Original Article

Neovascularization promoted by mononuclear cell transplantation after transient cerebral ischemia in mice

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Objectives: Ischemic stroke can cause major disability and a deteriorated quality of life. The purposes of this study were to examine whether neovascularization might be promoted by the intra-arterial transplantation of mononuclear cells (MNCs) in ischemic murine brain and to determine whether the new endothelial cells are derived from the donor or from the recipient.

Methods: Transient middle cerebral artery occlusion was accomplished using the intraluminal filament technique. Immediately after the embolus was withdrawn, MNCs harvested from C57BL/6J mice (wild-MNCs) or transgenic mice ubiquitously expressing green fluorescent protein (EGFP-MNCs) were infused intra-arterially. The brains of the mice were then sectioned and stained with anti-CD31 antibody to detect the endothelial cells.

Results: At 30 minutes after operation, transplanted EGFP-MNCs were found in the lung, spleen, and liver but not in the brain. At 6 weeks after operation, CD31-positive cells were evident in the murine brain transplanted with MNCs. Furthermore, the CD31-positive cells were stained with anti-GFP antibody in EGFP mice transplanted with wild-MNCs but not in wild-type mice transplanted with EGFP-MNCs.

Discussion: This animal model resembles the typical clinical course of acute cerebral infarction followed by thrombolytic therapies in humans and could be used to examine new therapies after intra-arterial thrombolysis. In cerebral ischemia, just as in limb ischemia, transplanted MNCs did not differentiate into endothelial cells but instead promoted the formation of new vessels.

Conclusion: In murine brain reperfused post ischemia, intra-arterial MNC transplantation promotes neovascularization. Furthermore, the new endothelial cells are derived from the recipient cells.

Rec.11/4/2008, Acc.12/2/2008, pp66-72

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Key words cerebral ischemia, intra-arterial transplantation, mononuclear cells, neovascularization, revascularization

Introduction

Ischemic stroke is a life-threatening event and continues to pose challenges in terms of both saving life and maintaining quality of life[1]. Tissue plasminogen activator (tPA) has been used as a therapy for cerebral infarction during the acute phase, but even if reperfusion is accomplished, paralysis persists in the majority of patients. As for ischemic stroke itself, angiogenesis is actually known to occur[2]. The number of new blood vessels is correlated with a longer survival period in patients with ischemic stroke. Therefore, angiogenesis is thought to be beneficial to the ischemic brain. In animal models, many kinds of therapies[3-6] to promote neovascularization in the ischemic brain have been reported. The purposes of this study were to examine whether neovascularization might be promoted by the intra-arterial transplantation of mononuclear cells (MNCs) after cerebral ischemia in mice and, if so, to determine whether the new endothelial cells are derived from the donor or from the recipient.

Materials and methods

1)Animals

Two types of adult 8- to 10-week-old mice (20-25 g) were used: wild-type C57BL/6J mice (wild-type mice) and transgenic mice ubiquitously expressing enhanced green fluorescent protein (EGFP) under a CAG-promoter (EGFP mice). Both types of mice were purchased from SLC (Shizuoka, Japan). All animal-related procedures were approved by the Laboratory Animal Care and Use Committee of Keio University and were conducted in accordance with the guidelines of the National Institutes of Health.

2)Mononuclear cells harvest

Bone marrow cells were obtained from wild-type mice or EGFP mice by crushing the tibias and femurs. Mononuclear cells (MNCs) were then isolated by density gradient centrifugation with Ficoll (Ficoll-Paque Plus; Amersham Pharmacia Biotech #17-1440-02, Sweden), as previously described[7]. For the intra-arterial transplantation, 2 x 10^5 MNCs were prepared in 100 μL of HBSS (wild-MNCs and EGFP-MNCs).

