UltraRapid Communication

Circulating Progenitor Cells Regenerate Endothelium of Vein Graft Atherosclerosis, Which Is Diminished in ApoE-Deficient Mice

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Abstract—Previously we showed that a large number of endothelial cells in vein grafts undergo apoptosis or necrosis during the first few days followed by endothelial regeneration. In the present study, we investigated endothelial cell death and regeneration in vein grafts using transgenic mice carrying LacZ genes driven by an endothelial TIE2 promoter. When a vein fragment from TIE2-LacZ was isografted into the carotid artery of wild-type mice, the number of β-gal$^+$ cells were reduced at 3 days and disappeared completely by 4 weeks after grafting. Conversely, β-gal$^+$ cells were observed on the surface of vein segments donated by wild-type mice isografted into TIE2-LacZ mice at 1 week and reached confluence by 4 weeks, suggesting recipient origins of endothelial cells. Interestingly, β-gal$^+$ cells were evenly distributed on the surface of the whole vein segment grafted into TIE2-LacZ mice, indicating a contribution of circulating progenitor cells. When wild-type veins were grafted into a chimeric mouse carrying TIE2-LacZ genes in bone marrow cells, a proportion of cells displayed a β-gal$^+$ staining. Furthermore, the number of CD34$^+$ and Flk$^+$ progenitor cells in blood of apoE-deficient mice were significantly lower than those of wild-type controls, which coincided with diminished β-gal$^+$ endothelial cells on the surface of vein grafts in TIE2-LacZ/apoE$^{-/-}$ mice. Thus, we provide the first evidence that endothelial cells of vein grafts are derived from circulating progenitor cells, of which one-third are derived from bone marrow progenitor cells. Hyperlipidemia due to apoE deficiency results in a lower number of endothelial progenitors in blood and correlated with enhanced atherosclerosis. The full text of this article is available online at http://www.circresaha.org. **(*Circ Res*. 2003;93:e76-e86.)**

Key Words: vein grafts ■ atherosclerosis ■ mouse model ■ endothelial cells ■ progenitor cells

Autologous vein grafts remain the only surgical alternative for many types of vascular reconstruction, although the patency rate is limited due to obliterative stenosis of the grafted vessels.[1,2] The occlusion of the vessel is due to the formation of atherosclerosis-like lesions in the intima, in which endothelial dysfunction or damage could be a crucial event in initiating the pathogenesis.[3] We demonstrated that the earliest cellular event in vein grafts is cell death, ie, apoptosis and necrosis.[4] Others have shown that an extensive loss of endothelial cells was observed in the intima at the early stage of vein grafts.[5] Although surgical process is a cause for the endothelial damage of vein grafts, it is believed that mechanical stress plays a major role,[6] because the grafted veins are subjected to increased biomechanical forces in the form of stretch stress due to blood pressure. The sudden elevation in mechanical forces could be a strong stimulus to the grafted vessel wall and result in activation of intracellular signal pathways leading to gene expression and cell death.[7] After endothelial death is cell regeneration, which was recently well documented by Ehsan et al.[8] However, it is unknown where regenerated endothelial cells in vein grafts are derived from, eg, vein segments per se or circulating endothelial progenitor cells.

Accumulating evidence indicates the presence of endothelial progenitor cells in the blood, which have the capacity to proliferate, migrate, and differentiate into mature endothelial cells.[9–11] Endothelial progenitor cells were characterized by expressing CD34 and vascular endothelial growth factor receptor-2 (VEGFR-2), 2 antigens shared by embryonic endothelial progenitors, and hematopoietic stem cells.[9] Recently, it was demonstrated that progenitor cells in bone marrow of apoE-deficient mice were significantly decreased during aging,[12] and endothelial progenitor cells contributed to repairing denuded endothelium of injured carotid arteries in animal models,[13–15] highlighting the potential role of these cells in cardiovascular diseases.

