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Impaired potency of bone marrow mononuclear cells for inducing therapeutic angiogenesis in obese diabetic rats

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Submitted 19 July 2005; accepted in final form 10 October 2005

Li, Tao-Sheng, Akira Furutani, Masaya Takahashi, Mako Ohshima, Shu-Lan Qin, Toshiro Kobayashi, Hiroshi Ito, and Kimikazu Hamano. Impaired potency of bone marrow mononuclear cells for inducing therapeutic angiogenesis in obese diabetic rats. Am J Physiol Heart Circ Physiol 290: H1362–H1369, 2006. First published October 14, 2005; doi:10.1152/ajpheart.00766.2005.—Using Zucker fatty rats, a strain characterized by diabetes and hyperlipidemia, we investigated the diabetes- and hyperlipidemia-related impairment of bone marrow mononuclear cells (BMCs) for inducing therapeutic angiogenesis. BMCs from Zucker fatty and normal Zucker lean rats were collected and cultured. Although the characterization and cell survival of BMCs did not differ, the VEGF production, endothelial differentiation, and endothelial cell colony-forming potential of BMCs from Zucker fatty rats were significantly lower than those of BMCs from lean rats. By using an ischemic hindlimb model, we found that the native recovery of induced limb ischemia in the Zucker fatty rats was also significantly worse than that in the lean rats. Furthermore, the expression of 5-hydroxytryptamine (5-HT2A) receptors was obviously higher in the Zucker fatty rats than in the lean rats and was enhanced after limb ischemia. Although the therapeutic potency was lower than with the implantation of BMCs from normal lean rats, the implantation of BMCs from fatty rats could also induce angiogenesis and increase blood flow significantly in the ischemic hindlimbs of Zucker fatty rats. Furthermore, the blood flow in the ischemic hindlimbs was increased by the administration of sarpogrelate, a selective 5-HT2A-receptor antagonist. Our results clearly show diabetes- and hyperlipidemia-related dysfunction and impaired potency for inducing angiogenesis of BMCs. However, the implantation of autologous BMCs into ischemic limbs of diabetic and hyperlipidemic rats has induced therapeutic angiogenesis effectively, and blood flow would be enhanced by the administration of a 5-HT2A-receptor antagonist.

diabetes; ischemia; blood flow; hyperlipidemia; 5-HT2A-receptor

CELL-BASED THERAPEUTIC ANGIOGENESIS has been demonstrated as an alternative method of treating several ischemic diseases. Most experimental investigations on cell-based therapeutic angiogenesis have achieved satisfactory results by using ischemic models in healthy animals (6, 11, 12). Clinical trials have also shown the safety and feasibility of implanting autologous peripheral blood or bone marrow-derived cells for the treatment of ischemic heart disease and peripheral arterial disease (1, 4, 5, 7, 17, 19, 20, 22, 24). Although these clinical trials clearly demonstrated improved clinical objective symptoms and regional blood flow in many patients after treatment, the effectiveness of cell-based therapeutic angiogenesis has yet to be confirmed in double-blind randomized clinical trials.

The condition of many patients with ischemic diseases is complicated by diabetes and hyperlipidemia, which lead to stem cell dysfunction and poor potency for inducing angiogenesis by cell-based therapies (8, 9, 18, 21, 23, 25, 27). Furthermore, plasma 5-hydroxytryptamine (5-HT) concentrations are elevated in patients with diabetes or peripheral arterial disease (3, 15), and the recruitment of collateral vessels could be compromised by excessive vasoconstrictor reactivity to 5-HT (14, 26). All of these factors suggest that the potency of therapeutic angiogenesis induced by the implantation of autologous peripheral blood or bone marrow-derived cells might be impaired in patients with diabetes and hyperlipidemia.

Therefore, the questions to be answered are what is the extent of diabetes- and hyperlipidemia-related impairment of bone marrow stem cells for inducing therapeutic angiogenesis and can implantation of autologous BMCs still induce angiogenesis effectively under conditions of diabetes and hyperlipidemia. Furthermore, it is also worthwhile to investigate whether the impaired therapeutic potency of BMCs for inducing angiogenesis can be remedied by combination therapy with certain drugs, such as the 5-HT2A-receptor antagonist.

