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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