5–7 days	Phase 1/2, NR/SB	China	MNC	Autologous	Bone marrow	IC
Wang et al.	2013	Ischemic	1–7 years	Phase 1, NR/OL	China	CD34+ positive cells	Autologous	Peripheral blood	IT
Prasad et al.	2014	Ischemic	18.5 days, median	Phase 2, R/SB	India	MNC	Autologous	Bone marrow	IV
Banerjee et al.	2014	Ischemic	Within 9 days	Phase 1/2, NR/OL	UK	CD34+ positive cells	Autologous	Bone marrow	IA
Chen et al.	2014	Ischemic	6 months to 5 years	Phase 2, R/SB	Taiwan	CD34+ positive cells	Autologous	Peripheral blood	IC

(continued)

Table 5.1 (continued)

Author	Year	Conditions being studied		Study type		Cell transplantation procedures			
		Stroke type	Timing of delivery after stroke	Phase category	Location	Cell type	Cell source		Delivery route
Sharma et al.	2014	Ischemic or hemorrhagic	4–144 months	Phase 1/2, NR/OL	India	MNC	Autologous	Bone marrow	IT
Taguchi et al.	2015	Ischemic	Within 10 days	Phase 1/2, NR/OL	Japan	MNC	Autologous	Bone marrow	IV

MSC mesenchymal stem cell, *MNC* mononuclear cell, *EPC* endothelial progenitor cell, *USA* United States of America, *UK* United Kingdom, *IV* intravenous administration, *IA* intra-arterial delivery, *IC* intracerebral delivery, *IT* intrathecal delivery

on a brain-weight, or body-mass, or mean-body-weight basis between human and rats. Some authors determined the cell dose based on other ongoing or previous clinical trials. Two trials employed allogeneic, pre-differentiated neuronal cells that were delivered directly into the brain of the stroke patient. One trial employed xenogeneic porcine brain cells that were directly transplanted into the brain of stroke patient. Thus, there are three trails that transplanted allo- or xenogeneic differentiated neuronal cells, but there are no published and completed trials employing neural stem/progenitor cells. Thirteen trials employed autologous BMMNCs. Of these, the cells were delivered directly into the brain in two trials, intra-arterially in five trials, intravenously in six trials, and intrathecally in one trial. On the other hand, five trials employed BMSCs. Of these, four trials delivered autologous BMSCs intravenously, and one trial delivered allogeneic BMSCs intra-arterially.

As shown in Table 5.2, there are 22 ongoing clinical trials regarding cell therapy against ischemic cerebral stroke being conducted all over the world, including the UK, France, Malaysia, China, Korea, the USA, Hong Kong, Spain, India, and Japan. Of these, 15 trials are currently recruiting participants and three trials are ongoing, but the recruitment of new participants was closed by the end of October 2015. The other four trials are expected to begin recruitment in the near future. The study subjects are all adults, including both sexes. The timing of cell delivery after stroke varies among these ongoing trials from the acute to chronic stages. Cell type also varies widely, including allogeneic neural stem cells (NSCs); autologous BMSCs; allogeneic MSCs derived from the bone marrow, umbilical cord, or adipose tissue; and autologous BMMNCs. One study is employing multipotent adult progenitor cells derived from allogeneic bone marrow. Some studies involve randomized, double-blind controlled trials to compare the safety, feasibility, and effect between intravenous transplantation of BMSCs or BMMNCs (or endothelial progenitor cells) and a placebo against ischemic cerebral stroke in the acute or subacute stage. The clinical trial phase category also varies from phase 1 to 3. Taken together, there are a total of 47 clinical trials worldwide that have been completed or are ongoing regarding cell therapy against cerebral stroke. The materials regarding cell culture, including the basic medium and supplements used for cell preparation, mainly depend on the cell type and timing of cell delivery. The following subsection will address this issue according to cell type.

5.2.1 Bone Marrow Stromal Cells

In the previous literature, “mesenchymal stromal cells,” “mesenchymal stem cells,” and “multipotent stem cells” are collectively abbreviated as “MSCs,” which are likely to lead some confusion. Similarly, “bone marrow stromal cells” and “bone marrow stem cells” are collectively abbreviated as “BMSCs.” In this subdivision, mesenchymal stromal cell is abbreviated as “MSC,” and bone marrow stromal cell is abbreviated as “BMSC” to avoid misunderstanding. According to a review article

Table 5.2 Ongoing clinical trials regarding "Cell Therapy against Cerebral Stroke" at the end of October 2015

	Conditions being studied		Study type					Cell transplantation procedures					
Clinical trial identifier	Stroke type	Timing of delivery after stroke	Phase category	Recruitment	Location	Start from	Last updated	Cell type	Cell source		Delivery route	Cell preparation	Published protocol
Allogeneic NSC													
NCT01151124	Ischemic	6–60 months	Phase 1, NR/OL	Ongoing but not recruiting	UK	2010/ Jun	2015/ May	NSC	Allogeneic	Human fetal brain cortical tissue	IC	Ex vivo derivation	Stem Cell Res Ther. 2014 Apr 11;5(2):49
NCT02117635	Ischemic	2–3 months	Phase 2, NR/OL	Recruiting	UK	2014/ Jun	2015/ Oct	NSC	Allogeneic	Human fetal brain cortical tissue	IC	n.d.	None
Autologous MSC													
NCT00875654	Ischemic	Less than 6 weeks of onset	Phase 2, R/OL	Ongoing but not recruiting	France	2010/ Aug	2015/ Feb	MSC	Autologous	Bone marrow	IV	n.d.	None
NCT01461720	Ischemic	2 weeks to 2 months	Phase 2, NR/SB	Recruiting	Malaysia	2012/ Mar	2015/ Mar	MSC	Autologous	Bone marrow	IV	Ex vivo expansion	None
NCT01714167	Ischemic or hemorrhagic	3–60 months	Phase 1, NR/OL	Recruiting	China	2012/ Jun	2015/ Jun	MSC	Autologous	Bone marrow	IC	n.d.	None
NCT01716481	Ischemic	Within 90 days of onset	Phase 3, R/OL	Recruiting	Korea	2012/ Nov	2014/ May	MSC	Autologous	Bone marrow	IV	Ex. vivo expansion	Trials. 2013 Oct 1;14:317
NCT02564328	Ischemic	6–60 months	Phase 1, R/SB	Recruiting	China	2014/ Nov	2015/ Sep	MSC	Autologous	Bone marrow	IV	n.d.	None
(Listed on clinical trials registry of Japan Medical Association as JMA-IIA00117)	Ischemic	Within 74 days	Phase 3, R/DB	Recruiting	Japan	2013/ Mar	2015/ Aug	MSC	Autologous	Bone marrow	IV	n.d.	Brain 134:1790–807, 2011
Allogeneic MSC													
NCT01297413	Ischemic	Over 6 months	Phase 1/2, NR/OL	Recruiting	USA	2011/ Feb	2015/ Sep	MSC	Allogeneic	Bone marrow	IV	n.d.	None

NCT01673932	Ischemic	6–60 months	Phase 1, R/OL	Recruiting	Hong Kong	2012/ Oct	2015/ Jul	MSC	Allogeneic	Umbilical cord blood	IC	Ex vivo expansion	None
NCT01678534	Ischemic	Within 2 weeks of onset	Phase 1/2, R/DB	Recruiting	Spain	2014/ Sep	2015/ Jan	MSC	Allogeneic	Adipose tissue	IV	Ex vivo expansion	J Stroke Cerebrovasc Dis. 2014 Nov-Dec;23 (10):2694–700
NCT02378974	Ischemic	Within 7 and 14 days	Phase1/2, R/DB	Recruiting	Korea	2015/ Feb	2015/ Mar	MSC	Allogeneic	Umbilical cord	IV	Ex vivo expansion	None
NCT01922908	Ischemic	3–10 days	Phase 1/2, R/DB	Not yet recruiting	USA	2015/ Jul	2015/ May	MSC	Allogeneic	Bone marrow	IV	Ex vivo expansion	None
NCT02448641	Ischemic	6–60 months	Phase 2, R/DB	Not yet recruiting	USA	2015/ Aug	2015/ May	MSC	Allogeneic	Modified human MSC line, SB623®	IC	Ex vivo expansion	Cell Therapy for Brain Injury. Springer International Publishing Switzerland; 2015. p.101
NCT01849887	Ischemic	24–72 h	Phase 1/2, R/DB	Not yet recruiting	USA	2017/ Jan	2014/ Dec	MSC	Allogeneic	Bone marrow	IV	Ex vivo expansion	None
Autologous MNC													
NCT02245698	Ischemic	Subacute/ chronic	Phase 1, NR/OL	Recruiting	India	2008/ Dec	2014/ Sep	MNC	Autologous	Bone marrow	IT	Ex vivo isolation	None
NCT01832428	Ischemic or hemorrhagic or other	N.D.	Phase1/2, NR/OL	Recruiting	India	2014/ Sep	2014/ Sep	MNC	Autologous	Bone marrow	IT	Ex vivo isolation	None
NCT02290483	Ischemic	1–7 days	Phase 2, R/OL	Recruiting	Spain	2015/ Apr	2015/ Jul	MNC	Autologous	Bone marrow	IA	Ex vivo isolation	None
NCT02178657	Ischemic	1–7 days	Phase 2, R/SB	Recruiting	Spain	2015/ Apr	2015/ Jul	MNC	Autologous	Bone marrow	IA	Ex vivo isolation	None

(continued)

Table 5.2 (continued)

Clinical trial identifier	Conditions being studied		Study type					Cell transplantation procedures					
	Stroke type	Timing of delivery after stroke	Phase category	Recruitment	Location	Start from	Last updated	Cell type	Cell source		Delivery route	Cell preparation	Published protocol
Allogeneic bone marrow-derived multipotent adult progenitor cells													
NCT01436487	Ischemic	1–2 days	Phase 2, R/DB	Ongoing but not recruiting	USA and UK	2011/ Oct	2015/ May	Multipotent adult progenitor cells	Allogeneic	Bone marrow-derived progenitor cell, MultiStem®	IV	Ex vivo expansion	Int J Stroke. 2014 Apr;9 (3):381–6
Comparative study between autologous MSC and MNC or EPC													
NCT01468064	Ischemic	Approximately 5 and 6 weeks	Phase 1/2, R/DB	Recruiting	China	2011/ Aug	2011/ Aug	MSC or EPC	Autologous	Bone marrow	IV	Ex vivo expansion	None
NCT00908856	Ischemic	Approximately 4 days for MNC and 23 days for MSC	Phase 1, R/DB	Not yet recruiting	USA	2016/ Jan	2014/ Dec	MSC or MNC	Autologous	Bone marrow	IV	Ex vivo isolation or expansion	Transfusion. 2009 Jul;49 (7):1471–81

R randomized, *NR* nonrandomized, *DB* double blind, *SB* single blind, *OL* open label, *USA* United States of America, *UK* United Kingdom, *NSC* neural stem cell, *MSC* mesenchymal stem cell, *MNC* mononuclear cell, *EPC* endothelial progenitor cell, *IV* intravenous administration, *IA* intra-arterial delivery, *IC* intracerebral delivery, *IT* intrathecal delivery, *n.d.* not described

by Charbord, the historical emergence of the concept of "mesenchymal stem cell" emerged in the 1960s [34]. Besides hematopoietic stem/progenitor cells, the bone marrow contains cells that form colonies consisting of plastic-adherent, elongated cells of fibroblastic appearance when cultured at low density in liquid medium containing serum. In 1991, Caplan first introduced the term "mesenchymal stem cell." To clarify the nomenclature for MSC, International Society for Cellular Therapy has proposed the term "multipotent mesenchymal stromal cell" in 2005. The accumulation of several years of solid and rigorous research indicates that MSCs are mesenchymal precursors with multipotency and self-renewal capacity that are present in the bone marrow of multiple species, including humans, as well as in other sources, including adipose tissue and the umbilical cord. MSCs can be extensively amplified ex vivo, which enables their use in cell therapy applications. Although the pluripotency of MSCs is somewhat controversial (criteria for differentiation need to be rigorously defined) [34, 35], MSCs, especially BMSCs, show several advantages (see Chap. 2) over other cell types as resources of cell therapy against cerebral stroke.

Regarding the cell culture of human BMSCs, previous basic research investigating human BMSC (hBMSC) therapy against cerebral stroke in animal models employed liquid culture media comprising a basic culture medium with supplements for cell growth [36–50]. Table 5.3 summarizes the basic culture medium and supplements used for ex vivo hBMSC expansion in these preclinical studies. Historically, animal serum (i.e., fetal bovine serum; FBS) or human serum was added to the basic medium, including Dulbecco's modified Eagle medium (DMEM) or alpha-minimal essential medium (αMEM) for ex vivo cell expansion. These basic media contain inorganic salts, amino acids, vitamins, nucleotides, glucose, and buffers. In most cases, antibiotic agents were also added. As previously discussed elsewhere, the use of FBS raises several concerns for preparations of clinical-grade hBMSC, including the potential for an immunologic reaction to the xenogeneic antigen and/or the potential risk for viral and prion contamination [51]. Animal or human serum has also been shown to have inconsistent lot-to-lot performance, which may cause variability in the cell expansion effect. To overcome the inconsistent performance associated with serum, the development of serum-free hBMSC culture medium has been warranted. Toward this end, human platelet lysate (PL) was recently tested for use in hBMSC expansion instead of FBS or autologous human serum. As a result, some researchers reported that autologous human PL was an efficient substitute for FBS in expanding hBMSCs [52, 53]. More recently, there have been attempts to establish another serum-free expansion system for hBMSCs. Chase et al. reported a serum-free medium containing xenogeneic components as a containing medium as a potential substitute for serum-containing medium in hBMSC expansion [54]. Thus, hBMSCs isolated and expanded in serum-free medium supplemented with recombinant human platelet-derived growth factor-BB (PDGF-BB), basic fibroblast growth factor (bFGF), and transforming growth factor-β1 (TGF-β1) were found to effectively retain their phenotypic, differentiation, and colony-forming potential. In addition, Yamauchi et al. reported that serum-free, allogeneic human PL-containing medium

Table 5.3 Past basic research regarding hBMSC therapy against cerebral stroke AND cell culture

		Animal		hBMSC culture		Cell transplantation	
Author	Year	Species	Stroke model	Basic medium	Supplement	Timing	Delivery route
Li et al.	2002	Rat	Transient MCAO	DMEM	FBS	1 day after MCAO	iv
Chen et al.	2003	Rat	Transient MCAO	DMEM	FBS	24 h after MCAO	iv
Kurozumi et al.	2004	Rat	Transient MCAO	DMEM	FBS	24 h after MCAO	IC
Kurozumi et al.	2005	Rat	Transient MCAO	DMEM	FBS	24 h after MCAO	IC
Honma et al.	2006	Rat	Transient MCAO	MSCBM	MSGS	12 h after MCAO	iv
Sasaki et al.	2011	African green monkey	Transient MCAO (2–4 h MCAO)	DMEM	Human serum and L-glutamine	1 h after reperfusion	iv
Steiner et al.	2012	Mouse	Transient MCAO	DMEM	FBS and L-glutamine	24 h after MCAO	iv
Mitkari et al.	2013	Rat	Transient MCAO	DMEM	Human platelet rich plasma	24 h after MCAO	ia
Ishizaka et al.	2013	Rat	Transient MCAO	DMEM	FBS and bFGF	1, 4, or 7 days after MCAO	ia
Onda et al.	2008	Rat	Permanent MCAO	MSCBM	MSGS	6 h after MCAO	iv
Omori et al.	2008	Rat	Permanent MCAO	MSCBM	MSGS	6–168 h after MCAO	iv
Toyama et al.	2009	Rat	Permanent MCAO	MSCBM	MSGS	6 h after MCAO	iv
Sugiyama et al.	2011	Rat	Permanent MCAO	αMEM	Human PL	7 days after MCAO	IC
Seyfried et al.	2006	Rat	Autologous whole blood injection (striatum)	n.d.	n.d.	1 day after ICH induction	iv
Seyfried et al.	2008	Rat	Autologous whole blood injection (striatum)	DMEM	FBS	1 day after ICH induction	ia

supplemented with granulocyte-colony stimulating factor (G-CSF) was safe and could accelerate the expansion of hBMSCs for cell therapy against cerebral infarct in rats [55]. Taken together, multiple culture media have been tested for hBMSC culture in basic research studies, including serum-containing media, serum-free and allogeneic human PL-containing media supplemented with or without G-CSF, and serum-free and xenogeneic recombinant human PDGF-BB-, bFGF-, and TGF-β-containing media.

In the completed and published clinical trials, autologous BMSCs were expanded ex vivo using a medium supplemented with FBS or autologous serum or using a serum-free medium supplemented with xenogeneic component as mentioned above (Table 5.4) [9, 11, 18, 19, 24]. It takes approximately 3–5 weeks to prepare approximately 0.5–1 $\times 10^8$ autologous hBMSCs for intravenous systemic cell delivery. In these phase 1 or 2 clinical trials, the safety and feasibility of the prepared hBMSCs were confirmed. In one trial employing human MSCs derived from allogeneic umbilical cords, the cells were expanded in FBS-containing media [26]. In this trial, the cells were provided from a cell bank, and there was no information provided as to the amount of time required for cell preparation after initiating harvest of the umbilical cord.

In the ongoing clinical trials focused on studying the safety, feasibility, and effect of hBMSC therapy against cerebral stroke, there is limited information regarding the hBMSC culture method provided (Table 5.4). For most of these studies, we could not obtain information from the literature reviews regarding how the investigators prepared the autologous or allogeneic hBMSCs. Thus, as shown in Table 5.2, there are 13 trials employing BMSCs or multipotent adult progenitor cells derived from autologous—or allogeneic—human bone marrow. Of these, only five of the ongoing studies have published their experimental protocols in advance. Two of these studies are employing autologous hBMSCs that are expanded using an autologous serum-containing medium [19, 56]. The other study is apparently employing FBS for ex vivo autologous hBMSCs expansion and using screened human serum albumin for ex vivo hBMSC cryopreservation [57]. Thus, these three studies are providing details on the hBMSC culture method. On the other hand, there are other two ongoing trials employing modified hBMSC lines. One study (NCT02448641) is using SB623®, an allogeneic cryopreserved hBMSC line transfected with a vector containing the Notch 1 intracellular domain [74]. Notch-induced human BMSC grafts were reported to reduce ischemic cell loss and ameliorate behavioral deficits in a chronic stroke animal model [58]. The other study (NCT01436487) is using another type of modified hBMSC which is allogeneic bone marrow-derived multipotent adult progenitor cells or MultiStem® [59]. Multipotent adult progenitor cells are known as an adherent population of adult stem cells, which are normally isolated from the bone marrow, and were originally characterized and described in 2002 [60]. Thus, the latter two studies published their study protocol, but did not provide information regarding culture media and growth media supplements. Taken together, in the clinical trials studying the safety, feasibility, and/or effect of hBMSC therapy against cerebral stroke with published protocol, hBMSCs are cultivated ex vivo using FBS-, autologous serum-,

Table 5.4 Completed or ongoing clinical hBMSC therapy trials against cerebral stroke with publication describing cell culture method

		hBMSC preparation				hBMSC transplantation	
Author	Year	Basic medium	Supplement for cell growth	Time periods for cell preparation	Transplanted cell number/patient	Delivery route	Cell source
Completed trials							
Bang et al.	2005	Low-glucose DMEM	10 % FBS	23–37 days	1×10^8	iv	Autologous
Lee et al.	2010	Low-glucose DMEM	10 % FBS	Approximately 5 weeks	1×10^8	iv	Autologous
Honmou et al.	2011	DMEM with 2 mM L-glutamine and 100 U/ml penicillin-streptomycin	10 % autologous serum	20.3 ± 7.1 days	$0.6–1.6 \times 10^8$	iv	Autologous
Bhasin et al.	2011	StemPro MSC SFM basal medium	Xenogeneic recombinant growth factors autologous serum	Around 23 ± 3 days	$50–60 \times 10^6$	iv	Autologous
Bhasin et al.	2013	StemPro MSC SFM basal medium	Xenogeneic recombinant growth factors autologous serum	23 ± 3 days	$50–60 \times 10^6$	iv	Autologous
Ongoing trials							
Kim et al.	2013	Low-glucose DMEM with 20 μg/ml gentamicin	10 % autologous serum	n.d.	1×10^6 cells/kg (maximum 1.2×10^8)	iv	Autologous
Honmou et al.	2011	DMEM with 2 mM L-glutamine and 100 U/ml penicillin-streptomycin	10 % autologous serum	n.d.	$0.6–1.6 \times 10^8$	iv	Autologous
Manley et al.	2015	n.d.	n.d.	n.d.		IC	Allogeneic (SB623®)
Hess et al.	2014	n.d.	n.d.	n.d.	4 or 12×10^6	iv	Allogeneic (MultiStem®)
Lane et al.	2009	DMEM	FBS	n.d.		iv	Autologous

or xenogeneic recombinant growth factor-containing medium and then transplanted intravenously or intracerebrally (Table 5.4). According to the best of published fact, it takes 3–5 weeks of expansion to obtain a sufficient number of cells for intravenous administration. Otherwise, allogeneic-derived modified hBMSC lines are employed; unfortunately, information on the detailed culture methods, including the culture media and supplements used, is hard to obtain for some reason. Thus, there is a paucity of detailed information regarding hBMSC culture methods even in human clinical trials, or at least there is a difficulty in obtaining this critical information, which should be corrected in the near future.