In this study, we investigated diabetes- and hyperlipidemia-related impairment of bone marrow cells for inducing therapeutic angiogenesis in vitro and in vivo by using the Zucker fatty rat, a strain characterized by obesity, hyperglycemia, hyperinsulinemia, and hyperlipidemia. We also tried to remedy diabetes- and hyperlipidemia-related impairment by combination therapy with sarpogrelate, a 5-HT2A receptor antagonist.

METHODS

Animals. Male 18-wk-old Zucker fatty (fa/fa) and Zucker lean (fa+/+) rats were used for these experiments (Charles River), which were approved by the Institutional Animal Care and Use Committee.
of Yamaguchi University and conform to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, Revised 1996). We measured blood glucose and triglycerides in Zucker fatty and lean rats before they were used in this study. The concentrations of blood glucose and triglycerides were 262 (SD 38) and 286 mg/dl (SD 53) in Zucker fatty rats (n = 56) and 115 (SD 21) and 66 mg/dl (SD 15) in Zucker lean rats (n = 15), respectively.

Separation and cultivation of bone marrow mononuclear cells. Bone marrow cells were collected from the femurs and tibias of Zucker fatty and lean rats. BMCs were isolated by density gradient centrifugation (13). Cells were collected and suspended at a density of 3 × 10^6 cells/ml in RPMI 1640 medium supplemented with 15% fetal bovine serum (GIBCO), 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO). Cells were cultured at 37°C in a humidified environment with 5% CO2.

Flow cytometry and RT-PCR analysis. To characterize these BMCs and determine the difference between Zucker fatty and lean rats, freshly collected BMCs were incubated for 30 min at 4°C with FITC-conjugated antibodies against CD34 (Pharmingen) and CD117 (Becton Dickinson). Isotype-identical antibodies served as controls. Quantitative flow cytometric analysis was done by using a FACSscan flow cytometer and Cell Quest software (Becton Dickinson).

The mRNA expression of VEGF in freshly collected BMCs was also detected by RT-PCR analysis as described previously (13).

Cell growth, endothelial differentiation, and VEGF production in vitro. BMCs were cultured on 24-well plates coated with 0.2 mg/ml fibronectin (Sigma) as described in Separation and cultivation of bone marrow mononuclear cells. After 1, 3, and 7 days of culture, the supernatant was collected and all cells were harvested. The total number of surviving cells was counted after staining with 0.4% Trypan blue solution (Sigma), and the cell survival rate was calculated by the percentage of total seeded cells on day 0. The concentration of VEGF in the supernatant was measured with a VEGF ELISA kit (R&D Systems) (12).

To observe the endothelial differentiation, BMCs were cultured on four-well chamber culture slides (Nalge Nunc International) coated with 0.2 mg/ml fibronectin and supplemented with 50 ng/ml vascular endothelial growth factor, 5 ng/ml fibroblast growth factor, and 5 ng/ml insulin-like growth factor I. After 7 days of culture, the cells were fixed in 1% formaldehyde, blocked with 2% BSA, and then incubated with FITC-conjugated antibody against vascular endothelial (VE)-cadherin (Pharmingen) (12).

 Colony-forming assay. Bone marrow mononuclear cells (3 × 10^6/well) were cultured in a BioCoat Cellware 24-well plate (Becton Dickinson Labware, Bedford, MA). Colony-forming units (CFU) were counted under phase-contrast microscopy after 10 days of incubation. The cells were fixed with 2% paraformaldehyde and then stained with FITC-labeled antibodies against Bandeiraea simplicifolia I (BS-1) lectin (Sigma) and VE-cadherin. Adherent colonies that stained positively with BS-1 lectin and VE-cadherin were defined as endothelial cell colonies (CFU-EC), which were also characterized by a few round cells in the center of the colonies. Inversely, these endothelial cell colonies (CFU-EC) and CFU-GM per well were counted, respectively, by two independent investigators.

