ORIGINAL RESEARCH

Treatment of ischemic limbs by transplantation of G-CSF stimulated bone marrow cells in diabetic rabbits

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Abstract

Purpose: To evaluate the efficacy of autologous transplantation of granulocyte colony-stimulating factor (G-CSF) stimulated bone marrow mononuclear cells in the treatment of diabetic lower limb ischemic disease in a rabbit model.

Methods: The diabetic model was produced by intravenous injection of 5% alloxan into New Zealand white rabbits. The lower limb ischemia model was created by femoral artery ligation in diabetic rabbits (n=50) 14 days after alloxan injection. Animals were then randomized into five groups (10 rabbits per group): group 1, transplanted with G-CSF immobilized peripheral blood mononuclear cells (PBMNC); group 2, transplanted with G-CSF stimulated bone marrow mononuclear cells (BMMNC); group 3, animals were transplanted with non-stimulated BMMNCs; group 4, G-CSF group injected with G-CSF alone without transplantation; and group 5, PBS group treated with PBS alone. Necrosis of foot or toes and blood flow recovery in ischemic limbs was assessed. Expression of von Willebrand factor (vWF) and vascular endothelial cell growth factor (VEGF) were measured in ischemic muscles by immunohistochemistry analysis.

Results: Blood flow in ischemic limbs was significantly improved in the G-BMMNC group (ratio of blood flows: 0.82 ± 0.06) in comparison with the G-PBMNC (0.61 ± 0.09) and BMMNC (0.62 ± 0.08) groups ($P < 0.001$). In the G-BMMNC group, the capillary density, a measure of vWF expression, was significantly higher than in either the G-PBMNC or BMMNC groups (47.9 ± 2.51 vs. 36.8 ± 4.16 and 39.6 ± 2.72 , respectively, $P < 0.05$). The expression of VEGF

in G-BMMNC animals was significantly increased in comparison with the G-PBMNC and BMMNC groups (16.93 ± 0.70 vs. 11.83 ± 0.98 and 12.32 ± 0.96 , respectively, $P < 0.05$).

Conclusion: A combination of G-CSF stimulation and autologous transplantation of bone marrow stem cells synergistically improved neovascularization and angiogenesis in ischemic limb tissues in diabetic rabbits.

One devastating complication of diabetes is peripheral arterial occlusive disease, including critical limb ischemia, which frequently leads to infection and even amputation.¹⁻³ Given the poor prognosis associated with lower limb ischemia in diabetic patients, numerous interventions have been attempted, primarily based on the stimulation of angiogenesis to trigger the formation of collateral blood vessels.¹⁻⁷ Stem cell transplantation-based therapy is a novel and attractive potential treatment strategy for diabetic patients with limb ischemia. It is based on the rationale that delivery of endothelial progenitor cells into areas of ischemia may result not only in differentiation of the progenitors into endothelium, thereby contributing to angiogenesis,^{8,9} but also produce numerous growth fac-

tors that can stimulate angiogenesis via paracrine interactions with adjacent cells.^{2,10,11}

Several clinical studies have suggested that the lower limb ischemia in diabetic patients could be improved by autologous transplantation of either bone marrow mononuclear cells (BMMNC) or granulocyte colony-stimulating factor (G-CSF) mobilized peripheral blood mononuclear cells (G-PBMNC).^{4-7,11-13} However, a proportion of these patients do not mobilize sufficient progenitors into the blood stream to produce adequate PBMNCs to allow for successful autologous transplantation.^{14,15} To develop a more effective approach, we evaluated the efficacy of autologous transplantation of G-CSF-stimulated bone marrow mononuclear cells (G-BMMNC) to treat diabetic lower limb ischemia in an alloxanate- (AXL) induced diabetic rabbit model.

Materials and Methods

Experimental diabetic model

All animals were treated humanely, and all procedures in the present study were conducted in accordance with institutional protocols and guidelines approved by the Hebei Medical University of China. New Zealand white rabbits (2-3 kg) were obtained from the animal facility of Hebei Medical University. Anesthesia was induced with pentobarbital (30 mg/kg of body weight) intravenously via a standard procedure similar to that used for humans. All surgical procedures were performed by physicians at the Third Hospital of Hebei Medical University in accordance with the standards for hospital operations on humans.

