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ORIGINAL ARTICLE

Platelet Lysate and Granulocyte-Colony Stimulating Factor Serve Safe and Accelerated Expansion of Human Bone Marrow Stromal Cells for Stroke Therapy

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Abstract Autologous human bone marrow stromal cells (hBMSCs) should be expanded in the animal serum-free condition within clinically relevant periods in order to secure safe and effective cell therapy for ischemic stroke. This study was aimed to assess whether the hBMSCs enhance their proliferation capacity and provide beneficial effect in the infarct brain when cultured with platelet lysate (PL) and granulocyte-colony stimulating factor (G-CSF). The hBMSCs were cultured in the fetal calf serum (FCS)-, PL-, or PL/G-CSF-containing medium. Cell growth kinetics was analyzed. The hBMSCs-PL, hBMSC-PL/G-CSF, or vehicle was stereotactically transplanted into the ipsilateral striatum of the rats subjected to permanent middle cerebral artery occlusion 7 days after the insult. Motor function was assessed for 8 weeks, and the fate of transplanted hBMSCs was examined using immunohistochemistry. As the results, the hBMSCs-PL/G-CSF showed more enhanced proliferation than the hBMSCs-FCS and hBMSCs-PL. Transplantation of hBMSCs expanded with the PL- or PL/G-CSF-containing medium equally promoted functional recovery compared with the vehicle group. Histological analysis revealed that there were no significant differences in their migration, survival, and neural differentiation in the infarct brain between the hBMSCs-PL and hBMSCs-PL/G-CSF. These findings strongly suggest that the combination of PL and G-CSF may accelerate hBMSC expansion and serve safe cell therapy for patients with ischemic stroke at clinically relevant timing.

Keywords Bone marrow stromal cell · Cell therapy · Granulocyte-colony stimulating factor (G-CSF) · Ischemic stroke · Platelet lysate

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Introduction

The bone marrow stromal cells (BMSCs) are believed as one of promising donor cells to enhance functional recovery after ischemic stroke. They can be harvested from the patients themselves without ethical or immunological problems. In addition, they have no tumorigenesis [1–4]. When the BMSCs are transplanted into the rodent model of ischemic stroke, they aggressively migrate toward the infarct rim through the chemokine system and express the phenotype of neural cells, yielding therapeutic effects on motor function within 1-month posttransplantation. They may also prevent excessive inflammatory response and attenuate further tissue damage in the infarct rim. Furthermore, the BMSCs release some neuroprotective molecules, including brain-derived neurotrophic factor (BDNF) and nerve cell growth factor (NGF), and ameliorate the neuronal damage in the host brain. Therefore, the BMSCs may contribute to post-stroke functional recovery through multiple mechanisms [3, 4].

However, several problems should be solved prior to clinical application of BMSC transplantation for ischemic stroke. First of all, the quality of donor cells should strictly be controlled. Thus, the BMSCs harvested from the animals and humans are cultured in the medium including fetal calf serum (FCS) in the majority of animal experiments and even in some of clinical trial [5–9]. However, the FCS may carry the potential risk of prion, viral, or zoonoses contamination and also provoke immunological reactions against xenogenic serum

antigens. Recent studies have shown that human platelet lysate (PL) may be a safe and effective substitute for FCS to expand the human BMSCs (hBMSCs) to treat the disorders of other organs and graft-versus-host disease. In fact, the hBMSCs cultured with PL maintain their capacity of proliferation, migration, BDNF/NGF production, and neural differentiation. They significantly serve therapeutic effects when transplanted into the infarct brain of rats [10–12].

Second, it is critical to transplant the donor cells into the host brain within therapeutic time window after the onset of ischemic stroke. Very recent study has shown that the beneficial effects of BMSC transplantation can be achieved when they are engrafted within 4 weeks after the onset of cerebral infarct in the rats [13]. However, in vitro cell expansion usually requires at least 4 to 6 weeks to obtain efficient number of autologous donor cells in previous clinical trials [8, 9]. Especially, this issue is very serious when considering clinical application of cell therapy for ischemic stroke, because the stem cells, including the BMSCs, suffer the effect of aging and reduce their capacity of self-renewal and differentiation [14]. Therefore, it is quite important to develop novel strategies to enhance biological features of the hBMSCs harvested from aged patients with ischemic stroke. Recent studies have shown that granulocyte-colony stimulating factor (G-CSF) would enhance the biological capacity of the BMSCs from both young and aged rats [14, 15].

Based on these considerations, this study was aimed to evaluate the hypothesis that the combination of PL and G-CSF enhances in vitro proliferation of the hBMSCs and serve beneficial effects on motor function when transplanted into the infarct brain of rats.

Materials and Methods

Preparation and Culture of Human BMSC

All experiments were approved by the Animal Studies Ethical Committee of Hokkaido University Graduate School of Medicine. The human BMSCs (hBMSCs) of three donors were purchased from Lonza Walkersville Inc. (Walkersville, USA). Their age ranged from 22 to 25 years. According to the manufacture's manual, the vial of the frozen hBMSCs was quickly thawed in a 37 °C water bath. Aseptically, the hBMSC suspension was transferred to a 15-ml tube with 10 ml of respective medium described below and was centrifuged at 200*g* for 5 min. After the supernatant was carefully removed, the cell number was counted. The hBMSCs were plated in non-coated 75-cm^2 culture flask (EasyFlask 159910; Nunc) at a density of 1,500 cells/cm^2. The hBMSCs were cultured in the culture medium (see below) at 37 °C in a humidified atmosphere containing 5 % CO_2. After 48 h, the non-adherent cells were washed off with phosphate-buffered saline (PBS). Cells were fed twice a week with fresh medium and incubated until reaching 90 % confluence. At this time point, the cultures were designed as P1. The hBMSCs were harvested after reaching 90 % confluence, passed by using 0.05 % Trypsin-EDTA (Gibco) for 5 min at 37 °C, and replated for expansion at a density of 1,500 cells/cm^2. The adherent cells were further passed three times.

