Angiogenesis Induced by Autologous Whole Bone Marrow Stem Cells Transplantation

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Background and Objectives: It has been presumed that unknown cells and growth factors in bone marrow might promote angiogenesis, so angiogenesis effect could be enhanced by autologous whole bone marrow (WBM) stem cell transplantation. We compared capillary ratio induced by autologous WBM and bone marrow-mononuclear cells (BM-MNCs) to evaluate the anigiogenic effect of autologous WBM. In addition, the combined effect of WBM transplantation and granulocyte colony-stimulating factor (G-CSF) injection was examined in an ischemic canine model.

Methods and Results: After creating ischemic limb model, autologous WBM and isolated BM-MNCs were transplanted into the ischemic muscle. In other experiments, autologous WBM with recombinant human G-CSF (rhG-CSF) and autologous WBM without rhG-CSF were transplanted into the ischemic muscle. In this study, normal saline was injected into the contralateral sites in each ischemic model as a control group. After 8 weeks of transplantation, angiography and muscle harvest were performed, and then the anigiographic findings and capillary density, as assessed by immunohistochemical staining, were investigated and analyzed. In comparison with the control group, BM-MNCs and WBM transplantation groups showed higher ratios of the capillary density (1.5 ± 0.01 times, $p < 0.001$ and 1.6 ± 0.15 times, $p = 0.005$, respectively). Between the BM-MNCs and WBM transplantation groups, the capillary ratio was 1.2 folds higher in the WBM group than that in the BM-MNCs group, but there was no significantly different ($p = 0.116$). The angiogenesis ratios of both the WBM without G-CSF group and the WBM with G-CSF groups were higher (1.6 ± 0.15 times, $p = 0.004$ and 1.8 ± 0.01 times, $p = 0.005$, respectively) than that of the control groups. In comparison with the WBM without G-CSF group, the WBM with G-CSF transplantation group revealed a 1.1 folds higher angiogenesis ratio, but there was no statistically significant difference ($p = 0.095$). **Conclusions:** Autologous WBM transplantation is a simpler method and it is not inferior for inducing therapeutic angiogenesis as compared with isolated BM-MNCs transplantation. In addition to autologous WBM transplantation, intravenous G-CSF injection enhances the angiogenic effect of autologous WBM in an ischemic limb.

Keywords: Stem cells, Bone marrow, Angiogenesis and ischemia

Introduction

Angiogenesis has been described as the development of new capillaries from pre-existing vessels and the final result is the formation of new capillary networks. This is preceded by either sprouting of endothelial cells or by intussusception (1). Angiogenesis can be achieved by the

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participation of several cytokines such as vascular endothelial growth factor (2) and granulocyte colony-stimulating factor (G-CSF) (3), and also by participation of stem cells that are endothelial progenitor cells (EPCs), including CD34-positive cells (4, 5).

G-CSF is known to mobilize peripheral blood stem cells and EPCs from the bone marrow (6, 7). Bone marrow (BM) derived cells have been reported to differentiate into the endothelial cells of blood vessels (4). Several recent studies have shown that autologous bone marrow derived mononuclear cells (BM-MNCs) transplantation increases the number of collateral vessels (8-10).

Bone marrow contains many kinds of immature cells like EPCs. Several cytokines are secreted from the BM components, including the stromal cells and various hematopoietic cells (11-13). Most of the previous studies have used the BM-MNCs or BM-EPCs, but the isolation of BM-MNCs or BM-EPCs is highly complex and expensive, and it incurs the potential danger of contamination.

In this study, it has been presumed that unknown cells and growth factors might also promote angiogenesis, so that the angiogenesis effect could be enhanced by autologous whole bone marrow (WBM) stem cells transplantation.

We compared the capillary ratio induced by autologous WBM and BM-MNCs to evaluate the angiogenic effect of autologous WBM and examined the combined effect of G-CSF in an ischemic canine model.