5.2.2 Neural Stem Cells

In the early 1990s, stem and progenitor cells in the adult mammalian central nervous system (CNS) were shown to be amenable to isolation, cultivation, and expansion [61, 62]. Thus, Reynolds and colleagues had first established a culture system, referred to as the neurosphere assay, that allowed for the isolation and expansion of cells derived from the embryonic and adult CNS that could retain the key capacity of stem cells for proliferation, self-renewal, and production of differentiated functional progeny, including neurons, astrocytes, and oligodendrocytes [61]. Since then, several studies have demonstrated that the fetal and adult human brain could also give rise to similar multipotent neurospheres [63, 64]. These discoveries have opened the door to the possibility of NSC therapy against cerebral stroke disability, especially in patients in the chronic stage. Thus, NSCs show innovative potential as an ideal cell type for cell replacement therapy for CNS injury (see, Chap. 3). Although this cell type is associated with a critical ethical issue with respect to cell source tissue acquisition, which requires resolution before clinical application, accumulating studies have described standardized and precise protocols for the culture and expansion of clinical-grade neural stem and precursor cells from human CNS tissue. Indeed, Reynolds and Deleyrolle edited the textbook *Neural Progenitor Cells: Methods and Protocols* in 2013, which provides practical techniques and protocols for producing neural stem and progenitor cells and highlights their promise toward NSC-based therapeutic applications for CNS disorders [75]. Therefore, in this section, the representative NSC lines employed in the ongoing clinical trials will be described. Then, practical techniques and protocols for the production of human NSCs or precursors under GMP conditions will be briefly summarized by focusing on the "clinical-grade" method described in the textbook.

As mentioned above, there are two ongoing clinical trials that are employing allogeneic NSCs as a cell source derived from human fetal brain cortical tissue (NCT01151124 and NCT02117635, Clinicaltrials.gov identifier). Stevanato and colleagues are studying the safety, feasibility, and effect of human NSC therapy against ischemic cerebral stroke. In brief, the investigators injected 2, 5, 10, or 20×10^6 cells directly into the damaged putamen region of 12 patients with chronic

unilateral ischemic stroke (6–60 months after the onset) affecting the subcortical white matter and/or basal ganglia (four dosage groups of three patients at each dosage level) as a phase 1 trial. Subsequently, a multicenter, open-label, single-arm phase 2 trial was initiated, which is now underway, in which a single dose of allogeneic human NSCs (20×10^6 cells) is administered to patients at 2–3 months postischemic stroke, in order to evaluate the safety, feasibility, and efficacy in patients with subacute stroke. They employed a genetically modified human NSC line (CTX0E03) that is derived from human fetal brain cortical tissue [65, 66]. Following genetic modification with a conditional immortalizing gene, c-mycER^{TAM}, a fusion protein is generated that stimulates cell proliferation in the presence of a synthetic drug, 4-hydroxy-tamoxifen (4-OHT). In the absence of growth factors and 4-OHT, the cells undergo growth arrest and differentiate into neurons and astrocytes. Fetal brain tissue was obtained following normal terminations and in accordance with nationally (the UK and/or USA) approved ethical and legal guidelines. Pollock K et al. described the precise methods used for this procedure in 2006 [65]. In brief, plasmid DNA encoding the myc-ER^{TAM} sequence was obtained, the sequence was cloned into the retroviral vector pLNCX-2, and an MMLV-type retrovirus encoding the myc-ER^{TAM} gene was generated. Primary cells were prepared by fine chopping the cortical region of the fetal brain followed by enzymatic dissociation. The cells were cultured on mouse-derived laminin-coated dishes in DMEM:F12 medium supplemented with human serum albumin, L-glutamine, human transferrin, putrescine dihydrochloride, human insulin, progesterone, sodium selenite, corticosterone, bFGF, and epidermal growth factor (EGF). They described this medium as "growth medium." At 50–60 % P0 confluency, the cells were infected by replacing the "growth medium" with a medium containing neat virus encoding the c-mycER^{TAM} gene, in the presence of polybrene. After the cells were exposed to the virus for 8–12 h, the virus-containing medium was replaced with fresh "growth medium" containing growth factors (bFGF and EGF) and 4-OHT. At 7–10 days post-infection, the cells were passaged (P1) and reseeded. The next day, individual dishes of cells were subjected to neomycin selection with a full medium change three times per week. Over the following 2–4 weeks, neomycin-resistant colonies emerged. Individual colonies were qualitatively identified to be positive with the c-mycER^{TAM} transgene and the neuroepithelial stem cell marker nestin by RT-PCR. Then, individual clones were expanded to reach five to ten million cells and frozen down as a seed stock of cells (CTX0E). Of these clones, the CTX0E03 line was exploited as the NSC source in the abovementioned clinical trials. The use of human fetal brain tissue, genetic modification of the transplanted cells, and animal and viral usage during cell production, even under GMP conditions, might raise some concern for future wide clinical use in patients with stroke. Nevertheless, the upcoming results of these NSC-based stroke therapy trials estimated to be completed between 2017 and 2023 must provide hope for disabled patients after stroke.

Aside from this NSC line that is already being employed in the clinical trials, there are several other methods being considered for clinical application. First, Steinberg and colleagues are developing a new NSC line, NR 1, which is derived

from human embryonic cell line H9 [67]. NR 1 is an allogeneic, non-genetically modified human NSC line with additional properties such as being euploid and amenable to large-scale production, which makes it an excellent candidate for clinical translation. Productive interaction with the Food and Drug Administration, including pharmacology and toxicology testing, along with further assay development is currently underway with the goal of formalizing their Investigational New Drug application. Their continuing efforts to develop this human stem cell product are supported with funding from the California Institute for Regenerative Medicine in the USA. This information was obtained from the Steinberg lab webpage (http://neurosurgery.stanford.edu/research/steinberg/translational.html), and regulatory aspects with respect to the manufacturing of safe cellular products for stroke cell therapy were further discussed in the literature [68]. Furthermore, Gelati and colleagues introduced some of the routinely used protocols into their GMP cell bank in Italy for the production of clinical-grade human NSC lines derived from the fetal human CNS [69]. Their protocol to isolate and expand human NSCs is fundamentally based on the neurosphere assay reported by Reynolds and colleagues [61]. In brief, primary cells were prepared by mechanical trituration of the neural tissue of human fetuses at 8–10 weeks gestation. The cells were seeded in preconditioned medium comprising DMEM/F12, bovine serum albumin, some hormones, and recombinant human growth factors (EGF, bFGF) and could proliferate to form a spherical cluster (neurosphere). Third, Siebzehnrubl et al. introduced a method for the isolation and culturing of adult human precursor cells derived from adult human brain specimens [70]. They reported that their technique could be applicable for both biopsy and autopsy specimens of a large number of brain regions, including the cortex, subventricular zone, hippocampus, and midbrain. Primary cells were prepared by mincing tissues followed by enzymatic dissociation using dispase II, DNAse I, and $MgSO_4$ dissolved in Hanks' balanced salt solution. Cells were plated in culture medium into laminin/poly-L-ornithine-coated culture dishes. The culture medium consists of N2 medium, FBS, and bovine pituitary extract supplemented with feeding solution containing recombinant human EGF, bFGF, leukemia inhibitory factor, and heparin. After 24–48 h of plating, the medium was replaced with fresh medium supplemented with the feeding solution. Subsequently, the feeding solution was added every day and the medium was changed once weekly until the cells reached confluence and adult human precursor cells were obtained. Finally, Bauchet et al. introduced a method to culture precursor cells from the adult human spinal cord donated from brain-dead patients who had agreed to be donors for organ transplantation [71]. A brain-dead patient with a beating heart met all of the clinical, biological, legal, and ethical criteria for organ donation according to the established guidelines of the French Biomedical Agency. The spinal cord was mechanically minced, weighed, and enzymatically dissociated using hyaluronidase, kynurenic acid, trypsin, and DNAse I for 30–50 min. After several steps to remove enzymes, neutralize the remaining trypsin, and filter the cellular suspension through a 100-μm cell strainer, the cells were resuspended in a sucrose solution and centrifuged for 30–40 min. After eliminating the top white layer (myelin) and the entire supernatant, the pellet was resuspended in a complete

medium. The medium comprised DMEM/F12 without glutamine but with glucose, $NaHCO_3$, pyruvate, N2 serum replacement, L-glutamine, insulin, ciprofloxacin, and gentamicin. Cells were seeded in poly-HEMA-coated flasks in the medium supplemented with EGF, fibroblast growth factor 2, and heparin. The neurospheres were typically observed after 2–4 weeks. Taken together, the culturing of human NSCs or precursor cells is basically performed using the conventional culture system known as the neurosphere assay. However, for some reason, the detailed protocol for the production of human NSCs, including xenogeneic animal-derived and/or recombinant product use or genetic modification of the cells, is difficult to obtain, possibly due to trade secrets. This might be a significant barrier for future wide propagation of NSC-based therapy against cerebral stroke.

5.2.3 Induced Pluripotent Stem Cell and Induced Neurons

As described in Sect. 5.2.2, NPCs and precursor cells show unique potential for replacement of the lost neuroglial tissue through their integration into the infarcted or peri-infarcted tissue; however, it remains a critical challenge to steadily obtain the cell source tissue. Thus, "clinical-grade" human NSCs can be isolated and expanded from the human fetal or adult brain or spinal cord and could otherwise be derived from a human embryonic stem cell line. However, ethical issues always accompany the acquisition and usage of human fetal tissues or embryos. In addition, the exploitation of healthy adult brain tissue is quite limited. Xenogeneic non-human primate tissue-derived NPCs might also be considered as an alternative source. However, this raises another concern, including the potential for viral and zoonose infection or allergic reaction. Thus, iPSCs generated from patients' own cells or from healthy human donor cells would be an ideal cell source to circumvent these difficulties. As reviewed by Malik and Rao, there are several methods for human iPSC derivation, including reprogramming by viruses with or without vector integration to the iPSC genome, nonviral reprogramming by plasma membrane-permeable bioactive proteins, mRNA transfection, microRNA infection/transfection, and insertion of PiggyBac or mobile genetic elements (transposons), minicircle vectors, or episomal plasmids [72]. These reprogramming methods can be classified based on the footprint of genetic modification, efficiency of iPSC deprivation, and number of somatic cell types known to be reprogrammed by the method. For example, viral reprogramming works well for many cell types, including fibroblasts and blood cells, and the reprogramming efficiency is high; however, viral vector sequences often integrate into the host cell genome. On the other hand, mRNA-based reprogramming methods work with zero footprint and good efficiency but are only effective in a few cell types. Sendai virus, an RNA virus, does not enter the nucleus, which allows for a zero-footprint reprogramming method, and has shown good efficiency for many cell types. However, there is still a disadvantage in Sendai-based reprogramming, in that it takes a long time for the virus to be completely lost from recently reprogrammed iPSCs. In addition to

these issues regarding reprogramming methods, there are other several issues to be overcome before clinical application of iPSC-based therapy for cerebral stroke. As Chen et al. stated, a xeno-free human iPSC culture is required for clinical application [7]. Thus, complete xeno-free derivation and maintenance of human iPSCs is required, including use of human-derived feeder cells and propagation/maintenance in xeno-free defined medium. This approach is time-consuming but is warranted for wide clinical application. In addition to these issues, there is an intrinsic disadvantage to the use of iPSCs, their high tumorigenesis potential, which needs to be excluded in cell transplantation therapy. Some interesting approaches have been proposed to circumvent this disadvantage. One idea is to convert somatic cells to various kinds of mature neuronal cells and NSCs without requiring iPSC fate (direct reprogramming, see Chap. 4) [73]. Another idea is the in situ reprogramming of reactive astrocytes into functional neurons after ischemic stroke. Taken together, iPSCs, induced neuronal cells, as well as NSCs show good potential for exploitation in clinical settings.

5.2.4 Bone Marrow-Derived Mononuclear Cell

As described in the introduction section, BMMNCs do not need to be cultivated ex vivo. Thus, this unique advantage of BMMNCs would allow them to be delivered almost immediately after the collection from patients without requiring expansion. Needless to say, this would eliminate any concerns mentioned for the other cell types regarding cell culture, including the use of xeno-derived material. However, as shown in Table 5.1, attention should be paid to the vehicle used for BMMNC delivery, including autologous serum and allogeneic human albumin. In addition, some studies have reported donor patients treated with consecutive G-CSF injections prior to bone marrow collection, which may be a burden to stroke patients at the acute phase [28, 31, 32].

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Chapter 6
Route, Cell Dose, and Timing

Masahito Kawabori

Abstract Cell transplantation therapy has been expected as one of the novel therapeutic strategies. However, there still exist several fundamental problems to be solved prior to clinical application of stem cell transplantation, such as optimal cell types, transplantation routes, cell dose, and transplantation timing. It is quite important to determine the most desirable and the maximal therapeutic effects of transplantation methods prior to clinical application of cell-based therapy, but there are not so many studies that scientifically determine the most favorable protocol even in animal experiments. Here, we will review and summarize the current experimental results focusing on the unsolved questions, optimal transplantation route, transplantation cell dose, and transplantation timings.

Keywords Stem cell therapy • Ischemic stroke • Transplantation route • Cell dose • Timing

6.1 Transplantation Route, Cell Dose, and Timing; Unsolved Questions

Cell transplantation therapy has been expected as one of the novel therapeutic strategies [1]. Various cell types including embryonic stem (ES) cells, induced pluripotent stem (iPS) cells, neural progenitor cells (NPC), umbilical cord blood stem cells (UCSC), adipose-derived stem cell (ADSC), bone marrow-derived mononuclear cells (BMMC), and bone marrow-derived stromal cells (BMSC) have been considered as candidates for the source of cell transplantation therapy. However, as also pointed out by one of the opinion leaders in this field, stem cell therapies as an emerging paradigm in stroke (STEPS) participants and the stroke therapy academic industry round table (STAIR), there still exist several problems to be solved prior to clinical application of stem cell transplantation [2–4]. These problems are optimal cell types, transplantation routes, cell dose, and

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transplantation timing. It is quite important to determine the most desirable and the maximal therapeutic effects of transplantation methods prior to clinical application of cell-based therapy, but there are not so many studies that scientifically determine the most favorable protocol [5–8]. In this section, the authors will summarize the current experimental results focusing on the unsolved questions, optimal transplantation route, dose, and timing.

6.2 Transplantation Route

Transplantation routes mainly examined in the experimental stem cell transplantation in the past are intravenous, intra-arterial, intracerebral, intraventricular, and intranasal (Fig. 6.1). In this section, we will review the different transplantation route and its strength and weakness and then review the literatures that are focused on comparison of different transplantation routes by both acute and chronic stage of ischemic stroke, since the previous reports have focused on transplantation route at either acute or chronic (including subacute) stage of ischemic stroke and it seems there are quite a difference in results between these stages.

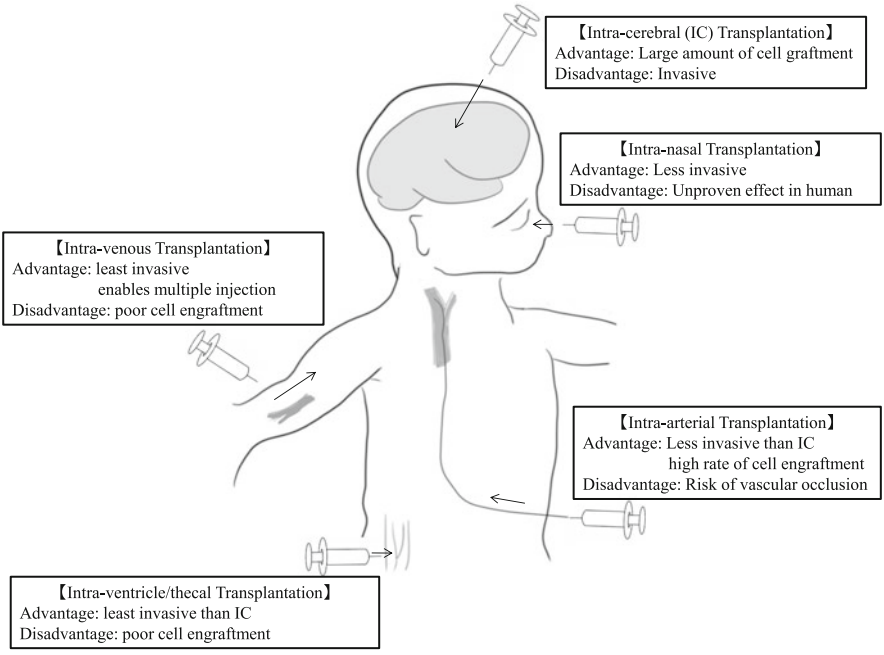

Fig. 6.1 Schematic drawing of the transplantation route with its advantage and disadvantage

6.2.1 Intravenous Route

One of the major advantages of using intravenous route for cell transplantation is its simplicity of administration with minimal invasiveness. Most of the cell transplantation in experimental research is conducted within 24 h from ischemic insult with favorable results; however, there are several reports showing that transplantation was also effective even 4 weeks after ischemia [9]. It is quite interesting that intravenously transplanted cells work not only by penetrating through blood-brain barrier (BBB) and settled in the brain but also work as exogenous supporter of the damaged cells. In fact, quite a few cells (approximately 0.4–4 %) or even no cells were found in the ischemic brain regardless of their neuroprotective results [10–15], and most of the transplanted cells are shown to be trapped in the peripheral organ such as the lungs and spleen [16, 17]. There are several possible mechanisms for the functional recovery without direct cell entry into the brain for intravenous transplantation, such as the secretion of trophic factor and cytokines which leads to facilitate damaged brain cell survival, and activation of neurogeneration [18–20]. There are interesting report saying transplanted cells trapped in the peripheral organ inhibited the secretion of tumor necrosis factor (TNF)-a and interleukin (IL)-6 from the spleen and prevented the systemic inflammatory response from progression [17]. Despite the advantage, intravenous routes have safety issues which cells may stick together and cause microemboli, including lethal pulmonary emboli.

6.2.2 Intra-arterial Route

Intra-arterial route has also been considered as less invasive method and contains several advantages over intravenous route. This method can deliver stem cells directly to the damaged area without trapped by the peripheral filtering organ, resulting in higher amount of cells delivered in the brain, and can distribute stem cells widely to the ischemic lesion [11, 21, 22]. However, recent reports have revealed that cells once injected and settled in the brain will move in the course of time and are trapped in the peripheral organ at the later time point [23–25]. There are also safety issues against intra-arterial route than microemboli causing higher mortality rate compared to other transplantation routes [26]. The use of microneedle injection methods might preserve anterograde blood flow throughout the transplantation process and may avoid the development of microstrokes [27].

6.2.3 Intracerebral Route

Intracerebral route results in most implanted cells delivered in the infarcted area compared with other delivery routes [28]. After injection, transplanted cells will migrate to the ischemic boundary zone navigated by chemotactic cytokines such as

SDF-1a [29–31]. The cells are shown to proliferate and differentiate to neuronal cells in order to cover the damaged neuronal cells [32] and also shown to ameliorate neurological damage by secreting trophic factors. Interestingly, Jin et al. have shown that the ratio of transplanted cells to neuronal cell transformation in the brain does not differ between different transplantation routes. In that case, intracerebral route can deliver most neuronal cells in to the brain compare to other routes [33]. Intracerebral route has also shown to have longer therapeutic time window that this method was effective 4 weeks after the ischemic insult [32]. On the other hand, it is worth noting that intracerebral injection at acute stage of ischemic stroke did not show good cell engraftment probably because the circumstances of the injured brain at acute stage contain abundant excitatory amino acid and reactive oxygen species (ROS), which is not suitable for transplanted cells to settle and proliferate. From these results, intracerebral transplantation might be more effective when it is transplanted between subacute and chronic stage of ischemia. However, the procedural risk for stereotaxic injection raises safety concern. Early clinical trials using intraparenchymal cell transplantation have reported severe adverse events involving motor worsening, seizures, syncope, and chronic subdural hematoma [34, 35].