Proliferation Kinetics

The hBMSC of three donors were cultured, using three different culture media. In the FCS group, the culture medium contained Dulbecco's modified Eagle medium/low glucose (D6046; Sigma-Aldrich), 1 % penicillin/streptomycin (P/S; Sigma-Aldrich), and 10 % FCS (Fetal Bovine Serum Qualified; Gibco). In this study, pooled thrombocyte concentrates were obtained from normal volunteers and were designed for clinical use. The platelet lysate (PL) was prepared by centrifuging at 200×*g* for 20 min. The platelet-rich plasma was aliquoted into small portions, frozen at −80 °C, thus obtaining PL, and thawed immediately before use. In the PL group, the culture medium contained alpha-minimum essential medium (M0894; Sigma-Aldrich), 1 % P/S, 2 IU/ml heparin (Mochida Pharmaceutical Co.), and 5 % PL. In the PL+ G-CSF group, 0.1 μM of G-CSF was added to the culture medium in the PL group.

At each passage, cumulative cell numbers were calculated as described previously, using trypan blue [7]. The time taken for the hBMSC culture to double in cell number (doubling time) was calculated, using the following formula: doubling time=$(t_2-t_1)\times \ln(n_2)/\ln(n_2/n_1)$, where t is consecutive time points (days) and n is respective cell numbers at these time points. The doubling time was determined from P2 to P4 where indicated. The cells harvested at P4 were used for subsequent experiments.

Rat Permanent Middle Cerebral Artery Occlusion Model

Male 9-week-old SD rats (n=27) were purchased from CLEA Japan, Inc. (Tokyo, Japan). Permanent middle cerebral artery (MCA) occlusion was induced as described previously. Briefly, anesthesia was induced by 4.0 % isoflurane in N_2O/O_2 (70:30), and was maintained with 2.0 % isoflurane in N_2O/O_2 (70:30). Core temperature was maintained between 36.5 and 37.0 °C through the procedures. The bilateral common carotid arteries (CCAs) were exposed through a ventral midline incision of the neck. Then, a 1.5-cm vertical skin incision was performed between the right eye and ear. The temporal muscle was scraped from temporal bone, and a 5×5-mm temporal craniotomy was performed, using a small dental drill. To prevent cerebrospinal fluid (CSF) leakage, the dura mater was carefully kept intact, and the right MCA was ligated using a 10-0 nylon thread through the dura mater. Then, the cranial

window was closed with the temporal bone flap. The temporal muscle and the skin were sutured with 4-0 nylon threads, respectively. Subsequently, the bilateral CCAs were occluded by surgical microclips for 1 h. Only animals that circled toward the paretic side after surgery were included in this study [10, 12, 16, 17].

Stereotactic Transplantation of hBMSC

The animals were randomly assigned, and the hBMSCs-PL, hBMSCs-PL/G-CSF, or vehicle (n=9 in each group) was stereotactically transplanted into the ipsilateral striatum at 7 days after the onset of permanent MCA occlusion, as described previously [10, 12, 16, 17]. Briefly, the animals were fixed to a stereotactic apparatus, and the cranium was exposed through midline skin incision. A burr hole was made 3 mm right to the bregma, using a small dental drill. Both the hBMSCs-PL and hBMSCs-PL/G-CSF were washed three times and suspended in phosphate buffered saline. A 25-μL Hamilton syringe with 23s gauge needle was inserted 5 mm into the brain parenchyma from the surface of the dura mater, and 10 μL of cell suspension (5.0×10^5 cells) or vehicle (PBS) was introduced into the striatum over a period of 5 min, using an automatic microinjection pump. All animals, including the vehicle-treated animals, were treated with 10 mg/kg of cyclosporine A (Novartis Pharma, Tokyo, Japan) subcutaneously every day after transplantation for 7 weeks. As reported previously, there were no obvious adverse effects of cyclosporine A [12, 18].

Behavioral Test

Motor function of the animals was serially assessed before and at 1, 7, 14, 21, 28, 35, 42, 49, and 56 days after the onset of ischemia, using a Rotarod treadmill. The Rotarod was set to the acceleration mode from 4 to 40 rpm for 3 min. The maximum time that the animal stayed on the Rotarod was recorded for each performance, as described previously [10, 12, 16, 17]. The researchers were blinded to the study groups.

Histological Analysis

At 7 weeks after transplantation, the animals were deeply anesthetized with 4.0 % isoflurane in N_2O/O_2 (70:30) and transcardially perfused. The brain was removed, immersed in 4 % paraformaldehyde for 2 days, and embedded in paraffin. The 4-μm-thick coronal sections were prepared for subsequent analysis.