The diabetic rabbit model was produced as reported previously, with minor modifications.¹⁶ Two doses of 5% alloxan monohydrate (Sigma, St Louis, MO), dissolved in PBS buffer, were administered intravenously to healthy rabbits. The first injection was done at a dosage of 80 mg/kg, and second injection was given 24 hours later at a dosage of 120 mg/kg. Blood glucose levels were measured 14 days after

ALX injection and diabetes was confirmed when fasting blood glucose levels were >16 mmol/L. Doubling the dose of ALX proved to be a safe, fast, economic and reliable way to create diabetic rabbit model, with more than 80% model success and a lower mortality than with previously published techniques.

Lower limb ischemic model

The lower limb model was created in diabetic rabbits (n=50) 14 days after ALX injection as described previously.^{17,18} Briefly, animals were pre-anesthetized and a skin incision was made in the right side of posterior limb. The femoral artery was then ligated and the distal portion and all the side branches were dissected free and excised. The left hindlimb was kept intact as an experimental control. After the establishment of ischemic lower limb model, the diabetic animals were randomized into five groups (10 rabbits per group) as follows. In the G-PBMNC group, diabetic animals were injected subcutaneously with G-CSF (10 µg/kg) daily for five consecutive days. On day six, peripheral blood was collected and mononuclear cells (PBMNCs) were separated and injected into the quadriceps and adductor muscles in the ischemic limbs, along the excised artery at 10 points; each point was injected with 5×10^6 PBMNCs in a 100 µL of PBS. Group G-BMMNC was stimulated with G-CSF for five days. Bone marrow (5 ml) was collected on the sixth day and mononuclear cells (BMMNCs) were separated and injected into the muscles in the ischemic limb in the same way as the G-PBMNC group, with each point injected with 5×10^6 BMMNCs. Group BMMNC animals were not stimulated subcutaneously by G-CSF. On the sixth day, 10 mL of bone marrow were drawn and BMMNCs were injected into the muscles of the ischemic limbs as described for the G-PBMNC group. In the G-CSF group, animals were stimulated G-CSF but had no additional treatment. For the control (PBS treated) group, animals were not stimulated by G-CSF, but 100 µL of

PBS was injected into the muscles of the ischemic limbs.

Blood flow measurements

Blood flow in the popliteal artery of ischemic limbs was measured at rest using an Aspen Advanced Doppler ultrasound device (Acuson, Siemens Medical Solutions, Mountain View, CA, USA) two weeks after transplantation. The blood flow recovery in the ischemic limbs were estimated by the ratio of blood flow in the affected limb in comparison with that in the normal left hindlimb. Data from three separate measurements for each rabbit in all groups were used for statistical analyses.

Tissue sampling and immunohistochemistry

The rabbits were anesthetized as before with pentobarbital 28 days after surgery, and adductor muscle was dissected, fixed with 4% paraformaldehyde and embedded in paraffin in a standard protocol. Histological tissue sections (5 μ m thick) were used for immunohistochemical analysis and were prepared using the three-step avidin-biotin immunoperoxidase method. The following markers were used: anti-von Willebrand factor (vWF) (NCSTAR Corporation, Stillwater, MN, USA) and anti-vascular endothelial cell growth factor (VEGF) (R&D Systems, Minneapolis, MN, USA).

Quantification of microvessel density

Microvessel density was determined by immunostaining against vWF and appropriate morphology.^{19,20} The number of capillaries in ischemic limb muscles was counted by the same observer blinded to the treatment regimen. A total of five fields of a 200X objective from each tissue section were randomly selected, and the number of vWF-positive stained vessel endothelial cells or cell clusters was counted for each field. The mean of three different tissue samples from each animal was used as the average for each animal. The

means for all 10 animals in each group were used to measure the final average capillary density and for statistical analyses.

Quantification of VEGF expression

Immunostained slides against VEGF antibody were visualized with a standard light and captured with a color image. Color images were then converted to grey-scale images and enhanced with the median filter. The interval of grey shades, corresponding to the reaction, was then defined and the area occupied by the reaction was calculated. The threshold operation converted foreground pixels into black color, while background pixels into white color. Thus the binary image represented the analyzed reaction. The area of VEGF positive reaction was estimated by the number of black pixels. The area fraction of VEGF positive reaction was determined as the percentage of black pixels in the binary image. For each slide, 5 fields of a 200X objective were randomly chosen for the above image analysis. The mean of three different tissue samples from each animal was used as the average for this animal. The means for all 10 animals in each group were used to measure the final average VEGF expression level for statistical analyses.