3)Transient cerebral ischemia

Before surgery, anesthesia was induced with 2.5% isoflurane and maintained with 1-1.5% isoflurane in 30% O_2 and 70% N_2O via a face mask. The rectal temperature was maintained at 37°C throughout the surgical procedure by placing the animals on a heating bed (Model MWT-100; Bio Research Center, Nagoya, Japan). A laser Doppler flowmeter probe (model ALF21; Advance, Tokyo, Japan) was attached to the surface of the ipsilateral cortex to monitor the regional cerebral blood flow. Transient middle cerebral artery occlusion (MCAo) was accomplished using the previously described intraluminal filament technique[3,8,9]. In brief, the right carotid bifurcation was exposed, and the external carotid artery (ECA) was coagulated distal to the bifurcation (Fig.1). A silicone-coated 8-0 monofilament was inserted through the stump of the ECA and gently advanced (9.0-10.0 mm) to occlude the MCA, as confirmed by the decrease in the regional cerebral blood flow. Thirty minutes after MCAo, reperfusion was effected by withdrawal of the embolus.

4)Intra-arterial transplantation

Immediately after the embolus was withdrawn, a polyethylene PE-10 catheter (Becton Dickinson & Co., USA; outer diameter, 0.61 mm; inner diameter, 0.28 mm) was inserted through the same incision, used to expose the ECA, and used to infuse the MNC suspension (EGFP mice transplanted with wild-MNCs [n=12] and wild-type mice transplanted with EGFP-MNCs [n=8]) or the same volume of HBSS (vehicle only) (control mice [n=14]). The MNCs or HBSS were infused gradually over ten minutes using a microdyalysis pump (The Univentor 801 Syringe Pump; Univentor, Malta). After transplantation, the incision was closed and the animals were allowed to recover from the anesthesia.

5)Histology

At an early time point (30 minutes after the operation; wild-type mice transplanted with EGFP-MNCs [n=4]) or at a chronic time point (6 weeks after operation; others), the animals were anesthetized with diethylether and then perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. The brains were then removed and postfixed with 4% PFA in PBS overnight at 4°C. Coronal sections of the brains (14 μm thick) were cut using a cryostat microtome (CM3000; Leica, Germany). Lung, spleen, and liver sections obtained at the early time point were also cut using a cryostat microtome to confirm the locations where the donor cells were trapped.

Each brain section obtained at the chronic time point was stained with cresyl violet to identify Nissl substance. This stain is used to differentiate between neurons and glial cells based on the amount and location of Nissl substance as well as the nuclear size and appearance. For the following immunohistochemical studies, we used rat anti-mouse CD31 antibody (BD Biosciences 550274, diluted 1:10) as the primary antibody and anti-rat HRP with Tyramide Signal Amplification (TSA kits; Molecular Probes, Oregon) as the secondary antibody. For another set of immunohistochemical experiments, rabbit polyclonal anti-GFP antibody (diluted 1:500) was used as the primary antibody and donkey biotinylated anti-rabbit IgG (Jackson 711-066-152, diluted 1:500) was used as the secondary antibody; the resulting

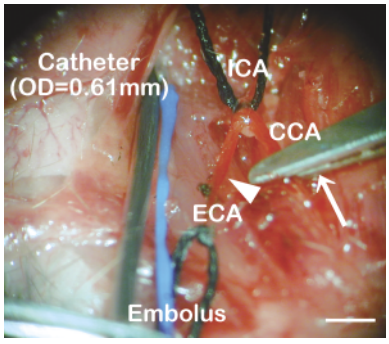

Fig.1 Right side of neck

After inducing anesthesia with isoflurane, the right side of the neck was opened. The common carotid artery was clipped with a temporary clip (arrow), and the distal end of the external carotid artery (ECA) was ligated and turned to the caudal side (arrow head). An embolus (silicone-coated 8-0 filament) and a catheter (polyethylene PE-10 catheter filled with India ink for demonstration) are shown on the left side. ICA, internal carotid artery; CCA, common carotid artery. Bar = 1 mm.