6.2.4 *Intraventricular/Intrathecal Route*

Intraventricular/intrathecal route seems less invasive than intracerebral route in which transplanted cells adhere to the ventricular wall and penetrate through the ventricular surface to the lesion [36]. However, there are conflicting results regarding the efficacy of intraventricular/intrathecal route in that, in one hand, intrathecal transplantation improved motor function and reduced ischemic damage with quite a small number of cells (0.5×10^6) [37], but on the other hand, no benefit of intraventricular transplantation was reported compared to the beneficial effect through intracerebral route [38].

6.2.5 *Intranasal Route*

Intranasal route has recently been recognized as an alternative route for cell delivery. Intranasally delivered cells can travel across the cribriform plate and migrates throughout the forebrain and olfactory bulbs by bypassing BBB [39]. Although the exact mechanism of intranasal delivery has not been elucidated, accumulating evidence suggests that several pathways such as olfactory nerve, trigeminal nerve, and vascular are involved. There has been accumulating evidence that intranasal transplantation is beneficial for stroke, especially in experimental neonatal hypoxia model [40, 41]. However, it is still unknown that intranasal delivery can be clinically applicable for the aged stroke patients.

6.2.6 *Comparing Transplantation Routes*

It is quite difficult to determine the optimal transplantation route because even the studies focused on direct comparison of the cell transplantation route adapts different stroke model, stem cell types, cell dose, and transplantation timing [42].

6.2.6.1 Comparing Routes at Acute Stage

Several reports have focused on the comparison of transplantation routes in acute stage (Table 6.1), and intravenous transplantation seems to be the most promising route compared to others [42, 43]. As aforementioned, the reason for effectiveness of intravenous transplantation might be from not only by direct cell migration and neural differentiation, but by bystander effect of stem cells, such as BBB stabilization, upregulating neurotrophic factors (VEGF, BDNF), and modulation of immune responses, also called as indirect paracrine mechanisms. Doeppner et al. have reported that intravenous, intra-arterial, and intra-striatum transplantation 6 h after ischemic stroke showed better neurological recovery compared to contralateral intra-striatum, intraventricular transplantation; however, intra-arterial transplantation had higher mortality rate and intra-striatum had shorter period of neurological recovery. They also reported that transplantation route did not affect endogenous angioneurogenesis but played important role in preserving BBB integrity and modulation of inflammatory responses [42]. Willing et al. have also compared intravenous vs. intracerebral transplantation 24 h after ischemia and found that intravenous was superior to intracerebral injection [44]. On the other hand, there are several reports showing that intra-arterial and intraventricular transplantation were as effective as intravenous transplantation and that intra-arterial and intraventricular showed even faster neurological recovery [43, 45–47]. Li et al. further have reported that intra-arterial transplantation showed significantly increased cell distribution compared to intravenous and intracerebral transplantation; however intra-arterial transplantation showed higher mortality [26].

6.2.6.2 Comparing Routes at Chronic Stage

There are quite a few reports focusing on the different transplantation route at chronic stage between a few days and 1 month after ischemic insult. Intravenous transplantation does not seem to be the optimal treatment strategy in this stage as is seen in the acute stage. Lim et al. have found that intrathecally injected stem cells showed similar neurological recovery compare to intravenous injection when transplanted 3 days from ischemia [37]. However, intravenous required larger cell dose to achieve recovery. Kawabori et al. have focused on the route problem and conducted a study comparing intravenous and intracerebral transplantation 7 days after permanent ischemia in rat. They adopted permanent ischemic model which

Table 6.1 Comparison of the different stem cell transplantation route in experimental stroke

Year	Author	Journal	Cell type	Ischemic model	Transplantation timing	Arterial	Venous	Striatal	Striatal (contralateral)	Cortical	Intraventricular
2015	Doeppner	Experimental Neurology	NPC		6 h	+	++	+	–	–	–
2014	Du	Acta Histochemica	ADSC	tMCAO	24 h	++	+				++
2013	Yang	Stroke	BMMC	tMCAO	24 h	+	+				
2012	Vasconcelo-dos-Santos	Stem Cell Research	BMMC	pMCAO	24 h	+	+				
2012	Zhang	Brain Research	hUCSC	tMCAO	24 h	+++	++	+++			+
2010	Li	J Cereb Blood Flow Metab	NPC	tMCAO	24 h	+	+++	++			
2003	Willing	J Neurosci Res	hUCSC		24 h		++	+			
2011	Lim	Stem Cell	hUCSC	tMCAO	Day 3		+				++
2012	Kawabori	Neuropathology	BMSC	pMCAO	Day 7		–	+			

ADSC adipose-derived stem cell, *BMMC* bone marrow-derived mononuclear cell, *BMSC* bone marrow-derived stromal cell, *hUCSC* human umbilical cord stem cell, *NPC* neural progenitor cell, *pMCAO* permanent middle cerebral artery occlusion, *tMCAO* transient middle cerebral artery occlusion

mimics the actual situation for ischemic stroke. They found that significant neurological recovery and intracerebral cell graft were seen in intracerebral transplantation, but not in intravenous group [32, 48]. They also found that this intracerebral transplantation was also effective 28 days after stroke with 1×10^6 cells but not with 1×10^5 cells. These differences are thought to be from the fact that neurological restore and replacement rather than neuroprotection seem to play important role at the chronic stage.

6.3 Transplantation Cell Dose

As Hess and Borlongan have mentioned in their report [49], it is quite difficult to determine the optimal stem cell transplantation dose for treating ischemic stroke patients from the experimental data available, since most of the reports are done by rodents. They mentioned that the current best approach to determine the optimal dose is to extrapolate the dose from rodents to humans based on their weight or brain size. However, there are a few studies which focused on this issue [14, 32, 50–55]. According to the limited data, it seems that the transplanted cell dosage is "the more, the better" (Table 6.2) [40, 56–59]. Most of the reports here found that cells more than 1×10^6 constantly showed better neurological recovery compare to lower doses. However, higher dose may cause unwanted complication such as plumbing of vessel, which Yavagal et al. reported that higher dose (1×10^6) showed decreased MCA flow up to 45 %, while low dose (1×10^5 cells) via intra-arterial transplantation did not compromise MCA blood flow in rat model [60].

6.4 Transplantation Timing

Timing of cell transplantation is very important for successful outcome in clinical treatment; however, it has not been fully investigated. There are no clearly defined therapeutic time windows for cell therapy with all routes of cell transplantation. There are quite a few studies which focus on the treatment efficiency between different timing points (Table 6.3). It seems that optimal transplantation timing differs between transplantation routes. There is a report that intravenous transplantation was effective at days 0–1 but not in the chronic stage (day 28) [42], while intra-arterial transplantation showed its peak efficacy at around days1–7 [25, 61, 62]. Furthermore, intracerebral transplantation showed functional recovery up to day 42 [63].

Table 6.2 Comparison of the different stem cell transplantation cell dose in experimental stroke

Year	Author	Journal	Cell type	Ischemic model	Transplantation timing	Transplantation route	1×10^5 cells/animal	1×10^6 cells/animal	1×10^7 cells/animal
2015	Cameron	*Mol Cell Neurosci*	BMSC	Hypoxia	Day 7	Parenchymal	+	++	
2014	Greggio	*Life Sci*	UCSC	Hypoxia	Day 1	Arterial		–	+
2014	Doeppner	*Cell Death Dis*	NPC	tMCAO	0 h	Venous		+	++
2014	Donega	*Plos One*	BMSC	Hypoxia	Day 10		–	+	
2013	Kawabori	*Neuropathology*	BMSC	pMCAO	Day 7	Cerebral	+	+	
2013	Kawabori	*Neuropathology*	BMSC	pMCAO	Day 28	Cerebral	–	+	
2013	Yang	*Stroke*	BMMC	tMCAO	Day 1	Venous	–	+	
2013	Yang	*Stroke*	BMMC	tMCAO	Day 1	Arterial	–	+	
2012	Shehadah	*Plos One*	UCSC	tMCAO	Day 1	Venous	+	+	
2011	Zhang	*Stroke*	UCSC	tMCAO	Day 1	Venous	–	+	+
2011	Lim	*Stem Cell Res Ther*	UCSC	tMCAO	Day 3	Venous	–	+	

BMMC bone marrow-derived mononuclear cell, *BMSC* bone marrow-derived stromal cell, *UCSC* umbilical cord blood stem cell, *NPC* neural progenitor cell, *pMCAO* permanent middle cerebral artery occlusion, *tMCAO* transient middle cerebral artery occlusion, – no difference against control, + moderate improvement than control, ++ better improvement than + group, +++ better improvement than ++ group

Table 6.3 Comparison of the different stem cell transplantation timing in experimental stroke

Year	Author	Journal	Cell type	Ischemic model	Transplantation dose	Transplantation route	0–2 h	Day 1	Day 2–6	Day 7–27	Day 28–
2016	Ishizaka	Stroke	BMSC	tMCAO	1×10^6	Arterial		++	+	−	
2014	Doeppner	Cell Death Dis	NPC	tMCAO	1×10^6	Venous	+	+			−
2014	Yavagal	Plos One	BMSC	tMCAO	1×10^6	Arterial	−	+			
2013	Mitkari	Behav Brain Res	BMMC	tMCAO	1×10^6	Arterial			+	+	
2013	Donega	Plos One	BMSC	hypoxia	0.5×10^6	Nasal			+	+	
2012	Rosenblum	Stroke	NPC	hypoxia	0.5×10^6	Arterial	−	+	++	−	
2011	Darsalia	J Cereb Blood Flow Metab	NPC	tMCAO	$3–15 \times 10^6$	Cerebral			++		+
2011	Zhang	Stroke	UCSC	tMCAO	5×10^6	Venous		+		+	+

BMMC bone marrow-derived mononuclear cell, *BMSC* bone marrow-derived stromal cell, *UCSC* umbilical cord blood stem cell, *NPC* neural progenitor/stem cell, *tMCAO* transient middle cerebral artery occlusion, − no difference against control, + moderate improvement than control, ++ better improvement than + group, +++ better improvement than ++ group

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Chapter 7
Role of Biomaterials as Scaffolding in Cell Therapy for Stroke

Toshiya Osanai

Abstract In this chapter, we review the application of scaffolds for cell therapy. Biological scaffolding carries many advantages for the treatment of not only stroke but also other neural disorders. First, we describe the role of cell therapy using multipotential cells such as embryonic stem cells, induced pluripotent stem cells, neuronal stem cells, and bone marrow stromal cells. Furthermore, we describe the ideal properties of a scaffold for cell therapy. Scaffolds for the central nervous system have requirements for various properties such as size, biocompatibility, mechanical compatibility, and biodegradability.

Second, we review current tissue engineering strategies for neuronal disorders, focusing on the use of specific materials, such as collagen, gelatin, alginate, hyaluronic acid, polyglycolic acid, poly(lactic glycolic) acid, poly 2-hydroxyethyl-methacrylate, and fibrin.

These scaffolds optimized for central nervous system cells or graft cells help promote survival, migration, and differentiation of grafted cells and contribute to the improvement of neurological function after transplantation. Biomaterials should be further studied to improve safety and efficacy.

Keywords Stroke • Scaffolding in cell therapy • Biomaterials

7.1 General Aspects

7.1.1 Stroke

Stroke is the third leading cause of death in the world. Recently, randomized clinical trials (RCTs) confirmed that endovascular treatment improved the outcomes of stroke patients [1–5]. However, 30–50 % of patients experience disabilities or death even after receiving endovascular treatment [1–5]. RCTs restrict the

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patient inclusion criteria for endovascular treatment. Thus, more patients need access to promising treatment alternatives for acute ischemic stroke.

It is believed that both the adult peripheral nervous system (PNS) and central nervous system (CNS) do not have the ability to regenerate. However, specific adult nerve cells have the instinctive ability to regenerate after damage. Researchers have shown that there are stem cells in the CNS, and in under some conditions, the CNS can self-renew. However, this phenomenon is highly limited. Thus, the main focus of basic research or preclinical research for the purpose of restoration of the CNS is cell transplantation.

Aguayo et al. reported that retinal cells, part of the CNS, were able to regenerate when used within a peripheral nerve graft, but they did not migrate beyond the graft into the CNS tissue [6]. Post-injury physiological responses and associated glial cell function inhibit the CNS from readily regenerating. Neurotrophic factor has positive anti-apoptosis and anti-inflammatory effects, but the appropriate administration protocol to achieve a satisfactory effect is unknown. In theory, direct administration will result in the maximum effect on neural function improvement. However, this method may also lead to secondary injury after operation due to injection. When neurotrophic factors are administered via a peripheral route such as transvenous delivery, they may cross the blood-brain barrier or blood-spine barrier, and adverse effects may occur.

7.1.2 Cell Therapy

Cell therapy has been successfully used for the treatment of specific organs such as the skin, heart, and cartilage. Many studies have applied cell therapy to treat central neural dysfunction after stroke. Embryonic stem (ES) cells, induced pluripotent stem (iPS) cells, neuronal stem cells (NSCs), and bone marrow stromal cells (BMSCs) have been employed as donor cells in past research. It is well known that the use of these cells results in improvements in animal models of cerebral infarction, cerebral trauma, spinal injury, and Parkinson's disease.

Multipotential cells like ES cells, iPS cells, and NSCs are promising candidates for cell therapy. These cells may be used to regenerate neurons and improve neuronal function. Very few studies have been performed using these multipotential cells. However, it was shown that adult NSCs derived from human wisdom teeth improved neurological function after implantation in a rat middle cerebral artery occlusion model.

BMSCs, adult stem cells, are the most extensively examined source for brain cell therapy to date. BMSCs are a promising cell source because of their ethical acceptability and low immune impact owing to the possibility of utilizing the patient's own BMSCs. BMSCs exhibit neural differentiation and produce growth factors to restore the CNS. Research has shown that cell therapy with BMSCs improved the outcome and cognitive function in animal stroke models for middle cerebral artery occlusion (MCAO) [7] and transient common carotid artery

occlusion [8]. Some studies have shown that BMSCs have the potential to aid in functional recovery regardless of the implantation route (intravenous or direct) [9]. However, the mechanism of functional recovery is unknown. Factors secreted by BMSCs are believed to promote neurological benefits [10–12]. However, the BMSC survival rate is low, and few differentiate into neural cells such as NeuN-positive cells. Thus, differentiation into neural cells is not believed to be the main factor aiding in neural function recovery.

While cell therapy is promising, satisfactory outcomes have not been achieved in more complex organs such as the brain. In addition, only 10–20 % of transplanted cells survive, and those that do may exhibit loss of cell function and uncontrolled cell differentiation. There are ethical issues associated with the use of embryonic cells, and optimal cell transplantation methods are yet to be unveiled.

The following approaches have been utilized for CNS BMSC transplantation: (1) direct transplantation, (2) transvenous transplantation, (3) the transventricle or transthecal approach, and (4) the transarterial approach. In direct transplantation, BMSCs are transplanted directly on or around the infarct lesions. These donor cells can migrate toward a damaged lesion from the peri-infarcted lesion in a couple weeks and remain there after administration [5–7]. The disadvantage of this method is the creation of new damage and the possibility of cell death before engraftment of donor cells.

Transvenous transplantation is a less invasive technique. Bang et al. applied transvenous administration in a clinical study on patients with cerebral infarction [8].

Studies on transthecal transplantation of BMSCs via the fourth ventricle or using lumbar puncture for rat spinal injury models have also been reported. BMSCs transplanted into the cerebrospinal fluid cavity remain in the damaged spine, and clinical studies on patients with spinal injury have been initiated. Moreover, transarterial transplantation via the cervical artery has been assessed by Shen [13], and clinical studies are underway. However, Lee used magnetic resonance imaging to show that multiple cerebral infarctions occurred after transplantation.

Although each method of transplantation has been studied independently, some newer reports compare various methods of administration mainly using spinal injury models in the same conditions. Bakshi et al. compared three methods of transplantation of BMSCs in a rat *semi spinal injury model*: venous, lumbar puncture, and ventricle, and it was shown that the transvenous approach was the least effective. Vaquero et al. transplanted BMSCs via a venous route or directly into a rat spinal injury model. The group that received BMSCs via the vein had improved neurological symptoms. The group that received direct BMSC transplantation also exhibited improvement, and the BMSCs stayed in the spine longer than in the transvenous group. In contrast, it has also been reported that only a small amount of BMSCs accumulate in the injured spine. A rat cardioinfarction model study indicated that many of the cells transplanted into the animal via the vein accumulated in the lung.

Based on the above articles, it is uncertain whether BMSC transplantation through the vein would be effective in a clinical setting. The optimal method of

cell transplantation needs to be elucidated in order to achieve satisfactory outcomes after brain injury due to stroke.

7.1.3 Nerve Guide Conduits (NGC)

Scaffold technology can be used to enhance the regeneration of both the PNS and CNS. Non-cell-carrying polymeric nerve guide conduits (NGCs) have been approved for promoting nerve regeneration in various countries [13, 14]. NGCs simulate the instinctual regeneration process by providing a proper environment for neuroregeneration and have achieved clinical success for PNS treatment (Wosnick, J.H.; Baumann, M.D.; Shoichet, M.S. 73 Tissue therapy: Central nervous system. In Principles of Regenerative Medicine; Atala, A., Lanza, R., Thomson, J.A., Nerem, R.M., Eds.; Academic Press: San Diego, CA, USA, 2008; pp. 1248–1269). NGCs were used to control glial scar formation, promote neuronal sprouting, and provide protection from the invading immune system in spinal cord injury [15]. These phenomena have been confirmed in scaffolds constructed of various materials and architectural features. However, scaffold technology is still less effective than autografting for long lesions.

7.1.4 Scaffold Properties for the CNS

Tissue engineering involves implantation of a scaffold made with biomaterials and seeded with transplanted cells. Nutrients and other bioactive elements may also be embedded within the scaffold. Surgical materials such as surgical sutures or artificial dura mater made from polymers, ceramics, and titanium are used widely in daily clinical work. Unlike the materials used for surgical procedures, the biomaterials used in regenerative medicine must be biodegradable, porous, and cytophilic. Such biomaterials have been used for adult cell therapy in the bone, cartilage, vessel, heart, and skin. However, these organs are less complex than the CNS. Adult cell therapy using scaffolds for the CNS has many limitations.

According to a previous study, scaffolds for the CNS have had various properties, compositions, and shapes [16]. Size is the most important factor for CNS applications because of the narrow space within the bone structure. Additionally, the CNS has small, sensitive tissues such as nuclei that are adjacent to each other. Therefore, scaffolds have to be small enough to not affect neighboring tissue. Small scaffolds have other advantages in regard to the CNS. Recently, advances in nanotechnology have enabled scaffolds to become finer and smaller [17]. Menei et al. reported that repeated implantations were possible without open surgery when using poly(lactide-co-glycolide) microspheres [18]. In addition, nanomaterials can have several advantageous properties, such as higher surface area and high porosity required for cell adhesion [19].

Another important consideration is biocompatibility. For a substance to be considered biocompatible, it must support the appropriate cellular behaviors without being toxic to living tissue. Scaffolds must elicit minimal adverse cell responses, such as glial scarring, inflammation, hemolysis, coagulation, thrombus formation, and immune cell invasion, including foreign body reaction [20, 21].

The biocompatibility of neural scaffolds can be evaluated based on three aspects: blood compatibility, histocompatibility, and mechanical compatibility [20]. Blood compatibility means that scaffolds do not induce hemolysis, destroy blood components, or promote coagulation and thrombus formation after coming into contact with blood. Histocompatibility is defined as compatibility between tissues of different individuals so that one may accept a graft from the other without having an immune reaction. For example, poly(lactic-co-glycolic acid) (PLGA) microspheres do not induce a specific astrocytic reaction.

Mechanical compatibility between the scaffolds and host tissues requires that the scaffold have the appropriate compressive and tensile properties. Mechanical properties are one of the most important parameters for successful implantation [22].