Statistical Analyses

Data were presented as mean $\pm$ SD. The data were analyzed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test. The statistical difference between two groups was determined by using two-sample t-test. All statistical analyses were carried out using SPSS 11.5, except for t-test, which was performed using Excel. The significance level was set at $P < 0.05$.

Results

Assessment of necrosis in ischemia limbs

To evaluate the efficacy of treatment by G-CSF stimulated bone marrow MNCs transplantation, the inci-

dence of necrosis of the ischemic lower limbs was first determined (Figure 1). Necrosis was graded on a 3-point scale: necrosis of the foot, necrosis of the toes, or the absence of necrosis. In control PBS-injected animals, 60% animals showed limb necrosis 20 days after femoral ligation, and half of these animals (25% of control group) had necrosis of the foot. G-CSF treatment decreased the incidence of necrosis of the foot to 20%. In G-PBMNC and BMMNC groups, the incidence of necrosis on toes was 30% for each group. Only one animal in the G-BMMNC group had toe necrosis and the remaining animals had neither necrosis nor limitation of movement. No deaths were observed after ligation of the proximal end of the femoral artery.

Improvement of blood flow

Two weeks after the transplantation, the blood flow in the popliteal artery of ischemic and normal limbs was assessed using Doppler ultrasound. As shown in Figure 2, the ratio of mean ischemic/normal blood flow in group G-BMMNC (0.823 ± 0.06) was the highest compared with groups G-PBMNC (0.612 ± 0.09), BMMNC (0.624 ± 0.08) G-CSF (0.483 ± 0.04) and PBS (0.427 ± 0.04) ($P < 0.05$), suggesting a combination of G-CSF stimulation and transplantation of bone marrow mononuclear cells could improve the blood flow of ischemic hind-limbs more efficiently than transplantation of G-CSF mobilized peripheral blood mononuclear cells or bone marrow stem cells alone.

Microvessel density in ischemic limb tissues

To compare the neovascularization levels of animals in G-BMMNC transplanted group to those in the G-PBMNC or BMMNC groups, microvessel density was determined by the expression level of vWF in adductor muscles in ischemic limbs using immunohistochemistry analysis. As expected (Figure 3), the number of positive vWF stained cells increased significantly in both the G-PBMNC and BMMNC groups in comparison with G-CSF alone or control PBS-treated

FIGURE 1. Decline in necrosis by transplantation with G-CSF stimulated bone marrow. Necrosis was graded on a 3-point scale: necrosis of the foot, necrosis on the toes or no necrosis. The incidence of necrosis was determined at 20 days after femoral ligation. G-BMMNC: G-CSF stimulated bone marrow mononuclear cells transplanted group; G-PBMNC: G-CSF mobilized peripheral blood mononuclear cells transplanted group; BMMNC: bone marrow mononuclear cells transplanted group without G-CSF stimulation; G-CSF: G-CSF alone treated group; PBS: PBS control injected group.

FIGURE 2. Mean blood flow in the ischemic limbs. Blood flow in the rabbit hind-limbs was measured by Doppler ultrasound. Two weeks after transplantation, blood flow of both ischemic and non-ischemic hindlimbs in the same animal was recorded. Values were expressed as a ratio of contralateral limbs (ischemic limb/normal limb). Data were shown as Mean $\pm$ SD. $n=10$. $P < 0.05$ was considered to be statistically significant.

B

Group	Microvessel Density (capillaries/microscopic field)
G-PBMNC	36.8±4.16
G-BMMNC	47.9±2.51
BMMNC	39.6±2.72
G-CSF	30.4±2.37
PBS	22.0±3.65

FIGURE 3. Microvessel density in ischemic limb muscles in diabetic rabbits using immunohistochemistry analysis against vWF antibody. **A.** Representative images (X400 magnification) of adductor muscles showed vWF positive stained cells (brown deposits). **B.** Quantitative analysis of microvessel density (capillaries/microscopic field) in the ischemic muscles. Method used for qualification was described in Materials and methods. Data are presented as mean ± SD. ANOVA test was performed for statistical comparisons of the means for each group followed by Student-Newman-Keuls test. P value <0.05 was considered to be statistically significant.

animals. There is no significant difference between these two latter groups; however, the capillary density in diabetic animals in the G-BMMNC group was significantly higher than in either the G-PBMNC or the BMMNC group (47.9 ± 2.51 vs. 36.8 ± 4.16 and 39.6 ± 2.72 , respectively, $P < 0.05$).

