Therapeutic Neovascularization Using Cord Blood–Derived Endothelial Progenitor Cells for Diabetic Neuropathy

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Diabetic neuropathy is based on the impairment of nerve blood flow and the metabolic disorder. Although the vasodilating agents and anticoagulants improve nerve function and symptoms in diabetic neuropathy, more effective treatments are needed. Because endothelial progenitor cells (EPCs) have been identified in adult human peripheral blood, many studies have shown that transplantation of EPCs improves circulation to ischemic tissues. In this study, we have demonstrated that therapeutic neovascularization using human umbilical cord blood–derived EPCs reversed diabetic neuropathy. EPCs were isolated and expanded on day 7 of culture from cord blood mononuclear cells. Unilateral intramuscular injection of EPCs into hindlimb skeletal muscles significantly ameliorated impaired sciatic motor nerve conduction velocity and sciatic nerve blood flow in the EPC-injected side of streptozotocin-induced diabetic nude rats compared with the saline-injected side of diabetic nude rats. Histological study revealed an increased number of microvessels in hindlimb skeletal muscles in the EPC-injected side of diabetic rats. These findings suggest that transplantation of EPCs from cord blood may be a useful treatment for diabetic neuropathy. *Diabetes* 54:1823–1828, 2005

Diabetic neuropathy, which is the most frequent and earliest diabetes complication, decreases the quality of life and increases the morbidity of diabetic patients (1) and occurs because of the impairment of nerve blood flow and metabolic imbalances in the neural compartment of the peripheral nerve. Disruption of the vasa nervorum has been reported in peripheral nerves in experimental diabetic neuropathy (2), and numerous studies indicate that reduced blood flow directly caused symptoms of diabetic neuropathy (3,4). We and others have demonstrated that vasodilatory and anticoagulant treatments, such as prostaglandin E_1/I_2, niceritrol, and cilostazol, improved nerve function and symptoms with diabetic neuropathy (5–8). Although these treatments ameliorate the symptoms of diabetic neuropathy, more effective treatments are needed clinically.

Since Asahara et al. (9) first demonstrated the existence of circulating endothelial progenitor cells (EPCs) in adult peripheral blood, the concept of adult vasculogenesis has been developed. When local ischemia occurs, EPCs are mobilized from bone marrow to the peripheral blood. Then EPCs are differentiated in the area of local ischemia, which induces neovascularization (10). This paradigm is termed "postnatal vasculogenesis." From the viewpoint of vasculogenesis, the ischemic diseases may be caused by an insufficient supply of EPCs. A number of experimental and clinical studies have revealed that ischemic heart diseases and arteriosclerosis obliterans can effectively be treated by EPC transplantation, causing postnatal neovascularization (11–14).

Schratzberger et al. (15) showed that vascular endothelial growth factor (VEGF) gene transfer significantly increased nerve conduction velocity and nerve blood flow as well as the amount of vasculature in the muscles and nerves, suggesting that the induction of local angiogenesis ameliorates experimental neuropathy. Therefore, we hypothesized that the transplantation of EPCs may promote local vasculogenesis and reverse diabetic neuropathy.

EPCs can be isolated from the bone marrow, cord blood, and peripheral blood. We and others have reported that EPCs isolated from cord blood have a greater proliferative potential and a higher cell cycle rate than EPCs from other sources, suggesting that cord blood–derived EPCs may more effectively contribute to therapeutic vas-

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DiI, 3,3′-dioctadecylindocarbocyanine; EPC, endothelial progenitor cell; MNC, mononuclear cell; MNCV, motor nerve conduction velocity; SNBF, sciatic endoneurial nutritive blood flow; STZ, streptozotocin; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor.

culogenesis (11,12,16). In this study, we examined whether transplantation of human umbilical cord blood–derived EPCs into the hindlimb skeletal muscles may prevent the development of diabetic neuropathy in streptozotocin (STZ)-induced diabetic immunodeficient nude rats.

RESEARCH DESIGN AND METHODS

Human umbilical cord blood. Human umbilical cord blood (50–120 ml) was obtained from each donor after the baby's delivery. Written informed consent was obtained from all mothers before labor and delivery. Protocols for sampling human umbilical cord blood were approved by the Institutional Review Board.