Biodegradability or degradation after implantation is also critical. Whether or not a biodegradable scaffold is required depends on the specific application [23]. If scaffolds are non-biodegradable or biodegrade slowly, they act as a barrier to protect transplanted cells from the host immune system and provide a proper environment for cells to survive for long periods. Moreover, it is easy to retrieve non-biodegradable scaffolds after prescribed treatment periods. Some clinical trials have shown that cells survived in non-biodegradable scaffolds and there was no evidence of immune cell infiltration [24, 25]. However, it is believed that non-degrading scaffolds often cause long-term complications that often require revision surgery for removal because they may become harmful and constrict nerve remodeling [26]. As a result, biodegradable scaffolds have become the main focus for neuronal regeneration applications. Biodegradable scaffolds allow for cells to differentiate into neuronal cells at the site while also replacing lost or injured tissues. The degradation products must also be tolerated by the transplanted cells and host and ideally be metabolized completely without toxic effects or adverse immune reaction [16]. The biodegradation rate is an important factor to control. If the degradation rate is too slow, chronic compression, foreign body reactions, and mild inflammatory reactions may occur [13, 20]. On the other hand, rapid degradation may result in failure to protect the regenerated axon. Keilhoff reported that neuroregeneration may be hampered by inadequate protection of transplanted cells from invading fibrous tissue or insufficient time for nerve fibers to mature [27]. Biodegradability depends on a number of factors, such as temperature, PH, ionic strength, hydrolysis (swelling), the presence of enzymes, and engulfing cells. In vitro degradability tests can be performed in water or phosphate-buffered saline (PBS).

7.1.5 Scaffold Advantages

The ways in which scaffolds affect transplanted cells and damaged tissue in the CNS have been described. The mechanisms by which scaffolds improve neurological function are unclear, but several have been proposed. First, placing a biomaterial scaffold into the damaged area or cavity may provide support for the surrounding brain tissue. The tissue around the cavity is damaged by secondary injury; thus, the biomaterial protects the peri-cavity tissue from consequent impairment. In addition, scaffolds act as supportive cells like astrocytes. These mechanisms are thought to improve neurological function after implantation of a biomaterial. The scaffold can also be used to deliver various promoting factors for the growth of cells such as drugs.

Scaffolds are known to improve the cell survival rate in vivo owing to the 3D environment as well as mechanical signal cues [16]. Scaffolds help to direct the enlargement of axial cells, function as a substrate for cells, promote neurite formation, and enable cell infiltration. Scaffolds also restrict astrocytosis, which has a deleterious effect on healthy tissue. Scaffolds should allow for graft integration while also promoting cellular differentiation and migration.

Finally, scaffolds aid in the development of extracellular matrix that controls the structure of tissues and helps regulate cell nutrition, humoral factors, and metabolites. Hence, scaffold technology is promising for clinical use to help improve cell replacement and tissue repair, and investigators are focused on further enhancing scaffold properties.

7.2 Scaffold Materials

Vacanti first introduced the concept of “tissue engineering” described as reconstruction of damaged tissue by combining donor cells and biomaterials into a scaffold. Biomaterials play the role of extracellular matrix and are expected to inhibit “anoikis” or cell death.

The ideal characteristics of biomaterials for tissue engineering are as follows:

1. Nontoxic
2. Easy to handle
3. Non-immunogenic
4. Biodegradable
5. Allow for the migration and growth of transplanted cells

Biomaterials for tissue engineering of the CNS should also exhibit adhesive properties and softness.

Biomaterials are roughly classified into two categories: “natural materials” such as peptides and polysaccharides and “synthetic materials” like polymers. Recently, nanotechnology has been applied to the development of biomaterials. Many studies

have been conducted to assess biomaterials, and they are discussed in the following sections.

7.2.1 Collagen

Collagen is a key component of extracellular matrix, and, thus far, 28 types of collagen have been discovered. Collagen I or II are mainly used as biomaterials. Ma et al. reported that collagen was useful for promoting the differentiation and growth of neural stem cells. Li et al. co-cultivated human neuroblastoma cells with collagen and reported that the biomaterial affected cellular gene expression and structure. Lu et al. transplanted BMSCs enclosed in collagen into rats with brain injury.

Growth inhibitory molecules are being used to increase our understanding of neuroplasticity [28] and are becoming the focus of potential treatments aimed at enhancing neural restoration. Asim et al. [29] reported that collagen-based scaffolds decreased the levels of Nogo-A, a type of growth inhibitory molecule, in the lesion boundary zone and downregulated Nogo-A gene expression. These reports also described how the scaffold and hMSCs were used to increase axonal density. Cytodex®, a collagen-coated dextran, enhanced the survival rate of cells transplanted into hemi-Parkinsonian rats [30, 31]. Furthermore, the use of Cytodex resulted in the retention of cell ability without immune suppression.

All of these studies indicate the need for cell attachment to a 3D complex to increase the survival rate of transplanted cells.

7.2.2 Gelatin

Gelatin is an irreversibly hydrolyzed form of collagen. Colloid forms a solid at high temperatures and gel at low temperatures. Deguchi et al. transplanted a porous gelatin compound into a cerebral cortex defect and gelatin contributed to the migration of cells and angiogenesis [32]. Gelfoam is also regarded as a promising material for regeneration of the myocardium. Bro et al. showed that a gelatin sponge scaffold (GS) with modified neuronal stem cells (NSCs) improved the survival of axotomized neurons, helped regenerate axons, promoted the differentiation and synaptogenesis of NSCs, and decreased cynic cavity formation. They concluded that GS is a promising scaffold material because of its low antigenicity, favorable biocompatibility, biodegradability, and low cost.

Spheramine® is a microsphere made of gelatin. The cross-linked porcine gelatin microcarriers with a mean diameter of 100 μm are biocompatible but not biodegradable [16]. Cepeda et al. reported that 6-OHDA rats recovered from functional deficit after implantation of human adrenal chromatin with Spheramine® [33]. Human retinal pigment epithelium (hPRE) cells have also been used with

Spheramine® in some studies. Spheramine® increases the survival rate of hPREs and increases long-term functional improvement [34]. hPREs implanted with Spheramine® in the brains of hemi-Parkinsonian monkeys also improved cell survival and long-term function [35]. hPREs with Spheramine® were evaluated in a phase II double-blind, randomized, multicenter, placebo-controlled study. This study showed the safety, tolerability, and efficacy of the implanted cell device into the postcommissural putamen of patients with severe Parkinson's disease [36]. In contrast, administration of hPREs alone did not have positive effects; thus, cellular attachment to micro carriers is important. However, the mechanism is still unclear.

7.2.3 Alginate

Alginate is a plant-based polysaccharide and is used as a source of dietary fiber. After the addition of a divalent ion such as calcium to an alginate solution, a gel is formed which can be used for biological scaffolding. Alginate has been extensively used as a synthetic extracellular matrix (Regenerative medicine research 2014). Kataoka et al. transplanted freeze-dried alginate sponges into rats with spinal dissection and regeneration of the axis was improved [37]. In addition, Novikova analyzed the biological behavior of BMSCs in alginate hydrogels. Cell encapsulation in alginate beads improves cell viability and prevents host rejection.

RGPS (Arg-Gly-Asp-Ser) is a peptide that mediates cell binding to fibronectin.

To treat spinal cord injury, implantation of polyhydroxybutyrate (PHB) fibers containing alginate hydrogel, fibronectin, and neonatal Schwann cells protected the neurons in the red nucleolus from secondary neuronal atrophy (*Journal of Neurotrauma*, vol 23 number 3/4, 2006). Alginate sponges also contributed to the survival and differentiation of rat hippocampus-derived neurosphere cells after transplantation into the injured rat spinal cord, as described above. Finally, neurotrophic factor-secreting epithelial cells immobilized in alginate capsules might be useful for preventing the degeneration of neurons observed in Huntington's disease [38].

7.2.4 Hyaluronic Acid (HA)

Hyaluronic acid (HA) is a mucopolysaccharide that consists of extracellular matrix and occurs in the vitreous body, joint, or skin. HA is biodegradable, biocompatible, and non-cytotoxic and can facilitate neural regeneration [39]. HA used to treat spinal defects reduced the lesion size in a spinal cord injury model (*JNS Spine* March 4, 1–11, 2016). Topical application of hyaluronic gel was found to prevent peripheral scar formation and enhance peripheral nerve regeneration [20]. A non-woven polymer made from hyaluronic acid called Hyaff is commercially available and can be used as an effective scaffold combined with BMSCs. Hyaluronic acid

supported cell migration and ameliorated the disabled function of the impaired forelimb in an MCAO model [40]. An HA-poly-D-lysine copolymer hydrogel was introduced as a scaffold to repair brain defects in rats.

7.2.5 Polyglycolic Acid (PGA)

PGA is a synthetic biodegradable polymer and is widely used in absorbable sutures. PGA has been used to prepare nanoparticles [38] and has demonstrated potential in combination with NSC transplantation.(*Journal of Neurotrauma* vol 23, number 3/4, 2006). Park et al. transplanted NSCs with PGA into massive tissue defects in hypoxia ischemic mice. PGA promoted cell survival and reciprocal interaction between NSCs and the host. Furthermore, they found that PGA reduced parenchymal loss after hypoxia injury [41]. The cellular matrix fostered the regrowth of cortical tissue and also reduced inflammation and scar formation within the brain [42]. PGA is approved by the United States Food and Drug Administration as a biomaterial for neural scaffolding.

7.2.6 Poly Lactic Glycol Acid (PLGA)

PLGA is one of the most promising candidates for tissue engineering and is known to be fully degradable with the end-product metabolites being CO_2 and H_2O [18]. PLGA provides the most suitable environment for ES cell colonization and PLGA scaffolds have been shown to help maintain transplanted cell morphology, viability, and growth kinetics [43].

PLGA is non-immunogenic and implantation of PLGA microspheres into the brain did not induce a specific astrocytic reaction [16]. PLGA scaffolds modified with laminin or fibronectin improved cell survival, and functional improvements were observed in an animal model [44]. The biomimic approach has also been applied to this synthetic polymer. Pharmacologically active microcarriers (PAMS) are biodegradable and biocompatible PLGA microspheres with cells on their surface and provide an adequate three-dimensional microenvironment in vivo [16]. When transplanted at 2 weeks post MCAO, Bible et al. noticed significant endothelial cell infiltration and neovascularization within the tissue formed by a conditionally immortalized human NP cell line that had been transplanted on the VEGF-PLGA micro particles into the damaged hemisphere [45].

7.2.7 *Poly(2-Hydroxyethyl-Methacrylate) (pHEMA)*

pHEMA is a non-biodegradable hydrogel and has been shown to be an effective scaffold material for cell transplantation therapy for BMSCs in animal spinal and brain injury models [46, 47]. A highly desirable property of nondegradable conduits for spinal cord injury is structural stability. This material has covalently cross-linked hydrogels that are widely used in bioengineering because of their ability to support cell growth and mimic the extracellular matrix [42]. However, nondegradable materials must be both biocompatible and nontoxic [48]. Implanted pHEMA elicited a modest cellular inflammatory response that disappeared after 4 weeks with minimal scarring around the matrix [22].

7.2.8 *Fibrin*

Fibrin is a fibrous blood protein formed of polymerized fibrinogen. This material is used as a hemostatic in operations and is also frequently used in regenerative medicine. Fibrin has desirable properties including adaptability and biodegradability. Fibrin causes few foreign body reactions and the concentration of fibrinogen or calcium can be adjusted to control polymerization time. These properties of fibrin make it useful for CNS applications.

Previous studies have used fibrin as scaffolding for the regeneration of the bone, cartilage, myocardium, skin, and bladder. Bhang et al. transplanted BMSCs with fibrin as a carrier of basic fibroblast growth factor (bFGF) into rats with brain injuries and reported a positive effect on tissue regeneration. Studies have also shown that BMSCs can grow in fibrin material and transplantation of BMSCs with fibrin promoted cell survival and migration.

7.3 Conclusion

We reviewed the role and the necessity of scaffolds in regenerative therapy of the CNS based on recent reports. Scaffolds optimized for central nerve cells or graft cells help promote survival, migration, and differentiation of grafted cells and contribute to the improvement of neurological function after transplantation. Various cell sources such as BMSCs, NSCs, and iPS cells have been assessed as potential candidates for the regeneration of the CNS. Biomaterial should be further studied in order to improve safety and efficacy.

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Chapter 8
In Vivo Cell Tracking Techniques for Applications in Central Nervous System Disorders

Taku Sugiyama, Satoshi Kuroda, and Kiyohiro Houkin

Abstract Cell therapy is expected to promote functional recovery in various kinds of central nervous system disorders. Many studies show beneficial effects of cell therapy, and several clinical studies have already been initiated worldwide. Although these results are encouraging, several problems remain, including elucidating the therapeutic mechanisms, treatment timing, optimal cell dose, type of cells, and cell delivery route. For further optimization of this therapy, it is essential to develop in vivo cell tracking techniques. Longitudinal and serial analyses of the fate of transplanted cells are quite important for solving these problems. There are several cell labeling techniques and imaging modalities, including magnetic resonance imaging, nuclear imaging, and optical imaging. However, any single imaging modality has its own distinct advantages and drawbacks. Proper understanding of each technique's characteristics is crucial for successful in vivo imaging. In this chapter, we present a literature survey of cell tracking techniques used in clinical settings and laboratories and introduce recent advances in this field.

Keywords Cell tracking • Central nervous system • Magnetic resonance imaging • Nuclear imaging • Optical imaging

8.1 Introduction

Because of the limited regenerative capacity of the central nervous system (CNS), cell therapy is increasingly expected to be a promising therapeutic strategy for treating various kinds of CNS disorders, including cerebral stroke, traumatic brain injury, spinal cord injury, and degenerative diseases [1, 2]. To date, various cell

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types have been investigated, including induced pluripotent stem (iPS) cells, embryonic stem (ES) cells, neural stem cell/neuronal progenitor cell, bone marrow-derived mononuclear cells (MNC), and bone marrow stromal cells (BMSC) [1, 2]. The majority of published studies show the efficacy of cell therapy [3–7]. As a result, several clinical trials have been initiated worldwide to assess the feasibility of this therapy for CNS disorders [8–13].

However, there is no consensus with regard to therapeutic mechanisms, treatment timing, optimal cell dose, type of cells, and cell delivery route. Further optimization of this therapy is still essential. For this purpose, noninvasive in vivo cell tracking using imaging techniques is expected to play a pivotal role in clarifying the mechanisms involved in recovery. Longitudinal and serial analyses of the fate of transplanted cells in living animals or humans are important in uncovering these pathways. Recent recommendations provided from "Stem Cells as an Emerging Paradigm in Stroke 3" (STEPS3) state the following [14]:

> Use of imaging in clinical trials is strongly encouraged to provide as much information as possible to assess vascular/structural lesions, infarct size, cell viability, location, the success and safety of implantation, and inflammation. Imaging should also be used to monitor safety and recovery and, when possible, to investigate mechanisms of action and provide information on surrogate markers of treatment effect. Imaging measures might also be useful to help stratify patients at baseline.

To date, several imaging approaches have been proposed, including magnetic resonance imaging (MRI), nuclear imaging, and optical imaging. However, it is quite important to understand the unique characteristics of the different imaging modalities in order to foster the most appropriate usage of each, as they all possess advantages and drawbacks. Therefore, in this chapter, we conduct a literature survey of cell tracking technique used in clinical settings and laboratories and introduce recent advances in this field.

8.2 Cell Labeling Technique

Generally, cell labeling techniques are classified as exogenous labeling (direct labeling) and endogenous labeling (reporter gene labeling) as summarized in Table 8.1.

In exogenous labeling, the cellular marker (e.g., MR contrast agents, radiotracer, or fluorescence probes) is taken up into the cell or attaches to its surface. Usually, exogenous labeling is performed in vitro prior to transplantation. There are several kinds of probes for each modality and each has specific characteristics. Although cytotoxicity and labeling efficiency vary for each probe, currently, exogenous labeling is considered the first choice for clinical applications. This method has several favorable features including requiring only simple incubation with the probe according to standardized protocols, and it is capable of being applied to allogenic or autologous cells without gene transfection. However, it also has fundamental limitations when it comes to transplanted cell quantification and

Table 8.1 Methods of labeling donor cells in each imaging modality

		Exogenous labeling	Endogenous labeling
MRI	T1WI	*Gd*, *Mn*	
	T2WI	*SPIO*	MR reporter gene
Nuclear imaging	SPECT	^{111}In-oxine, ^{99m}Tc-HMPAO	Reporter gene
	PET	^{18}F- FDG	
Optical imaging	FI	QD, organic dye	GFP
	BI		Luciferase reporter

BI bioluminescence imaging, ^{18}*F-FDG* ^{18}F-fluorodeoxyglucose, *FI* fluorescence imaging, *GFP* green fluorescent protein, *Gd* gadolinium, *In* indium, *Mn* manganese, *PET* positron emission tomography, *SPECT* single-photon emission computed tomography, ^{99m}*Tc-HMPAO* ^{99m}technetium-hexamethylproplyleneamine

long-term monitoring, due to the dilution of tracer by cell proliferation and cell death, and possible transfer to other phagocytic cells such as macrophages [15]. Another problem is that the persistence of the tracer, which can be detected by imaging, does not directly indicate the viability of transplanted cells. Moreover, radiotracers for nuclear imaging have a short half-life, and fluorescent probes for optical imaging exhibit photobleaching or degradation [16–18].

On the other hand, endogenous labeling is usually performed by genetic manipulation of transplanted cells ex vivo, so that they are able to produce certain proteins that can later be used as markers. This method is free of the abovementioned problems. The inclusion of genes means that only living cells can be detected by imaging, because dead cells will no longer synthesize proteins as a marker. Moreover, there is no dilution of a tracer by cell division because a parent cell will supply each daughter cell with the same gene. Reporter genes are widely used for cell tracking in the field of nuclear imaging [19–21]. In this technique, the manipulated gene in the transplanted cell produces a particular protein that is involved in the uptake or accumulation of the tracer, and the tracer itself is then administered immediately before imaging to label target cells in vivo. However, this method is unlikely to be used for human application because of the ethical problems and possible functional changes resulting from gene transfection [22].

Alternatively, receptor-based in vivo labeling techniques are also available. However, there is the possibility that cell surface markers might change when transplanted cells undergo differentiation. Therefore, this technique has not been widely used for in vivo cell tracking to date.

8.3 Imaging Modality

The characteristics of each imaging modality are summarized in Table 8.2. Appropriate choice of imaging system is crucial for successful in vivo imaging.

Table 8.2 Comparison of each imaging modalities

Modality	MRI	Nuclear imaging	Optical imaging
Cost	High	Medium	Low
Acquisition	Minutes-hours	Minutes	Seconds-minutes
Radiation	No	Yes	No
Stability	Weeks	Minutes-days	Weeks
Labeling toxicity	Safe	Yes	Varied
Quantifiable	No	Yes	No
Sensitivity	>1,000 cells	single cell	$>1.0 \times 10^5$ cells
Penetration depth	No limit	No limit	<1 cm (FI), 3 cm (BI)
Resolution	10–100 μm	1–2 mm	2–3 mm
Visualization	3D, any model	3D, any model	2D, only small animal

BI bioluminescence imaging, *FI* fluorescence imaging, *MRI* magnetic resonance imaging

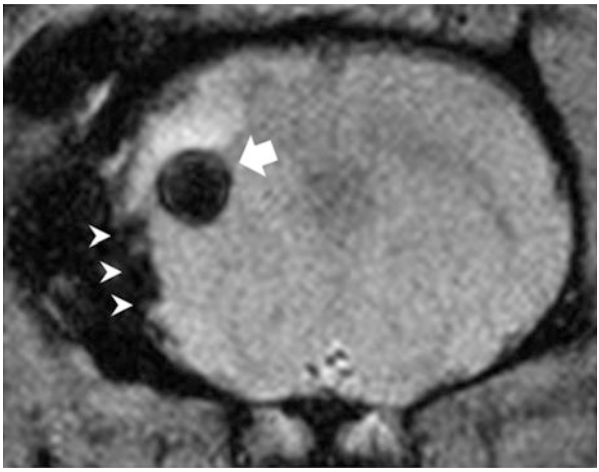

Fig. 8.1 T2*-weighted MR images of the rat subjected to permanent middle cerebral artery (MCA) occlusion reveal that the transplanted superparamagnetic iron oxide (SPIO)-labeled bone marrow stromal cells (BMSC) (*arrow*) migrate toward the cerebral infarct 2 weeks after transplantation (*arrow heads*)

8.3.1 MRI

MRI has clear advantages over the other imaging modalities because it has the highest spatial resolution and is widely available in clinical situations. MRI is an excellent modality for detailed demonstration of cell location after transplantation.