VEGF expression in ischemic limb muscles

VEGF is an endothelial cell-specific mitogen that is considered to be a critical growth factor in pathological and therapeutic angiogenesis.²¹⁻²³ As summarized in Figure 4, both G-PBMNC and BMMNC transplanted animals showed increased expression levels of

B

Group	Expression of VEGF (%)
G-PBMNC	11.83±0.98
G-BMMNC	16.93±0.70
BMMNC	12.32±0.96
G-CSF	10.15±0.46
PBS	8.77±0.65

FIGURE 4. VEGF expression level in ischemia limb muscles in diabetic rabbits using immunohistochemistry analysis against VEGF antibody. **A.** Representative images (X200 magnification) of adductor muscles showed VEGF positive stained cells (brown deposits). **B.** Quantitative analysis of VEGF protein expression in the ischemic muscles. Method used for qualification was described in Materials and methods. Data are presented as mean ± SD. ANOVA test was performed for statistical comparisons of the means for each group, followed by Student-Newman-Keuls test. P value <0.05 was considered to be statistically significant.

VEGF in comparison with G-CSF alone or control PBS-treated animals. No significant difference was observed between the G-PBMNC and BMMNC groups; however, the expression of VEGF in animals of group G-BMMNC was significantly higher than in the G-PBMNC and BMMNC groups (16.93 ± 0.70 vs 11.83 ± 0.98 and 12.32 ± 0.96 , respectively, $P < 0.05$). This suggests that the combination of bone marrow stem cell transplantation and hypodermic injection of G-CSF had a significant synergistic effect in promoting angiogenesis.

Discussion

Treatment of lower limb ischemia in diabetic patients remains a clinical challenge due to impaired angiogenic response to ischemia.²⁴⁻²⁶ Endothelial progenitor cells (EPCs) have been identified as circulating precursors of adult neovasculogenesis and vascular homeostasis. EPCs usually reside in the bone marrow and are mobilized to the peripheral blood upon stimulation, including tissue ischemia and the local release of cytokines and growth factors.^{27,28} It has been shown that the number of circulating EPCs is greatly reduced in diabetic patients;²⁴⁻²⁶ thus, making peripheral vascular complications more difficult to treat.

Peripheral blood mobilized EPCs autologous transplantation has been recently applied in clinical trials in the treatment of critical lower limb ischemia diseases.¹³ Unfortunately, up to 30% of patients are unable to mobilize adequate progenitors for successful autologous transplantation.^{14,15} In this study, we showed that transplantation coupled with G-CSF stimulated bone marrow mononuclear cells attenuated the symptoms of necrosis in ischemia limbs of diabetic rabbits. We also demonstrated a significant improvement of blood flow recovery, neovascularization and angiogenesis in G-CSF stimulated bone marrow mononuclear cells transplanted animals as determined by indices of capillary density and VEGF expression in ischemic tissues. Our data strongly suggested that combination of G-CSF stimulation and autologous transplantation of bone marrow stem cells might predispose a more favorable outcome in the treatment for lower limb ischemia symptoms in diabetic patients.