Ischemic hindlimb model. The rat ischemic hindlimb model was created as described previously (6, 13). Briefly, after the rats were given general anesthesia, the left femoral artery was exposed and ligated, and its branches were dissected free and excised. Six Zucker fatty rats and six lean rats were examined to assess the potency of natural recovery of acute induced tissue ischemia. Another 38 Zucker fatty rats were used for cell implantation and control treatment as described below.

Cell transplantation and chronic block of 5-HT2A receptor. After the ischemic hindlimb model was established, the 38 Zucker fatty rats were divided randomly into five groups. One group was injected with PBS only (PBS group, n = 7); one group was injected with a total of 3 × 10^7 freshly isolated BMCs from Zucker fatty rats, as well as the daily oral vehicle (fatty BMC group, n = 8); one group was given daily oral sarpogrelate (10 mg/kg; Sarp group, n = 7); one group was given both the injection of BMCs and daily oral sarpogrelate (fatty BMC+Sarp group, n = 8); and one group was injected with a total of 3 × 10^7 freshly isolated BMCs from normal Zucker lean rats, as well as the daily oral vehicle (lean BMC group, n = 8). The PBS and BMCs were injected intramuscularly at six points (10 μl PBS or 5 × 10^6 cells/point) in the quadriceps and adductor muscles of the ischemic hindlimb by using a 100-μl microsyringe and 27-gauge needles.

Monitoring of circulating CD34-positive cells. To measure the circulating CD34-positive cells in Zucker fatty and lean rats, ~0.2 ml of peripheral blood were collected before and 3, 7, and 14 days after limb ischemia. freshly collected peripheral blood nuclear cells were stained with FITC-conjugated antibody against CD34, and quantitative flow cytometric analysis was done as described in Flow cytometry and RT-PCR analysis.

Measurement of blood flow in ischemic hindlimbs. Blood flow in the ischemic hindlimb was measured by using a laser-Doppler perfusion imaging system (PeriScan PIM II, Liska, Sweden) before and 3, 7, 14, and 28 days after treatment, as described previously (12). Briefly, both normal and ischemic feet were scanned with the animal under light anesthesia, and mean perfusion scores were obtained for each foot (below the knee joints). To minimize data variables, the recovery of perfusion in the ischemic hindlimb of each rat was estimated by the percentage of limb blood flow (%LBF), which was calculated as a percentage by the mean perfusion score in the left hindlimb compared with that in the normal right hindlimb (12).

Histological analysis of microvessel density. All rats were killed 28 days after treatment, and the quadriceps and adductor muscles were harvested. Each collected sample was halved for histological and Western blot analysis, respectively. To detect the development of microvessels in ischemic muscles, 5-μm-thick frozen sections were stained for alkaline phosphatase with an indoxyl tetrazolium method as described previously (12, 13). The number of microvessels and muscle fibers was counted under a microscope at 200-fold magnification by a single observer blind to the treatment regimen, and a total of 20 different fields on four independent slides from different cross sections were randomly selected for each rat. The density of microvessels was estimated by the microvessel-to-muscle fiber ratio (12, 13).

Western blot analysis of 5-HT2A receptors and endothelial nitric oxide synthase. To estimate the levels of 5-HT2A receptors and endothelial nitric oxide (NO) synthase (eNOS) in the ischemic hindlimbs, total tissue proteins were extracted from the collected muscles. Protein samples (100 μg) were separated by 10% SDS-PAGE and then transferred to a polyvinylidene difluoride membrane. These membranes were blocked for 45 min with blocking buffer (5% nonfat dry milk in Tris-buffered saline, pH 7.5) and then incubated for 1 h with the goat polyclonal antibody against 5-HT2A receptors (Santa Cruz) or the rabbit monoclonal antibody against eNOS (IBL, Gunma, Japan). Signals were detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL, Amersham). Quantification of bands was done by using NIH Image 1.60 software, and the loading differences were normalized by using the β-actin antibody.