Cell culture. Mononuclear cells (MNCs) were isolated from umbilical cord blood using the Histopaque-density centrifugation method (9). The MNC layer was collected, washed twice with 1 mmol/l EDTA in PBS, and suspended in degassed PBS with 0.5% BSA and 2 mmol/l EDTA. Total MNCs were cultured in M-199 medium with 20% fetal bovine serum (Sigma, St. Louis, MO) and bovine brain extract on human fibronectin-coated plastic plates. EPCs from attached cells and cell clusters were expanded under the condition described above. To confirm that these cells had the character of endothelial cells, separate coverslips were incubated with 10 mg/ml 3,3′-dioctadecylindocarbocyanine (DiI)-acetylated LDL for 4 h at 37°C and observed under fluorescent microscope.

Tube formation in basement matrix gel. At day 7 of culture, cord blood–derived EPCs were transferred to basement membrane matrix gel (Matrigel; BD Biosciences, San Jose, CA). At days 3–7 of culture, the formations of angiogenesis-like endothelial cell networks were seen under a fluorescence microscope.

Flow cytometry. Cord blood–derived EPCs at day 7 of culture were subjected to flow cytometric analysis as described previously (17). Cells were then stained with phycoerythrin-conjugated CD34 (clone 8G12; BD Biosciences) or fluorescein isothiocyanate-conjugated CD45 (clone 2D1; BD Biosciences) antibody. Isotype-identical antibodies served as control. After staining, cells were fixed with 1% paraformaldehyde and analyzed by flow cytometry.

Rats. Male immunodeficient nude rats (F344/N rnu/rnu) at 6 weeks of age were provided by Cler Japan (Tokyo, Japan). All protocols were approved by the Nagoya University Institutional Animal Care and Use Committee.

Induction of diabetes. Diabetes was induced after an overnight fast with a single intraperitoneal injection of STZ (60 mg/kg in 0.9% sterile saline). Serum glucose levels were measured every week, and rats with serum glucose 12.5 mmol/l were used as the diabetic rats. Age- and weight-matched nude rats were used as control animals.

Transplantation of ex vivo expanded EPCs for therapeutic neovascularization. Eight weeks after the induction of diabetes, control and diabetic anesthetized nude rats (5 mg/100 g pentobarbital i.p.) were injected with EPCs (1×10^6 cells/rat) intramuscularly in the unilateral femoral quadriceps muscle, the femoral biceps muscle, and the soleus muscle using a 26-gauge needle. Saline was injected into the contralateral hindlimb skeletal muscles in both normal and diabetic nude rats as controls. Four weeks after the treatments, physiological and histological assessments were performed under anesthesia as follows.

Motor nerve conduction velocity. Rats were anesthetized with pentobarbital (5 mg/100 g) by intraperitoneal injection and placed on a heated pad in a room maintained at 25°C to ensure a constant rectal temperature of 37°C. Motor nerve conduction velocity (MNCV) in sciatic nerves between the ankle and sciatic notch was measured as described previously (18). MNCV was determined with a Neuropak NEM-3102 instrument (Nihon-Koden, Osaka, Japan) by methods described previously (6).

Sciatic endoneurial nutritive blood flow. Rats were anesthetized with pentobarbital (5 mg/100 g) by intraperitoneal injection and placed on a heated pad in a room maintained at 25°C to ensure a constant rectal temperature of 37°C. Sciatic endoneurial nutritive blood flow (SNBF) was measured by the hydrogen clearance technique with an analog recorder BW-4 (Biochemical Science, Kanawasa, Japan) and electrolysis tissue blood flow meter RBA-2 (Biochemical Science), as described previously (15), and calculated with the equation of Koshu et al. (19).