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References

1. Dormandy JA, Rutherford RB: Management of peripheral arterial disease (PAD). TASC Working Group.

- TransAtlantic Inter-Society Consensus (TASC). *J Vasc Surg*; 2000; 31: S1-S296.
2. Murphy MP, Wang H, Patel AN, *et al*. Allogeneic endometrial regenerative cells: an "Off the shelf solution" for critical limb ischemia? *J Transl Med*; 2008; 6: 45.
3. Kügler CF, Rudofsky G. The challenges of treating peripheral arterial disease. *Vasc Med*; 2003; 8: 109-14.
4. Franz RW, Parks A, Shah KJ, *et al*: Use of autologous bone marrow mononuclear cell implantation therapy as a limb salvage procedure in patients with severe peripheral arterial disease. *J Vasc Surg*; 2009; 50: 1378-90.
5. Procházka V, Gumulec J, Chmelová J, *et al*: Autologous bone marrow stem cell transplantation in patients with end-stage chronic critical limb ischemia and diabetic foot. *Vnitr Lek*; 2009; 55: 173-8.
6. Kirana S, Stratmann B, Lammers D, *et al*: Wound therapy with autologous bone marrow stem cells in diabetic patients with ischaemia-induced tissue ulcers affecting the lower limbs. *Int J Clin Pract*; 2007; 61: 690-2.
7. Esato K, Hamano K, Li TS, *et al*: Neovascularization induced by autologous bone marrow cell implantation in peripheral arterial disease. *Cell Transplant*; 2002; 11: 747-752.
8. Hill JM, Zalos G, Halcox JP, *et al*: Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med*; 2003; 348: 593-600.
9. Waltenberger J. Impaired collateral vessel development in diabetes: potential cellular mechanisms and therapeutic implications. *Cardiovasc Res*; 2001; 49: 554-560.
10. Asahara T, Murohara T, Sullivan A, *et al*: Isolation of putative progenitor endothelial cells for angiogenesis. *Science*; 1997; 275: 964-7.
11. Hamano K, Li TS, Kobayashi T, *et al*: Angiogenesis induced by the implantation of self-bone marrow cells: a new material for therapeutic angiogenesis. *Cell Transplant*; 2000; 9:439-43.
12. Tateishi-Yuyama E, Matsubara H, Murohara T, *et al*: Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. *Lancet*; 2002; 360: 427-35.
13. Huang P, Li S, Han M, *et al*: Autologous transplantation of granulocyte colony-stimulating factor-mobilized peripheral blood mononuclear cells improves critical limb ischemia in diabetes. *Diabetes Care*; 2005; 28: 2155-60.
14. Jansen J, Thompson JM, Dugan MJ, *et al*: Peripheral blood progenitor cell transplantation. *Ther Apher*; 2002; 6: 5-14.

15. Lemoli RM, de Vivo A, Damiani D, et al: Autologous transplantation of granulocyte colony-stimulating factor-primed bone marrow is effective in supporting myeloablative chemotherapy in patients with hematologic malignancies and poor peripheral blood stem cell mobilization. *Blood*; 2003; 102:1595-600.
16. F. Weekers, A. Giulietti, M. Michalaki, *et al.* Metabolic, endocrine, and immune effects on stress hyperglycemia in a rabbit model of prolonged critical illness. *Endocrinology*; 2003; 144: 5329-5338.
17. Takeshita S, Zheng LP, Brogi E, et al. 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: 662-70.
18. Murohara T, Asahara T, Silver M, et al. Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. *J Clin Invest*; 1998; 101: 2567-78.
19. Li TS, Hamano K, Nishida M, Hayashi et al: CD117⁺ stem cells play a key role in therapeutic angiogenesis induced by bone marrow cell implantation. *Am J Physiol Heart Circ Physiol*; 2003; 285: H931-H937.
20. Li TS, Hamano K, Suzuki K, et al: Improved angiogenic potency by implantation of ex vivo hypoxia prestimulated bone marrow cells in rats. *Am J Physiol Heart Circ Physiol*; 2002; 283: H468-H473.
21. Takeshita S, Zheng LP, Brogi E, et al: 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: 662-70.
22. Pearlman JD, Hibberd MG, Chuang ML, et al: Magnetic resonance mapping demonstrates benefits of VEGF-induced myocardial angiogenesis. *Nat Med*; 1995; 1: 1085-9.
23. Aiello LP, Avery RL, Arrigg PG, et al: Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N Engl J Med*; 1994; 331: 1480-7.
24. Loomans CJ, de Koning EJ, Staal FJ, et al: Endothelial progenitor cell dysfunction: a novel concept in the pathogenesis of vascular complications of type 1 diabetes. *Diabetes*; 2004; 53: 195-9.
25. Tamarat R, Silvestre JS, Le Ricousse-Roussanne S, et al: Impairment in ischemia-induced neovascularization in diabetes: bone marrow mononuclear cell dysfunction and therapeutic potential of placenta growth factor treatment. *Am J Pathol*; 2004; 164: 457-466.
26. Tepper OM, Galiano RD, Capla JM, et al: Human endothelial progenitor cells from Type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. *Circulation*; 2002; 106: 2781-2786.
27. Jackson KA, Majka SM, Wang H, et al: Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. *J Clin Invest*; 2001; 107: 1395-402.
28. Schatteman GC, Hanlon HD, Jiao C, et al: Blood-derived angioblasts accelerate blood-flow restoration in diabetic mice. *J Clin Invest*; 2000; 106: 571-8.

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