Statistical analysis. All data are expressed as means (SD). Statistical significance was evaluated by ANOVA followed by Scheffe’s procedure by using the StatView software (version 5.0). Values of P < 0.05 were considered significant.
RESULTS

Dysfunction of BMCs in Zucker fatty rats. More than 98% of the freshly isolated BMCs survived. CD34 and CD117 were expressed in 2.5% (SD 0.6) and 4.3% (SD 1.1) of the freshly isolated BMCs from the Zucker fatty rats, which was not significantly different from the lean rats [2.3% (SD 0.8) and 4.0% (SD 1.3)]. The survival rate of the BMCs after cultivation also did not differ significantly between the Zucker fatty and lean rats (Fig. 1A). To detect the endothelial differentiation in vitro, BMCs were cultured with the supplement of several growth factors. After 7 days of cultivation, ~40% of the BMCs were stained positively for VE-cadherin in the Zucker lean rats, but only ~15% were stained positively in the Zucker fatty rats (Fig. 1B).

However, the concentration of VEGF in the supernatant 3 and 7 days after BMC cultivation was significantly lower in the Zucker fatty rats compared with the lean rats (Fig. 1C). Furthermore, the VEGF mRNA expression in freshly collected BMCs was also decreased in the Zucker fatty rats compared with the lean rats (Fig. 1D).

The colony-forming assay revealed significantly less CFU-EC formation in the BMCs of Zucker fatty rats than in those of the lean rats (P < 0.01, Fig. 2). However, the number of CFU-GM did not differ significantly between the Zucker fatty and lean rats. All these results reflect the dysfunction of BMCs in inducing angiogenesis in the Zucker fatty rats.

Impaired potency of native recovery to acute induced ischemia in Zucker fatty rats. By monitoring the natural recovery from acute induced ischemia, we found that the ischemic hindlimbs of all the healthy Zucker lean rats recovered well.
However, recovery of the ischemic hindlimbs was poor in the Zucker fatty rats, two of which had necrotic change in the toe 28 days after the creation of the ischemic hindlimb model (Fig. 3A). The blood flow in the ischemic hindlimb recovered to ~60% of that of the nonischemic hindlimb in the lean rats but recovered to <40% in the fatty rats, 28 days after acute induced ischemia (Fig. 3B). Histological analysis confirmed that the microvessel density was significantly lower in the fatty rats than in the lean rats ($P < 0.05$, Fig. 3C).

When compared with the baseline, the CD34-positive cells were increased after limb ischemia in both the Zucker fatty and lean rats. Although there were fewer circulating CD34-positive cells in the Zucker fatty rats than in the lean rats at both the baseline level and after limb ischemia, there was no significant difference between the two groups (Fig. 3D). However, compared with lean rats, the expression of the 5-HT$_{2A}$ receptor in normal nonischemic hindlimbs was obviously increased in Zucker fatty rats. Furthermore, the expression of the 5-HT$_{2A}$ receptor was enhanced after limb ischemia in both the fatty and lean rats (Fig. 3E).

*Inducing therapeutic angiogenesis by BMCs implantation in ischemic hindlimb of Zucker fatty rats.* After 28 days of treatment, the muscle fiber of ischemic limb showed normal histological findings, and the scoring of the physical appearance of ischemic limb did not differ among these groups. However, the microvessel density, which is an index of neo-vascularization, was significantly higher in the fatty BMC, fatty BMC+Sarp, and lean BMC groups than in the Sarp and PBS groups ($P < 0.01$, Fig. 4). Furthermore, the microvessel density in the ischemic muscle was also significantly higher in the lean BMC groups than in the fatty BMC group ($P < 0.05$). However, there was no significant difference in microvessel density between the Sarp and PBS groups or between the fatty BMC and fatty BMC+Sarp groups.