Gadolinium chelates [23] and manganese [24], which are T1 contrast agents that generate positive contrast, were used in cell labeling in early studies. Superparamagnetic iron oxide (SPIO) nanoparticles have become the most widely used agents for cell labeling, because SPIO is more sensitive and biologically compatible than other contrast agents. One of the SPIO formulations has been approved by the Food and Drug Administration (FDA) for human use, and SPIO has been used in a clinical study [10, 12]. SPIO is a T2 contrast agent that generates negative contrast (Fig. 8.1).

Hoehn et al. tracked murine ES cells using MRI after direct grafting them into rat brains subjected to focal cerebral ischemia. They found that the ES cells labeled with ultrasmall SPIO started to migrate toward the lesion within a few days and that they accumulated in large numbers in the border zone of the damaged brain tissue 3 weeks after transplantation [25]. Zhang et al. intracisternally transplanted rat subventricular zone cells labeled with ferromagnetic particles into the infarcted rat brain. Serial MRI tracking revealed that the engrafted cells migrated toward the ischemic parenchyma at a mean speed of 65 ± 14.6 μm/h in the living rats [26]. As mentioned above, long-term monitoring of this technique was problematic; however, Kim et al. directly transplanted the SPIO-labeled human BMSCs (hBMSCs) into rodent brains subjected to cerebral infarction and were able to track them using MRI 10 weeks after transplantation [27]. Our group also succeeded in tracking hBMSCs up to 8 weeks after transplantation [28]. As with the nuclear imaging method detailed below, MR reporter gene assays were developed as a novel endogenous labeling technique geared toward long-term and quantifiable cell tracking study [20].

MRI is not without limitations. First, the sensitivity to detect transplanted cells is generally lower as compared to nuclear imaging and bioluminescence imaging. Although MRI could detect 100 cells when using high-field (17.6 T) MRI [29], our recent study showed that 1×10^3 cells were needed to reach detection threshold using a clinical (3.0 T) MRI apparatus [30]. MRI is also sometimes impeded by imaging artifacts, such as an intracranial hemorrhage, thus the specificity of MR signal is not always excellent [12].

8.3.2 Nuclear Imaging

Nuclear imaging, including positron emission tomography (PET) and single-photon emission computed tomography (SPECT), is characterized by excellent in vivo sensitivities and whole-body imaging capabilities. Nuclear imaging can detect even a single cell. Therefore, this modality is useful in quantifiable analysis of transplanted cells. Brenner et al. and Aicher et al. transplanted ^{111}indium (In) - oxine-labeled endothelial progenitor cell and hematopoietic progenitor cell into rats subjected to myocardial infarction intravenously and monitored them using SPECT [31, 32]. They reported that only 1 % of transplanted cells engrafted to the myocardial lesion. de Haro et al. transplanted intravenously ^{111}In oxine-labeled BMSC into rats with spinal cord injury and detected accumulation of the cells at the injured lesion using SPECT [33]. Correa et al. and Barbosa da Fonseca et al. transplanted and monitored ^{99m}technetium-hexamethylproplyleneamine oxine-labeled MNCs into a patient with cerebral infarction through the internal carotid artery [8, 9, 11].

The most commonly used radiotracer for PET is ^{18}F-fluorodeoxyglucose, which is FDA approved. Thus, several studies have used this technique in human clinical trials related to myocardial infarction [13].

The short half-life of radiotracers is a major limitation of this method. To overcome this limitation, reporter gene assays have been used for long-term monitoring and assessment of transplanted cell viability. Cao et al. injected ES cells labeled with this method into the myocardium of adult nude rats and succeeded in monitoring viability, engraftment, and proliferation of the transplanted cells at least 4 weeks after transplantation [19, 21]. As an another approach, longer half-life tracers, for example, ^{89}Zr-oxinate$_4$, have recently detected cells up to 14 days after labeling and administration [34].

Another major disadvantage of a radiotracer is radiation toxicity to the labeled cell. Therefore, knowledge of maximum safe doses of radiotracers is crucial for clinical applications. A recent study showed that ^{18}F-fluoro-2-deoxy-D-glucose may label cells safely at concentrations up to 25 Bq/cell without compromising cellular function [35].

8.3.3 Optical Imaging

Because of light scattering and absorption by tissue, the use of optical imaging for cell tracking is limited to only small animals. However, optical imaging has some advantages including lower cost and rapid acquisition time.

"Bioluminescence" refers to light generated by intrinsic properties of organisms in nature, such as fireflies. Bioluminescence reporter gene luciferase assays have been applied to cells through genetic manipulation before transplantation. When the luciferase substrate (luciferin) is systemically injected, light photons are produced by transplanted cells [36]. Using this technique, in one study, transplanted neural progenitor cells were monitored 21 days after stroke in both rats and mice [37].

Fluorescence imaging has also been attempted in cell tracking for CNS disorders, using green fluorescence protein [38–40]. However, it was difficult to detect green fluorescence through the bone and skin because its short wavelength had low penetration depth of the tissue. Recently, using near-infrared (NIR) emitting quantum dots, our group succeeded in monitoring transplanted BMSC in the rat brain subjected to cerebral infarct (Fig. 8.2) [18]. Interestingly, they could be monitored up to 8 weeks after transplantation. Although the sensitivity is still quite low (approximately 2×10^5 cells were needed to be detectable), the results opened up new opportunities to track transplanted cells in rodent brains [41, 42]. Recently, fluorescent nanodiamonds are attracting a great deal of attention because they have favorable characteristics such as photostability, chemical non-reactivity, biocompatibility, and emission in the NIR band. However, they have not yet been used for in vivo cell tracking [43].

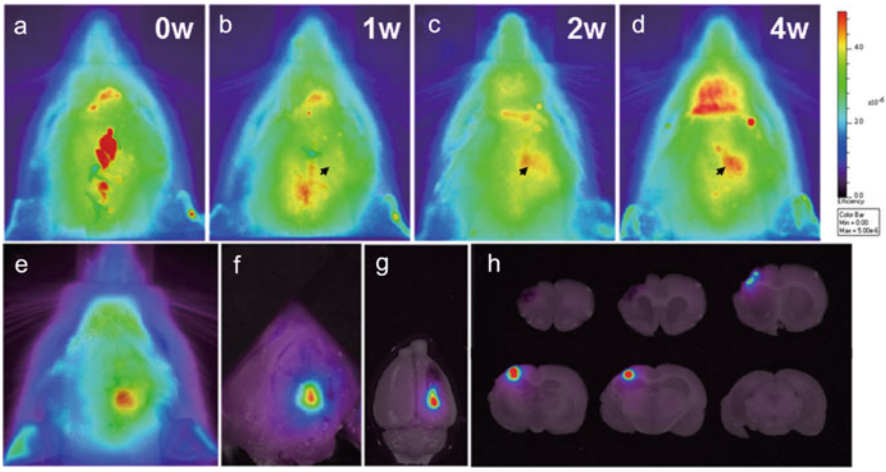

Fig. 8.2 Serial in vivo fluorescence optical images after quantum dot (QD) 800-labeled BMSC transplantation in a rat subjected to permanent MCA occlusion. The near-infrared (NIR) fluorescence emitted from QD800-labeled BMSC could not be detected through the scalp immediately after transplantation into the right striatum (**a**). The NIR fluorescence, however, could be visualized in the right parietal region 1 week after transplantation (**b**, *arrow*). The intensity significantly increased by 4 weeks after transplantation (**c**, **d** *arrow*). Representative fluorescence optical images in the living animal (**e**), after the removal of scalp (**f**) and after the removal of skull (**g**), clearly show that the NIR fluorescence is emitted from the brain infarct. A fluorescence optical image of the 2-mm-thick coronal brain slices at 2 weeks after transplantation shows that the NIR fluorescence is emitted from the peri-infarct neocortex (**h**)

8.3.4 Other Modalities

CT is characterized by excellent temporal resolution, high spatial resolution, and satisfactory anatomical and topographical depiction with relatively low soft tissue contrast. Therefore, it is a potentially promising candidate for cell tracking in the CNS. Gold nanoparticle can also be used to image cells in vivo [44]. However, compared to MRI, cell tracking with CT is far less developed at this point.

The major advantage of ultrasonography (USG) is low cost. USG may be useful in navigating the device for cell injection. However, the major limitation of USG is that ultrasound images are severely attenuated by bones, making cell tracking within the CNS quite difficult.

8.4 Perspectives

The ideal imaging system is one that does not produce radiation but has high spatial resolution, temporal resolution, contrast, and sensitivity to a small number of cells. It is also low cost and commonly available in clinical situations. However, any

single imaging modality has its advantages and drawbacks. Therefore, multimodality approaches are the current trend in cell tracking [19, 45]. Multimodal contrast agents and reporter gene assays are increasingly being developed for this purpose, with new developments expected for future studies.

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Chapter 9
Functional Bio-imaging

Hisayasu Saito, Michiyuki Miyamoto, Hideo Shichinohe, Kiyohiro Houkin, and Satoshi Kuroda

Abstract Cell transplantation therapy has been expected to promote functional recovery in various kinds of central nervous system (CNS) disorders, including cerebral stroke. However, there are several concerns to be resolved before clinical application of cell therapy for CNS disorders. The issues include the development of imaging techniques to monitor the response of the host CNS. It would be essential to establish functional bio-imaging technique serially and noninvasively validating the effects of cell therapy on the host CNS in order to achieve clinical application of cell therapy for cerebral stroke. Nuclear imaging technique is one of the most useful methods to assess the functional change in various kinds of CNS disorders, including cerebral stroke. Very recently, using a small-animal SPECT/CT apparatus, we could serially visualize the effects of BMSC transplantation on the distribution of ^{123}I-IMZ in the infarct brain of the living rodents longitudinally and noninvasively. Furthermore, we serially assessed local glucose metabolism in the rats subjected to permanent MCA occlusion and found that BMSC transplantation significantly enhances the recovery in the peri-infarct area, using small-animal ^{18}F-FDG PET/CT system. The BMSCs may enhance the recovery of local glucose metabolism by improving neuronal integrity in the peri-infarct area, when directly transplanted into the infarct brain. Although there are few studies that indicate the utility of imaging techniques to monitor the response of the host CNS after cell therapy and further investigation is needed, ^{123}I-IMZ SPECT and ^{18}F-FDG PET may be promising modalities to assess the therapeutic benefits of cell therapy for ischemic stroke without subjective bias in clinical situation.

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Keywords FDG • Functional imaging • Iomazenil • PET • SPECT

9.1 Introduction

Cell transplantation therapy has been expected to promote functional recovery in various kinds of central nervous system (CNS) disorders, including cerebral stroke [1]. A variety of cell types have been studied as cell source of transplantation in animal models of CNS disorders, including embryonic stem cells (ESCs), neural stem cells (NSCs), induced pluripotent cells (iPSCs), and bone marrow stromal cells (BMSCs) [2]. Recent animal studies have demonstrated that cell transplantation therapy significantly enhances functional recovery after ischemic stroke and several clinical trials have already been started in patients with ischemic stroke [3–9]. As pointed out by several investigators, however, there are several concerns to be resolved before clinical application of cell therapy for CNS disorders [10, 11]. The issues include the development of imaging techniques to monitor the response of the host CNS. These techniques would enable validation of the therapeutic benefits of cell transplantation therapy without subjective bias. However, there are few studies that indicate the use of imaging technique to monitor the response of the host CNS after cell therapy [5, 6, 12–14]. Based on the history of preclinical studies for neuroprotective drugs, noninvasive imaging technique may provide *biologically relevant end point*, although functional outcome was only end point in previous clinical testing of cell therapy [1]. It would be essential to bridge the still existing gap between preclinical studies and clinical investigations in order to achieve clinical application of cell therapy for ischemic stroke. From this viewpoint, it would be essential to establish functional bio-imaging technique serially and noninvasively validating the effects of cell therapy on the host CNS.

Common methods of functional neuroimaging include functional magnetic resonance imaging (fMRI), magnetoencephalography (MEG), near-infrared spectroscopy (NIRS), positron emission tomography (PET), and single-photon emission computed tomography (SPECT). The major attribute of nuclear imaging, such as PET and SPECT, that distinguishes them from the other imaging methods is the high sensitivity with which they can detect metabolic activity and trace the concentration of specific proteins in the body (e.g., neuroreceptor proteins of the brain) [15]. Thus, nuclear imaging technique is one of the most useful methods to assess the functional change in various kinds of CNS disorders, including cerebral stroke. In this chapter, we present recent progress in translational research about nuclear imaging of cell therapy for stroke.

9.2 Nuclear Imaging of Cell Therapy for Ischemic Stroke

Ischemic cerebral stroke mainly affects the blood supply to the brain, which is high for providing the oxygen and glucose demand required for neuronal function of nervous tissue. Therefore, the measurement of cerebral blood flow (CBF) was and is a central task in research [16]. SPECT studies in ischemic cerebral stroke are confirmed mainly to the imaging of perfusion and regional CBF (rCBF) using several tracers, such as ^{123}I-isopropyl iodoamphetamine (IMP), ^{99m}Tc-hexamethylpropyleneamine oxime (HMPAO), and ^{99m}Tc-ethyl cysteinate dimer (ECD) [16–18]. ^{15}O-PET is able to quantify rCBF and regional cerebral blood volume (rCBV), regional cerebral metabolic rate of oxygen ($rCMRO_2$), and oxygen extraction fraction (OEF) [19]. In clinical situation, they are very useful tools to identify ischemic penumbra which is characterized by the potential for functional recovery without morphologic damage [16, 20–26]. PET with ^{18}F-FDG, an analog of glucose, provides valuable functional information of glucose metabolism in ischemic core and peri-ischemic regions. A few other tracers, including ^{11}C-flumazenil (FMZ), ^{123}I-iomazenil (IMZ), ^{18}F-misonidazol, and ^{11}C-N-butan-2-yl-1-(2-chlorophenyl)-N-methylisoquinoline-3-carboxamide (PK11195), have special research-oriented applications [16]. ^{11}C-FMZ and ^{123}I-IMZ are radioactive ligands selective for the central type of benzodiazepine receptor. They are known useful to visualize the neuronal integrity on PET and SPECT, because the central type of benzodiazepine receptor is specifically expressed in neurons. With these tracers, ischemic penumbra and selective neuronal loss can be visualized in acute or chronic stages of ischemic stroke [27–29]. ^{18}F-misonidazol, a marker of hypoxic tissue, may also be able to identify penumbral tissue [30–32]. ^{11}C-PK11195, a peripheral benzodiazepine receptor, is known as a biomarker of inflammation. PET with ^{11}C-PK11195 may be able to detect reactive microglias and macrophages in the ischemic core and peri-infarct zones [16, 33–36]. Thus, PET and SPECT with several tracers are used to assess functional status after stroke in basic research and in clinical situation. However, there are few studies that indicate the utility of radionuclide imaging to monitor the response of the host CNS after cell therapy.

9.2.1 Effects of Cell Therapy on Neuronal Integrity

As aforementioned, ^{123}I-IMZ is a ligand displaying high affinity for central-type benzodiazepine receptors. The benzodiazepine receptor is a part of the postsynaptic GABA receptor complex and presents in high concentration on all intact cortical neurons [29]. According to previous studies, ^{123}I-IMZ is known as a useful tracer to assess neuronal viability in various kinds of CNS disorders such as Alzheimer's disease, epilepsy, and ischemic stroke [37–42]. Animal experiments have also shown that ^{123}I-IMZ is a useful marker of neuronal viability on autoradiography. Thus, Kuge et al. reported that ^{123}I-IMZ uptake markedly decreased in the infarct

regions at 4 and 24 h after the onset of MCA occlusion [39]. Kaji et al. also showed that neuronal DNA was still intact in the ischemic regions where ^{123}I-IMZ uptake was preserved [38]. Using autoradiography, we have previously shown that the engrafted BMSCs express the marker protein specific for $GABA_A$ receptor and significantly improve the distribution of ^{125}I-IMZ in the peri-infarct area [6]. Similar results have been obtained in the rat model of spinal cord injury [13]. These results strongly indicate the utility of nuclear imaging to evaluate the beneficial effects of cell therapy. However, autoradiography allows observation at only one time point by postmortem study and cannot serially evaluate brain function in the living rodents. Although previous PET/SPECT scanners could not assess cerebral function in the small animals because of their limited spatial resolution, the recent improvement in scanner technology has made it possible to evaluate it [43]. Very recently, using a small-animal SPECT/CT apparatus, we could serially visualize the effects of BMSC transplantation on the distribution of ^{123}I-IMZ in the infarct brain of the living rodents longitudinally and noninvasively. The rats were subjected to permanent middle cerebral artery occlusion. The BMSCs or vehicle was stereotactically transplanted into the ipsilateral striatum at 7 days after the insult. Using small-animal SPECT/CT apparatus, the ^{123}I-IMZ uptake was serially measured at 6 and 35 days after the onset of ischemia. As shown in Fig. 9.1, visual observations revealed a marked decrease in the distribution of ^{123}I-IMZ in the ipsilateral neocortex at 6 days postischemia. In the vehicle-transplanted animals, the distribution of ^{123}I-IMZ did not change in the peri-infarct neocortex at 35 days postischemia. However, BMSC transplantation improved the distribution of ^{123}I-IMZ in the peri-

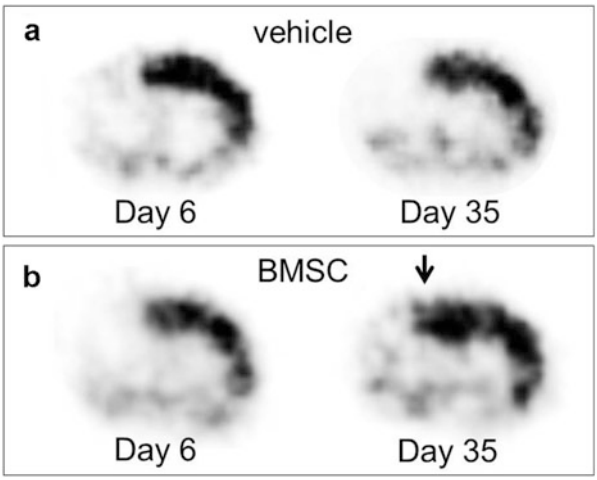

Fig. 9.1 Representative findings of ^{123}I-IMZ SEPCT. *White*-and-*black* images of the vehicle- (**a**) and bone marrow stromal cell (BMSC)-transplanted rats (**b**). In both vehicle- and BMSC-treated rats, a marked decrease in the uptake of ^{123}I-IMZ is observed in the ipsilateral neocortex at 6 days postischemia. The uptake of ^{123}I-IMZ significantly improves in the peri-infarct neocortex of the BMSC-transplanted rats at 35 days postischemia (*arrow*). The finding cannot be observed in the vehicle-transplanted rats [14]

infarct neocortex at the same timing. As the results, the engrafted BMSCs improve neuronal integrity in the peri-infarct area and enhance functional recovery after ischemic stroke. The BMSCs are densely distributed in the peri-infarct area and some of them express the neuronal phenotype [14].

9.2.2 Effects of Cell Therapy on Glucose Metabolism

Using an autoradiography technique, Mori et al. found that BMSC transplantation significantly improves glucose metabolism in the thalamus and barrel cortex in response to whisker stimulation after neocortical freezing injury [12]. The study by Dr. Wang et al. demonstrated functional recovery in a rat stroke model treated by intraventricularly administered ESCs and iPSCs along with increased ^{18}F-FDG uptake in stroke lesions depicted by small-animal PET and autoradiography. Immunohistochemistry examination at 4 weeks after cell transplantation also indicated the presence of neuronal differentiation among injected cells [44, 45]. Du et al. reported that the BMSCs delivered via the intra-arterial route promoted angiogenesis and improved functional recovery in a rat transient MCA occlusion model, using ^{99m}Tc-ECD SPECT and ^{18}F-FDG PET [46]. We also serially assessed local glucose metabolism in the rats subjected to permanent MCA occlusion and found that BMSC transplantation significantly enhances the recovery in the peri-infarct area, using small-animal ^{18}F-FDG PET/CT system. As shown in Fig. 9.2, glucose utilization was markedly decreased in the ipsilateral neocortex at 6 days after ischemia. In the vehicle-treated animals, glucose utilization improved to some extent in the peri-infarct neocortex at 35 days after ischemia, that is, 28 days after transplantation. However, BMSC transplantation significantly enhanced the recovery in the peri-infarct neocortex at the same time point [5]. Considering together with ^{123}I-IMZ study, the BMSCs may enhance the recovery of local glucose metabolism by improving neuronal integrity in the peri-infarct area, when directly transplanted into the infarct brain, because oxidative glucose metabolism is quite high in the neurons. Therefore, BMSC transplantation may possibly contribute to accelerate functional recovery by improving neuronal integrity and local metabolism in the peri-infarct brain. However, some alternative possibilities are not completely excluded. The increase in ^{18}F-FDG uptake might indicate not only neural but also glial proliferation by cell therapy, neovascular growth facilitated by treatment, or a simple reflection of macrophage migration and microglial activation after ischemia. Although the mechanisms of ^{18}F-FDG uptake in the stem cell-treated lesions are still not clear, the uptake and functional recovery measured by behavioral testing appear to correlate in some studies [45].