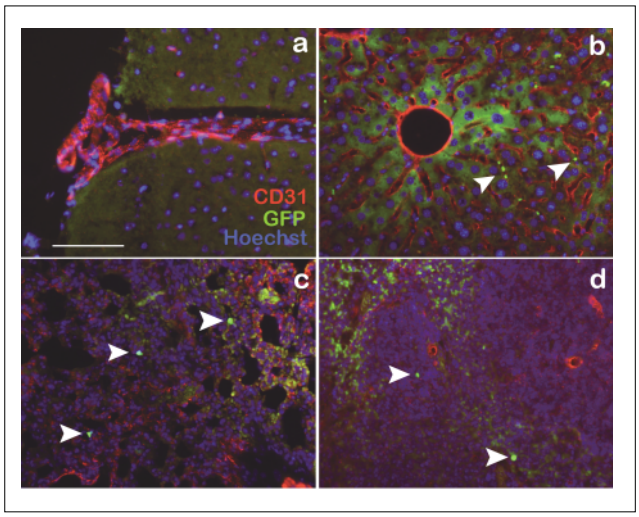

Fig.2 Early detection of transplanted mononuclear cells from EGFP mice

After transient middle cerebral artery occlusion, a polyethylene catheter was inserted through the same incision to transplant the mononuclear cells (MNCs) suspension harvested from EGFP mice. Thirty minutes after the transplantation, the mice were sacrificed and dissected to identify the locations of the MNCs from the EGFP mice. The slices were stained immunohistochemically with anti-CD31 antibody (red), anti-GFP antibody (green), and Hoechst (blue). MNCs (arrow head) from EGFP mice were detected in the liver (b), lung (c), and spleen (d), but not in the brain (a). Bar = 100 μm.

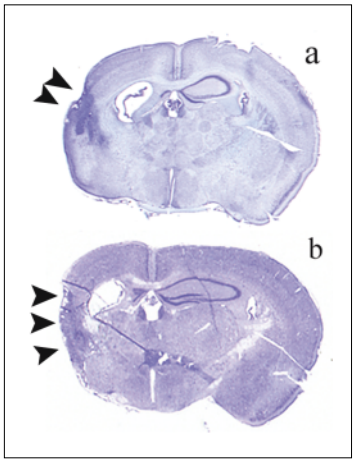

Fig.3 Cerebral infarct area at a chronic time point

After thirty minutes of transient middle cerebral artery occlusion, reperfusion was performed by withdrawal of the embolus. Coronal sections of the brain stained with cresyl violet show the infarct area appearing as a cicatrix (arrow head) with compensatory dilatation of the lateral ventricle on the ischemic side. The shape of the infarct was similar in the transplanted (a) and control mice (b).

color was enhanced using ABC-DAB (Vectastain Elite ABC Kit Standard, PK-6100 and Vectastain stain, SK-4100; Vector Laboratories). Adjacent sections were stained with cresyl violet to facilitate the identification of the infarct area.

The volumes of the endothelial cells marked by the anti-CD31 antibody were determined offline after the images had been transferred to an image analysis software (Image-J freeware, downloaded from the NIH website[10]; National Institute of Health, USA). The infarct area was divided into three random small squares in each slice, and the area (%) of CD31-positive cells was counted and expressed as the mean ± SD. A p value less than 0.05 was considered significant.

Results

It took about 16.5 minutes (13 to 20 minutes) from the start of anesthesia to accomplish the MCAo using an intraluminal filament via the right ECA. During the early phase, EGFP-MNCs were detected in the lung, spleen, and liver but not in the brain (Fig.2).