*Increased blood flow of ischemic hindlimbs by BMCs implantation and sarpogrelate administration.* Blood flow of the ischemic hindlimb was measured by a noninvasive method with the use of a laser-Doppler perfusion imaging system. Although the blood flow of ischemic limbs measured by the laser-Doppler perfusion imaging was, in fact, skin blood flow...
(~1 mm depth of penetration by laser), it agreed well with the blood flow measured by the method of microsphere (12). We found that the %LBF of the ischemic hindlimb was remarkably better in the fatty BMC group than in the PBS group (Fig. 5). However, the %LBF of ischemic limb in lean BMC group by implanting BMCs from normal Zucker lean rats was significantly better (~5–10% up) than that in fatty BMC group by implanting BMCs from Zucker fatty rats. Furthermore, blockade of the 5-HT2A receptor by the oral administration of sarpogrelate also increased the %LBF of the ischemic hindlimb significantly, from 14 days after treatment onward. The %LBF was significantly higher in the fatty BMC+Sarp group than in the fatty BMC group 28 days after treatment and was the same as in the lean BMC group by implanting BMCs from normal rats. These indicated that partial impairment of the angiogenic potency of BMCs was related to diabetes and hyperlipidemia in Zucker fatty rats, but the impaired angiogenic potency could be covered by the combination therapy with a 5-HT2A-receptor blocker.

Increased eNOS expression of ischemic hindlimbs by BMCs implantation and sarpogrelate administration. Western blot analysis showed that the expression of eNOS in the ischemic hindlimbs was significantly higher in the Sarp, fatty BMC, and fatty BMC+Sarp groups than in the Ischemia (untreated) and PBS groups 28 days after treatment (Fig. 6A). Furthermore, the expression of eNOS in the ischemic hindlimbs was significantly lower in the Sarp, fatty BMC, and fatty BMC+Sarp groups than in the Ischemia and PBS groups 28 days after treatment (Fig. 6A). Conversely, the expression of 5-HT2A receptor in the ischemic hindlimb was significantly lower in the Sarp, fatty BMC, and fatty BMC+Sarp groups than in the Ischemia and PBS groups.

Fig. 4. Microvessel density in ischemic muscle of Zucker fatty rats 28 days after treatment. A: representative image. B: quantitative analysis showed that microvessel density in ischemic muscle was significantly higher in the fatty BMC, fatty BMC+Sarpogrelate (fatty BMC+Sarp), and lean BMC groups than in PBS and Sarp groups, but it was not significantly different between the PBS and Sarp groups or between the fatty BMC and fatty BMC+Sarp groups. Microvessel density in ischemic muscle was also significantly higher in lean BMC groups than fatty BMC groups.

Fig. 5. Laser-Doppler analysis of perfusion in ischemic hindlimbs of Zucker fatty rats. A: representative color-coded image representing blood flow distribution (maximal perfusion is shown as red). B: quantitative analysis showed that blood flow in ischemic hindlimbs was significantly better than that of PBS group after implantation of autologous BMCs and administration of sarpogrelate, although blood flow in ischemic hindlimb was ~5–10% lower in fatty BMC groups than in lean BMC groups.
PBS groups (Fig. 6B). There was no significant difference between the Ischemia and PBS groups.

**DISCUSSION**

Experimental and clinical evidence suggests that diabetes and hyperlipidemia are related to endothelial cell dysfunction, peripheral blood and bone marrow stem cell dysfunction, and excessive vasoconstriction (8, 9, 18, 21, 23, 25, 27). All of these factors may reduce the vascular regenerative potential and thereby contribute to the pathogenesis of vascular complications. These factors are also thought to result in a reduced capacity to augment therapeutic angiogenesis by the implantation of autologous bone marrow or peripheral blood-derived stem cells. By using Zucker fatty rats, a strain characterized by Type 2 diabetes and hyperlipidemia, we investigated whether the implantation of autologous BMCs would effectively induce therapeutic angiogenesis.

First, we investigated the dysfunction of BMCs related to diabetes and hyperlipidemia by using various in vitro assessments. We have found that the expression of VEGF mRNA in BMCs was decreased in Zucker fatty rats. Although the characterization of freshly isolated BMCs did not differ between the Zucker fatty and lean rats, significantly decreased VEGF production (~70% that of the lean rats) and endothelial differentiation (~40% that of the lean rats) were observed in the BMCs of the Zucker fatty rats 7 days after cultivation. Furthermore, there was ~50% less endothelial cell colony formation from the BMCs in the Zucker fatty rats than in the healthy lean rats. However, the cell survival and the formation of granulocyte-macrophage colonies of BMCs did not differ significantly between the Zucker fatty and lean rats, suggesting that hematogenesis was normal in the Zucker fatty rats. Because the production of angiogenic cytokines and endothelial differentiation both play important roles in inducing angiogenesis after the implantation of BMCs into ischemic tissues, our data indicate that the functions of bone marrow mononuclear cells for inducing therapeutic angiogenesis are impaired by Type 2 diabetes and hyperlipidemia.