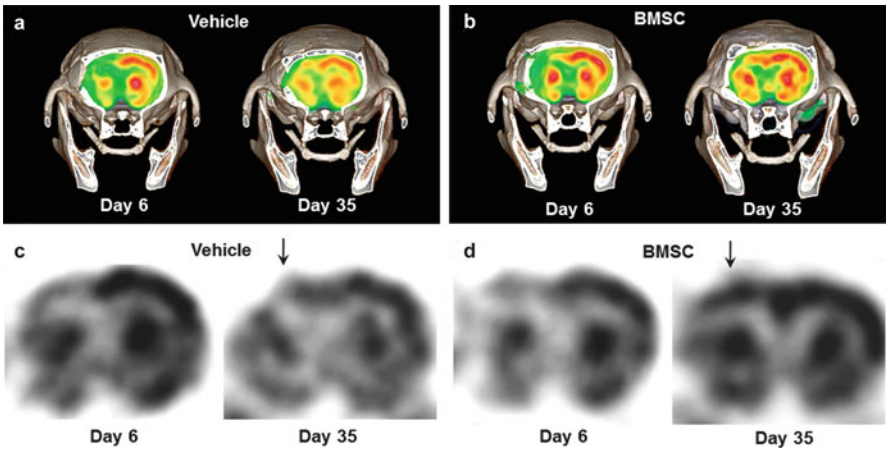

Fig. 9.2 Representative findings of ^{18}F-FDG PET at 6 and 35 days after ischemia. Color (**a**) and *black*-and-*white* (**c**) images of vehicle-transplanted animals. Color (**b**) and *black*-and-*white* (**d**) images of BMSC-transplanted rats. There was significant increase in local glucose metabolism in peri-infarct neocortex (*arrows*) [5]

9.3 Conclusion

It would be essential to establish functional bio-imaging technique serially and noninvasively validating the effects of cell therapy on the host CNS in order to achieve clinical application of cell therapy for cerebral stroke. Although there are few studies that indicate the utility of imaging techniques to monitor the response of the host CNS after cell therapy and further investigation is needed, ^{123}I-IMZ SPECT and ^{18}F-FDG PET may be promising modalities to assess the therapeutic benefits of cell therapy for ischemic stroke without subjective bias in clinical situation.

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Part III
Clinical Trials

Chapter 10
Review of Previous Clinical Trials and Guidelines of Cell Therapy

Hideo Shichinohe

Abstract Stroke is still a leading cause of death and disability, and despite intensive research, few treatment options exist. A recent breakthrough in cell therapy is expected to reverse the neurological sequelae of stroke. In the present article, we aim to review the previous clinical trials of cell therapy. Although some pioneer studies on the use of cell therapy for treatment of stroke have been reported, certain problems still remain unsolved. Moreover, we review Stem cell Therapeutics as an Emerging Paradigm in Stroke (STEPS) group and the guidelines for the development of cell therapy for stroke in the United States as well as introduce the development of new guidelines in Japan. These guidelines are expected to encourage the development of cell therapy for stroke management.

Keywords Stroke • Cell therapy • Regenerative medicine • Clinical trial • Guidelines

10.1 Introduction

Stroke is still a leading cause of death and disability [1]. Despite intensive research, few treatment options exist. Once the central nervous system (CNS) is damaged, it is difficult for the tissue to regenerate. Therefore, many patients with aftereffects of cerebral infarction also have difficulty in daily life activities. However, a lot of data on how to overcome the difficulties that occur after stroke have sequentially been obtained. In particular, the most important finding is the possibility of using regenerative medicine against CNS disorders [2]. Regenerative medicine has rapidly progressed in the recent years; therefore, it is believed that it could revive hard-to-cure neurological sequelae. The therapeutic potential of cell transplantation has been declared in various pathological CNS conditions, including traumatic brain [3, 4] and spinal cord injuries [5–9], degenerative [10] and demyelinating diseases [11], and ischemic stroke [12–16].

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Various cells could be used as a cell source in cell therapy [17]. For example, we can consider pluripotent stem cells, including embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC), and somatic stem cells, including neural stem cell, neuronal precursor cell, and mesenchymal stem cells (marrow stromal cell or MSC). Furthermore, MSC has various origins, such as bone marrow, fat, pulp, and umbilical cord [18]. In addition, bone marrow mononuclear cells (BMMNC) are also used as a source for cell therapy, although their characteristics do not classify them as stem cells.

Each cell source has a different characteristic. Studies with ESC have played a leading role in regenerative medicine because of its pluripotency. However, there is an ethical problem because to obtain ESC, an embryo has to be destroyed [19]. iPSC reported by Yamanaka et al. in 2006 attracted worldwide attention [20]. It is artificially made from somatic cells, such as epidermal or white blood cells, and has self-replication ability and pluripotency. An important characteristic of iPSC is that an embryo does not have to be destroyed at the time of procurement. However, further studies are necessary to reduce risks, such as post-transplant tumorigenesis. MSC attracted much attention after Azizi et al. reported in 1998 that MSC could differentiate into neural cells [21]. The advantages of MSC for clinical use are as follows: cell collections and culture methods are simple and established, the autologous cell source is available, and there are fewer problems, such as bioethics, immunoreaction, and tumorigenesis compared with those observed with ESC or iPSC.

10.2 Pioneer Studies on Cell Therapy for Ischemic Stroke

In 2005, some research groups reported pioneering studies concerning the use of cell therapy against cerebral stroke. Kondziolka et al. reported a phase 2 trial with LBS-Neurons (human teratocarcinoma cell line origin, Layton BioScience Inc, CA, USA) [22]. They tested the usefulness of neuronal cell transplantation in patients with substantial fixed motor deficits associated with a basal ganglia stroke. The trial included 18 patients with completed stroke duration of 1–6 years. Nine strokes were ischemic in origin and nine were hemorrhagic. Patients were randomized for stereotactic implantation of either five or ten million cells in 25 sites in the brain followed by participation in a stroke rehabilitation program or to serve as a nonsurgical control group (rehabilitation only). The primary efficacy measure was a change in motor score at 6 months. There were no significant changes in the motor scores in patients who received cell implants compared with that in control. Serial evaluations demonstrated that three patients suffered complications, including single seizure, syncope, and asymptomatic chronic subdural hematoma.

In the same year, Savitz et al. reported an open-label trial concerning stereotactic transplantation with LGE cells (fetal porcine striatum-derived cells, Genvec Inc., MD, USA) in five patients with basal ganglia infarcts and stable neurological deficits [23]. The study initially planned to enroll 12 patients with ischemic stroke.

All patients received the same number of cells per volume of infarct. Two patients showed improvements in speech, language, and/or motor impairments. However, two patients had adverse effects; one had temporary worsening of motor deficits 3 weeks after transplantation and the other had seizures 1 week after transplantation. The study was terminated by Food and Drug Administration (FDA) after the inclusion of five patients.

On the other hand, in East Asia, Bang et al. reported in 2005 the feasibility and safety of cell therapy using autologous MSC for patients with ischemic stroke [24]. They prospectively and randomly allocated 30 patients with cerebral infarcts within the middle cerebral arterial region and with severe neurological deficits to the treatment group, who received intravenous infusion of 1×10^8 autologous MSCs ($n = 5$) and the control group ($n = 25$). They reported that serial evaluations showed no adverse cell-related, serological, or imaging-defined effects. Meanwhile, outcomes improved temporarily in MSC-treated patients compared with control patients, but there was no significant change in the motor score during a 12-month period.

Honmou et al. reported a phase one half trial using autologous MSC [25]. Twelve patients with cerebral infarction received intravenous cell infusion in acute phase. They showed that there was improvement in neurologic symptoms in 11 patients, without adverse cell-related, serological, or imaging-defined effects. They concluded that intravenous administration of autologous MSC appeared to be feasible and safe and merited further study as a therapy that may improve functional recovery.

10.3 Guidelines Encourage the Development of Cell Therapy Against Stroke

Because people had high hopes for regenerative medicine, the failure of these pioneer studies in the United States disappointed them [26]. When we look back at the history of neuroprotective drugs development, there were few approved drugs, although many promising chemical compounds were developed [27]. Researchers accepted the necessity to set standards for developing neuroprotective drugs. The working group, which was named as Stroke Therapy Academic Industry Round-table (STAIR), was organized in the US, and the first recommendation was published in 1999 [28]. Thereafter, the recommendations were published continuously, and the aim of the latest STAIR VIII was the development of neurothrombectomy devices [29]. Modeled on the STAIR format, some researchers in the US intended to formulate guidelines for the development of cell therapy against stroke. In 2007, Stem cell Therapeutics as an Emerging Paradigm in Stroke (STEPS) group, whose members belonged to academia, industry, and National Institutes of Health (NIH), launched an effort to set the standards for the development of cell therapy. The first recommendation, STEPS-I, was

published in 2009 [30]. The guidelines included the design of the pre-clinical studies, such as types of animals, stroke models, behavior and imaging analyses, and moreover, the design of the early phase of clinical trials, such as the end-point of the trials, choice of cell delivery methods, cell dose, patient selection, and treatment duration. In 2011, STEPS-2 was published, and FDA also participated in the working group at the moment [31]. In 2014, STEPS-3 was published as the latest guidelines that included the design of the later phase of the clinical trials [32].

Since a series of STEPS, many clinical trials concerning cell therapy against stroke have been initiated worldwide. When we tried comprehensively searching for clinical trials based on cell therapy against stroke using ClinicalTrials.gov, 30 trials were found using the keywords, "stem cell" and "stroke" (Table 10.1). We found several cell sources; however, most of them were derived from bone marrow. The use of allogeneic bone marrow derived-cell sources has been regarded as the mainstream. These clinical trials started in not only the United States but also Europe, India, China, and Korea. These results show that a series of STEPS has encouraged the development of new cell therapies all over the world.

STEPS members also started new trials in rapid succession. At the International Stroke Conference 2014 (San Diego, CA), Steinberg et al. described a phase 1/2A study, with SB623 cells (SanBio Inc, CA, USA, NCT01287936 in Table 10.1). The trials were two-center, open-label, safety, and dose escalation feasibility studies. The cell source was genetically modified-bone marrow cells. Eighteen patients with ischemic stroke took the stereotactic transplantation in chronic phase. It was noteworthy that they showed the potential to improve motor function according to the European stroke scale, NIH stroke scale (NIHSS), and Fugl-Meyer scale.

Hess et al. also reported the study with MultiStem (Athersys Inc, Cleveland, USA, NCT01436487 in Table 10.1) at the European Stroke Organization Conference 2015 (Glasgow, UK). Thirty-three hospitals in both the US and UK participated in the double-blind, randomized, placebo-controlled, phase 2 safety and efficacy trial. The cell source was allogeneic bone marrow derived-cells. A total of 126 patients with ischemic stroke took intravenous transplantation in the acute phase. The primary endpoint was the modified Rankin scale, NIHSS, and Barthel index at 90 days after the treatment. They showed favorable recovery in the earlier therapeutic time window (24–36 h after stroke).

10.4 New Guidelines in Japan

To date, there are only two clinical trials using the autologous bone marrow derived-cells in Japan, including the phase 2 trial with autologous MSC by Honmou et al. [25] and the phase 1/2A trial with autologous BMMNC by Taguchi et al. [33]. When compared with successful basic research concerning stem cells, such as the establishment of iPSC [20], the numbers are less. To encourage basic science to translate into bedside treatments, Japanese researchers and regulatory agencies need to think about what they need to do. In 2012, Ministry of Health, Labour

Table 10.1 List of 30 completed or ongoing trials for cell therapy against stroke on ClinicalTrials.Gov

ID	Study	Sponsor (country)	1st received	Condition of patients	Cell source (product code)	Delivery route	Phase	Status of trials
NCT00473057	Study of Autologous Stem Cell Transplantation for Patients With Ischemic Stroke	Universidade Federal do Rio de Janeiro (Brazil)	2007	Acute/ subacute	Autologous BM-MNC	I.A.	1	Completed
NCT00535197	Autologous Bone Marrow Stem Cells in Ischemic Stroke	Imperial College London (UK)	2007	Acute	Autologous CD34(+) cells	I.A.	1/2	Completed
NCT00761982	Autologous Bone Marrow Stem Cells in Middle Cerebral Artery Acute Stroke Treatment	Hospital Universitario Central de Asturias (Spain)	2008	Acute	Autologous CD34(+) cells	I.A.	1/2	Completed
NCT00859014	Safety/Feasibility of Autologous Mononuclear Bone Marrow Cells in Stroke Patients	The University of Texas Health Science Center, Houston (USA)	2009	Acute	Autologous BM-MNC	I.V.	1	Completed
NCT00875654	Intravenous Stem Cells After Ischemic Stroke (ISIS)	University Hospital, Grenoble (France)	2009	Acute	Autologous MSC	I.V.	2	Active, not recruiting
NCT00950521	Efficacy Study of CD34 Stem Cell in Chronic Stroke Patients	China Medical University Hospital (China)	2009	Chronic	Autologous CD34(+) cells	I.C.	2	Completed
NCT01151124	Pilot Investigation of Stem Cells in Stroke (PISCES)	ReNeuron Limited (UK)	2010	Chronic	Allogeneic NSC (CTX0E03)	I.A.	1	Active, not recruiting
NCT01287936	A Study of Modified Stem Cells in Stable Ischemic Stroke	SanBio, Inc. (USA)	2011	Chronic	Allogeneic MSC (SB623)	I.A.	1/2	Active, not recruiting
NCT01297413	A Study of Allogeneic Mesenchymal Bone Marrow Cells in Subjects With Ischemic Stroke	Stemedica Cell Technologies, Inc. (USA)	2011	Chronic	Allogeneic MSC	I.V.	1/2	Active, not recruiting
NCT01310114	Study of Human Placenta-derived Cells (PDA001) to Evaluate the Safety and Effectiveness for Patients With Ischemic Stroke	Celgene Corporation (USA)	2011	Acute	Allogeneic placenta-derived cells (PDA001)	I.V.	2	Terminated

(continued)

Table 10.1 (continued)

ID	Study	Sponsor (country)	1st received	Condition of patients	Cell source (product code)	Delivery route	Phase	Status of trials
NCT01436487	Study to Examine the Effects of MultiStem in Ischemic Stroke	Athersys, Inc (USA)	2011	Acute	Allogeneic MSC (MultiStem)	I.V.	2	Completed
NCT01461720	Intravenous Autologous Mesenchymal Stem Cells Transplantation to Treat Middle Cerebral Artery Infarct	National University of Malaysia (Malaysia)	2011	Acute	Autologous MSC	I.V.	2	Recruiting
NCT01468064	Autologous Bone Marrow Stromal Cell and Endothelial Progenitor Cell Transplantation in Ischemic Stroke (AMETIS)	Southern Medical University (China)	2011	Acute	Autologous MSC, autologous EPC	I.V.	1/2	Recruiting
NCT01501773	Intravenous Autologous Bone Marrow-derived Stem Cells Therapy for Patients With Acute Ischemic Stroke	Manipal Acunova Ltd. (India)	2011	Acute	Autologous BM-MNC	I.V.	2	Completed
NCT01678534	Reparative Therapy in Acute Ischemic Stroke With Allogenic Mesenchymal Stem Cells From Adipose Tissue, Safety Assessment, a Randomised, Double Blind Placebo Controlled Single Center Pilot Clinical Trial (AMASCIS-01)	Instituto de Investigación Hospital Universitario La Paz (Spain)	2012	Acute	Allogenic AT-MSC	I.V.	2	Recruiting
NCT01714167	Autologous Bone Marrow Mesenchymal Stem Cell Transplantation for Chronic Stroke	Wenzhou Medical University (China)	2012	Subacute/chronic	Autologous MSC	I.C.	1	Recruiting

NCT01716481	The STem Cell Application Researches and Trials In NeuroloGy-2 (STARTING-2) Study	Samsung Medical Center (Korea)	2012	Acute/ subacute	Autologous MSC	I.V.	3	Recruiting
NCT01832428	A Clinical Trial to Study the Safety and Efficacy of Bone Marrow Derived Autologous Cell for the Treatment of Stroke (BMACS)	Chaitanya Hospital, Pune (India)	2013	Cerebral infarct or hemorrhage	Autologous BM-MNC	I.T.	1/2	Recruiting
NCT01922908	Mesenchymal Stromal Cells for Ischemic Stroke (SAMCIS)	The University of Texas Health Science Center, Houston (USA)	2013	Acute	Allogeneic MSC	I.V.	1/2	Not yet recruiting
NCT02065778	Safety and Efficacy of Autologous Stem Cell Therapy in Chronic Stroke	Neurogen Brain and Spine Institute (India)	2014	Chronic	Autologous BM-MNC	I.T.	1	Completed
NCT02117635	Pilot Investigation of Stem Cells in Stroke Phase II Efficacy (PISCES-II)	ReNeuron Limited (UK)	2014	Subacute	Allogeneic NSC (CTX DP)	I.C.	2	Recruiting
NCT02245698	Autologous Stem Cell Therapy in Stroke	Neurogen Brain and Spine Institute (India)	2014	Subacute/ chronic	Autologous BM-MNC	I.T.	1	Recruiting
NCT02283879	Human Umbilical Cord Mesenchymal Stem Cell in Cerebral Hemorrhage Sequela	Shenzhen Hornetcorn Bio-technology Company, LTD (China)	2014	Chronic cerebral hemorrhage	Allogeneic UC-MSC	I.V.	1	Active, not recruiting
NCT02378974	Evaluation of the Safety and Potential Therapeutic Effects After Intravenous Transplantation of Cordstem-ST in Patients With Cerebral Infarction	CHABiotech CO., Ltd (Korea)	2015	Acute	Allogeneic UC-MSC (Cordstem-ST)	I.V.	1/2	Recruiting
NCT02397018	Cord Blood Infusion for Ischemic Stroke	Duke University Medical Center (USA)	2015	Acute	Allogeneic UCB	I.V.	1	Recruiting

(continued)

Table 10.1 (continued)

ID	Study	Sponsor (country)	1st received	Condition of patients	Cell source (product code)	Delivery route	Phase	Status of trials
NCT02425670	Stem Cell Therapy For Acute Ischemic Stroke Patients (InVeST)	All India Institute of Medical Sciences, New Delhi (India)	2015	Acute	Autologous MSC	I.V.	2	Completed
NCT02448641	Study of Modified Stem Cells (SB623) in Patients With Chronic Motor Deficit From Ischemic Stroke (ACTIsSIMA)	SanBio, Inc. (USA)	2015	Chronic	Allogeneic MSC (SB623)	I.A.	2	Recruiting
NCT02564328	Autologous Bone Marrow Mesenchymal Stem Cell Transplantation for Chronic Ischemic Stroke	Southern Medical University (China)	2015	Acute/ subacute	Autologous MSC	I.V.	1	Recruiting
NCT02580019	Umbilical Cord Derived Mesenchymal Stem Cells Treatment in Ischemic Stroke	Affiliated Hospital to Academy of Military Medical Sciences (China)	2015	Acute	Allogeneic UC-MSC	I.V.	2	Not yet recruiting
NCT02605707	Autologous Endothelial Progenitor Cells Transplantation for Chronic Ischemic Stroke	Southern Medical University (China)	2015	Subacute/ chronic	Autologous EPC	I.V.	1/2	Recruiting

BM-MNC bone marrow-derived mononuclear cells, *MSC* (bone marrow-derived) mesenchymal stem cells, *NSC* neural stem cells, *EPC* endothelial progenitor cells, *AT-MSC* adipose tissue-derived MSC, *UC-MSC* umbilical cord-derived MSC, *UCB* umbilical cord blood, *I.V.* intravenous, *I.C.* intra-cerebral, *I.A.* intra-arterial, and *I.T.* intra-thecal

and Welfare in Japan started the new project, "Initiative for Accelerating Regulatory Science in Innovative Drug, Medical Device, and Regenerative Medicine." The project promoted a personal exchange between the developers and Pharmaceuticals and Medical Devices Agency (PMDA), which is the regulatory agency in Japan and a counterpart of FDA or European Medicines Agency (EMA), to establish various guidelines for the development of new drugs, medical devices, and cell-products. As a part of the project, the working group (chairman, Dr. Kiyohiro Houkin, Hokkaido University, Sapporo, Japan) established new guidelines for cell therapy against stroke, which started in November 2013. The members consisted of neurosurgeons, neurologists, a neuro-radiologist, a physician for neuro-rehabilitation, basic and regulatory scientists, PMDA staffs. It was important to show an original stance in Japan because of domestic regulations for regenerative medicine, although a series of STEPS was used as a reference. The scope of the guidelines consisted of cell therapy against ischemic stroke. Regarding somatic stem cells, MSC, MNC, and neural stem cells were included. It was noteworthy that not only the researchers but also PMDA could use the guideline for reviews. In 2016, the guidelines will be launched in Japanese, and then the text will be translated into English to propagate it worldwide. It is believed that the new guidelines will promote the development of new cell therapies in Japan and will be established for stroke management in the future.