Immunohistological staining. At the end of the experiments, rats were killed with an overdose of pentobarbital. After perfusion-fixation in 4% paraformaldehyde, the soleus muscles from both sides of eight rats (four normal rats and four diabetic rats) were immersed in 4% paraformaldehyde overnight. All of the samples were embedded in paraffin and cut into 5-μm sections for hematoxylin-eosin staining and immunohistochemical staining with primary antibody. The sections were cleared of paraffin in xylene and rehydrated through decreasing concentrations of ethanol. The slides were treated with a solution of 0.3% hydrogen peroxide (H_2O_2) in methanol for 30 min at room temperature to abolish endogenous peroxidase activity. Sections were then incubated overnight at 4°C with the primary antibody (anti–von Willebrand factor (vWF) polyclonal antibody, DAKO Japan, Tokyo, Japan) diluted 1:600. Sections were subsequently stained by Simplestain rat system (Nichirei, Tokyo, Japan) according to the manufacturer's instructions. Negative control was performed by omitting anti–factor VIII antibody. The capillary endothelial cells were counted under light microscopy (×200) to determine the capillary density. Five fields from the muscle samples were randomly selected for the capillary counts. To avoid overestimating the capillary density because of muscle atrophy or underestimating it because of interstitial edema, the capillary density was expressed as the capillary-to-muscle fiber ratio.

Statistical analysis. All group values were expressed as means ± SE. Statistical analyses were made by a one-way ANOVA with the Bonferroni correction for multiple comparisons.

TABLE 1
Body weights and blood glucose concentrations of nude rats

	n	Body weight (g)	Blood glucose concentration (mmol/l)
Normal nude rats	6	246.0 ± 8.0	5.31 ± 0.50
Diabetic nude rats	6	180.0 ± 6.8*	19.23 ± 1.54*

Data are expressed as means ± SE. *$P < 0.001$ vs. normal rats.

RESULTS

Body weights and blood glucose concentrations of nude rats. Diabetic nude rats showed significant decrease in body weights and significant increase in blood glucose concentrations compared with normal nude rats (Table 1). The transplantation of EPCs into unilateral hindlimb skeletal muscles did not change body weights and blood glucose concentrations compared with untreated nude rats (data not shown).

Culture of EPCs from cord blood. EPCs were expanded under culture conditions from MNCs isolated from umbilical cord blood. EPCs were expanded from attached cells (Fig. 1*A*) and cell clusters (Fig. 1*B*) isolated from culture of total cord blood MNCs. After 7 days of culture, cells showed enhanced differentiation, such as cord-like structure (Fig. 1*C*) and cobble stone pattern (Fig. 1*D*). Furthermore, the evaluation of the tube formation and cell surface antigen of the cells obtained after 7-day culture of total MNCs were performed. Cells were collected at day 7 of culture, cultured on basement membrane matrix gel in DMEM containing 20% fetal bovine serum and brain extract. Within 3 days, tube formation was identified in the matrigel (Fig. 1*E*). More than 90% of the isolated cells were identified by DiI-acetylated LDL, which is a marker of vascular endothelial cells (Fig. 1*F*).

Because CD34 antigen is thought to be an appropriate marker for the identification of EPCs from human umbilical cord blood (17,20,21), enumeration of $CD34^+$ cells was performed after 7-day culture of total MNCs. As shown in Fig. 1*G*, 93.6 ± 5.3% of cells were $CD34^+$. On the other hand, only a few cells expressed CD45, which is a common leukocyte antigen (Fig. 1*H*)

MNCV. The sciatic MNCV of the saline-injected side in diabetic rats was significantly delayed compared with that in the saline-injected side of normal rats (diabetic saline-injected, 29.0 ± 1.0 m/s; normal saline-injected, 50.7 ± 2.7 m/s, $P < 0.001$) (Fig. 2*A*). Transplantation of EPCs signif-

