Autologous bone marrow cell implantation as therapeutic angiogenesis for ischemic hindlimb in diabetic rat model

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Hirata, Ken, Tao-Sheng Li, Masahiko Nishida, Hiroshi Ito, Masunori Matsuzaki, Shunji Kasaoka, and Kimikazu Hamano. Autologous bone marrow cell implantation as therapeutic angiogenesis for ischemic hindlimb in diabetic rat model. Am J Physiol Heart Circ Physiol 284: H66–H70, 2003. First published September 19, 2002; 10.1152/ajpheart.00547.2002.—The angiogenic effect induced by autologous bone marrow cell implantation (BMCI) was examined in the ischemic hindlimbs of diabetic and nondiabetic rats. Diabetes mellitus was induced by the systemic administration of streptozotocin. We investigated the production of angiogenic factors and endothelial differentiation from bone marrow cells and the native recovery of blood flow in the ischemic hindlimbs. To observe the angiogenic effect induced by BMCI treatment, 6 × 107 bone marrow cells were injected intramuscularly at six points into the ischemic limbs, and regional perfusion recovery was evaluated with colored microspheres 2 wk later. No difference was found between diabetic and nondiabetic rats in the release of angiogenic factors or endothelial differentiation from bone marrow cells in vitro. The levels of nitric oxide in plasma were significantly lower, and native perfusion recovery in the ischemic hindlimbs was significantly slower in the diabetic rats than in the nondiabetic rats. However, although perfusion recovery was achieved in the ischemic hindlimbs, there was no significant increase in systemic VEGF after BMCI treatment in either the diabetic or nondiabetic rats. Therefore, therapeutic angiogenesis induced by BMCI could be a safe and effective treatment for ischemic limb disease in diabetic patients.

diabetes mellitus; angiogenic factor; endothelial differentiation; colored microsphere

THE CONCEPT OF “therapeutic angiogenesis” has become widely accepted (8, 10, 11). Severe ischemic disease is often complicated by diabetes mellitus, which accelerates vasculopathy (9). Moreover, it has been reported that loss of the modulatory role of the endothelium in diabetic patients leads to insufficiencies of the coronary and peripheral collateral vessels (1, 7, 21). Several treatments have been attempted to ameliorate tissue ischemia in diabetic animals, such as intramuscular adeno-VEGF gene transfer and the direct injection of exogenous basic FGF (bFGF) (21, 26). However, because it is well known that diabetes predisposes to microvascular proliferative disease, these treatments might also increase the plasma levels of VEGF or bFGF, thereby accelerating microvascular proliferation with unfavorable consequences. Thus it is necessary to find a safe, feasible method of inducing therapeutic angiogenesis in patients with diabetes mellitus.

Bone marrow consists of multiple cell populations, including endothelial progenitor cells, which can differentiate into endothelial cells and release several angiogenic factors in a milder way than the direct injection or gene transfer of some angiogenic growth factors. Therefore, BMCI could be a promising, safe method of inducing therapeutic angiogenesis in patients whose condition is complicated by diabetes mellitus.

Although therapeutic angiogenesis was successfully induced by the intramuscular injection of blood-derived CD34-positive cells into the ischemic hindlimbs of diabetic mice (22), the cells used for implantation were derived from healthy human beings. Therefore, it is unknown whether therapeutic angiogenesis could also be induced effectively by implanting CD34-positive cells, or other cells, derived from patients with diabetes mellitus. Furthermore, the advantages and safety of this cell-based therapy have not yet been fully elucidated. In the present study, we investigated the safety and effectiveness of BMCI for inducing therapeutic angiogenesis in the ischemic hindlimbs of diabetic rats.
MATERIALS AND METHODS

Animals and experimental diabetic rat model. This experiment was approved by the Committee of Ethics on Animal Experiments at Yamaguchi University School of Medicine and was conducted according to the Guidelines for Animal Experiments in Yamaguchi University School of Medicine. Inbred male dark agouti (DA) rats (8 wk of age and weighing 220–260 g) were used in all experiments. The rats were fed normal chow and allowed water ad libitum.

The diabetic rat model was produced by a single intravenous injection of 55 mg/kg streptozotocin (STZ; Sigma, St. Louis, MO) in DA rats (18) that had been anesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital sodium. The blood glucose level was measured with Advantage II and test strips (Roche Diagnostics, Tokyo, Japan) 2, 7, 14, and 28 days after STZ injection, and diabetes was confirmed when the blood glucose level was >220 mg/dl. Age-matched nondiabetic rats were used as controls.