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Chapter 11
Intravenous Autologous Bone Marrow Mononuclear Cell Transplantation for Stroke Patients

Yukiko Kasahara, Tomohiro Matsuyama, and Akihiko Taguchi

Abstract Therapeutic angiogenesis mediated by hematopoietic stem cell transplantation has been initiated in patients with ischemic diseases and has shown promising results. We previously demonstrated that therapeutic angiogenesis is essential for neurogenesis after stroke and that intravenous administration of hematopoietic stem cells improves functional recovery through enhanced angiogenesis in an experimental stroke model. Based on these observations, we initiated a clinical trial of cell therapies with the aim of achieving functional recovery in patients with cerebral ischemia through regenerative microcirculation in the brain following a stroke.

This review summarizes recent findings from basic and clinical research on stroke and introduces our own clinical trial aimed at enhancing functional recovery in stroke patients using bone marrow mononuclear cells.

Keywords Autologous bone marrow-derived mononuclear cells • Clinical trial • Stroke

11.1 Introduction: Current Therapy in Stroke Patients

Stroke is the third leading cause of death in developed countries after heart disease and cancer [1] and is associated with a negative impact on activity and quality of daily life. Thrombolytic therapy is effective for the functional outcomes of stroke patients, but it can only be applied to certain patients because it must be given within 4.5 h after stroke onset [2], and no definitive treatment exists after that period other than rehabilitation. Consequently, more than 50 % of stroke survivors cannot

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recover completely, and 20 % of stroke patients need help with their everyday activities [3]. Thus, development of novel and safe therapies to regenerate neuronal function after stroke is eagerly awaited.

To improve functional recovery after stroke, clinical trials of various drugs have been conducted, but have achieved either only mild or no significant therapeutic effects, or have sometimes even had serious adverse effects [4, 5]. Furthermore, clinical trials of neural stem cell transplantation in stroke patients have been conducted, but such treatments have also shown only mild or nonsignificant therapeutic effects [6], while some have had adverse effects [7].

Transplantation of bone marrow-derived mononuclear cells has been shown to reduce ischemic damage and enhance functional recovery in experimental models, including limb [8–11], myocardium [12–15], and cerebral ischemia [16, 17] models, and various clinical trials using bone marrow-derived mononuclear cells are ongoing with promising results so far [18–20]. Recently, we showed that therapeutic angiogenesis by bone marrow-derived mononuclear cells enhances neurological recovery in experimental stroke model [21], and we initiated cell-based therapy using autologous bone marrow mononuclear cells for patients after cerebral embolism as a phase 1/2a clinical trial, which we have now completed [22].

This chapter focuses on the clinical application of autologous bone marrow-derived mononuclear cells in stroke patients, in particular the findings from our clinical trial, and discusses the future of cell-based therapy for stroke patients.

11.2 Bone Marrow-Derived Mononuclear Cells as Candidate Cells to Improve Stroke Outcome

To develop a novel therapeutic strategy for stroke, we investigated the relationship between bone marrow-derived cells and cerebral microvasculature in the poststroke brain. Observational studies have demonstrated that decreased levels of circulating immature bone marrow-derived cells are associated with impaired cerebrovascular function [17] and reduced cognition [23] and, in contrast, high levels of bone marrow-derived immature cells are associated with neovascularization of the ischemic brain [24]. On the basis of these observations, we demonstrated that the intravenous transplantation of bone marrow-derived mononuclear cells [25] and hematopoietic stem cells [21] after stroke activates the cerebral microvasculature in the poststroke brain, followed by enhanced endogenous neurogenesis and functional recovery in an experimental model.

Besides the bone marrow mononuclear cell, many kinds of stem cells have been tested in clinical trials to enhance endogenous neurogenesis after stroke, such as mesenchymal stem cells. Although some clinical trials of mesenchymal stem cells have demonstrated safety, feasibility, and preliminary efficacy in stroke patients [26, 27], autologous mesenchymal stem cells require cell culture to obtain the

required dose and unable treatment of patients in the acute/subacute stage of stroke. In addition, in vitro expansion of the cell population may bear the risk of contamination and/or malignant transformation [28]. In contrast, mononuclear cells can be prepared rapidly within a few hours and permit autologous administration, thereby avoiding the problem of immunological rejection. In addition, compared with mesenchymal stem cells, bone marrow mononuclear cells may be expected to have a lower risk of pulmonary embolism after intravenous injection because of the smaller cell size, as suggested by results in a rodent model [29].

Granulocyte colony-stimulating factor (G-CSF) has been shown to promote angiogenesis in ischemic tissue via mobilization of hematopoietic stem cells [30, 31], but it has not been shown to have therapeutic benefit in patients after stroke [32] because G-CSF exaggerated the inflammatory response, a key element that induces neural stem/progenitor cell death and negates the therapeutic effects associated with angiogenesis [33].

11.3 Clinical Trials of Bone Marrow-Derived Mononuclear Cells in Stroke Patients

A list of major clinical trials using bone marrow mononuclear cells is shown in Table 11.1. All clinical trials have shown that transplantation of bone marrow mononuclear cells in stroke patients is feasible and safe, though the clinical protocols have varied among these clinical trials, especially with regard to the time window and route for administration.

11.3.1 Therapeutic Time Window

Previously, we reported that histopathological studies in stroke patients have pointed out the presence of neural stem/progenitor cells in the poststroke human cerebral cortex and that the peak in endogenous neurogenesis occurs approximately 1–2 weeks after a stroke [34]. Consistent with this, analysis of the therapeutic time window in murine stroke model revealed that administration of bone marrow-derived mononuclear cells within 24 h after stroke had a mild and nonsignificant effect on brain regeneration/protection following ischemia [35], but administration of these cells between day 2 and day 14 after the ischemic event showed significant positive effects [36]. Recently, Prasad et al. reported that intravenous administration of bone marrow-derived cells for stroke patients in the chronic period is safe, but there was no beneficial effect of treatment on stroke outcome [37]. The time window after stroke onset in their study was 18.5 days (median), which seems too late to support endogenous neurogenesis after stroke. Their results may be attributed to the time lag between the onset of stroke and the peak of neurogenesis.

Table 11.1 Clinical trials of bone marrow mononuclear cells in stroke patients

Institute	Country	Total	Stroke type	Cell dose	Administration	Status	Identifier
Universidade Federal do Rio de Janeiro	Brazil	20	Chronic (2–3 months)	$1–5 \times 10^8$	Intra-arterial	Complete (2011) [39]	NCT00473057
The University of Texas Health Science Center	USA	10	Acute (24–72 h)	1×10^7/kg	Intravenous	Complete (2011) [46]	NCT00859014
Hospital Sao Lucas	Brazil	20	Subacute (3–7 days)	2.2×10^8	Intra-arterial	Complete (2012) [38]	N/A
NeuroGen Brain and Spine Institute	India	24	Chronic	1×10^6/kg	Intrathecally	Complete (2014) [47]	NCT02065778
All India Institute of Medical Sciences	India	48	Chronic (18.5 days)	2.8×10^8	Intravenous	Complete (2014) [48]	NCT0150177
National Cerebral and Cardiovascular Center	Japan	12	Subacute (7–10 days)	2.5×10^8 and 3.4×10^8	Intravenous	Complete (2015) [22]	NCT01028794
Andalusian Initiative for Advanced Therapies	Spain	76	Subacute (1–7 days)	2×10^6/kg and 5×10^6/kg	Intra-arterial	Recruiting	NCT02178657
University of California	USA	33	Subacute (4 days)	N/A	Intravenous	Not yet recruiting	NCT00908856

11.3.2 Delivery Route

The intra-arterial route is well known to result in higher cell counts at the target site compared to intravenous infusion [38, 39]. Although some clinical trials of intra-arterial administration of bone marrow mononuclear cells have demonstrated feasibility and safety [38, 40], several studies reported that intra-arterial injection was not superior to intravenous injection in experimental stroke models [41, 42]. Friedrich et al. reported that there were no significant differences in neurological function with patients undergoing intra-arterial bone marrow mononuclear cells treatment, compared with the control group [39]. Moreover, generalized seizures developed in patients after intra-arterial injection of bone marrow mononuclear cells, though the relation between seizures and intra-arterial treatment is unknown [38]. Intra-arterial injection would permit a substantial lowering of the number of cells, but caution might be exercised in intra-arterial administration bone marrow mononuclear cells for stroke patients compared with intravenous administration. Results of the therapeutic and adverse effects of these treatments and different modes of cell administration are expected to emerge over the next several years. More definitive conclusions regarding differences between the intravenous and intra-arterial administration of bone marrow mononuclear cells for stroke patients await the results of an ongoing randomized and controlled multicenter trial in Spain [43].

11.3.3 Cell Doses

In Table 11.1, the number of cells (from 1×10^7 to 5×10^8 cells) given to stroke patients in clinical trials is relatively small. A growing body of clinical and experimental evidence indicates that the number of injected cells reaching the brain parenchyma seems to be very small, i.e., preclinical studies indicate that approximately 0.02–1 % of injected cells home into the brain [42, 44, 45] and, probably, the differentiation of the stem cells into endothelial cells in the ischemic brain may not play a critical role in angiogenesis after stroke. We found that administration of a relatively small number of bone marrow-derived mononuclear cells had significantly beneficial effects on regeneration/protection of injured brain tissue in an experimental model [36].

11.4 Introduction of Our Phase 1/2a Clinical Trial

Based on these observations, we conducted a clinical trial to enhance functional recovery through activating angiogenesis by autologous bone marrow mononuclear cells in patients with cerebral infarction. Our clinical trial was an unblended,

uncontrolled phase 1/2a clinical trial. This clinical protocol was approved by the Ministry of Health, Labour and Welfare and the institutional review board of the National Cerebral and Cardiovascular Center (ClinicalTrials.gov Identifier: NCT01028794). The aim of this clinical trial was to assess the feasibility, safety, and efficacy of the intravenous transplantation of autologous bone marrow mononuclear cells into patients with stroke. The clinical trial employed a nonrandomized open-label study design for 12 stroke patients (25 ml, low-dose group, $N = 6$; 50 ml, high-dose group, $N = 6$). The outline of this protocol is shown in Fig. 11.1. Major inclusion criteria were patients with cerebral embolism, National Institute of Health Stroke Scale (NIHSS) score more than 9 on day 7 after stroke, and improvement in NIHSS in the first 7 days after onset less than 6 points. To improve the sensitivity for detecting efficacy signals, the enrollment was restricted to patients diagnosed with cerebral embolism and those expected to exhibit very poor outcomes during the chronic period at day 7 after onset.

On day 7–10 after stroke, patients had 25 ml (low-dose group) or 50 ml (high-dose group) of bone marrow cells aspirated from their iliac bone. Autologous bone marrow-derived mononuclear cells were purified by the density gradient method and administered intravenously on the day of the bone marrow aspiration. The primary endpoint was safety and improvement in the NIHSS score compared with our historical control. No side effects or safety problems were observed. A comparison of the results from patients receiving the two doses (25 and 50 ml) of bone marrow mononuclear cells showed that the higher dose was superior to the lower one in terms of showing a trend toward improved response (without statistical significance). Similarly, in comparison to historical control group, autologous bone marrow cell transplantation also showed significantly better outcomes in NIHSS score. Most of the patients showed a significant improvement in neurological function at 6 months after cell transplantation. Furthermore, analysis of cerebral blood flow and metabolism in patients after cell transplantation showed a trend favoring an increase in rCBF and $rCMRO_2$ in both ipsilateral and contralateral hemispheres at 6 months, compared to 1 month, after cell transplantation. Our study demonstrates that administration of autologous bone marrow mononuclear cells to patients with severe embolic stroke was feasible and safe. Furthermore, the positive trends favoring neurologic recovery in a dose-dependent manner and improvement in cerebral blood flow and metabolism in poststroke patients receiving cell therapy emphasized the potential of this approach.

11.5 Future Cell Therapy for Stroke Patients

For accurate assessment of the therapeutic effects of hematopoietic stem cell transplantation for stroke, improvements in clinical trial design are desirable. Fugl-Meyer Assessment is one of the most widely used quantitative measures of motor impairment after stroke [49], and assessment of motor function by Manual Muscle Testing at enrollment and follow-up with the Fugl-Meyer test would be one

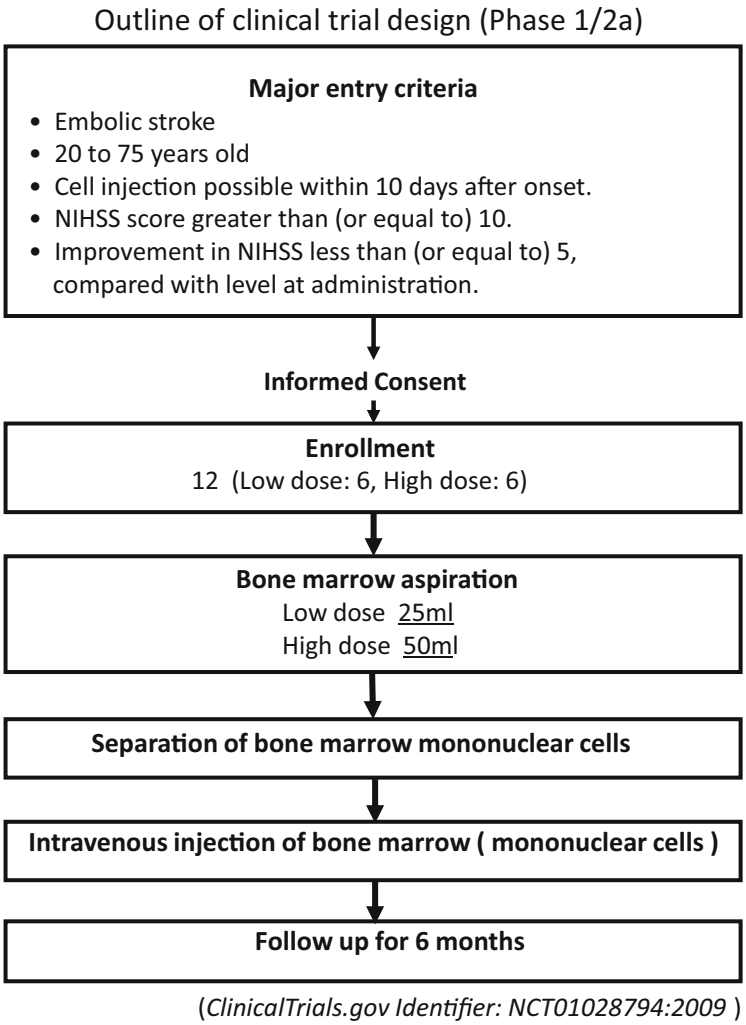

Fig. 11.1 Outline of clinical trial design. Our clinical trial was an unblended, uncontrolled phase 1/2a clinical trial. On days 7–10 after first detection of stroke, autologous bone marrow was aspirated. Separation of bone marrow mononuclear cells and transplantation of purified bone marrow mononuclear cells were both performed on the day of the bone marrow aspiration

of the most workable designs to evaluate the effect of cell therapy. In addition, image assessment is another important component in evaluating the effect of cell therapy. Resting-state functional magnetic resonance imaging (fMRI) is one possible candidate, because it is applicable to patients with stroke who are not capable of proper performance of motor tasks [50]. Furthermore, systematic assessment of initial resting-state functional connectivity using resting-state fMRI will be able to provide prognostic prediction of later motor recovery in stroke patients. Although

the presence of "responder" and "nonresponder" patients in the context of cell therapy has been reported [51], information obtained by MRI images may also provide the criteria to select only responder for enrollment in clinical trial.

11.6 Conclusion

The results from clinical trials, including our study, indicate that autologous hematopoietic stem cell transplantation is feasible and safe in patients with stroke and encourage the performance of randomized clinical trials to clearly prove the effect of cell therapy. In addition to hematopoietic stem cells, many kinds of stem cells have been tried in clinical trials, including autologous mesenchymal stem cells [52], allogeneic mesenchymal-derived stem cells [53], allogeneic teratocarcinoma-derived neuronal cells [6], and fetal porcine-derived neural stem cells [7]. Though the source of transplanted cells and the route for injection have varied, the major target of cell transplantation is, we believe, the modulation of the healing process after stroke (Fig. 11.2), which is similar to the wound healing process that consists

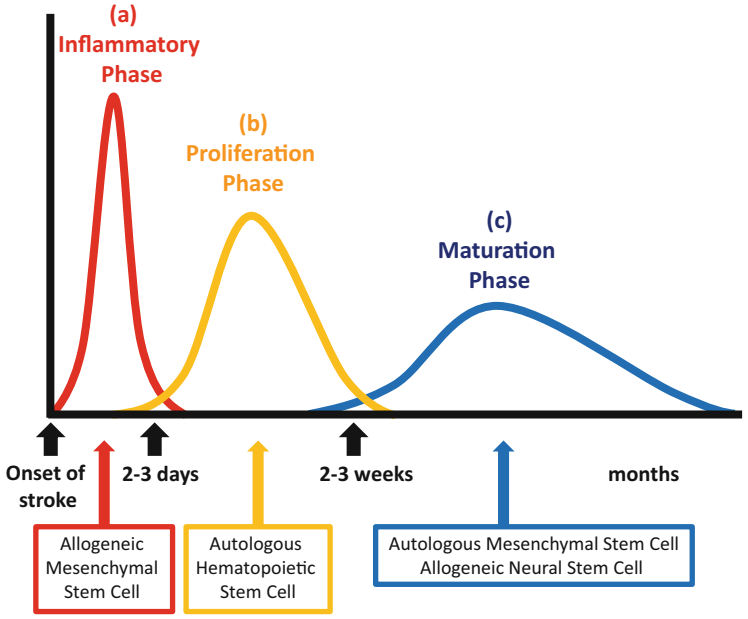

Fig. 11.2 Major cell therapies and their target phase. (**a**) During the inflammatory phase, allogeneic mesenchymal-derived stem cell transplantation has been shown to modulate inflammatory response. (**b**) In the proliferation phase, hematopoietic stem cell transplantation has been shown to activate angiogenesis and endogenous neurogenesis. (**c**) During the maturation phase, transplanted stem cells have been shown to differentiate into neuronal cells and/or release neurotropic factors

of inflammatory, proliferative, and remodeling phases [54]. Optimal treatment during each phase would maximize functional recovery after stroke, and the combination of cell therapies at different phases could be one of the approaches for best recovery in the future.

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Chapter 12
Mesenchymal Stem Cells

Masanori Sasaki and Osamu Honmou

Abstract Mesenchymal stem cells (MSCs) derived from adult human bone marrow hold a spectrum of functional properties. Intravenous infusion of MSCs provides functional improvements in animal models including cerebral stroke and spinal cord injury. Suggested mechanisms may include neuroprotection, angiogenesis, induction of axonal sprouting, and remyelination.

Therapeutic effects have been reported in animal models of stroke after intravenous infusion of human MSCs derived from adult human bone marrow. Initial clinical studies on intravenously infused MSCs have now been completed in human stroke patients. Here, we review the reparative and protective properties of infused MSCs in stroke models, describe initial human studies on intravenous infusion of MSC in stroke, and provide a perspective on prospects for future progress with MSCs.

Keywords Mesenchymal stem cell • Stroke • Clinical trial

12.1 Introduction

Transplantation of MSCs derived from adult rat bone marrow after induction of experimental cerebral ischemia can reduce infarct volume and improve behavioral function in cerebral ischemia models (ref). Lesion volume as estimated from magnetic resonance imaging (MRI; Fig. 12.1a, b) or histological analysis (Fig. 12.1c) is reduced after intravenous infusion of MSCs, and there is therapeutic efficacy [1–3] (Fig. 12.1d). Suggested mechanisms for these therapeutic effects of MSCs include neuroprotection, angiogenesis, stimulation of neurogenesis and axonal sprouting/regeneration, and remyelination. We focus on the potential reparative effects in stroke of an operationally defined MSC derived from bone marrow. These cells are $CD34^{-}$, $CD45^{-}$, $CD73^{+}$, and $CD105^{+}$, providing a basis for isolation by flow cytometry [1, 4].