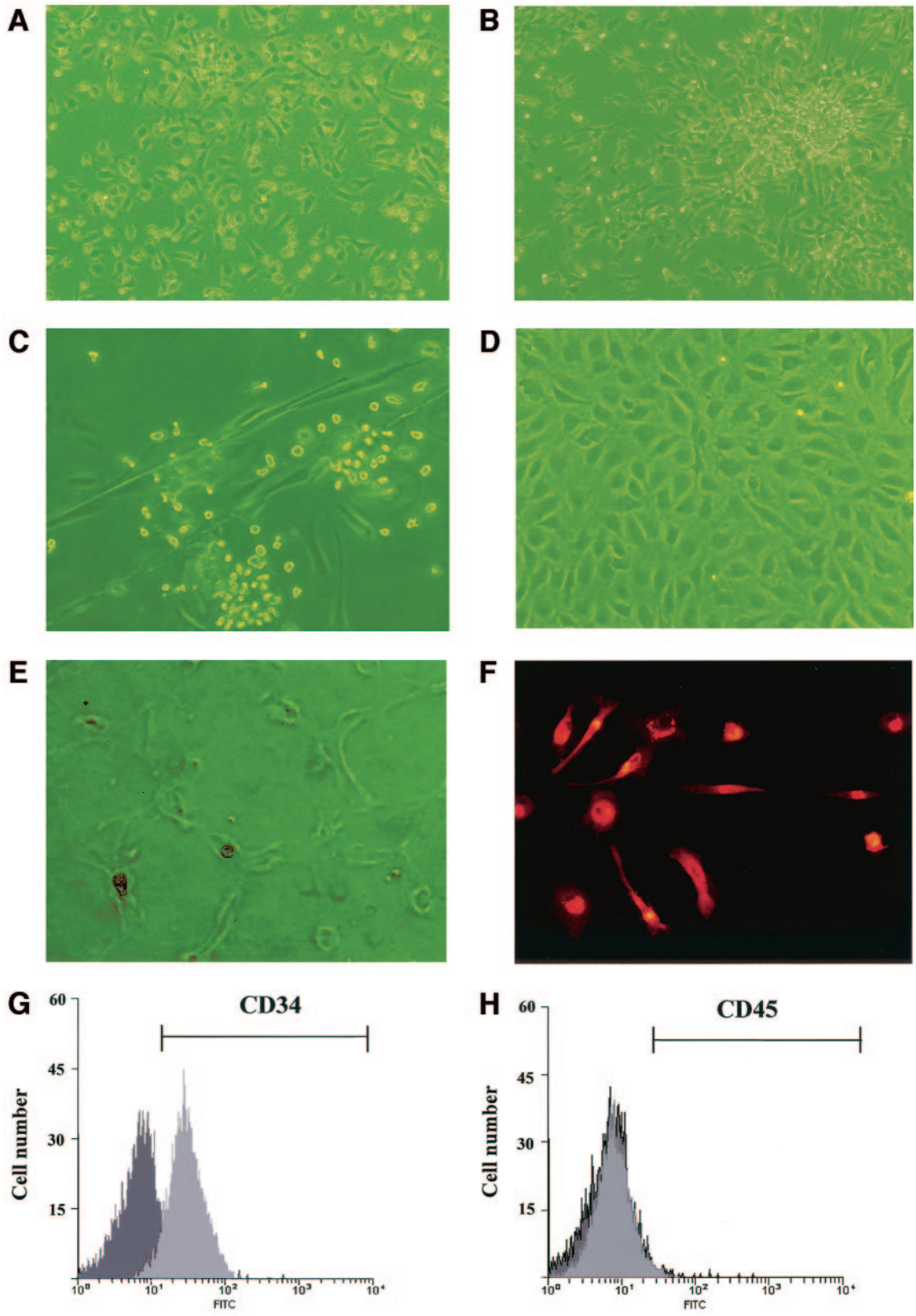

FIG. 1. Differentiation and identification of EPCs from human umbilical cord blood MNCs in vitro. Differentiation of EPCs: when cord blood MNCs were cultured on fibronectin, attached cells (*A*) and cell clusters (*B*) sprouted. *C*: After 7 days of culture, the cells showed enhanced differentiation, such as cord-like structure. *D*: Cells showed a cobblestone pattern when they matured. *E*: Identification of EPCs. When cells were isolated from culture plates on basement membrane matrix gel, tube formation was identified in the matrigel. *F*: More than 95% of the isolated cells showed the uptake of DiI-acetylated LDL. FACS analyses of isolated cells were conducted after 7 days of culture. Cells were labeled with fluorescent antibodies to CD34 (*G*) or CD45 (*H*).