To compare the effect of hBMSC transplantation on infarct volume among three experimental groups, the total volume of cerebral infarct was calculated as described previously [12]. To evaluate the fate of the engrafted hBMSC, double fluorescence immunohistochemistry was performed, as previously described [10, 12, 16, 17]. The deparaffinized sections were processed through antigen retrieval for 2 min by pressure pot. For blocking nonspecific immune reaction, the sections were treated with 10 % goat serum for 30 min. Each section was treated with a primary mouse antihuman nuclei monoclonal antibody (MAB 1281, dilution 1:100, Chemicon), which is specific for all human cell types, at 4 °C overnight [12]. Then, the sections were treated with a rhodamine-conjugated goat antimouse antibody (dilution 1:200, Chemicon) as the secondary antibody at room temperature for 1 h. Subsequently, the sections were treated with primary mouse IgG antibodies, conjugated with Zenon Alexa Fluor 488 (Mouse IgG Labeling Kit; Molecular Probes Inc.), against neuronal nuclear antigen (NeuN, dilution 1:200, Chemicon) and glial fibrillary acidic protein (GFAP, dilution 1:500, Chemicon) at 4 °C overnight. The fluorescence emitted was observed through an appropriate filter on a fluorescence microscope (BX61, Olympus, Tokyo, Japan) and was digitally photographed using a cooled charge-coupled device camera connected to the microscope (model VB-6000/6010; Keyence Co., Osaka, Japan). To semiquantitatively determine the migratory capacity of the engrafted cells toward the peri-infarct area, the number of the MAB 1281-positive cells were counted in the randomly selected five regions of interest (ROIs, 280×370 μm) placed on the areas adjacent to cerebral infarct. To analyze their capacity of neural differentiation, the randomly selected five ROIs were placed on the same areas, and the percentages of the cells doubly positive for MAB 1281 and NeuN or GFAP were calculated as described previously [19].

Statistical Analysis

All data were expressed as mean±standard deviation (SD). Continuous data were compared, using a paired t test between two groups and one-factor ANOVA followed by Bonferroni's test among three groups. $P<0.05$ was considered statistically significant.

Results

Platelet Lysate and G-CSF Enhance hBMSC Proliferation

Figure 1 shows representative microphotographs of hBMSCs when cultured with FCS, PL, and PL/G-CSF. Although they exhibited the various morphologies, they became comparatively homogeneous in appearance, which was the relatively elongated and fibroblast-like morphology under all culture conditions, as the cells were

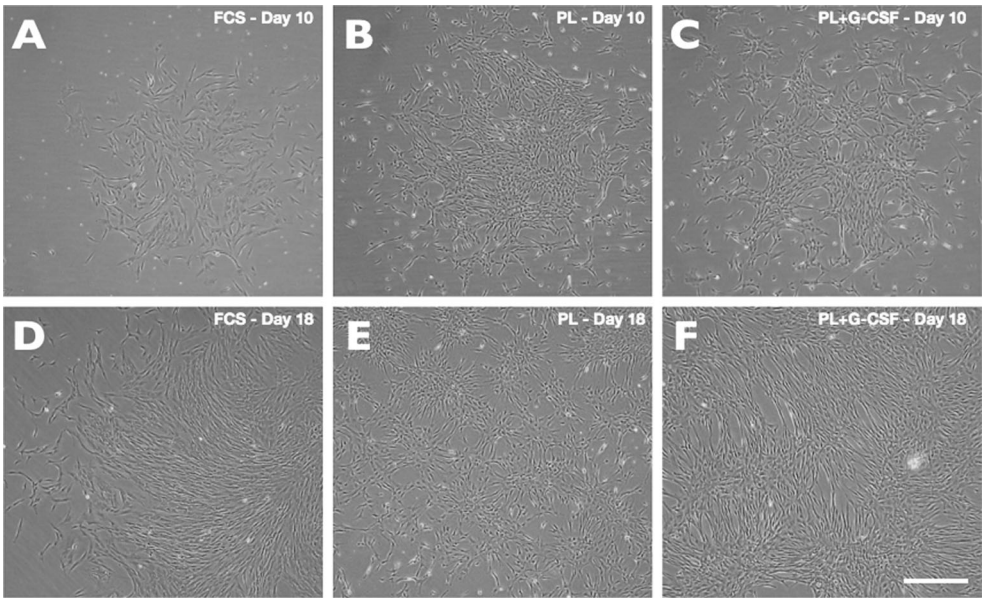

Fig. 1 Photomicrographs of human bone marrow stromal cells (hBMSCs) cultured in the fetal calf serum (FSC, **a** and **d**), platelet lysate (PL, **b** and **e**), or PL plus granulocyte-colony stimulating factor (G-CSF, **c** and **f**). *Scale bar*=200 μm

passed. There were no significant differences in their morphology among three groups.

Figures 2 and 3 summarizes the cumulative cell numbers of hBMSCs and their doubling time in each subject, respectively. As the results, the cells cultured with PL showed more enhanced growth than did cells cultured with FCS in two of three subjects at P4. On the other hands, the cells cultured with PL/G-CSF showed more enhanced growth than did cells cultured with PL in all three subjects at P4.

