CD117$^+$ stem cells play a key role in therapeutic angiogenesis induced by bone marrow cell implantation

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Submitted 31 December 2002; accepted in final form 13 May 2003

Therapeutic angiogenesis has been successfully induced by various methods and developed clinically as a new treatment for several ischemic diseases (4, 21, 31). We previously reported satisfactory angiogenic potency in vivo by local autologous bone marrow cell implantation in a corneal model, an ischemic heart model, and an ischemic hindlimb model in rats (9, 10, 12, 14, 17, 20), which was related to both the secretion of angiogenic growth factors and the endothelial differentiation from bone marrow cells.

Because CD34$^+$ cells (also called "endothelial progenitors") have been found to differentiate into endothelial cells in vivo and in vitro, the majority of researchers use CD34$^+$ cells from peripheral blood for implantation to induce therapeutic angiogenesis (1, 15, 16, 18). Unlike peripheral blood cells, the cell fractions in bone marrow are extremely complex (19), and total bone marrow cells, or their various fractions, have been selected for implantation to induce therapeutic angiogenesis in many clinical and experimental studies (3, 7–12, 14, 17, 20, 24–26, 29, 30). To obtain a consensus on the selection of cell fractions from bone marrow for implantation, it is important to clarify the angiogenic potency induced by different cell fractions in bone marrow cells. We hypothesized that the stem cells in bone marrow may be the best cell fraction for inducing therapeutic angiogenesis because they maintain good potency in differentiation and produce multiple cytokines.

However, the most suitable marker to use for stem cell selection is still unclear. CD34 is the most characterized marker for the selection of hematopoietic stem cells and may also be the best marker of cell selection from peripheral blood for inducing therapeutic angiogenesis because CD34$^+$ stem cells are extremely rare in peripheral blood. We selected CD34, but not CD34, as the marker for stem cell selection from bone marrow in this study because 1) some of the bone marrow stem cells that express CD117$^+$ and CD34$^+$ might generate angiogenic cytokines and differentiate into endothelial cells (13); and 2) the CD34$^+$ cell fraction from bone marrow shows 60–80% coexpression of CD117 (18).

In this study, we investigated the differences in the secretion of angiogenic factors and endothelial differentiation by stem cells and mature bone marrow mononuclear cells in vitro. We also analyzed the angiogenic potency of the two different cell fractions in vivo using a mouse ischemic hindlimb model.
METHODS

Animals. Male 12- to 15-mo-old C57BL/6 mice were used for these experiments, which were approved by the Institutional Animal Care and Use Committee of Yamaguchi University. The animals were bred in clean conditions and allowed free access to food and water in a temperature-controlled environment with a 12:12-h light-dark cycle.

Separation of stem cells from bone marrow cells. Mice were killed under deep general anesthesia, and bone marrow cells were collected from the femur and tibia. Bone marrow mononuclear cells were isolated by a Histopaque-1077 gradient (Sigma). Stem cells were separated from matured bone marrow mononuclear cells using the antibody to the stem cell receptor (CD117). Briefly, cells were incubated with FITC-conjugated rat anti-mouse CD117 (c-Kit) monoclonal antibody (Pharimingen) at 4°C for 60 min. After being washed, the cells were incubated with anti-FITC microbeads (Miltenyi Biotech) according to the manufacturer’s instructions. We separated CD117-positive (CD117+/-) cells and CD117-negative (CD117-) cells from the bone marrow cells by passing them through a magnetic cell sorting system (MACS). About 2% of the bone marrow cells expressed CD117, and the purity of CD117+ cells collected by MACS was about 90%. The viability of CD117+ and CD117- cells was >99%, as evaluated by staining the cells with 0.4% Trypan blue solution (Sigma). These freshly separated and collected cells were used for the following studies.

Angiogenic factor production and endothelial differentiation in vitro. To observe the production of angiogenic factors, CD117+ (3 × 10^6 cells/ml) and CD117- (1.5 × 10^6 cells/ml) cells were cultured on 24-well culture plates coated with 1% gelatin (Sigma) in RPMI 1640 (GIBCO) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO) at 37°C. The medium was changed 7 days after culture. The supernatants were collected, and the surviving cells were counted 1, 3, 7, and 14 days after culture. The levels of VEGF and IL-1β in the supernatants were measured in triplicate with a mouse VEGF and IL-1β ELISA kit (R&D Systems). Different numbers of CD117+ and CD117- cells were seeded for culture because the numbers of surviving CD117+ and CD117- cells were similar after ~7 days of culture and also because the VEGF concentration in the medium was too low to be measured by ELISA when fewer CD117- cells were seeded.