Materials and Methods

Ischemic canine model

Mongrel dogs, weighing 23~25 kg each, were anesthetized with propofol (6 mg/Kg, DongKook Pharm, Korea) and norcuron (1 cc/10 kg, N.V. Organon, Halland, Korea). The femoral arteries in both the lower limbs were encircled by using an ameroid constrictor (RISW[®], CA, USA) with an inner diameter of 1.5 mm. After suture and disinfection of the incision sites, all the animals were intramuscularly injected with cefazolin (20 mg/kg, Chong Kun Dong, Korea) for 5 days. After 4 weeks, we identified the complete occlusion of a femoral artery by performing Doppler examination. This study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Samsung Biomedical Research Institute (SBRI). SBRI is a member of the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC international) accredited facilities and it abides by the Institute of Laboratory Animal Resources (ILAR) guidelines.

WBM aspiration and BM-MNCs isolation

Four weeks after creating the ischemic model (at 3 days of rhG-CSF injection), 20 ml of WBM was aspirated from the right femurs with using a 30 ml syringe that contained 2,000 units of unfractionated heparin. The aspirated WBM was mixed with the same volume of 1x phosphate buffer saline (PBS, Gibco, CO). The mixture was then layered onto the top of Histopaque-1077 and this was followed by centrifugation at $\times 400$ g for 30 minutes. The BM-MNCs were isolated from the layers between the Histopaque-1077 reagent and the blood plasma by performing density gradient centrifugation. The red blood cells (RBCs) were completely removed with RBC-lysis buffer and they were washed two times each for 10 minutes in PBS.

WBM and BM-MNCs transplantation

Control versus BM-MNCs and WBM: $3 \times 10^7 \sim 5 \times 10^7$ cells of autologous BM-MNCs ($n=4$) or 20 ml of WBM ($n=4$) were injected into 4 sites of the muscle in the left ischemic limb, and 20 ml of normal saline was injected into the contralateral sites of the canine models as a control.

BM-MNCs versus WBM: $3 \times 10^7 \sim 5 \times 10^7$ cells of BM-MNCs ($n=4$) were injected into 4 sites of the muscle in the left ischemic limb, and 20 ml of autologous WBM ($n=4$) was injected into contralateral sites of the canine models. The angiogenic effect was compared with the autologous WBM transplantation group and the BM-MNCs transplantation group by performing immunohistochemical analysis for determining the number of the capillary endothelial cells.

WBM with G-CSF versus WBM without G-CSF: For testing the combined effect of G-CSF, recombinant human G-CSF (rhG-CSF; 1 ug/kg/day, Leucostim 300 mcg, CJ, Korea) was injected intravenously from 2 days before to 3 days after transplantation, and then 20 ml of autologous WBM ($n=4$) were transplanted into 4 sites of the ischemic muscle of the limb. In addition, 20 ml of autologous WBM without injection of rhG-CSF ($n=4$) were transplanted in another ischemic model. As a control group, normal saline was injected into the contralateral sites of an ischemic canine model. After that, the angiogenesis ratio was analyzed and compared with the WBM with G-CSF transplantation group, the WBM without G-CSF transplantation group and the control group.

Angiography and muscle harvest

After 8 weeks of autologous WBM or BM-MNCs transplantation, the dogs were re-anesthetized. Following exposure of the abdominal aorta, the contrast dye (Ultravist

300 150 ml, Schering, Korea) was injected into the abdominal aorta and then the angiograms were obtained. The angiographic images were recorded on 43×35 cm X-ray films. After angiography, the muscles of the ischemic limbs were harvested for histological analysis.

Immunohistochemical staining

The harvested muscles were fixed in 10% formalin solution that was neutral buffered (SIGMA-AIDRICH CHEMIE, Steinheim, Germany); the muscles were next dehydrated with using a graded series of ethanol solutions and then embedded in paraffin. The specimens were cut into 4 μ m-thick sections and stained with hematoxylin and eosin (H&E). The sections were also followed up via performing immunohistochemistry with von Willebrand Factor (DAKO, Glostrup, Denmark). After deparaffinization with xylene, all the sections were dehydrated and treated with proteinase K (DAKO, Glostrup, Denmark) for 12 minutes. The sections were then treated with protein-blocking solution (DAKO, Glostrup, Denmark) for 30 minutes and they were next treated with normal goat serum (Vector Laboratories, Burlingame, U.S.A.) for 1 hour. The sections were incubated with the primary antibody for 1 hour at room temperature and they were next washed three times. The sections were then incubated for 30 minutes with EnVision+Systems polymer-conjugated secondary antibody (DAKO, Glostrup, Denmark) and they were finally incubated with diaminobenzidine (DAB) for 10 minutes.

