Improved angiogenic potency by implantation of ex vivo hypoxia prestimulated bone marrow cells in rats

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Li, Tao-Sheng, Kimikazu Hamano, Kazuhiro Suzuki, Hiroshi Ito, Nobuya Zempo, and Masunori Matsuzaki. Improved angiogenic potency by implantation of ex vivo hypoxia prestimulated bone marrow cells in rats. Am J Physiol Heart Circ Physiol 283: H468–H473, 2002; 10.1152/ajpheart.00261.2002.—Therapeutic angiogenesis can be induced by local implantation of bone marrow cells. We tried to enhance the angiogenic potential of this treatment by ex vivo hypoxia stimulation of bone marrow cells before implantation. Bone marrow cells were collected and cultured at 33°C under 2% O2-5% CO2-90% N2 (hypoxia) or 95% air-5% CO2 (normoxia). Cells were also injected into the ischemic hindlimb of rats after 24 h of culture. Hypoxia culture increased the mRNA expression of vascular endothelial growth factor (VEGF), vascular endothelial (VE)-cadherin, and fetal liver kinase-1 (Flk-1) from 2.5- to fivefold in bone marrow cells. The levels of VEGF protein in the ischemic hindlimb were significantly higher 1 and 3 days after implantation with hypoxia-cultured cells than with normoxia-cultured or noncultured cells. The microvessel density and blood flow rate in the ischemic hindlimbs were also significantly (P < 0.001) higher 2 wk after implantation with hypoxia-cultured cells (89.7 ± 5.5%) than with normoxia-cultured or noncultured cells (70.4 ± 7.7%). Ex vivo hypoxia stimulation increased the VEGF mRNA expression and endothelial differentiation of bone marrow cells, which together contributed to improved therapeutic angiogenesis in the ischemic hindlimb after implantation.

endothelial differentiation; vascular endothelial growth factor; vascular endothelial-cadherin; ischemic hindlimb.

induction of therapeutic angiogenesis by various methods has recently been developed to treat ischemic diseases (4, 12, 19, 22, 24). Both the increase of multiple angiogenic cytokines and the proliferation or migration of endothelial cells are critically important for improving angiogenesis (5, 6, 21). Bone marrow cell implantation is considered an ideal method for inducing therapeutic angiogenesis, because bone marrow cells secrete multiple angiogenic cytokines and differentiate into endothelial cells (2, 9).

We have shown previously that therapeutic angiogenesis can be induced by local implantation of bone marrow cells, and this finding was related to both the endothelial differentiation and the secretion of multiple angiogenic cytokines from the implanted bone marrow cells (10, 14, 17). We have also begun a clinical trial to induce therapeutic angiogenesis by local implantation of autologous bone marrow cells in patients with ischemic heart and limb diseases (11). Our preliminary clinical outcome indicates that the effectiveness of therapeutic angiogenesis induced by autologous bone marrow cell implantation was insufficient and required further study.

Theoretically, the angiogenic potency induced by bone marrow cell implantation could be enhanced by increasing the production of angiogenic cytokines or by improving the endothelial differentiation from bone marrow cells. mRNA expression of vascular endothelial growth factor (VEGF) can be increased in various types of cells when cultured under hypoxia (13, 20, 26). We have found that ex vivo hypoxic stimulation increases the production of VEGF from bone marrow cells (9). The effect of therapeutic angiogenesis induced by bone marrow cell implantation might be enhanced by ex vivo hypoxic stimulation of bone marrow cells before implantation. In the present study, we investigated whether ex vivo hypoxic stimulation would influence the level of mRNA expression of VEGF and the endothelial differentiation of bone marrow cells. We also observed whether the angiogenic potency could be enhanced by

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the implantation of hypoxia-cultured bone marrow cells into the ischemic hindlimb of rats.

MATERIALS AND METHODS

Animals. Male Dark Agouti rats, 6–8 mo old, were used for all experiments, which were approved by the institutional Animal Care and Use Committee of Yamaguchi University School of Medicine. Animals were housed under clean conditions and allowed free access to food and water in a temperature-controlled environment with a 12:12-h light-dark cycle.