Measurement of plasma nitric oxide level of diabetic rats. As an index of endothelial dysfunction, the plasma nitric oxide (NO) level was measured in the diabetic rats at 4, 8, and 12 wk after STZ injection (n = 5 in each group). Briefly, after the induction of general anesthesia, arterial blood was collected from the aorta. After centrifugation, plasma samples were stored at −80°C until analysis. Capillary zone electrophoresis (28) was used to determine the concentration with a model 805 data station (Waters-Millipore, Milford, MA). NO was measured as nitrite and nitrate, which are stable metabolites of NO, and the concentration of NO in the blood was assessed by the sum of nitrate plus nitrite. For control, the plasma NO level in age-matched normal rats was also measured. On the basis of plasma NO level, diabetic rats and nondiabetic rats were used for the following study 12 wk after STZ injection.

VEGF production and endothelial differentiation of bone marrow cells in vitro. Bone marrow cells were harvested from diabetic rats 12 wk after STZ injection and from age-matched nondiabetic rats as described previously (12, 13). Cells were suspended in RPMI 1640 medium that contained 10% FBS and 1% penicillin-streptomycin and cultured at 37°C with 5% CO2. To investigate the potency of VEGF production, cells were seeded into 24-well plates (5 × 10^6 cells·well−1) for culture. The supernatant of the culture medium was collected after 1, 3, and 7 days of culture. The VEGF concentration in the supernatant of the culture medium was measured with a mouse VEGF enzyme-linked immunosorbent assay kit (Quantikine M; R&D Systems) according to the manufacturer’s instructions. Data from six independent experiments were used for statistical analysis.

To investigate the endothelial differentiation from bone marrow cells in vitro, cells were cultured in a four-well Lab-Tek II chamber slide (Nalge Nunc) as described above. After 14 days of culture, the cells were fixed in 1% formaldehyde for 5 min and then blocked with 2% bovine serum albumin in PBS for 90 min. The cells were then incubated with rabbit polyclonal antibodies against CD31 (Pharmingen, Becton Dickinson) or Flk-1 or Tie-2 (Santa Cruz Biotechnology) for 90 min at room temperature. After being washed three times with PBS, cells were incubated with FITC-conjugated goat anti-rabbit IgG antibody. The percentage of positively stained cells was calculated by counting under microscopy at 200-fold magnification. A single observer blinded to the treatment regimen counted at least 1,000 cells in random fields. Data from six independent experiments were used for statistical analysis.

Ischemic hindlimb model and BMC1 treatment. The ischemic hindlimb model was created in diabetic rats (n = 17) 12 wk after STZ injection and age-matched nondiabetic rats (n = 17) as previously reported (17, 26). Briefly, animals were anesthetized and a skin incision was made in the left hindlimb. After ligation of the proximal end of the femoral artery at the level of the inguinal ligament, the distal portion and all the side branches were dissected free and excised. The right hindlimb was kept intact to control the original blood flow.

After the establishment of the ischemic hindlimb model, both diabetic and nondiabetic rats were randomized into three groups as follows. In the BMCI group (n = 7 for both diabetic and nondiabetic rats), the ischemic hindlimbs were given BMCI treatment as previously described (13). Briefly, bone marrow cells were collected from the diabetic rats 12 wk after STZ injection and age-matched nondiabetic rats as described in VEGF production and endothelial differentiation of bone marrow cells in vitro. The quadriceps and adductor muscles in the ischemic hindlimb were injected along to the excised artery at six points, each point injected with 1 × 107 bone marrow cells in 10 μl of PBS. The ischemic hindlimbs in the diabetic rats were injected with bone marrow cells derived from the diabetic rats, and the ischemic hindlimbs (n = 7) in nondiabetic rats were injected with bone marrow cells derived from the nondiabetic rats without crossover. For control, the ischemic hindlimbs of diabetic and nondiabetic rats were injected with PBS only (PBS group; n = 5 for both diabetic and nondiabetic rats) or were not injected at all (Control group; n = 5 for both diabetic and nondiabetic rats).