After the operation, the mice exhibited paralysis on the left

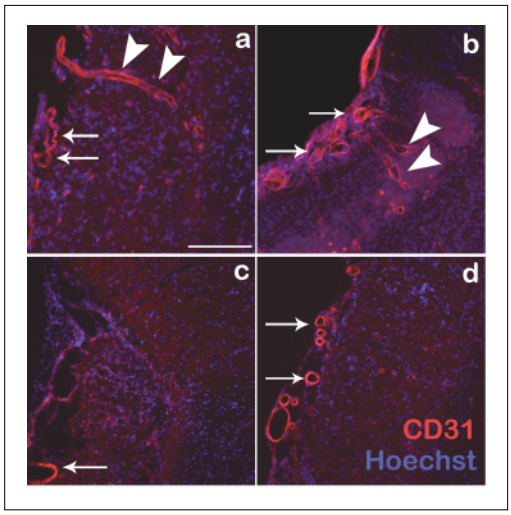

Fig.4 Immunohistochemical staining for anti-CD31 antibody in the area of the cerebral infarction

Coronal brain sections, 14 μm thick, were prepared using a cryostat 6 weeks after the intraarterial transplantation of MNCs following transient middle cerebral artery occlusion. The sections were stained immunohistochemically for anti-CD31 antibody (red) to identify the vascular endothelial cells and Hoechst (blue) to identify the nuclei. In the transplanted group (a,b), CD31-positive cells were seen not only around (arrow), but also within (arrow head) the infarct area in the brains of MNCs transplanted mice; in the brains of the control mice (c,d), CD31-positive cells were seen only around the infarct area. (4a-4d are from different mice.) Bar = 200 μm.

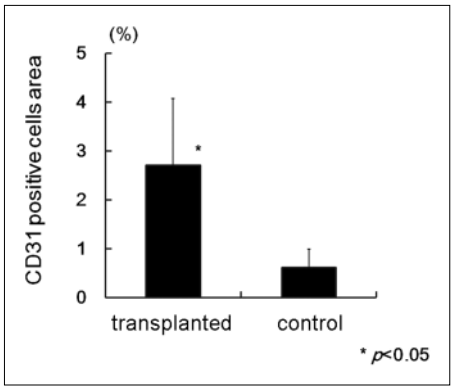

Fig.5 Area of CD31-positive lesions in the infarct area

The images of coronal sections stained with anti-CD31 antibody were transferred to an image analysis software program, Image-J. The infarct area was divided into three random small squares in each slice, and the area of CD31-positive lesions was counted and expressed as the mean ± SD. The area of the endothelial cells increased more in the transplanted mice than in the control mice (2.71 ± 1.37 vs. 0.62 ± 0.38; * p<0.05)

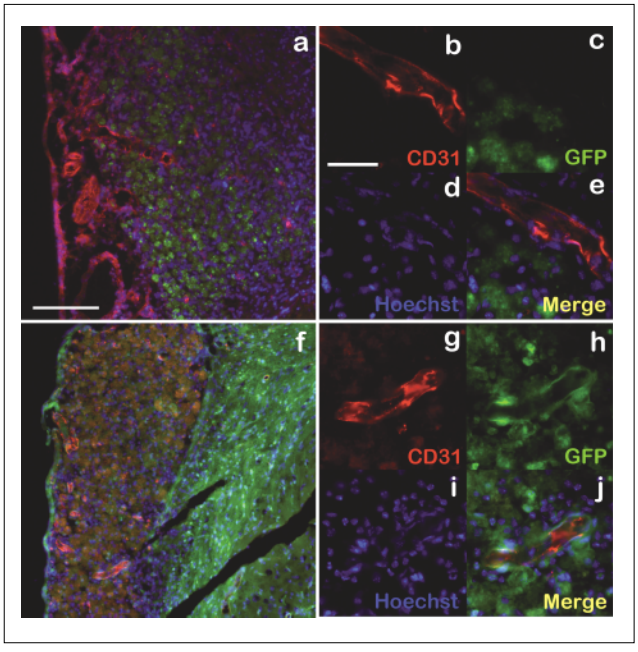

Fig.6 New endothelial cell derived from recipient cells

Six weeks after the transplantation of EGFP-MNCs (a-e) or wild-MNCs (f-j) in ischemic murine brain (transient MCAo to wild-type mice [a-e] or EGFP mice [f-j]), numerous endothelial cells stained with anti-CD31 antibody were seen around and in the infarct area. In the brain of wild-type mice transplanted with EGFP-MNCs, CD31-positive cells (red) were negative for GFP (green) (b-e). In the brain of EGFP mice transplanted with wild-MNCs, CD31-positive cells were also positive for GFP (g-j). Bars = 200 μm (a,f), 50 μm (b-e, g-j).