Ex vivo hypoxic stimulation of bone marrow cells. Rats were killed under deep general anesthesia induced by intraperitoneal pentobarbital injection. Bone marrow was collected from the femur and tibia and placed in PBS. Simple bone marrow cell suspensions were prepared by gently pressing bone marrow segments through fine wire mesh. Bone marrow cells were separated from red blood cells by 1,800 rpm centrifugation for 20 min in Histopaque 1077 (Sigma, St. Louis, MO). Collected cells were washed two times, and the survival rate of cells was higher than 98%, as evaluated by staining the cells with 0.4% trypan blue solution (Sigma). Purified bone marrow cells were then resuspended at a density of 1 × 10^7/ml in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (all from GIBCO). Cells were cultured at 33°C (7), under 2% O2-5% CO2-90% N2 (hypoxia) or under 95% air-5% CO2 condition, in a gas-tight humidified incubator (ASTEC, Fukuoka, Japan).

RT-PCR Analysis. Cells were collected after 0 (baseline), 4, 8, 12, 24, and 48 h of culture, and five separate cultures were performed for each time point. Total RNA was extracted from cells with the use of an EZNA total RNA kit (Omega Bio-Tek; Doraville, GA) according to the manufacturer’s instructions. cDNA was synthesized and amplified by PCR with the BcaBEST RNA PCR Kit Version 2.1 (Takara, Osaka, Japan) according to manufacturer’s instructions. We used the following primers: VEGF (353 bp), sense 5'-CCCTGAGGCACTTTC-CAGG-3' and reverse 5'-CCCTGGTGGACATCTTC-3; VE-cadherin (336 bp), sense 5'-AACATGACAACCACGCCC-3' and reverse 5'-CTCGTGAATCTCCAGCGC-3; fetal kidney kinase-1 (Fkl-1) (434 bp), sense 5'-TGCTCTCAAGACATC-3' and reverse 5'-TTCTTCTCCTACCATCGT-3; GAPDH (726 bp), sense 5'-CTCATTTGACCTCATTACATTG-3' and reverse 5'-CTCACTGATCCCAGGATGCTT-3', was used as a positive standard control for mRNA quantitation and for determining stability between specimens.

Amplifications of these cDNAs were performed under the following conditions: denaturing for 1 min at 94°C, annealing for 1 min at 60°C, and elongation for 1 min at 72°C. Optimal numbers of cycles were determined to be 28 for VEGF, 30 for VE-cadherin, 27 for Flk-1, and 25 for GAPDH. The PCR products were labeled with [α-32P]dCTP and separated on 5% polyacrylamide gels. Relative radioactivity of the bands was estimated with an imaging analyzer (model BAS-2000; Fuji Photo Film, Tokyo, Japan). Expressions of mRNA were normalized to constant GAPDH expression, and for statistical analysis, the expression level for each factor measured was calculated as the percentage of baseline (i.e., 0 h) level for that factor.

Bone marrow cell implantation in the ischemic hindlimb of rats. Bone marrow cells were collected after 24 h of culture under hypoxia or normoxia. Survival rate of these collected cells was higher than 98% according to the results of trypan blue staining as described in Ex vivo hypoxic stimulation of bone marrow cells. Cells were suspended in PBS at the density of 1 × 10^7/ml for injection. The induction of angiogenesis by bone marrow cell implantation was investigated in a rat ischemic hindlimb model described previously (10). Briefly, rats were anesthetized with 50 mg/kg of pentobarbital given intraperitoneally. A vertical longitudinal incision was made in the left thigh. The femoral artery and its branches were dissected, ligated, and completely excised. The bone marrow cells (1 × 10^7 in 10 μl of PBS) with 24 h of culture under hypoxia (hypoxia group, n = 23) or under normoxia (normoxia group, n = 23) were then injected with a 26-gauge needle into the ischemic muscles in the thigh of rats at six points. For control, uncultured (control group, n = 23) bone marrow cells were also injected into the ischemic hindlimb of rats. Finally, the incision was closed by suture.