The angiogenesis ratio

Five fields of each muscle sample were randomly selected and the number of capillaries was counted and measured using the Image-pro plus 5.1 program (Media Cybernetics). In addition to this, the angiogenesis ratio

of the treated limbs was measured and compared with that of the control limbs.

Statistical analysis

The results are expressed as means $\pm$ SDs. Comparison of the results between the different groups was performed by using unpaired Student's t-tests. All the calculations were performed with the computer program SPSS 8.0 (SPSS Inc, Chicago, IL).

Results

Control *versus* BM-MNCs group

Angiography and histological evaluations were performed at 8 weeks after the BM-MNCs transplantation. The limbs treated with BM-MNCs showed more abundant capillaries than did the controlled limbs (Fig. 1A). Immunohistochemical staining for von Willebrand Factor (vWF) also revealed the presence of numerous capillary endothelial cells (ECs) in the BM-MNCs treated limbs, but the controlled limbs showed a lower number of capillary ECs (Fig. 1B). The ratio of the capillary density was higher (1.5 ± 0.01 times) for the treated limbs than that for the control limbs ($p<0.001$) (Fig. 2).

Control *versus* WBM group

Angiography and immunohistochemical staining revealed greater development of new collateral vessels in the WBM transplantation group than that in the control group. The ratio of the capillary density (Fig. 2) was significantly higher (1.6 ± 0.15 times) in the WBM transplantation group than that in the control group ($p=0.005$).

BM-MNCs *versus* WBM group

On comparing the BM-MNCs group and the WBM

Fig. 1. Bone marrow-mononuclear cells (BM-MNCs) along with recombinant human granulocyte colony-stimulating factor (rhG-CSF) injection into the muscle. (A) Angiography. (B) Immunohistochemical staining for von Willebrand Factor (vWF).

Fig. 2. Comparison of capillary density ratio: Control group versus bone marrow-mononuclear cells (BM-MNCs) transplantation group and whole bone marrow (WBM) transplantation group.

Fig. 3. Comparison of capillary density: Bone marrow-mononuclear cells (BM-MNCs) transplantation group versus whole bone marrow (WBM) transplantation group.

transplantation group, the capillary ratio was 1.2 fold higher for the WBM group than that for the BM-MNCs group. Yet, there was not significant difference ($p=0.116$) between the two groups (Fig. 3).

WBM without G-CSF *versus* WBM with G-CSF

Under the assumption that G-CSF has a synergic effect with WBM for angiogenesis, we compared angiogenesis ratios of the WBM without G-CSF and with G-CSF transplantation groups with the control group, respectively, and then we compared the angiogenesis ratio of the WBM without G-CSF transplantation group with the angiogenesis ratio of the WBM with G-CSF transplantation group. As a result, the angiogenesis ratio was higher for the WBM without G-CSF group (1.6 ± 0.15 times, $p=0.004$) and the WBM with G-CSF group (1.8 ± 0.01 times, $p=0.005$) compared with the control group (Fig. 4). On comparison with the WBM without G-CSF group, the

Fig. 4. Comparison of capillary density: Whole bone marrow (WBM) without G-CSF transplantation group versus WBM with G-CSF transplantation group.

WBM with G-CSF transplantation group showed a 1.1 folds higher angiogenesis ratio, but there was not a statistically significant difference ($p=0.095$).