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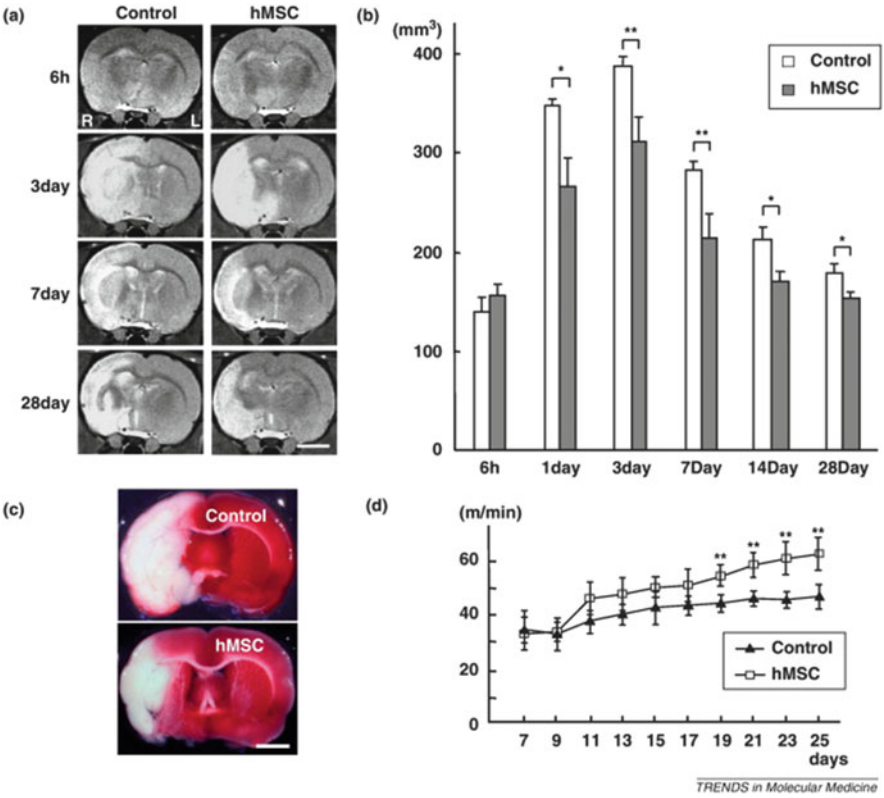

Fig. 12.1 Ischemic lesion volume is reduced and functional improvement is observed after human MSC (hMSC) injection. MRI imaging (**a**) of the rat brain at various times after systemic infusion of hMSCs indicates reduced lesion volume compared with control animals (control) without MSC infusions (**b**). Reduced lesion (*white area*) is evident in the TTC-stained sections (**c**). Behavioral testing indicates that the maximum speed on a treadmill test was greater in the MSC-infused group (**d**). Thus, lesion volume was reduced, and functional improvement was obtained in the MSC group (Modified from Honmou et al. [36])

Stroke patients display spontaneous functional improvements in various degrees [5] and even if the lesion volume may increase during recovery or not change in experimental models of stroke [6]. These phenomena indicate that functional improvements may be contributed by compensatory neural plasticity or brain remodeling over the time-dependent manner.

Transplantation of exogenous cells into a stroke may produce new mechanisms that will facilitate functional improvements or enhance endogenous recovery [7]. Current thinking is that several mechanisms, including the possibility that stem cells may release or stimulate release of trophic factors that may be neuroprotective and/or promote neovascularization, axonal sprouting, and remyelination, although an early assumption in cellular therapy for neurological diseases was that transplanted cells would reconstruct injured tissues/cells.

These different mechanisms are not mutually exclusive, raising the possibility that a cell-based approach may display multiple therapeutic effects at various lesions and times in the injured tissue, as the cells respond to a damaged microenvironment. We describe in this chapter experimental work and the initial clinical studies of intravenous MSC infusions in human stroke patients.

12.2 Neuroprotective Effects of MSCs

It has been suggested that the MSCs have the capacity to release growth and trophic factors or to stimulate their release from resident neural tissue and to contribute to produce the therapeutic benefit in cerebral stroke [8]. Intravenous infusion of MSCs in experimental stroke models leads to inhibit apoptosis of cells at the lesion boundary [9] and promotes endogenous cellular activities such as proliferation [10]. Low-level basal secretion of multiple neurotrophic factors by MSCs has been observed in vitro, and it has been reported that ischemic rat brain extracts can produce neurotrophins and angiogenic growth factors in MSCs [8]. Brain-derived neurotrophic factor (BDNF) is constitutively expressed at low levels in primary human MSCs in vitro and is increased in ischemic lesions after intravenous infusion of MSC in the rat middle cerebral artery occlusion (MCAO) model [3, 11]. Transplantation of BDNF gene-modified human MSCs increased BDNF levels in ischemic lesions and stronger therapeutic effects than MSCs alone [3, 11]. Enhanced benefit was also observed with human MSC genetically modified to express GDNF (glial cell-line derived neurotrophic factor) [12]. Transplantation of BDNF-secreting MSCs into a spinal cord injury model promotes functional recovery and facilitates sprouting of corticospinal and raphespinal axons [13]. One potential advantage of a cellular therapy that delivers trophic factors to damaged neural tissue rather than systemic pharmacological approach is the reduction in potential adverse effects of systemic drug delivery.

12.3 Angiogenic Stimulation

MSCs derived from bone marrow secrete angiogenic cytokines such as vascular endothelial growth factor (VEGF) [14] and angiopoietin-1 (Ang-1) in vitro [14, 15]. VEGF has angiogenic property in nervous tissue [16] and initiates to form immature vessels by vasculogenesis/angiogenesis [17]. However, VEGF increases vascular permeability within shortly after an ischemic injury [18], which induces cerebral edema. Direct injection of VEGF into central nervous system (CNS) tissues results to open the blood-brain barrier (BBB) [19]. Ang-1 contributes maturation, stabilization, and remodeling of blood vessels [20, 21] and promotes angiogenesis in the nervous tissue [15, 22]. Ang-1 protects the vasculature from leakage [23], an action that may contribute to anti-edematic effects

following cerebral ischemia. Ang-1, which is produced by pericytes [24], signals through the Tie2 family of tyrosine kinase receptors on endothelial cells to enhance blood vessel stabilization and could maintain BBB integrity and reduce "leakiness" [25, 26].

Following traumatic brain injury, pericytes migrate from the vascular wall [27], and the neurovascular unit (endothelial cell-pericyte-astrocyte-neuron) is compromised. If a similar disruption of the neurovascular unit occurs following stroke, it would be expected that MSCs might provide support for the microvasculature via Ang-1 signaling to vulnerable endothelial cells.

We have found that the infused Ang-1-MSCs genetically modified to express Ang-1 results in greater neovascularization and functional improvements than MSCs alone in MCAO rat [15]. By contrast, intravenous infusion of genetically modified MSCs that hypersecrete VEGF into MCAO model resulted in deterioration of neurological function [14], consistent with VEGF leading to increased vascular permeability. Miki et al. [28] reported that marrow stromal cells genetically modified to express VEGF, however, may have greater therapeutic effect than non-modified cells. Therefore, the level of VEGF expression may be critical in terms of potential therapeutic effects. Intravenous injection of MSCs genetically modified to express both Ang-1 and VEGF resulted in the greatest neovascularization and functional recovery [14]. Thus, an orchestrated expression of VEGF and Ang-1 may be important for appropriate neovascularization.

It has been suggested that pericytes are a source of MSCs [29, 30]. Because pericytes are disrupted after neural insult and that transplanted MSC may have therapeutic effects on microvasculature, repair of microvasculature will be an important therapeutic target for cerebral ischemia.

12.4 Stimulation of Neurogenesis and Axonal Sprouting

New neurons from progenitor cells are generated within the subventricular zone (SVZ) of the lateral ventricle and the dentate gyrus of the hippocampus in the adult mammalian brain [31]. Neural progenitor cells in the SVZ migrate through the rostral migratory stream (RMS) to the olfactory bulb where they differentiate into interneurons [32]. The number of progenitor cells within the SVZ (doublecortin-positive cells) increases after cerebral ischemic insult. It has been reported that the transplanted MSCs contribute to increase cell number [7, 10]. Shen et al. [33] demonstrated that expression of synaptophysin, a presynaptic marker, increases in MSC-treated ischemic brains, suggesting synaptogenesis.

12.5 Clinical Studies on Intravenously Delivered Human MSCs

Bang et al. [34] recruited 30 patients prospectively and randomly with cerebral infarcts in the middle cerebral artery (MCA) territory, all of whom showed severe neurological deficits in the first study to examine the feasibility, efficacy, and safety of a cell therapy approach in stroke patients using culture-expanded autologous MSCs. Of these, 5 patients received intravenous administration of 1×10^8 MSCs and 25 did not. The pretreatment characteristics (clinical and radiological) were similar in both the control- and MSC-infused groups. Bone marrow collection was performed 1 week after admission and mononuclear cells were isolated. Plastic-adherent cells were cultured and expanded in fetal bovine serum. MSCs as $CD34^-$, $CD45^-$, $SH2^+$, and $SH4^+$ were delivered via two infusions (5×10^7 cells per infusion) at 4–5 and 7–9 weeks after stroke onset. The patients were observed over the course of a year.

The patients in the first study by Bang et al. [34] had large infarctions within the MCA territory, evaluated by diffusion-weighted MRI. The MSC-injected group showed greater functional recovery as measured by the Barthel index. The MSC group showed no deaths, stroke recurrence, or serious adverse events. This study demonstrated safety and indicated modest functional improvement, but it was emphasized that double-blinded studies with larger cohorts would be necessary to reach a definitive conclusion. A 5-year follow-up study confirmed that there were no adverse events after injection of human MSCs [35].

We reported a Phase I/II study describing a series of 12 stroke patients who received intravenous infusions of autologous bone marrow-derived MSCs [36] (Fig. 12.2). First, safety was confirmed with human MSCs in a nonhuman primate MCAO model [37]. The overall protocol of the study is outlined in Fig. 12.3. In this study, bone marrow collection was carried out within weeks after admission of the patients into our hospital. Plastic-adherent cells were cultured with patient-derived autoserum using methodologies that allowed culturing of autologous human MSCs (ahMSCs) to very high homogeneity [4, 38]. The expression pattern of cell surface antigen ($CD34^-$, $CD45^-$, $CD73^+$, and $CD105^+$) was consistent between patients. After the cells were expanded and safety and antigenic phenotype analyses were performed, the ahMSCs were cryopreserved and stored until use. On the day of infusion, cryopreserved cells were thawed and infused intravenously.

MRIs after cell infusion showed no tumor or abnormal cell growth in any of the 12 patients over 7 years. There was a trend of correlation between improvements of the National Institutes of Health Stroke Score (NIHSS) and the reduction of lesion volume within the first weeks after cell infusion, suggesting a therapeutic benefit from intravenous infusion of ahMSCs [36]. Notably, in some of these patients, the recovery rate dramatically improved within the first 2 weeks after ahMSC infusions (Fig. 12.3a). Moreover, there was a steep reduction in lesion volume during the first 2 weeks after cell infusion (Fig. 12.3b), and the reduction in lesion volume correlated with functional improvement (Fig. 12.3c) [36]. Serial evaluations

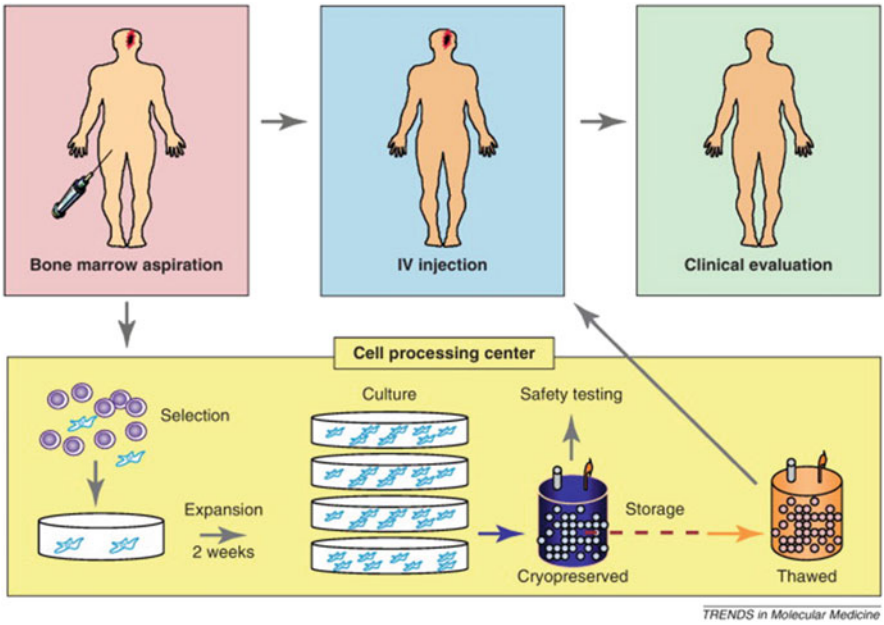

Fig. 12.2 Schematic drawing of the sequence of events for a clinical study systemically delivered autologous human MSCs. After stroke and study enrollment, bone marrow collection was performed from each patient. The cells were processed in a cell tissue processing center where they were cultured and cryopreserved. The cells were tested for safety and, after thawing, were used for intravenous delivery. Clinical evaluation was performed over 1 year [36]

showed no severe adverse effects by cell-related, serological, or imaging-defined events.

As a limitation of the Phase I/II study, these initial cases were not blinded and did not include placebo controls. The results should be interpreted with caution. A contribution of spontaneous recovery after cell infusion in these patients cannot be excluded. Nonetheless, the time-locked increase in the rate of recovery and lesion volume in patients who received ahMSCs 36–136 days after stroke is an initial suggestion of the possible therapeutic benefit of ahMSC infusions into stroke patients and encourages a future double-blinded placebo control study.

12.6 Concluding Remarks and Future Prospects

Intravenously infused MSCs have been studied in clinical studies for a number of neurological diseases [34, 36]. Their relatively benign safety properties support the prospect of potential therapeutic use of MSCs for several CNS disorders. Optimal therapeutic protocols should be established as future studies in terms of cell number

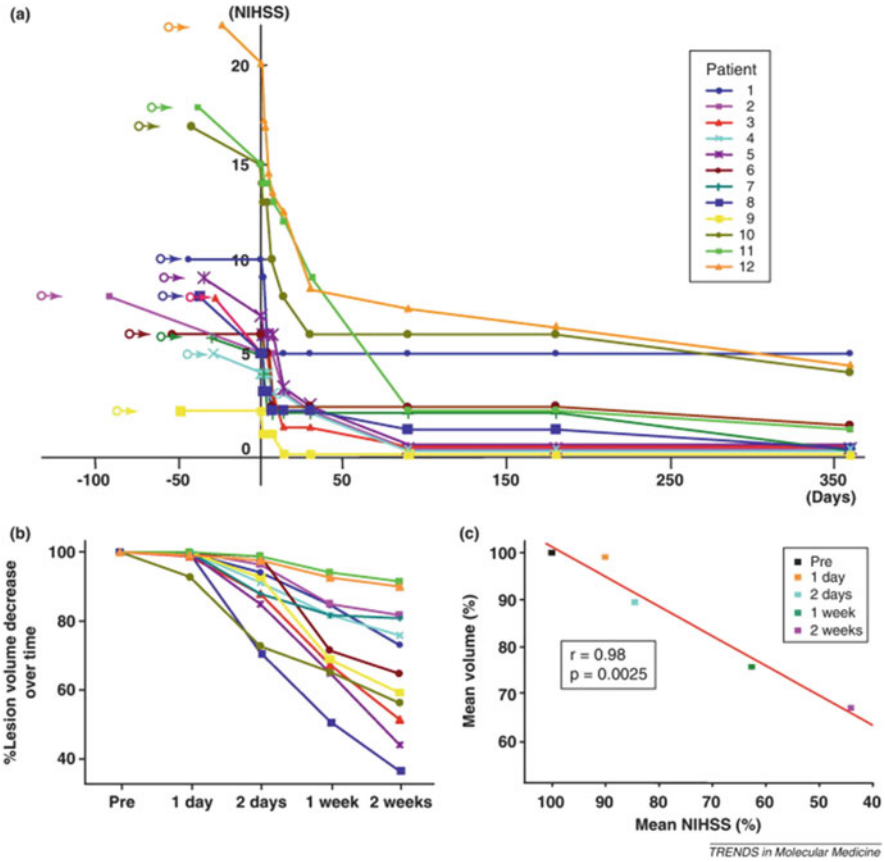

Fig. 12.3 Data summary for 12 stroke patients infused with autologous human MSCs (*hMSCs*). (**a**) NIHSS at the time of infusion and for 1 year following infusion. (**b**) Summary of lesion volumes calculated from high-intensity area in MRI FLAIR (fluid attenuation inversion recovery) images for all cases at pre-infusion and 1, 2, 7, and 14 days post-infusion. (**c**) Mean % change in lesion volume plotted against mean change in NIHSS, compared with pre-infusion values (Modified with permission from Honmou et al. [36])

preparation. A hypothetical sequence of potential therapeutic mechanisms in neurological disorders is presented in Fig. 12.4.

At early phase of post-cell infusion times (days), beneficial effects may be the result of excitability modulation by secreted neurotrophic factors from MSCs, such as BDNF. MSCs could also provide trophic support for vulnerable neurons, particularly in the penumbra, and anti-inflammatory responses with reduction of edema, thus leading to enhanced tissue sparing. With increased time, MSCs may contribute to angiogenesis, vascular stabilization, and maintaining of integrity of the BBB, thereby protecting CNS tissue and limiting cerebral edema. MSCs may also facilitate local axonal sprouting with new synaptic connections. Finally, the MSCs could mobilize endogenous progenitor cells that may contribute to neurogenesis

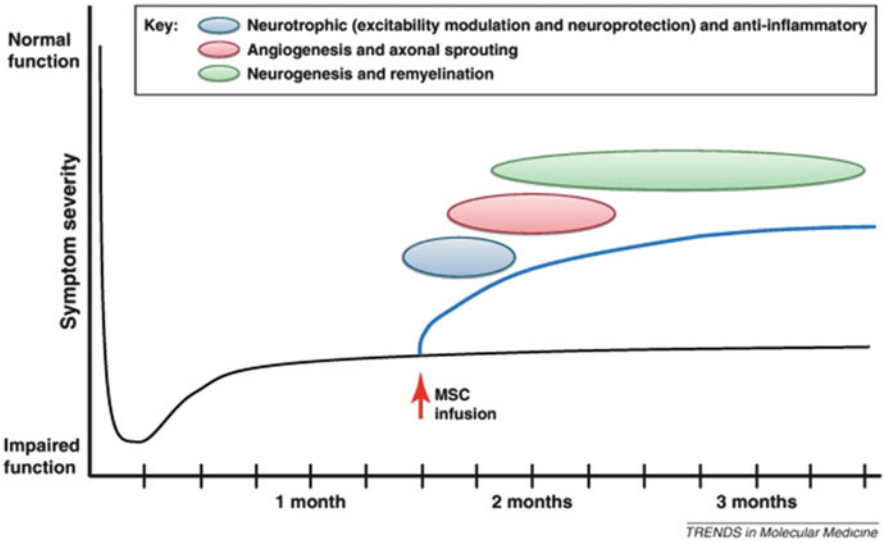

Fig. 12.4 Schematic representation of potential therapeutic mechanisms following systemic administration of hMSCs in stroke. The *black line* indicates an idealized spontaneous recovery curve following ischemic insults with initial severe deficits that demonstrate some endogenous recovery which subsequently plateaus. The *blue line* indicates incremental recovery of neurological function after MSC therapy (*red arrow*). Early improvement in function may result from neurotrophic effects that may modulate excitability and provide neuroprotection and anti-inflammatory reactions. An intermediate phase of recovery may result from angiogenesis, axonal sprouting, and remyelination. If neurogenesis and remyelination contribute to functional improvements, it would probably contribute to the later phase of recovery

and axonal remyelination. Each of these potential mechanisms should be investigated. It is hoped that future clinical studies will conclusively determine whether therapeutic intervention, via either cellular or noncellular approaches, in the subacute and early chronic phase can positively provide improved clinical outcome in stroke.

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