Next, we investigated the natural recovery of acute induced ischemia by using an ischemic hindlimb model. We found that the blood flow and microvessel density in ischemic limbs were significantly lower in the Zucker fatty rats than in the lean rats. The impaired capacity of ischemic-induced neovascularization in the Zucker fatty rats could be explained by the dysfunction of endothelial cells, the decrease of circulating endothelial progenitors, and other factors.

Our data clearly showed that diabetes and hyperlipidemia were associated with bone marrow cell dysfunction and impaired capacity of ischemia-induced neovascularization. Thus we suggest that the implantation of autologous BMCs in patients with diabetes and hyperlipidemia will result in a reduced capacity to augment therapeutic neovascularization or have no therapeutic merit at all. However, we found that the implantation of autologous BMCs in Zucker fatty rats increased the microvessel density significantly and contributed to >20% improvement in blood flow of the ischemic hindlimb, from 38.9% in the PBS group to 62.5% in the fatty BMC group, 28 days after treatment. This indicates that the implantation of autologous BMCs into ischemic limbs has induced therapeutic angiogenesis effectively in diabetic and hyperlipidemic rats. However, the lower microvessel density and blood flow in ischemic limbs achieved by implanting BMCs from Zucker fatty rats compared with BMCs from healthy Zucker lean rats suggest that angiogenic capacity is impaired to some degree in the presence of diabetes and hyperlipidemia (9, 18, 21, 23, 27).

It is well known that the increased 5-HT concentrations in the plasma of patients with peripheral artery disease and diabetes induce excessive vasoconstriction of the collateral vessels (3, 14, 15, 26) and that serotonin receptor blockade improves distal perfusion after limb ischemia (10). In fact, our investigation showed that the expression of the 5-HT \textsubscript{2A} receptor in hindlimbs was obviously increased in Zucker fatty rats and enhanced further after limb ischemia. Therefore, we also investigated whether sarpogrelate, a 5-HT \textsubscript{2A} receptor antagonist, could improve endothelial function and increase regional
perfusion. We found that sarpogrelate increased the blood flow of ischemic hindlimbs significantly but that it did not increase the microvessel density. Our data suggest that the administration of sarpogrelate could be beneficial for improving regional perfusion. Although the mechanism is still unclear, the improvement of regional perfusion achieved by the administration of sarpogrelate is probably related to the blockade of 5-HT-induced excessive vasoconstriction and the improvement in endothelial function. The implantation of BMCs and the administration of sarpogrelate both contributed to increased expression of eNOS, but decreased expression of 5-HT<sub>2A</sub> receptor, in the ischemic hindlimbs. Because we only measured the eNOS and 5-HT<sub>2A</sub> receptor expression 28 days after treatment, we could not determine whether the administration of sarpogrelate had a direct effect on the improvement of endothelial function or whether the increased expression of eNOS and the decreased expression of 5-HT<sub>2A</sub> receptor in the ischemic hindlimbs were simply the result of improved tissue ischemia.

In summary, our results show the dysfunction of BMCs and the impaired potency of ischemia-induced angiogenesis in the Zucker fatty rats. Although the therapeutic capacity seems to be reduced, the implantation of autologous BMCs into ischemic hindlimbs has also induced angiogenesis and increased blood flow effectively in diabetic and hyperlipidemic rats. Several methods, including the amendment of BMC dysfunction by ex vivo pretreatment (2, 13, 16), the combined treatment of targeting endothelial dysfunction and excessive vasoconstriction of collateral vessels, just as presented in this study, may increase the potency of therapeutic neovascularization by the implantation of autologous BMCs. Further clinical trials are required to confirm our experimental findings in patients with diabetes and hyperlipidemia.

GRANTS
This work was supported by Grant-in-Aid for Scientific Research 16390397 and 16591259 from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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