Discussion

Many researchers have created ischemic animal models by excision or tying an artery (3). These methods have provided an acute arterial occlusive model, and so how to make a chronic ischemic model is very important. As was previously published, we made an ischemic model that was similar to a naturally created chronic ischemic model by using an ameroid constrictor in a medium-sized animal (14). In addition to, we transplanted WBM and BM-MNCs via intramuscular injection. It has been thought that muscle injection may help promote angiogenesis due to the partial existence of several cell sources. Intra-venous (I.V.)

and intra-arterial (I.A.) injection has been employed in the cell transplantation for cell therapy (15). However, it was reported that the cells via I.V. and I.A. injection may be delivered to organs other than the target tissue (16). These results indicate that I.V. and I.A. injection of cells has the danger to induce another disease according to the non-specific adhesion that's due to the poor homing of the injected cells. Haakon K et al. have also reported that the accumulation of cells was significantly greater after intra-coronary (I.C.) injection than after I.V. injection (17).

Asahara et al. reported that angiogenesis is induced by EPCs in the same manner as that for the blood-derived CD34-positive cells (5), and many other studies have demonstrated that angiogenesis is induced by BM-MNCs and several growth factors such as VEGF, Ang-1 and G-CSF (2, 5, 10-12).

WBM contains many kinds of immature cells, including EPCs and various hematopoietic cells and stromal cells that secrete several growth factors, so angiogenesis might be induced by these unknown cells and the cytokines released from WBM. In this study, we hypothesized that autologous WBM produces a more potent angiogenesis than BM-MNCs. First, we compared the angiogenesis ratio of the BM-MNCs transplantation group and WBM transplantation groups with the control group, and the BM-MNCs and WBM transplantation groups showed higher angiogenesis ratios than did the control group. In addition, the WBM transplantation group revealed higher rates of the development of new collateral vessels as compared with the BM-MNCs transplantation group. These results suggest that the isolation of BM-MNCs may remove a positively-influential angiogenesis source during the sorting processes.

We previously reported that WBM-treated limbs with rhG-CSF injected into the muscle have a higher capillary ratio (1.8 folds) compared with the control limbs (14). rhG-CSF has been frequently employed for increasing the white blood cells in the peripheral blood (PPB) and it induced endothelial cells to express an activation program related to angiogenesis (18). It is known that CD34⁺ cells were transferred from the bone marrow to the PPB by using rhG-CSF.⁴ However, we showed in a previous paper (19) that WBM has many CD34⁺, CD133⁺, CD34⁻CD133⁺ and CD34⁺CD133⁺ cells. We experimented on animals to determine the effectiveness of injecting rhG-CSF with WBM into the muscles of ischemic limbs. The angiogenic effect of the WBM revealed a difference depending on whether or not rhG-CSF was present. With using rhG-CSF, the WBM treatment showed a higher capillary density than WBM treatment without rhG-CSF

(Fig. 4). According to these results, we suggest that the rhG-CSF helps the angiogenesis process.

Our results indicated that the prominent effect of WBM for angiogenesis could be achieved by the primitive cells, the EPCs and unknown growth factors in autologous WBM.

In conclusion, autologous WBM transplantation is a simpler method and it is not inferior for inducing therapeutic angiogenesis as compared with isolated BM-MNCs transplantation. In addition to, autologous WBM transplantation with G-CSF injection enhances the angiogenic effect of autologous WBM in an ischemic limb.

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Potential Conflict of Interest

The authors have no conflicting financial interest.

References

1. Risau W. Mechanisms of angiogenesis. *Nature* 1997;386: 671-674
2. Takeshita S, Zheng LP, Brogi E, Kearney M, Pu LQ, Bunting S, Ferrara N, Symes JF, Isner J. Therapeutic angiogenesis. A single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model. *J Clin Invest* 1994;93:662-670
3. Minamino K, Adachi Y, Okigaki M, Ito H, Togawa Y, Fujita K, Tomita M, Suzuki Y, Zhang YM, Iwasaki M, Nakano K, Koike Y, Matsubara H, Iwasaka T, Matsumura M, Ikehara S. Macrophage colony-stimulating factor (M-CSF), as well as Granulocyte colony-stimulating factor (G-CSF), Accelerates Neovascularization. *Stem Cells* 2005;23:347-354
4. Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, Kearne M, Magner M, Isner JM. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res* 1999;85:221-228
5. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997;275:964-967
6. Yamamoto Y, Yasumizu R, Amou Y, Watanabe N, Nishio N, Toki J, Fukuhara S, Ikehara S. Characterization of peripheral blood stem cells in mice. *Blood* 1996;88:445-454

7. Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, Homma S, Edwards NM, Itescu S. Neovascularization of ischemic myocardium by human bone marrow derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nature Medicine* 2001;7:430-436
8. Shintani S, Murohara T, Ikeda H, Ueno T, Sasaki K, Duan J, Imaizumi T. Augmentation of postnatal neovascularization with autologous bone marrow transplantation. *Circulation* 2001;103:897-903
9. Tateishi-Yuyama E, Matsubara H, Murohara T, Ikeda U, Shintani S, Masaki H, Amano K, Kishimoto Y, Yoshimoto K, Akashi H, Shimada K, Iwasaka T, Imaizumi T. Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomized controlled trial. *Lancet* 2002;360:427-435
10. Hernandez P, Cortina L, Artaza H, Pol N, Lam RM, Dorticos E, Macias C, Hernandez C, del Valle L, Blanco A, Martinez A, Diaz F. Autologous bone-marrow mononuclear cell implantation in patients with severe lower limb ischaemia: A comparison of using blood cell separator and Ficoll density gradient centrifugation. *Atherosclerosis* 2007; 194(2):e52-56
11. Bikfalvi A, Han ZC. Angiogenic factors are hematopoietic growth factors and vice versa. *Leukemia* 1994;8:523-529
12. Rohde D, Wickenhauser C, Denecke S, Stach A, Lorenzen J, Hansmann ML, Thiele J, Fischer R. Cytokine release by human bone marrow cells; analysis at the single cell level. *Virchows Arch* 1994;424:389-395
13. Prockop DJ. Marrow stromal cells as stem cells for non-hematopoietic tissues. *Science* 1997;276:71-74
14. Kim DI, Im RJ, Lim JE, Kim JS, Jeon HJ, Jang IS, Kim BS, Cho SW. Angiogenesis induced by the implantation of autogenous whole bone marrow stem cells in an ischemic animal model. *J Korean Soc Vasc Surg* 2005;21:113-117
15. Tatenos K, Minamino T, Toko H, Akazawa H, Shimizu N, Takeda S, Kunieda T, Miyauchi H, Oyama T, Matsuura K, Mishi JI, Kobayashi Y, Nagai T, Kuwabara Y, Iwakura Y, Nomura F, Saito Y, Komuro I. Critical roles of muscle-secreted angiogenic factors in therapeutic neovascularization. *Circ Res* 2006;98:1194-1202
16. Tolar J, O'Shaughnessy MJ, Panoskaltsis-Mortari A, McElmurry RT, Bell S, Riddle M, McIvor RS, Yant SR, Kay MA, Krause D, Verfaillie CM, Blazar BR. Host factors that impact the biodistribution and persistence of multipotent adult progenitor cells. *Blood* 1996;107:4182-4188
17. Groggaard HK, Sigurjonsson OE, Brekke M, Klow NE, Landsverk KS, Lyberg T, Eriksen M, Egeland T, Ilebakk A. Cardiac accumulation of bone marrow mononuclear progenitor cells after intracoronary or intravenous injection in pigs subjected to acute myocardial infarction with subsequent reperfusion. *Cardiovasc Revasc Med* 2007;8:21-27
18. Bussolino F, Ziche M, Wang JM, Alessi D, Morbidelli L, Cremona O, Bosia A, Marchisio PC, Mantovani A. In vitro and in vivo activation of endothelial cells by colony-stimulating factors. *J Clin Invest* 1991;87:986-995
19. Kim DI, Kim MJ, Joh JH, Shin SW, Do YS, Moon JY, Kim NR, Lim JE, Kim AK, Eo HS, Kim BS, Cho SW, Yang SH, Park CJ, Shim JS. Angiogenesis facilitated by autologous whole bone marrow stem cell transplantation for Buerger's disease. *Stem Cells* 2006;24:1194-1200