To investigate endothelial differentiation, the CD117+ and CD117- cells were seeded on four-well chamber culture slides (Nalge Nunc) coated with 1% gelatin and cultured as described above. After 14 days of culture, the cells were fixed in 1% formaldehyde, blocked with 2% BSA, and then incubated with FITC-conjugated antibodies against CD34, VE-cadherin (Pharimingen), and fetal liver kinase-1 (Flk-1) (Santa Cruz Biotechnology), respectively. After the cells were washed, the percentage of positively stained cells was calculated by counting under a microscope at 200-fold magnification. A single observer blind to the treatment regimen counted at least 1,000 cells in random fields.

Ischemic hindlimb model and cell implantation. The mouse ischemic hindlimb model was created as described previously (6). Briefly, after the mice were given general anesthesia, the left femoral artery was exposed and ligated, and its branches were dissected free and excised. On the basis of the percentages of each cell fraction, the quadriceps and adductor muscles of the ischemic hindlimb were injected at four points in five groups of mice. One group was injected with 2 × 10^5 CD117+ cells (CD117+ group, n = 14), one group with 9.8 × 10^6 CD117- cells (CD117- group, n = 14), and one group with 1 × 10^7 total bone marrow cells (total group, n = 14). There were two control groups: one injected with 10 μl PBS (PBS group, n = 10), and one group left untreated (control group, n = 10), respectively. The cells of the different fractions used for injection were separated and collected fresh the same day. The induction of angiogenesis was analyzed 2 wk after treatment.

To monitor the fate of cells after implantation, the CD117+ and CD117- cells were labeled with an intracellular fluorescent dye, either 5,6-carboxyfluorescein diacetate succinimidyloxyl-ester (CFSE) or fluorescent carbocyanine Dil dye (Molecular Probes) as described previously (10), and then injected into the ischemic hindlimbs of 16 supplementary mice (n = 8 in each group) as described above. Four mice from each group were killed 7 and 14 days after implantation, and the samples of harvested muscle were embedded in OCT compound and snap frozen in liquid nitrogen. Frozen sections were used to detect the survival of cells, and the microvessels were stained with FITC-conjugated anti-mouse CD34 antibody (Pharimingen) to examine the endothelial differentiation and incorporation.

Histological analysis of microvessel density. Mice were killed 2 wk after treatment (n = 5 or 7 in each group), and the quadriceps and adductor muscles were harvested. The samples of harvested muscle were embedded in OCT compound and snap frozen in liquid nitrogen. To detect the development of microvessels in ischemic muscle, 5-μm-thick frozen sections were stained for alkaline phosphatase using indoxyl tetrazolium methods and then counterstained with eosin (33). The number of microvessels and muscle fibers was counted under a microscope using 200-fold magnification by a single observer blind to the treatment regimen, and a total of 20 different fields on two independent slides from different cross sections was randomly selected for each mouse. The density of microvessels was estimated by the microvessel-to-muscle fiber ratio.

Measurement of blood flow in the ischemic hindlimbs. Five or seven mice from each group were reanesthetized 2 wk after treatment and placed on a heating plate set at 37°C to record perfusion in the left hindlimb compared with that in the normal right hindlimb. After the laser-Doppler perfusion recording was stopped, 6 × 10^9/20 μl of eosin Dye-Trak microspheres (15 μm in diameter, Triton Technology) were injected into the abdominal aorta, and the mice were killed by severing the abdominal aorta ~30 s later. Tissue specimens were collected from the hindlimb, weighed, and then digested in 1 M KOH for 48 h at 60°C. The microspheres in the tissues were recombined 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 estimated by determining the percentage of limb blood flow compared with that in the normal right hindlimb, which was calculated as (OD of the ischemic limb/OD of the normal limb) × (tissue weight of the normal limb/tissue weight of the ischemic limb) × 100 (28).

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Data analysis. Data are presented as means ± SD. Statistical significance was evaluated by ANOVA, followed by Scheffe’s procedure and by repeated ANOVA to test for interactions. P < 0.05 was considered significant.

RESULTS

Survival, endothelial differentiation, and secretion of angiogenic factors in vitro. Figure 1A shows that the CD117+ cells survived and grew well, adhering to the culture flask 2 wk after culture. Conversely, many of the CD117− cells died within 14 days after culture. Quantitative analysis showed that the number of CD117− cells increased, but the number of CD117+ cells decreased significantly with the time of culture (Fig. 1B). Furthermore, the CD117+ cells expressed positivity for ~80% of CD34, 65% of VE-cadherin, and 70% of Flk-1, but almost no positively stained cells were found in the CD117− cells after 14 days of culture (Fig. 1A).

The concentration of VEGF in the medium increased in the CD117+ cells but decreased in the CD117− cells with time. Although CD117+ cells accounted for only ~2% of the CD117− cells, VEGF secretion by the CD117+ cell fraction was significantly higher than that by the CD117− cells after 7 days of culture (P < 0.01; Fig. 2A). In contrast, IL-1β secretion by the CD117+ cells was significantly lower than that by the CD117− cells in the first 3 days (P < 0.01), but no difference was seen after 7 days of culture (Fig. 2B).