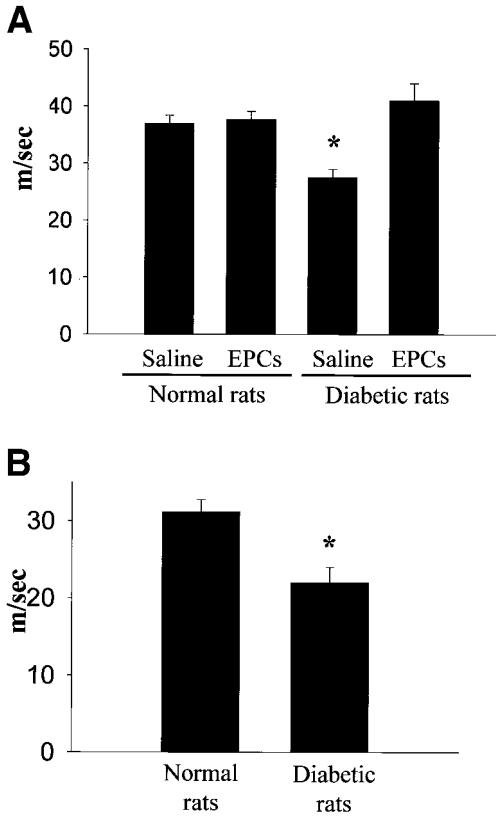

FIG. 2. The effects of EPC transplantation on sciatic and tail nerve conduction velocity (NCV) in normal and diabetic rats. EPCs were transplanted into unilateral hindlimb skeletal muscles 8 weeks after STZ injection. Sciatic and tail NCV were measured 4 weeks after EPC transplantation. *A*: Sciatic NCV. $P < 0.001$ vs. saline-injected normal. *B*: Tail NCV. $P < 0.05$ vs. saline-injected normal rats.

icantly ameliorated the sciatic MNCV of the EPC-injected side compared with that of the control side in diabetic rats (diabetic EPC-injected, 46.7 ± 1.9 m/s, $P < 0.001$). There was no significant difference between the sciatic MNCV of EPC-injected side in diabetic rats and that in normal rats. The transplantation of EPCs in normal rats did not show significant increase in sciatic MNCV.

On the other hand, the transplantation of EPCs into the unilateral hindlimb did not reverse diabetes-associated tail MNCV deficit (Fig. 2*B*). A significant decrease in tail MNCV was observed in diabetic rats compared with that in normal rats (diabetic tail, 22.0 ± 2.0 m/s; normal tail, 31.2 ± 1.6 m/s, $P < 0.05$).

SNBF. SNBF in the saline-injected side of diabetic rats was reduced compared with that in normal rats (diabetic saline-injected, 7.1 ± 1.4 ml · min^{-1} · 100 g^{-1}; normal saline-injected, 16.6 ± 0.3 ml · min^{-1} · 100 g^{-1}, $P < 0.05$) (Fig. 3). Transplantation of EPCs significantly augmented SNBF in the EPC-injected side of diabetic rats compared with the control side of diabetic rats (diabetic EPC-injected, 15.8 ± 0.8 ml · min^{-1} · 100 g^{-1}, $P < 0.05$). The transplantation of EPCs in normal rats did not show significant differences in SNBF.

Immunological staining of vasculature. Vasculature was visualized by vWF immunostaining, a specific marker for endothelial cells (Fig. 4). Quantitative analyses revealed that the capillary–to–muscle fiber ratio in the saline-injected side of diabetic rats was significantly reduced compared with that of normal rats ($P < 0.001$). Transplantation of EPCs significantly augmented the capillary–to–muscle fiber ratio of the EPC-injected side compared with that of the saline-injected side in diabetic rats ($P < 0.001$). There were no significant differences between the capillary-to-muscle fiber ratio of the EPC-injected side in diabetic rats and that of normal rats. The injection of EPCs into normal rats did not show significant differences in the capillary-to-muscle fiber ratio.

DISCUSSION

The present study first demonstrates that ex vivo cell therapy, consisting of culture-expanded EPC transplantation, may be a novel therapy for diabetic neuropathy. The transplantation of cord blood–derived EPCs into unilateral hindlimb skeletal muscles reversed the impairment of sciatic MNCV and SNBF in the EPC-injected side of diabetic animal. The immunohistological study revealed that the number of the vessels was increased in the EPC-injected side of hindlimb skeletal muscle.