Blood flow recovery of ischemic hindlimbs and systemic VEGF levels. Blood perfusion in the ischemic hindlimbs was assessed with the use of colored microspheres 2 wk after treatment as described previously (16, 27). Briefly, animals were anesthetized and warmed on a heater at 37°C. A polyethylene catheter was introduced through the left carotid artery to the aortic arch. Dye-Trak microspheres (9 × 10^6, 15-μm diameter; Triton Technology, San Francisco, CA) were injected over 6 s. After 30 s, the animals were killed by cutting the inferior vena cava and the gastrocnemius muscle was harvested and weighed. The muscle sample was processed according to the protocols recommended by the manufacturer. The optical density (OD) of dye derived from these samples was measured with a spectrophotometer. The recovery of perfusion in the ischemic hindlimb was estimated by the percentage of blood flow in the affected limb compared with that in the normal right hindlimb, which was calculated as (OD of ischemic limb/OD of normal limb) × (tissue wt of normal limb/tissue wt of ischemic limb) × 100 (27).

To detect the systemic influence of BMCI, peripheral blood was also collected from these rats 2 wk after treatment and the plasma VEGF levels were measured with a mouse VEGF enzyme-linked immunosorbent assay kit (Quantikine M) according to the manufacturer’s instructions.

Statistical analysis. Statistical analysis was performed by unpaired t-test. Data are presented as means ± SD, and significance was defined as P < 0.05.

RESULTS

Downregulation of NO release in diabetic rats. The blood glucose level increased immediately after the STZ injection, and hyperglycemia (blood glucose >220 mg/dl) continued throughout the period of observation in the diabetic rats. Compared with the age-matched nondiabetic rats, the plasma NO levels in diabetic rats were not changed significantly at 4 and 8 wk after the STZ injection. By 12 wk after the STZ injection, how-
However, the plasma NO level in the diabetic rats was significantly lower than that in the nondiabetic rats \([8.26 \pm 1.91 \text{ vs. } 12.10 \pm 1.37 \text{ pg/\mu L}}; P < 0.01]; \text{Fig. 1}\). This indicated that endothelial dysfunction in the diabetic rats was not raised until \(\sim 12\) wk after STZ injection, and the following studies were performed in these diabetic rats \(12\) wk after STZ injection.

**VEGF production and endothelial differentiation from bone marrow cells in vitro.** The VEGF production from the bone marrow cells of the diabetic and nondiabetic rats is shown in Fig. 2. The VEGF concentration in the medium increased with the time of culture in bone marrow cells from both the diabetic and nondiabetic rats. Although VEGF release from the bone marrow cells was greater in high-glucose culture than in normal-glucose culture in the diabetic rats and the reverse in the bone marrow cells from the nondiabetic rats, the potency of VEGF production from the bone marrow cells of the diabetic rats did not differ significantly from that of the nondiabetic rats.

Furthermore, the positive expression of CD31, Tie-2, and Flk-1 in the bone marrow cells of the diabetic rats also did not differ significantly from that in the bone marrow cells of the nondiabetic rats after 14 days of culture (Fig. 3). This indicated the possibility that bone marrow cells from diabetic rats have potency in endothelial differentiation similar to those from nondiabetic rats.

**Blood flow recovery of ischemic hindlimbs and systemic VEGF levels.** As shown in Fig. 4, the percentage of blood flow in the ischemic hindlimbs of rats in the Control group was significantly lower in the diabetic rats than in the nondiabetic rats \(2\) wk after the operation \((52.0 \pm 13.5\% \text{ vs. } 78.6 \pm 20.9\%; P < 0.05)\). This indicated that the native restoration of perfusion in the ischemic hindlimbs was slower in the diabetic rats than in the nondiabetic rats. However, BMCI treatment (BMCI group) accelerated the recovery of blood flow in the ischemic hindlimbs in both diabetic and nondiabetic rats compared with injection of PBS only (PBS group) and no injection (Control group) \((91.4 \pm 1.7\% \text{ vs. } 63.4 \pm 4.5\% \text{ and } 45.0 \pm 5.1\% \text{ in diabetic rats and } 94.9 \pm 1.8\% \text{ vs. } 75.3 \pm 1.8\% \text{ and } 51.8 \pm 5.1\% \text{ in nondiabetic rats, respectively}; P < 0.05)\).

Furthermore, a similar recovery of blood flow in the ischemic hindlimbs of the diabetic and nondiabetic rats was achieved \(2\) wk after BMCI treatment, although the native recovery of blood flow in the ischemic hindlimbs was significantly slower in the diabetic rats than the nondiabetic rats. The percentage of blood flow of the ischemic hindlimbs in both diabetic and nondiabetic rats was not significantly different between the PBS group and the Control group, also indicating that the recovery of blood flow in the ischemic hindlimbs could not be accelerated by PBS injection alone. The plasma concentration of VEGF was not significantly different among the BMCI, PBS, and Control groups \(2\) wk after treatment (Fig. 5), and no significant difference was detected between diabetic and nondiabetic rats.