Fig. 1. Survival and endothelial differentiation of CD117+ and CD117− cells after culture. A: CD117+ cells (top) survived and grew well, with strong expression of CD34, VE-cadherin, and fetal liver kinase-1 (Flk-1). On the other hand, many of the CD117− cells (bottom) died and were negative for CD34, VE-cadherin, and Flk-1 after 14 days of culture. B: Quantitative analysis showed that the number of CD117− cells decreased rapidly, but the number of CD117+ cells increased slowly after culture.

Fig. 2. ELISA analysis of the concentration of angiogenic cytokines in culture supernatants. A: VEGF secretion by the CD117+ cells was 10-fold higher than that by the CD117− cells after 7 days of culture. B: a higher level of IL-1β was seen in the culture supernatants of CD117+ cells than in those of CD117− cells during the first 3 days of culture. Open bars, CD117+ cells; solid bars, CD117− cells. *P < 0.01 vs. CD117− cells.
Survival and endothelial differentiation of cells after implantation into ischemic hindlimbs. Most of the CD117<sup>+</sup> cells survived (A), but few of the CD117<sup>-</sup> cells (E) were detected 7 days after implantation. Histological examination of microvessel staining with the anti-CD34 antibody (B and F) and the Dil-labeled CD117<sup>+</sup> (C) and CD117<sup>-</sup> cells (G) showed that the implanted CD117<sup>+</sup> cells were incorporated in microvessels (arrows in D), but the CD117<sup>-</sup> cells were not (H).

**Survival and endothelial differentiation of cells after implantation into the ischemic hindlimbs.** Figure 3 shows that the CFSE-labeled CD117<sup>+</sup> cells survived well 7 days after implantation. These CD117<sup>+</sup> cells were also differentiated into endothelial cells and incorporated in microvessels by 14 days after implantation. However, only a few of these implanted CD117<sup>-</sup> cells were detected in the ischemic muscles 7 days after implantation, and there were no signs of endothelial differentiation and incorporation.

**Microvessel density in the ischemic hindlimbs.** The microvessel-to-muscle fiber ratio was significantly higher in the CD117<sup>+</sup> group than in the other groups (1.26 ± 0.08 in the CD117<sup>+</sup> group vs. 0.84 ± 0.11 in the CD117<sup>-</sup> group, P < 0.01; and vs. 1.02 ± 0.22 in the total group, P < 0.05; Fig. 4). A higher density of microvessels in ischemic muscle was also seen in the total group than in the CD117<sup>-</sup> group, although the difference between the two groups was not significant (P = 0.09). No significant difference was seen among the CD117<sup>-</sup> group, the PBS group (0.89 ± 0.12), and the control group (0.79 ± 0.15).

**Blood flow recovery of the ischemic hindlimbs after treatment.** Blood perfusion of the ischemic hindlimbs recovered well after the implantation of CD117<sup>+</sup> cells (Fig. 5A) or the total bone marrow mononuclear cells (Fig. 5C), but there was poor recovery after implantation of the CD117<sup>-</sup> cells (Fig. 5B) compared with the PBS injection (Fig. 5D) or without treatment (Fig. 5E). Quantitative analysis showed that the blood perfusion of the ischemic hindlimbs was significantly better in the CD117<sup>+</sup> cell-implanted mice than in the CD117<sup>-</sup> cell-implanted mice according to laser-Doppler analysis (P < 0.01; Fig. 5). The percent limb blood flow was also ~25% greater in the CD117<sup>+</sup> cell-implanted mice than in the CD117<sup>-</sup> cell-implanted mice according to...
the method of microsphere blood flow measurement (92.7 ± 8.9% in the CD117+ group vs. 65.8 ± 7.4% in the CD117- group, \(P < 0.001\); Fig. 6). Blood perfusion of the ischemic hindlimb was well restored by both the CD117+ cell and total bone marrow cell transplantation. No significant difference in perfusion recovery of the ischemic hindlimb was found between the CD117+ group and the total group 2 wk after treatment.

**DISCUSSION**

Bone marrow is mesenchymal tissue, and bone marrow cells are composed of extensive complex cell fractions containing many kinds of undifferentiated stem cells and differentiated cells (19). The implantation of autologous bone marrow cells has been demonstrated to be an effective and feasible technique of inducing therapeutic angiogenesis in both clinical and experimental studies (3, 7–12, 14, 17, 20, 24–26, 29, 30). However, the angiogenic potency might differ among the cell fractions of bone marrow cells, and which of these play a key role remains unelucidated. It is important to understand the angiogenic potency induced by different cell fractions because selecting the most effective cell fraction for implantation could improve this new treatment. Considering that the risk of ischemic diseases increasing with age, we used aged mice in this experiment.