After the discovery of EPCs in adult peripheral blood in 1997, many experimental studies revealed that EPCs have a potent ability for neovascularization and that the transplantation of EPCs improves ischemic tissue (11,17). Because EPCs exist in bone marrow and cord blood and small percentages are found in peripheral blood, clinical trials of the transplantation of MNCs from bone marrow or peripheral blood were performed for the treatment of ischemic diseases. The Therapeutic Angiogenesis Using Cell Transplantation study has demonstrated the safety and efficacy of therapeutic angiogenesis stimulated by transplantation of autologous bone marrow MNCs into patients with peripheral arterial disease (13).

On the other hand, the impairment of neovascularization by bone marrow–derived MNCs was reported in diabetic mice (22). Recent human studies revealed that proliferation, adhesion, and incorporation of EPCs into vasculature were impaired in type 1 and type 2 diabetic patients (23,24). Our results indicate that the highly proliferative cord blood–derived EPCs were maintained even in the diabetic condition, which confirmed that EPCs from cord blood have a greater proliferative potential and a higher cell cycle rate than EPCs from other sources (11,12,16). In the absence of adverse immunological reaction to allografts, transplantation of cord blood–derived

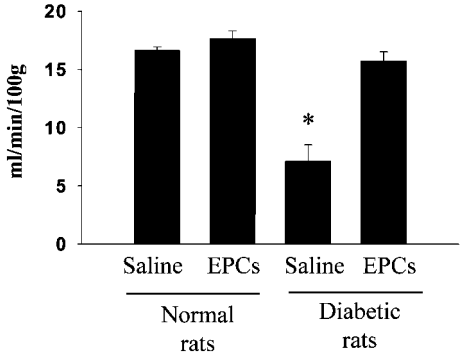

FIG. 3. The effects of EPC transplantation on SNBF in normal and diabetic rats. EPCs were transplanted into unilateral hindlimb skeletal muscles 8 weeks after STZ injection. SNBF was measured 4 weeks after EPC transplantation. $P < 0.001$ vs. saline-injected normal rats.