The hBMSCs Enhance Functional Recovery after Ischemic Stroke

All animals survived throughout the experiment and were used for subsequent analysis. As shown in Fig. 4, all animals exhibited severe neurological deficit during 1 week after the onset of focal cerebral ischemia. Vehicle, hBMSCs-PL, or hBMSCs-PL/G-CSF was stereotactically transplanted at 7 days post-ischemia. There was no

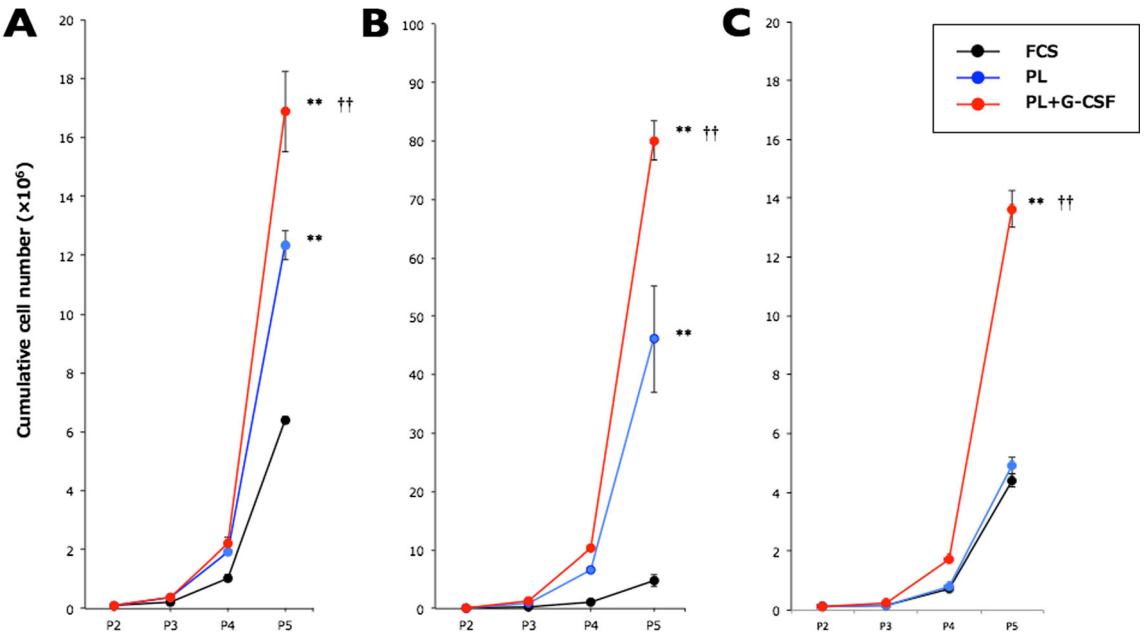

Fig. 2 The graph shows the cumulative cell number of hBMSCs cultured in FCS (*black*), PL (*blue*), or PL plus G-CSF (*red*). Each graph shows the data from the hBMSCs harvested from three donors, including a 25-year-old male (**a**), a 22-year-old male (**b**), and a 25-year-old female (**c**). $^{**}P<0.01$ compared with hBMSC-FCS group. $^{††}P<0.01$ compared with hBMSC-PL group

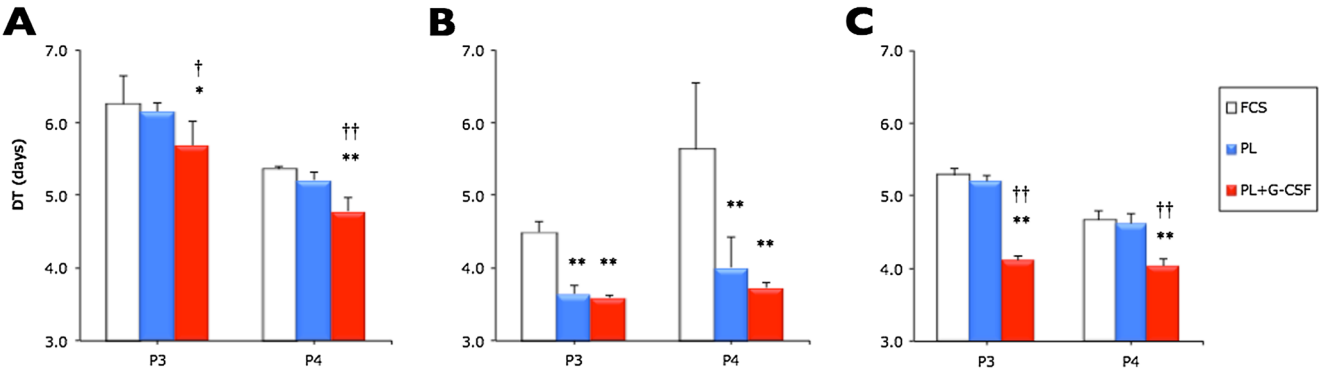

Fig. 3 The bar graph shows the doubling time (DT, days) of hBMSCs cultured in FCS (*white column*), PL (*blue column*), or PL plus G-CSF (*red column*). Each graph shows the data from the hBMSCs harvested from three donors, including a 25-year-old male (**a**), a 22-year-old male (**b**), and a 25-year-old female (**c**). *$P<0.05$ compared with hBMSC-FCS group. **$P<0.01$ compared with hBMSC-FCS group. †$P<0.05$ compared with hBMSC-PL group. ††$P<0.01$ compared with hBMSC-PL group

significant difference in motor function among three groups during 2 weeks after transplantation. Subsequently, the vehicle-transplanted animals did not show any significant improvement of motor function until the endpoint. However, the hBMSCs-PL-treated animals showed significantly enhanced functional recovery at 4 to 7 weeks posttransplantation, when compared with the vehicle-treated animals ($P<0.01$). Likewise, the hBMSCs-PL/G-CSF-treated animals showed significantly enhanced functional recovery at 3 to 7 weeks posttransplantation, when compared with the vehicle-treated animals ($P<0.05$ at 3 weeks and $P<0.01$ at 4 to 7 weeks). There were no significant difference in motor function between hBMSC-PL group and hBMSC-PL/G-CSF group throughout the experiment except for at 6 weeks posttransplantation ($P<0.05$).