Measurement of VEGF level in ischemic hindlimb by ELISA. On days 1, 3, and 7 after the implantation of bone marrow cells, five rats per group per day were killed, samples of the adductor muscles were harvested, and wet weights were measured. All samples were minced and homogenated with 2.5 ml of PBS on ice. These samples were centrifuged at 10,000 g for 5 min at 4°C, and the resulting supernatants were stored at −80°C. VEGF in the supernatant was measured with VEGF ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. The detection limit was 5 pg/ml. Levels of VEGF per gram wet tissue was used for statistical analysis.

Histological analysis of microvessel density. Five rats from each group were killed 2 wk after treatment (n = 7 or n = 5 in each group), and the quadriceps and adductor muscles were harvested. Samples of harvested muscle were embedded in optimum cutting temperature compound, and snap-frozen in liquid nitrogen. To detect the development of microvessels, 5-μm-thick frozen sections were stained for alkaline phosphatase with an indoxyl tetrazolium method and then were counterstained with eosin. The number of microvessels was counted under a microscope using ×200 magnification by a single observer blind to the treatment regimen, and a total of 20 different fields were randomly selected. Density of microvessels was estimated by the microvessel/muscle fiber ratio.

Evaluation of blood flow recovery in the ischemic hindlimb. Eight rats from each group were reanesthetized 2 wk after the implantation of bone marrow cells. Rats were placed on a heating plate set at 37°C, and then 6 × 10^4/200 μl of eosin Dye-Trak microspheres (15 μm in diameter; Triton Technology, Loughborough, UK) were injected into the abdominal aorta. Rats were killed by cutting the abdominal aorta ~30 s after the injection of the microspheres. Tissue specimens were collected from the hindlimb, weighed, and then digested in 16N KOH for 48 h at 60°C. The microspheres in the tissues were reclaimed with a vacuum filter, and dye from the microspheres was extracted with dimethyl formamide. The optical density (OD) of these dye samples was measured with a spectrophotometer. The recovery of perfusion in the ischemic hindlimb was evaluated by determining the percentage of limb blood flow in relation to that of a normal right hindlimb, which was calculated as (ischemic limb OD/normal limb OD) × (normal limb tissue wt/ischemic limb tissue wt) × 100 (23).

Statistical analysis. All data are expressed as means ± SD. Statistical significance was evaluated with unpaired Student’s t-test for comparisons between two means, with ANOVA, followed by Scheffé’s procedure for more than two means, and with repeated-measures ANOVA to test for interaction. Data were considered significant when the P value was <0.05.

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RESULTS

Expression of VEGF, VE-cadherin, and Flk-1 mRNA in bone marrow cells. The level of expression of VEGF mRNA in bone marrow cells increased rapidly after culture under hypoxia and showed a peak at ~24 h of culture. However, the level of VEGF mRNA expression in bone marrow cells did not change significantly within the first 12 h of culture under normoxia and only increased slightly after 24 h of culture under normoxia. Quantitative analysis showed that the level of VEGF mRNA expression in bone marrow cells was increased about fivefold after 24 h of culture under hypoxia but only ~1.2-fold after 48 h of culture under normoxia (Fig. 1).

Levels of VE-cadherin mRNA expression in bone marrow cells also increased rapidly and significantly after culture under hypoxia but only increased slightly after culture under normoxia. Quantitative analysis showed that the level of VE-cadherin mRNA expression in bone marrow cells was increased ~2.5-fold at 24 h and ~3.3-fold at 48 h of culture under hypoxia, but only ~1.3-fold at 48 h of culture under normoxia (Fig. 1).

Similar to the results for expression of VEGF and VE-cadherin mRNA, the mRNA expression of Flk-1 increased significantly and rapidly in bone marrow cells cultured under hypoxia. Quantitative analysis showed that the level of Flk-1 mRNA expression in bone marrow cells increased ~2.5-fold after 48 h of hypoxic culture but did not change significantly after culture under normoxia (Fig. 2).