**DISCUSSION**

Diabetic vasculopathy is a systemic disease characterized by severe impairment of the development of collateral vessels \((4, 9)\). Several mechanisms have been suggested to explain this disorder including endothelial dysfunction \((1)\), downregulation of VEGF by hyperglycemia \((21)\), and impairment of monocyte migration \((29)\). According to other investigators, diabetic vascular disease is based on endothelial dysfunction pathophysiologically induced by hyperglycemia \((7)\). Furthermore, diabetes could affect NO production through generalized actions on the endothelium or through specific effects on the NO pathway \((5, 6, 15)\).

In the present study, plasma NO was remarkably depressed \(12\) wk after STZ injection in diabetic rats. We considered that this decrease was affected by the endothelial dysfunction induced by hyperglycemia.

Our previous investigations \((12, 13, 16, 17)\) showed that therapeutic angiogenesis induced by BMCI treatment was related to the production of angiogenic cytokines, including some inflammatory cytokines, and to endothelial differentiation from the bone marrow cells.
It is important to clarify the potency of angiogenic cytokine production and endothelial differentiation from bone marrow cells derived from diabetic rats. Although it has been reported that VEGF is downregulated by hyperglycemia (19, 21), our investigation in vitro showed that the potency of VEGF secretion and endothelial differentiation from the bone marrow cells of diabetic rats did not change significantly compared with those of nondiabetic rats. This finding provided basic evidence that angiogenesis could be effectively induced by the implantation of autologous bone marrow cells in patients with diabetes mellitus.

The native blood flow recovery in the ischemic hindlimbs was impaired in the diabetic rats. According to the results of this study and of others (21, 22), endothelial dysfunction might contribute to slow the recovery of perfusion of the ischemic hindlimbs in diabetic rats.

However, BMCI accelerated the recovery of blood flow in the ischemic hindlimbs of diabetic rats, similarly to when BMCI treatment was given to nondiabetic rats. The blood flow in the ischemic hindlimbs of both diabetic and nondiabetic rats recovered to >90%, which was significantly higher than that seen after the injection of PBS alone, at ~60%. Although the native recovery of blood flow in the ischemic hindlimbs of diabetic rats was significantly lower than that in the nondiabetic rats, the blood flow 2 wk after BMCI treatment did not differ significantly between the two groups. The mechanism of angiogenesis induced by BMCI in diabetic rats is speculated to be the same as that in normal rats, and previous studies showed that it is related to the release of angiogenic growth factors, including some inflammatory cytokines, and to endothelial differentiation from the implanted bone marrow cells (13, 16). Interestingly, the angiogenesis induced by BMCI treatment in diabetic rats seems to be more sensitive than that in nondiabetic rats, which might relate to the overexpression of VEGF receptors in the diabetic animal (19).

The potential problems of inducing therapeutic angiogenesis by the direct administration or gene transfer of angiogenic growth factors include a theoretic risk of neoplasms and plaque angiogenesis that must not be ignored (3, 20, 24). A paradoxical situation makes therapeutic angiogenesis more complicated in diabetes (8), in that both microvascular insufficiencies and microvascular proliferative diseases, sometimes simultaneously, create events such as deteriorating proliferative retinopathy, nephropathy, or malignancy. Thus it is critically important to confirm the safety of inducing
therapeutic angiogenesis in patients with diabetes mellitus. We measured the plasma level of VEGF 2 wk after BMCI treatment and found that it was not increased significantly compared with that 2 wk after PBS injection, indicating that therapeutic angiogenesis induced by BMCI would not affect systemic microvascular proliferation. Although the safety of BMCI must be investigated further, it could be the ideal effective method of inducing therapeutic angiogenesis to treat local ischemia in diabetic patients.

In conclusion, bone marrow cells derived from diabetic rats showed good potency in the production of angiogenic factors and endothelial differentiation. Therefore, the local implantation of these bone marrow cells into the ischemic hindlimbs of diabetic rats could induce therapeutic angiogenesis effectively and safely. Our results provide experimental evidence that BMCI treatment could be an effective, feasible method of inducing therapeutic angiogenesis in diabetic patients with ischemic disorders.

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