Histological Analysis

To evaluate the effect of hBMSC transplantation on infarct volume, the brains were perfused and removed at 7 weeks posttransplantation. As a result, infarct volume was 23±3.9 %, 23±3.5 %, and 23±4.9 % in the vehicle-, hBMSC-PL-, and hBMSC-PL/G-CSF-treated animals, respectively ($n=9$ in each group). There was no significant difference in infarct volume among three groups.

Fluorescence immunohistochemistry was performed to define the distribution and fate of engrafted hBMSCs in infarct brain. The MAB1281-positive cells were densely found in the peri-infarct area. The number of the MAB1281- positive cells in the peri-infarct area were $2.1\pm0.6\times10^2$ and $2.6\pm0.8\times10^2/mm^2$ in the hBMSC-PL- and the hBMSC-PL/G-CSF-transplanted animals, respectively ($n=9$

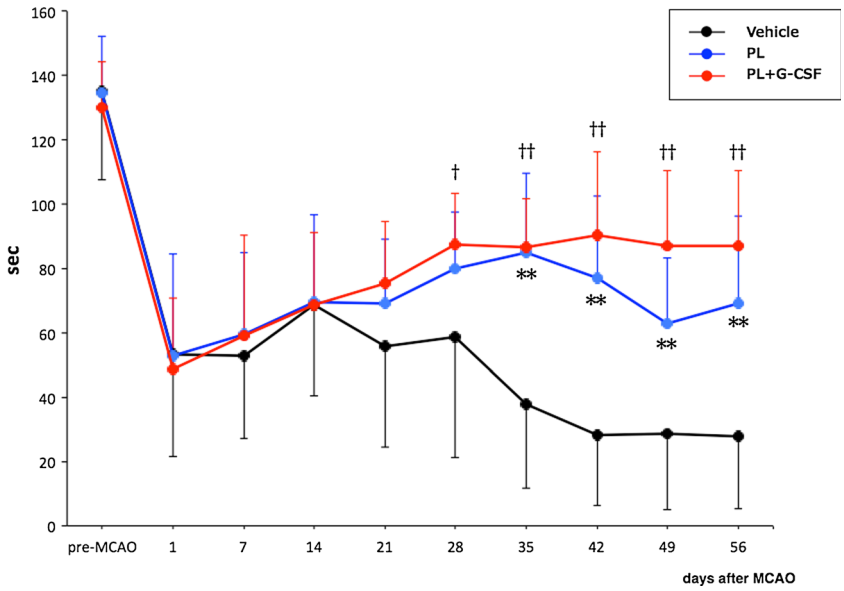

Fig. 4 Rotarod treadmill performance. Line graph shows temporal profile of functional recovery in vehicle- (*black*), hBMSC-PL- (*blue*), or hBMSC-PL/G-CSF-transplanted (*red*) rats subjected to permanent middle cerebral artery occlusion (MCAO). **$P<0.01$ (hBMSC-PL vs. vehicle). †$P<0.05$ (hBMSC-PL/G-CSF vs. vehicle) ††$P<0.01$ (hBMSC-PL/G-CSF vs. vehicle)