side and a left-rotated gait. However, no differences in the neurological deficits of mice transplanted with MNCs and the deficits of those treated with vehicle alone were observed. The paralysis improved after 2 weeks in both types of mice. Six weeks after the operation, the mice were sacrificed and their brains were removed. Cresyl violet staining of the brain sections showed the infarct area as a cicatrix with compensatory dilatation of the lateral ventricle on the same side (Fig.3). Numerous CD31-positive cells were identified in the vascular structures around and inside the infarct area in wild-type mice transplanted with EGFP-

MNCs and in EGFP mice transplanted with wild-MNCs (Fig.4a,b). On the other hand, in the control mice, vascular structures were seen only on the surface of the scar and not seen inside the infarct area (Fig.4c,d). The area of the CD31-positive cells was 2.71 ± 1.37 % in the transplanted mice and 0.62 ± 0.38 % in the control group, respectively ($p<0.05$) (Fig.5).

During the chronic phase, GFP-positive cells were not found in the infarct area of wild-type murine brain transplanted with EGFP-MNCs (Fig.6a-e). However, in the brains of EGFP mice transplanted with wild-MNCs, CD31-positive cells were also positive for EGFP (Fig.6f-j).

Discussion

Our study showed that neovascularization was promoted in ischemic murine brain after the intra-arterial transplantation of MNCs. Furthermore, the new-endothelial cells were derived from the recipient, not the donor. Some previous studies have demonstrated that neovascularization after cerebral ischemia can be promoted by the intracerebral injection of embryonic stem (ES) cells[4] or the intravenous transplantation of bone marrow-derived cells[5] in mouse models. Intra-arterial transplantation provides an efficient route for the treatment of stroke in rat models[6]. On these grounds, we transplanted the cells intra-arterially in the mice model used in the present study. To the best our knowledge, this is the first report of intra-arterial transplantation in a mouse model. This mouse model exhibits three characteristic features. First, we transplanted the MNCs intra-arterially. Intra-arterial transplantation is useful when only a small volume of cells or drugs needs to be introduced, with an intravenous route being selected for higher volumes of cells or drugs. In the early phase of ischemic stroke, the intravenous infusion of tPA allows early reperfusion to be achieved in only 30-50% of patients, with even lower rates in cases with proximal large vessel occlusions (i.e., middle cerebral, basilar artery, and carotid terminus)[11,12]. Our model could be used for the development of new therapies after intra-arterial local thrombolysis.

Second, the occluded middle cerebral artery is reperfused by the withdrawal of the embolus after 30 minutes. This setting resembles the typical clinical course of acute cerebral infarction followed by thrombolytic therapies in humans. Intravenous thrombolysis with tPA within the first 3 hours of symptom onset has been reported to improve the clinical outcome significantly in case with acute ischemic stroke[13,14]. Intra-arterial local thrombolysis with urokinase has also been shown to be effective, especially when administered within 6 hours after the occurrence of infarction[15-17]. Furthermore, the combined use of tPA and antithrombotic agents may allow the safe prolongation of the time window for treatment[18]. We showed that neovascularization was promoted according to a course resembling the typical clinical course in humans.