VEGF levels in ischemic hindlimb after bone marrow cell implantation. Levels of VEGF protein in the ischemic hindlimb of rats decreased with time after treatment in all groups (Fig. 3). The VEGF level in ischemic hindlimb of rats was significantly higher in the hypoxia group than in the normoxia or control groups at 1 and 3 days after treatment (245.7 ± 46.6 vs. 119.0 ± 25.3 and 127.4 ± 24.6 pg/g tissue on day 1, *P < 0.001; 173.2 ± 30.4 vs. 88.1 ± 24.9 and 89.9 ± 15.4 pg/g tissue on day 3, **P < 0.01). However, no significant difference was found among the three groups 7 days after treatment. VEGF concentration in the ischemic hindlimb of rats was not significantly different between the normoxia and control groups at any of the time points after treatment.

Fig. 1. Expression of vascular endothelial growth factor (VEGF) mRNA in bone marrow cells after culture under hypoxia and normoxia. Expression level was measured in fresh bone marrow cells (baseline) and in cells after 4, 8, 12, 24, and 48 h of culture. Results of quantitative analysis show that the VEGF mRNA in bone marrow cells is increased significantly after culture under hypoxia, but is only increased slightly under normoxia (*P < 0.05, **P < 0.01, ***P < 0.001 vs. baseline; †P < 0.05, ††P < 0.01, †††P < 0.001 vs. normoxia).

A

Fig. 2. Expression of vascular endothelial (VE)-cadherin (A) and fetal liver kinase-1 (Flk-1) mRNA (B) in bone marrow cells after 0 (baseline), 4, 8, 12, 24, and 48 h of culture under hypoxia and normoxia. Quantitative analysis results show that both VE-cadherin and Flk-1 mRNA in bone marrow cells is increased significantly after culture under hypoxia, but is increased mildly under normoxia (*P < 0.05, **P < 0.01, ***P < 0.001 vs. baseline; †P < 0.05, ††P < 0.01, †††P < 0.001 vs. normoxia).
Microvessel density in ischemic hindlimb. The microvessel-to-muscle fiber ratio was significantly higher in the hypoxia group (1.78 ± 0.09) than in the control (1.41 ± 0.18) and normoxia (1.34 ± 0.22) groups (P < 0.01, Fig. 4). No significant difference was seen between the control and normoxia groups.

Blood flow recovery of ischemic hindlimb after bone marrow cell implantation. Blood flow recovery in the ischemic hindlimb was evaluated and expressed as the percentage of normal blood flow in the right (nonischemic) hindlimb. At 2 wk postimplantation with bone marrow cells, recovery of blood flow in the ischemic hindlimb was significantly greater in the hypoxia group (89.7% ± 5.5%) than in either the normoxia group (67.0% ± 9.6%) or the control groups (70.4% ± 7.7%) (P < 0.001; Fig. 5).

DISCUSSION

We have safely performed local implantation of autologous bone marrow cells to induce therapeutic angiogenesis for patients with ischemic heart or limb disease (11). To find a safe, easy, and simple method for improving the angiogenic effect of this treatment, we undertook the present investigation. We cultured bone marrow cells under 33°C (7), which might depress the metabolism of cells and prevent the rapid maturation or differentiation of these stem cells during culture. Cells were subjected to mild hypoxic stimulation under 2% O2 rather than full hypoxia under 0% O2, because we wanted to maintain the cells in good bioactivity for...
Implantation in vivo after culturing. Under such culture conditions, the survival rate of bone marrow cells was higher than 98% even after 24 h of hypoxia, which was equivalent to the rate for freshly collected bone marrow cells. This result indicates that it might also be safe and reasonable to implant the bone marrow cells even after 24 h of culture under 2% O2 at 33°C.