The stem cells in bone marrow have the potential to proliferate and differentiate, and the fact that they can be differentiated into blood cells and also interstitial cells, including endothelial cells (1, 18), suggests that they play an important role in inducing therapeutic angiogenesis in both clinical and experimental studies (3, 7–12, 14, 17, 20, 24–26, 29, 30). However, the angiogenic potency might differ among the cell fractions of bone marrow cells, and which of these play a key role remains unelucidated. It is important to understand the angiogenic potency induced by different cell fractions because selecting the most effective cell fraction for implantation could improve this new treatment. Considering that the risk of ischemic diseases increasing with age, we used aged mice in this experiment.

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angiogenesis. We selected CD117 as the marker for stem cell separation in this study, and ~2% of the bone marrow mononuclear cells showed positive expression of CD117. The purity of the separated CD117+ cells are near 90%, and ~30% of CD117+ cells expressed CD34 (data not shown).

Without a specific supplement of exogenous growth factors, we found that many of these CD117+ cells died within 14 days after culture. However, the CD117+ cells survived well and proliferated mildly after culture, even if no exogenous growth factors were supplemented. These CD117+ cells also showed strong positive expression of several endothelial specific markers of CD34 and VE-cadherin after 14 days of culture, indicating endothelial differentiation. Furthermore, the level of VEGF was more than 10-fold higher in the medium of the CD117+ cell fraction than in the medium of the CD117− cell fraction after 7 days of culture, indicating that there was more VEGF secretion from the CD117+ cells than from the CD117− cells. Because the increase in angiogenic cytokines and the proliferation and migration of endothelial cells have both been found critical for inducing angiogenesis (5, 23), our findings indicate that the CD117+ cell fraction might play a more important role in therapeutic angiogenesis than the CD117− cells of bone marrow.

After implantation into ischemic hindlimbs, the CD117+ cells survived well and differentiated into endothelial cells, to be incorporated in microvessels. Conversely, the survival and endothelial differentiation of the implanted CD117− cells was very poor, which correlated well with the in vitro results. The microvessel density in the ischemic hindlimbs was significantly higher in the CD117+ cell-implanted mice than in the CD117− cell-implanted mice. The recovery of blood perfusion was also better in the CD117+ cell-implanted mice than in the CD117− cell-implanted mice. These results of our in vivo investigation also demonstrated that CD117+ cells play a major role in the therapeutic angiogenesis induced by bone marrow cell implantation.

Although the density of microvessels in ischemic hindlimbs was significantly greater in the CD117+ cell-implanted mice than in the total bone marrow-implanted mice, similar recovery of blood flow in the ischemic limbs was observed after the implantation of CD117+ cells and total bone marrow cells. The reason for this is unknown. Because we have only followed up to 2 wk after cell implantation in this study, it is possible that many of these new microvessels have not formed networks as functional vessels within 2 wk. We expect that the majority of microvessels in the CD117+ cell-implanted mice will remodel and form functional vessel networks with time and then contribute further improvement of blood flow in ischemic limbs after treatment. So, it is possible that the higher density of microvessels in the CD117+ cell-implanted mice will result in significantly higher blood flow than in the total bone marrow-implanted mice later than 2 wk after treatment.

About 30% of CD117+ cells are also CD34+ cells, and the role played by these CD34+ cells in inducing therapeutic angiogenesis is still unknown. We found that the VEGF produced by the 30% subpopulation of CD117+/CD34+ cells was significantly less than that produced by the total CD117+ cells, about 15% of the level in the total CD117+ cells (data not shown). Further studies are also needed to clarify the best marker, evaluating CD117, CD34, and CD133, for selecting stem cells from bone marrow for implanting to induce therapeutic angiogenesis.

Previous studies have demonstrated that stem cells can be mobilized by the administration of cytokines and drugs or by ischemic stimulation (2, 18, 22, 27, 32). In the present study, only ~2% of the total bone marrow cells were CD117+ in the untreated mice. We believe that the therapeutic angiogenesis induced by bone marrow cell implantation would be enhanced by increasing the number of stem cells, achieved by in vitro expansion of CD117+ cells, by the systemic administration of cytokines and drugs, or by ischemic stimulation.

In conclusion, we demonstrated that the CD117+ stem cell fraction in bone marrow plays a key role in therapeutic angiogenesis induced by bone marrow cell implantation, which might be related to the high level of VEGF generation and good endothelial differentiation from stem cells.

DISCLOSURES

This work was supported by Grant-in-Aid for Scientific Research (C) 13671391 from the Ministry of Education, Culture, Sports, Science, and Technology and by Research Grant for Cardiovascular Diseases 13C-1 from the Ministry of Health, Labour and Welfare.
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