Finally, in the present experiments, the transplanted cells were harvested from the bone marrow. In humans, while ES cells or other cultured cells have ethical and technical barriers for clinical application, requiring a Good Manufacturing Practice (GMP)-level culture facility, bone marrow-derived fresh cells can be used as for bone marrow transplantation and do not pose ethical problems. Bone marrow-derived autologous cells harvested from an ischemic stroke patient are also not immunologically rejected. However, this technique has three limitations that must be pointed out before our findings can be applied to humans. In our study, we transplanted MNCs harvested from a syngenic donor animal, because autologous MNCs were not available from the recipient mouse. Therefore, it is possible that the beneficial effects were derived from the syngenic transplantation, and autologous MNC transplantation may not induce neovascularization. The second limitation is the risk of hemorrhage during bone marrow aspiration after reperfusion has been achieved through the use of a thrombolytic agent. The third limitation is that there were no differences in the paralysis experienced by the mice transplanted with MNCs and those treated with the vehicle alone in our experimental model. Even with these limitations, however, we believe that our method could represent a novel additional modality for the treatment of cerebral infarction.

We hypothesized that the transplanted MNCs differentiated into new endothelial cells. CD31-positive cells, which exhibited endothelial cell markers, located in the infarction area were thought to be new endothelial cells because the vessels that formed from the CD31-positive cells in the infarction area had a larger diameter and meandered more than those in the ipsilateral normal area. However, GFP-positive cells were not found in the infarction area of wild-type murine brain transplanted with EGFP-MNCs. GFP-positive cells were found in the infarction area of EGFP-mice transplanted with wild-MNCs, and these cells were also stained by the CD31 antibody. Furthermore, transplanted EGFP-MNCs were not found in the brain at 30 minutes after operation. This result implies that the new endothelial cells were not derived from the donor cells, but from recipient cells. In an animal model of limb ischemia, MNC transplantation promoted neovascularization and the transplanted MNCs did not differentiate into the endothelial cells; the stimulated muscle cells produced angiogenic factors, thereby promoting neovascularization[19]. In cerebral ischemia, as in limb ischemia, the transplanted MNCs

did not differentiate into the endothelial cells but instead promoted the formation of new endothelial cells. In an ischemic limb model, IL-1β secreted from muscle cells promoted neovascularization[19]. Thus, the transplanted MNCs might not be necessary for the differentiation of new endothelial cells, but might instead exert trophic actions that stimulate some kinds of host cells. Thus, we speculated that by performing an intra-arterial transplantation, a larger number of MNCs might stimulate host cells in ischemic brain than by an intra-venous transplantation, and some angiogenic factors may promote neovascularization. We have not yet been able to identify what kinds of cells were stimulated by the transplanted MNCs and what kinds of angiogenic factors were produced, but we have confirmed that the new endothelial cells were not derived from the donor in our model. Further work is required to determine what kinds of cells and/or factors may contribute to neovascularization after ischemic stroke and to demonstrate the differences in MNC volume between intra-arterial and intra-venous routes of administration. Furthermore, additional therapies may be required to improve paralysis.

In our study, neovascularization was promoted in the ischemic brain after MNC transplantation; however, a relation between neurogenesis and neovascularization was not confirmed in this report. The microenvironment, or vascular niche, is thought to be a key regulator of stem cell behavior[20]. Regarding adult neurogenesis, microanatomical studies of the subventricular zone have recently revealed that the vasculature is a key component of the adult neural stem cell niche[21-23]. Furthermore, neural regeneration occurs at the vascular niche after an insult to the mouse brain[22]. Regarding cicatrix formation, we could not compare the infarct volumes but the shape of the infarct was similar in mice transplanted with MNCs and in control mice. In mice transplanted with MNCs, neovascularization was promoted in the infarct area, possibly leading to neurogenesis. In the future, we intend to examine whether the neovascularization around the infarct area promotes neurogenesis.

Conclusions

Neovascularization was promoted by the intra-arterial transplantation of MNCs after cerebral ischemia and reperfusion in mice, and the new endothelial cells were derived from the recipient, and not the donor cells.

Acknowledgement

We thank Professor Hideyuki Okano for his instructions and supervision regarding this report. This work was supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) (to T.F., J.N., and Y.M.) and was also supported in part by a grant-in-aid from the Global COE program of the Ministry of Education, Science, and Culture of Japan to Keio University.

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