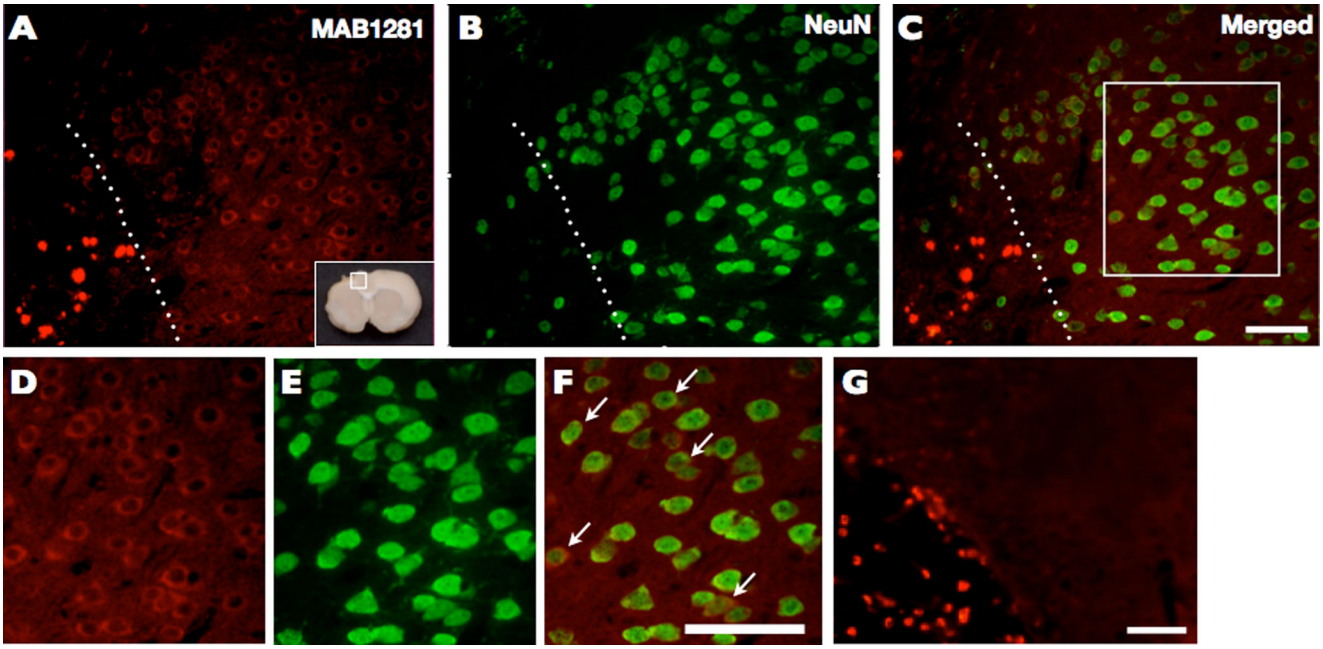

Fig. 5 Photomicrograph of double fluorescence immunohistochemistry, using monoclonal 1281 (MAB1281, **a** and **d**) and neuronal nuclear antigen (NeuN, **b** and **e**), in the peri-infarct area of hBMSC-PL/G-CSF-transplanted rats at 8 weeks after permanent middle cerebral artery occlusion. The merged image (**c** and **f**) shows the cells with the nuclei doubly positive for MAB1281 and NeuN (*arrows*). The *white square* in panel **c** represents the location of panels **d-f**. *Dotted line* indicates the border between cerebral infarct (*left*) and peri-infarct area (*right*). Panel **g** represents the photomicrograph of negative control for MAB1281 immunostaining. Very bright *red dots* on panels **a** and **g** indicate autofluorescence from the necrotic cells. *Scale bar*=200 μm

in each group). There was no significant difference in their number between two groups (Fig. 5a).

Double fluorescence immunohistochemistry was employed to assess the fate of engrafted hBMSC in the infract brain. The MAB1281-positive cells were also positive for NeuN or GFAP in the peri-infarct area at 7 weeks after transplantation. The percentages of cells doubly positive for MAB1281 and NeuN were 45.2±23.4 % and 48.4±20.5 % in the hBMSC-PL- and hBMSC-PL/G-CSF-treated animals, respectively (n=9 in each group, Figs. 5 and 6). Likewise, the percentages of cells doubly positive for MAB1281 and GFAP were 24.3±14.4 % and 26.1±16.9 % in the hBMSC-PL- and hBMSC-PL/G-CSF-treated animals, respectively (n=9 in each group, Fig. 7).

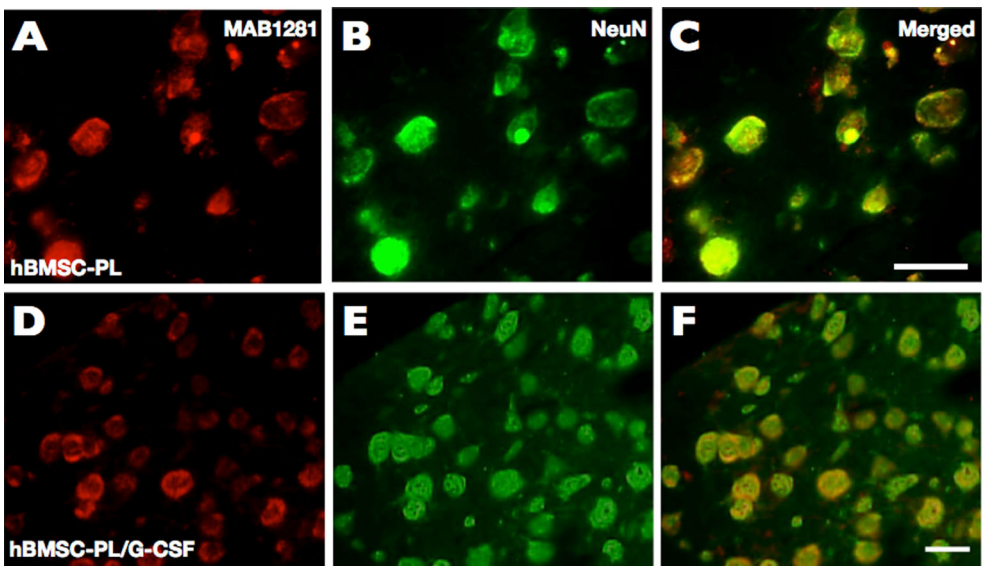

Fig. 6 High-power photomicrograph of double fluorescence immunohistochemistry, using monoclonal 1281 (MAB1281, **a** and **d**) and neuronal nuclear antigen (NeuN, **b** and **e**), in the peri-infarct area of hBMSC-PL- (**a-c**) or hBMSC-PL/G-CSF-transplanted rats (**d-f**) at 8 weeks after permanent middle cerebral artery occlusion. The merged image (**c** and **f**) shows some of MAB1281-positive cells also express NeuN. *Scale bar*=25 μm