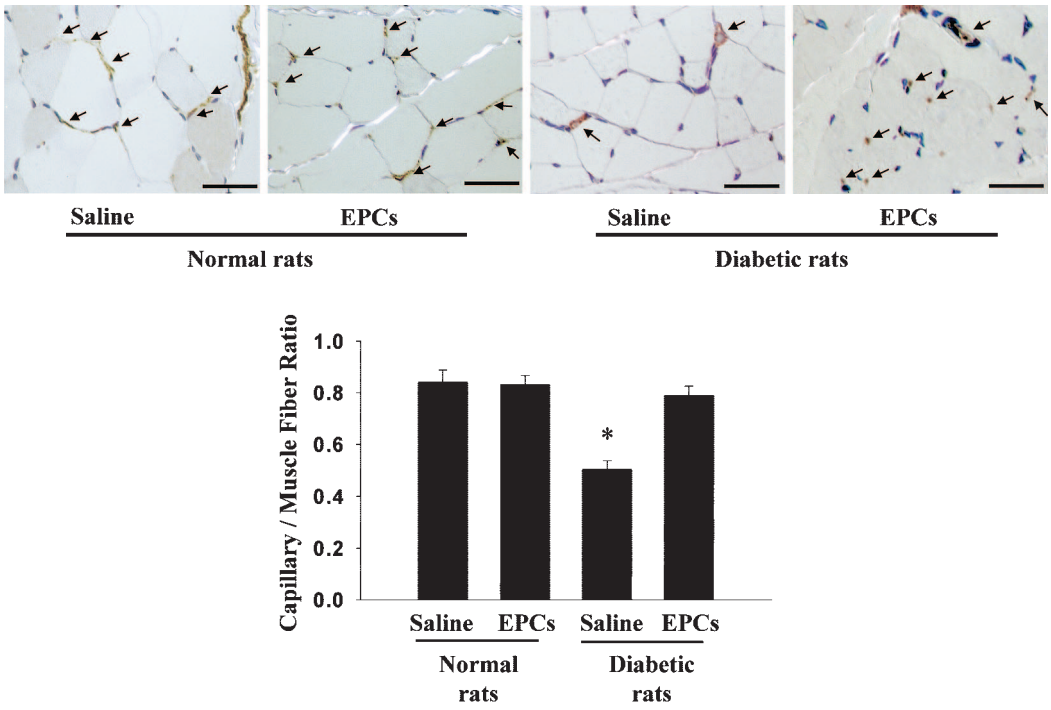

FIG. 4. ***Top panel*****: Representative photomicrographs of histological sections in each saline-injected and EPC-injected sides of skeletal muscles of normal and diabetic rats. Arrowheads indicate vascular endothelial cells detected by immunohistological staining for vWF. Bar = 50 μm.** ***Bottom panel*****: Quantitative analyses for capillary–to–muscle fiber ratio of the saline-injected and EPC-injected sides of skeletal muscles in normal and diabetic rats. $P <$ 0.001 vs. saline-injected normal rats.**

EPCs with high proliferative capacity may have an effective treatment of human diabetic neuropathy.

We have demonstrated the effects of therapeutic vasculogenesis on diabetic neuropathy 4 weeks after the transplantation of EPCs in diabetic rats, in which diabetes duration was 12 weeks, in this study. Because the improvement of local circulation by therapeutic angiogenesis using bone marrow–derived MNCs was reported to be sustained for at least for 24 weeks in clinical study, a prolonged effect on diabetic neuropathy by EPC transplantation may be expected. In fact, our preliminary study revealed that the therapeutic effect on diabetic neuropathy continued 12 weeks after EPC transplantation in diabetic mice (data not shown). Further study with long-term observation is under way in our laboratory.

Pathological characteristics of diabetic neuropathy include various abnormalities, such as loss of myelinated nerve fiber, axonal degeneration, segmental demyelination, and nerve fiber regeneration as it progresses. Because axonal degeneration of peripheral nerves is observed by 5 months after STZ injection in rat (25), the delayed MNCV of diabetic animals in this study may be due to the dysfunction of Schwann cells. Although the pathological examination revealed that human diabetic neuropathy is largely an axonal disease, the dysfunction of Schwann cells, which play an important role in maintenance of axonal caliber, perineurial blood-nerve barrier, and nerve regeneration, is one of the essential abnormalities in diabetic neuropathy (26). The therapeutic vasculogenesis in diabetic subjects may conserve Schwann cell function. Further study is required.

The impairment of blood flow is one of the major factors in diabetic neuropathy. Our study revealed that the capillary-to-muscle ratio was significantly lower in diabetic rats compared with nondiabetic controls, which is consistent with human studies (27). In fact, previous studies have reported the salutary effects of various vasodilatory and anticoagulant agents, such as prostaglandin E_1/I_2, niceritrol, and cilostazol; ACE inhibitors; and protein kinase C β inhibitor on diabetic neuropathy (5–8,28–30). Despite the beneficial effects of these agents in experimental diabetic neuropathy, these monotherapies were not sufficient for neuropathic symptoms in humans. Our study revealed that the injection of EPCs into the hindlimb skeletal muscle of diabetic rats contributed increased neovascularization in the muscles, leading to an increase in the SNBF. The reason that EPCs did not affect vasculogenesis in normal rats is unclear. Because the upregulation of VEGF gene expression was reported in ischemic skeletal muscle and diabetic peripheral nerves (31,32), local VEGF may contribute to the formation of vasculature by EPCs. Further studies are in progress in our laboratory.

VEGF gene transfer was reported to increase nerve conduction velocity and nerve blood flow in diabetic animals (15). We have demonstrated that the transplantation of EPCs derived from cord blood has successfully recovered impaired nerve conduction velocity and nerve blood flow in diabetic condition. The major difference of these gene/cell therapy and conventional vasodilatory/anticoagulant therapy is the induction of new vessels in target organs. These results suggest that gene/cell therapy may become the novel strategies for the treatment of diabetic neuropathy.

In conclusion, we have first documented the beneficial effect of transplantation of cord blood–derived EPCs on diabetic neuropathy. Although further study with a long duration of diabetes will be required, the effect was localized in the injected-side hindlimb, and no adverse effects were detected in the present study, suggesting the safety and usefulness of EPC transplantation for the treatment of diabetic neuropathy. The ex vivo cell therapy, consisting of culture-expanded EPC transplantation, may be a novel therapy for diabetic neuropathy.

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