The endothelium as a barrier between blood and the subendothelial matrix proteins is essential for preventing thrombus formation and subsequent atherosclerosis development in vein grafts. It is a key issue to know how denuded endothelium is replaced and which cells are responsible for regenerating the endothelium. Due to a lack of appropriate

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animal models, it has been difficult to answer these questions. To take advantage of transgenic animals, we recently developed and characterized a new animal model of vein graft atherosclerosis in wild-type[16] and apoE-deficient mice.[17] The lesion displayed classical complex morphological features and a heterogeneous cellular composition. Furthermore, transgenic mice expressing LacZ genes controlled by specific endothelial or housekeeping gene promoters are now available.[18,19] These mice express β-galactosidase (β-gal) only in endothelial cells (TIE2-LacZ)[18] or all types of cells (ROSA26).[19] When these mice are crossed with apoE knockout mice, which develop spontaneous atherosclerosis,[20] then staining the tissue with X-gal enables the detection of endothelial origins in vein grafts. The present study was designed to clarify the endothelial origins in vein grafts because it is of fundamental importance with regard to the pathobiology of vein graft atherosclerosis. Using our animal models for vein graft atherosclerosis,[17,21] we performed vein isografts in two types of transgenic mice expressing β-gal in endothelial cells, including TIE2-LacZ, TIE2-LacZ/apoE$^{-/-}$, and wild-type mice. We demonstrated that regenerated endothelial cells were derived from circulating endothelial progenitor cells.

Materials and Methods

Mice and Vein Graft Procedure

All animal experiments were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals. Transgenic TIE2-LacZ mice expressing β-gal under the control of the endothelial-specific protein TIE2 promoter[18] and ROSA26[19] mouse were purchased from The Jackson Laboratory, Bar Harbor, Maine. An endothelial-specific enhancer was introduced into the first intron of the mouse *TIE2* gene. A combination of the *TIE2* promoter with the intron fragment containing this enhancer allows gene expression specifically and uniformly in virtually all vascular endothelial cells throughout embryogenesis and adulthood.[18] The ROSA26 mouse is a β-gal transgenic mouse produced by random retroviral LacZ gene insertion into embryonic stem cells and expresses β-gal activity in adult tissues.[19] The β-gal activity of cells from both mice is mainly localized in the nucleus. ApoE$^{-/-}$ mice (Jackson Laboratories) were crossed with TIE2-LacZ mice in our laboratory, and heterozygous offspring were mated to produce apoE-deficient mice expressing β-gal in endothelial cells (TIE2-LacZ/apoE$^{-/-}$). Three genotypes of LacZ$^{-/-}$, $^{+/-}$, and +/+ mice were identified using The Jackson Laboratory's PCR protocol (primers, 5′-ATCCTCTGCATGGTCAGGTC-3′ and 5′-CGTGGCCTGATTCATTCC-3′). For apoE$^{-/-}$ mice genotyping, a similar protocol was used with primers: oIMR180 5′-GCCTAGCCGAGGGAGAGCCG-3′, oIMR181 5′-TGTGACTTGGGAGCTCTGCAGC-3′, and oIMR182 5′-GCCGCCCCGACTGCATCT-3′. The mice were maintained on a light/dark (12/12 hour) cycle at 22°C receiving food and water ad libitum. The genetic constitution of all mice used in the present study was C57BL/6, which were bred in our laboratory.

The vein graft procedure was similar to that described previously.[22,23] Briefly, 3-month old mice were anesthetized with sodium pentobarbital (50 mg/kg body weight, IP). The vena cava was harvested from the donor. In the recipient, the right common carotid artery was mobilized free from the bifurcation at the distal end toward the proximal, cut in the middle, and a cuff placed at the end. The cuff was made of an autoclavable nylon tube 0.63 mm in diameter outside and 0.5 mm inside (Portex LTD). The artery was turned inside out over the cuff and ligated. The vein segment was grafted between the two ends of the carotid artery by sleeving the ends of the vein over the artery-cuff and ligating them together with the 8-0 suture. The complete grafting procedure required 30 to 40 minutes.