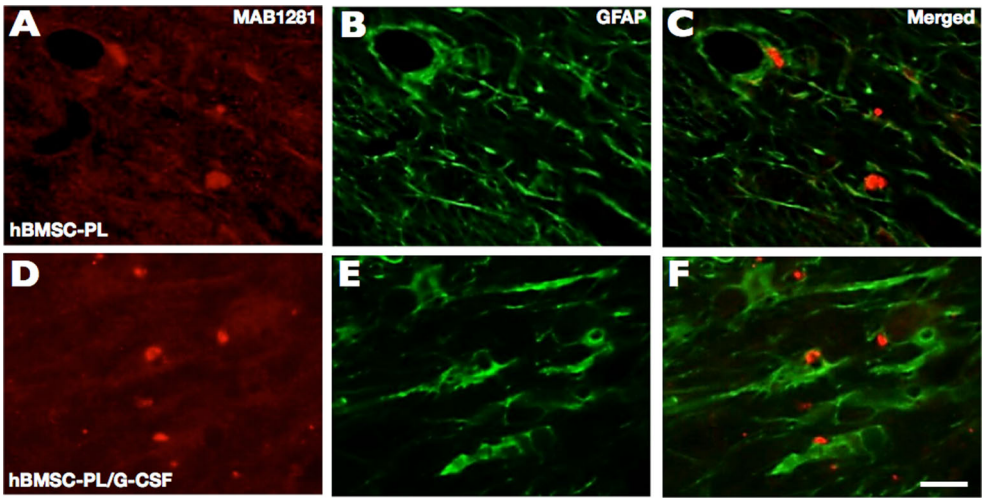

Fig. 7 High-power photomicrograph of double fluorescence immunohistochemistry, using monoclonal 1281 (MAB1281, **a** and **d**) and glial fibrillary acid protein (GFAP, **b** and **e**), in the peri-infarct area of hBMSC-PL- (**a-c**) or hBMSC-PL/G-CSF-transplanted rats (**d-f**) at 8 weeks after permanent middle cerebral artery occlusion. The merged image (**c** and **f**) shows some of MAB1281-positive cells also express GFAP. *Scale bar*=25 μm

As the results, there were no significant differences in the capacity to differentiate into the neural cells between the two groups.

Discussion

This study clearly shows that PL can be a clinically valuable and safe substitute for FCS in expanding the hBMSCs for cell therapy. Furthermore, the addition of G-CSF into the PL-containing medium further accelerates their proliferation activity. The hBMSCs-PL or hBMSCs-PL/G-CSF yields almost same therapeutic effects when stereotactically transplanted into the infarct brain. Animal protein-free culture medium may promote the clinical testing of hBMSC transplantation for patients with ischemic stroke, because the presence of FCS in the culture medium was one of major obstacles for their clinical use. The PL/G-CSF-containing culture medium may also enhance the in vitro growth of hBMSCs harvested from the patients and shorten the interval between the onset of ischemic stroke and cell therapy. This is the first study to prove the possibility to provide safe and effective cell therapy in a clinically relevant period after the onset of ischemic stroke, using the hBMSCs cultured under animal protein-free condition.

Platelet Lysate—An Alternative to Fetal Calf Serum

There are no significant differences in the cell morphology among FCS group, PL group, and PL+ G-CSF group. The finding partly correlates with previous data from the authors' laboratory. Thus, the hBMSCs-FCS and hBMSCs-PL were morphologically similar and expressed identical phenotypic markers [11, 12]. Their production of BDNF, NGF, and hepatocyte growth factor (HGF) did not differ between two groups. Furthermore, the hBMSCs-PL maintains their capacity of in vitro differentiation into the neurons and astrocytes [11].

Previously, several investigators have performed similar studies. Thus, Meuleman et al. (2006) cultured the hBMSCs in a commercial serum-free medium (UC) supplemented with a serum substitute (ULROSER) [20]. All of these studies have suggested that these media may be suitable for clinical scale expansion of hBMSCs. Subsequent studies have also revealed that PL increases cumulative cell numbers after expansion and maintain all biological features of hBMSCs, including plastic adherence, spindle-shaped morphology, surface marker expression, and differentiation capacity to multiple lineages [21–24]. In fact, both PL and platelet-rich plasma include much higher concentrations of growth factors including platelet-derived growth factor (PDGF) than FCS [25, 26]. Ng et al. (2008) have recently shown that PDGF signaling is one of essential pathways for the growth and differentiation of hBMSCs [27].

In spite of these biological analyses, however, there are few studies that denote whether the hBMSCs can serve therapeutic effects in animal model of neurological disorders when cultured with animal serum-free medium. The authors have previously shown that transplantation of hBMSCs expanded with the FCS- or PL-containing medium equally promotes functional recovery compared with the vehicle group. Histological analysis also revealed no significant differences in their migration, survival, and neural differentiation in the infarct brain between hBMSC-FCS and hBMSC-PL groups [12]. Using a 7.0-T MR apparatus, they have also demonstrated that the hBMSCs-PL maintain their migratory capacity toward cerebral infarct in the living rodents. Histological analysis also revealed that the engrafted hBMSCs-PL acquired the phenotypes of neural cells in the peri-infarct area. These therapeutic effects were identical to those obtained with hBMSCs-FCS

[10]. In this study, therefore, therapeutic effects on motor function were not compared between the hBMSCs-FCS and hBMSCs-PL (Fig. 4).