The main aim of the present study was to develop a method for increasing the angiogenic effect of bone marrow cell implantation. Therapeutic angiogenesis can be induced by direct administration or gene transfer of VEGF or basic fibroblast growth factor (bFGF) (4, 19, 21, 24). One potential method for increasing the angiogenic potency of bone marrow cell implantation is to increase the production of angiogenic cytokines, such as VEGF and bFGF, from the implanted bone marrow cells. In the present study, we tried to enhance VEGF mRNA expression of bone marrow cells ex vivo before implantation, because the enhanced VEGF mRNA expression of bone marrow cells might result in higher VEGF production and more effective angiogenesis in vivo after implantation. As with other cells cultured under hypoxia (13, 20, 26), 2% O2 hypoxic stimulation rapidly increased the expression of VEGF mRNA in bone marrow cells with the expression level increased fivefold after 24 h of hypoxic culture.

Bone marrow as an origin of endothelial progenitor cells has been shown to contribute to postnatal vasculogenesis in physiological and pathological neovascularization (1, 3). Implantation of ex vivo expanded endothelial progenitor cells has also been shown to induce therapeutic angiogenesis effectively for ischemic tissues in experimental models (15, 16). For increasing the angiogenic effect of bone marrow cell implantation, we believed that increasing the endothelial differentiation of bone marrow cells might provide more endothelial cells and thus improve angiogenesis. Ex vivo hypoxic stimulation is known to upregulate the expression of VE-cadherin and Flk-1 in some cells (8, 25). We investigated whether mild hypoxia stimulation would also influence the endothelial differentiation of bone marrow cells, which should be a key factor for improving angiogenesis. Expression of VE-cadherin, a special surface antigen of endothelial cells, increased rapidly to ~3.3-fold of baseline after 48 h of culture under 2% O2 hypoxia. Flk-1, a receptor of the most important angiogenic growth factor of VEGF, is a marker used to evaluate the endothelial differentiation of bone marrow cells (2, 18). In the present study, we observed that the expression of Flk-1 mRNA in bone marrow cells increased significantly after culture under 2% O2 hypoxia, showing the level increased ~2.5 of baseline after 48 h of hypoxic culture. mRNA expression of VE-cadherin and Flk-1 increased to only ~1.3-fold or less of baseline in the bone marrow cells after culture under normoxia. Significant increase of VE-cadherin and Flk-1 gene expression in bone marrow cells after culture under hypoxia indicates the possibility that ex vivo hypoxia stimulation could improve the endothelial differentiation of bone marrow cells.

To determine whether the over expression of VEGF mRNA in hypoxia-cultured bone marrow cells would also contribute to higher VEGF production in vivo after implantation, we injected bone marrow cells into the ischemic hindlimb of rats and then measured the local level of VEGF protein in the ischemic hindlimb. We found a significantly higher VEGF concentration in the ischemic hindlimb after implantation with hypoxia-cultured bone marrow cells than with normoxia-cultured or noncultured bone marrow cells. This result indicates clearly that the overexpression of VEGF mRNA in bone marrow cells contributed to higher VEGF production in vivo after implantation into ischemic tissue.

The final purpose of our investigation was to develop a method for inducing an improved therapeutic angiogenesis in ischemic tissues by bone marrow cell implantation. Using an ischemic hindlimb model, we found that the microvessel density was significantly higher in the hypoxia group than in the control and normoxia groups. Furthermore, blood flow in the ischemic hindlimb recovered to ~90% of normal level after implantation with hypoxia-cultured bone marrow cells, which was much higher than the recovering level after implantation with normoxia-cultured (67%) or noncultured (70%) bone marrow cells. The significant enhancement of angiogenic potency after implantation of hypoxia-cultured bone marrow cells is apparently associated with both the upregulation of VEGF mRNA and the improvement of endothelial differentiation of bone marrow cells by ex vivo hypoxia stimulation before implantation.

In conclusion, we found that ex vivo mild hypoxia stimulation of bone marrow cells increased their level of VEGF mRNA expression and of endothelial differentiation. Furthermore, blood flow recovery in the ischemic hindlimb was improved more in rats implanted with ex vivo hypoxia-stimulated bone marrow cells than in those implanted with noncultured bone marrow cells. Our results indicate that improved effective-
ness of therapeutic angiogenesis might be induced by ex vivo hypoxia prestimulation of bone marrow cells before implantation.

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