Histology

Mice were anesthetized and perfused with 0.9% NaCl solution via cardiac puncture in the left ventricle and subsequently perfusion fixed with 4% phosphate-buffered formaldehyde (pH 7.2) for 2 and 5 minutes, respectively, as described previously. The grafts were harvested at 1, 3, and 7 days, 4 and 8 weeks postoperatively (five to eight mice per group) by cutting the grafted segments from the native vessels at the cuff end. Femurs and tibias were harvested for either section or bone marrow cell preparations. For sections, bones were fixed with 4% phosphate-buffered formaldehyde at 4°C in the presence of EDTA for 1 week. Vessel samples were fixed with 4% phosphate-buffered formaldehyde at 4°C for 24 hours. The grafts were processed by routine histology and embedded in paraffin. Sections (4 μm) begun at the center of the graft were stained with hematoxylin and eosin (HE) for histological evaluation.[16]

En Face Preparation and X-Gal Staining

Mice were anesthetized and perfused with 0.9% NaCl solution and subsequently perfusion-fixed with 2% formaldehyde and 0.2% glutaraldehyde (pH 7.2) for 2 and 10 minutes, respectively. The procedure for en face preparation is similar to that described elsewhere.[21,24] In short, vein segments were harvested and the samples were fixed with 2% formaldehyde and 0.2% glutaraldehyde at 4°C for 24 hours. Each vessel segment (about 5×5 mm^2) was prepared free from the adventitia and cut open. The procedure for X-gal staining was similar to that described previously.[25] Briefly, vein segments were incubated at 37°C for 18 hours in PBS supplemented with 1 mg/mL X-Gal (Sigma), 5 mmol/L potassium ferricyanide, 5 mmol/L potassium ferrocyanide, and 2 mmol/L $MgCl_2$. Vessel segments were rinsed with 3% DMSO in PBS and mounted with the endothelium up on a glass slide (2.6×7.5 cm). For X-gal staining of other tissues, bone sections and cultured cells were fixed in 2% formaldehyde and 0.2% glutaraldehyde in PBS for 5 minutes and stained similarly as described for vein segments. Positive cells were enumerated under the microscope.

Immunofluorescent Staining

For en face immunostaining, vein grafts were harvested without perfusion, mounted on a silicone plate, and fixed with acetone. For frozen section preparation, vein grafts were harvested without perfusion and immediately frozen in liquid nitrogen. The procedure used for immunofluorescent staining was similar to that described previously.[23] Briefly, both en face grafts and frozen sections were labeled with a rabbit anti–von Willebrand factor antibodies, rat monoclonal antibodies against CD31, CD34 (Abcam Ltd), VE-cadherin, MAC-1 (CD11a/18b), and ICAM-1 (PharMingen) and visualized with swine anti-rabbit Ig or rabbit anti-rat Ig conjugated with FITC (Dakopatts). After blocking with rabbit serum, sections were stained with rabbit biotin-labeled Ig against β-gal and then with streptavidin-Cy3.

Bone Marrow Transplantation

The procedure used for creating chimeric mice was similar to that described previously.[26,27] Briefly, donor mice were killed and the femurs and tibias were removed aseptically. Marrow cavities were flushed with Ca,Mg-free Hanks' Balanced Salt Solution (HBSS) (GIBCO-BRL) using a 25-gauge needle attached to a syringe. Single cell suspensions were prepared by repeat pipetting, and the cell preparations passed through a nylon mesh to remove particulate matter. Cells were washed twice in HBSS, counted using a hemocytometer, and resuspended at 3×10^7 cells/mL before transplantation. Six- to eight-week-old mice received a lethal dose of whole body X-ray irradiation (950 Rads) as described previously.[26] The irradiated recipients received 1×10^7 bone-marrow cells in 0.3 mL RPMI 1640 by tail vein injection. Vein grafts was performed 4 weeks after bone marrow transfer. The efficiency of bone marrow transplantation was monitored by β-gal staining and Y-chromosome

in situ hybridization for bone marrow sections of chimeric mice.[26,27] Two types of chimeric mice were created, ie, wild-type mice with TIE2-LacZ bone marrow and TIE2-LacZ mice with wild-type bone marrow.