The human PL can easily be obtained from human platelet concentrates after freeze/thaw process [12]. Previous studies have clearly shown that allogeneic human PL allows expansion and clinical grade production of hBMSCs from small samples of bone marrow aspirates [28]. Therefore, it would be practical to culture the hBMSC harvested from the patients themselves with the autologous or allogeneic PL. Transplantation of syngeneic cells would be ideal, but human BMSCs were transplanted into the rat brain under the use of cyclosporine A in this study, because this study was aimed to assess whether human PL can be the substitute for FCS in donor cell culture.

G-CSF—An Effective Promoter for hBMSC Expansion

G-CSF is a 20-kDa glycoprotein that stimulates the proliferation, survival, and maturation of cells committed to the neutrophil precursors and mature neutrophils through binding to specific G-CSF receptors [29]. The BMSCs highly express the G-CSF receptor and significantly enhance their proliferation capacity and growth factor production when cultured with 0.1 μM of G-CSF. FACS analysis confirms that G-CSF significantly increases the population of BMSCs in S phase [15]. Furthermore, G-CSF significantly improves the proliferation capacity and BDNF production of BMSCs isolated from the aged rats. G-CSF also accelerates their survival and functional recovery after BMSC transplantation into the infarct brain of rats [14]. These findings strongly suggest that G-CSF may pharmacologically modify the biological features of BMSCs and improve clinical results of BMSC transplantation for ischemic stroke. Furthermore, the proliferation capacity and BDNF/NGF production of hBMSCs-PL are quite similar to those of hBMSCs-FCS [11, 12].

In this study, therefore, G-CSF was added to the FCS-free, PL-containing medium to assess whether a combination of PL with G-CSF further enhance the proliferation activity of hBMSCs. As the results, the hBMSCs-PL/G-CSF show an increased cumulative cell number and decreased doubling time, compared with the hBMSCs-FCS and hBMSCs-PL. There are no previous studies that evaluate the effects of a combination of PL with G-CSF on in vitro expansion of hBMSCs. Previous study only revealed that the addition of 1-ng/ml basic fibroblast growth factor (bFGF) to PL-containing medium did not significantly accelerate the growth of hBMSCs [24]. Therefore, this new protocol of hBMSC culture with PL and G-CSF may serve accelerated and safe expansion of hBMSCs for their clinical scale use.

Clinical Application of Cell Therapy for Ischemic Stroke

The present findings may provide valuable information to the investigators who are considering translational testing of BMSC transplantation for patients with ischemic stroke. Thus, there are still surprising discrepancies of the protocols between animal experiments and clinical trials. In most of animal experiments, the BMSCs are transplanted into the infarct brain within 24 h after the onset. However, the BMSCs are transplanted several weeks after the onset in a majority of previous clinical trials, because the expansion of BMSCs requires several weeks for clinical use. However, there are few studies that assess whether the BMSCs significantly enhance functional recovery when transplanted into the infarct brain several weeks after the insult. When considering to scientifically applying cell therapy into clinical situation, we should learn from a history of clinical development of neuroprotective agents for acute ischemic stroke [3, 30]. Thus, a large number of neuroprotective agents have shown significant effects in animal experiments, but failed to achieve beneficial results when brought forward into clinical development. Despite encouraging preclinical data, none of agents was shown to improve outcome in phase III clinical trials. These disappointing data may partially result from the lack of the guideline concerning how to perform preclinical development of purported neuroprotective agents for acute ischemic stroke. Based on these observations, the first Stroke Therapy Academic Industry Roundtable (STAIR) meeting was organized and published the recommendations for preclinical stroke drug development in 1999 [31, 32]. The guideline strongly recommends careful assessment of therapeutic time window, that is, the interval after the onset of ischemia when the drug can be successfully administered, to obtain positive results in clinical trials. Subsequently, similar recommendation has also been published in the field of cell therapy for stroke [33].

From this viewpoint, the present results strongly suggest that a combination of PL with G-CSF may be one of the useful strategies to enable BMSC transplantation in clinical situation within effective therapeutic time window in animal experiments by accelerating their proliferation. In this study, the hBMSCs were transplanted into the brain at 7 days after the onset of ischemia because of the following reasons. First, Hofstetter et al. (2002) transplanted the BMSCs into the injured spinal cord and found that the BMSCs given 1 week after injury led to significantly larger numbers of surviving cells and better functional outcome than immediate treatment [34]. Second, Baksi et al. reported that BMSC transplantation within 14 days of injury provided significantly greater grafting efficacy than more delayed delivery [35]. Kawabori et al. also transplanted the BMSCs into the infarct brain at 4 weeks post-ischemia, and concluded that higher dose of donor cells required to yield therapeutic effects similar to BMSC

transplantation at 1 week [13]. In addition, the hBMSCs were obtained from young volunteers. Further studies would be warranted to confirm the present results by using the cells obtained from elder volunteers at higher risk for ischemic stroke, because adult stem cells, including BMSCs, suffer the effect of aging and reduce their self-renewal and differentiation capacity [14].

Conclusion

In this study, the combination of PL and G-CSF may accelerate in vitro expansion of hBMSCs under the condition without animal serum and serve safe cell therapy for patients with ischemic stroke at clinically relevant timing. The present knowledge would promote the clinical testing of safe and effective hBMSC transplantation for patients with ischemic stroke in very near future.

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Conflict of Interest Tomohiro Yamauchi, Hisayasu Saito, Masaki Ito, Hideo Shichinohe, Kiyohiro Houkin, and Satoshi Kuroda declare that they have no conflict of interest.

Compliance with Ethics Requirements All institutional and national guidelines for the care and use of laboratory animals were followed.

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