Bone Marrow Cell Culture

Harvested bone marrow cells (1×10^6/mL) were plated on 8-well slide chambers in RPMI 1640 supplemented with 20% fetal calf serum and incubated at 37°C in 5% CO_2 for 3 hour. Nonadhered cells were washed away using serum-free RPMI 1640. Adhered cells were fixed and stained for β-gal. Another proportion of cultured cells were incubated at 37°C in the presence of vascular endothelial growth factor (VEGF) and PDGF-BB (10 ng/mL; Sigma) for 7 days, fixed, and stained for β-gal. β-Gal$^+$ cells from both TIE2-LacZ and chimeric mice were counted under the microscope.

Characterization of Blood Cells in ApoE-Deficient Mice

Heparin blood was collected from apoE$^{-/-}$ and apoE$^{+/+}$ mice. Red blood cells were removed as described previously.[28] White blood cells were incubated at 37°C for 12 hours in RPMI 1640 medium supplemented with 20% FCS in a chamber bottle coated with 0.02% gelatin. Nonattached cells were washed away with the medium. Attached cells were fixed with acetone, labeled with rat monoclonal antibodies against cell marker proteins, CD31, CD34 (Abcam Ltd), CD45, VEGFR2 (PharMingen), and visualized by rabbit anti-rat Ig conjugated with FITC. Cells were counterstained with Hoechst 33258 (1 μg/mL) for 5 minutes. Positive cells and total nuclei were enumerated under the microscope.

LDL Isolation and Oxidation

EDTA plasma was pooled from normolipemic, fasting (12 to 14 hour) humans, aged 20 to 30 years. Lipoproteins were prepared by differential centrifugation using solid KBr to adjust the density, as described previously.[29,30] LDL were obtained in fractions between 1.020 to 1.050 g/mL. Concentrations of LDL were determined gravimetrically by aliquot weight after drying, and quantities of lipoproteins were expressed as total weights. LDL oxidation was performed by incubation of LDL (1 mg/mL PBS) with 10 μmol/L $CuCl_2$ at 37°C for 18 hour.[19] The extent of oxidation was assessed by measurement of TBARS (9.8 ± 1.3 nmol/mg).[31,32]

Cell Viability Assays

Bone marrow cells were isolated from apoE$^{-/-}$/TIE2-LacZ and apoE$^{+/+}$/TIE2-LacZ mice and cultivated in the presence of VEGF (10 ng/mL) for 7 days as described above. Cells were fixed and stained for β-gal, and β-gal$^+$ cells were counted under the microscope. Another proportion of cultured cells were cultivated in the presence or absence of TNF-α (100 ng/mL) or oxidized-LDL (200 μg/mL) at 37°C for 24 hours. Cells were fixed and stained for β-gal, and remaining β-gal$^+$ cells were counted under the microscope. Untreated controls were taken as 100% of survival cells.

Statistical Analysis

Statistical analyses were performed on Macintosh Computer using the Mann-Whitney *U* test and ANOVA, respectively. A value of $P<0.05$ was considered significant.

Results

Endothelial Damage and Neointimal Lesions

In these experiments, the vena cava was used as the donor vessel for isografting into the carotid arteries of littermates. Figure 1a shows HE-stained sections from freshly harvested vena cava showing an integrated endothelium plus 2 layers of other cells in the intima and media of the vessel wall. One day after vein graft surgery, mononuclear cell attachment to the surface of the intima was observed (Figure 1b), but a large number of cell nuclei were lost in the 3-day graft (Figure 1c). Cells appeared on the surface of the vein graft 1 week postoperatively, although few cells within the intima and media were seen (Figure 1d). After cell death, a massive mononuclear cell infiltration into the vessel wall was observed.[16,21] Four weeks after grafting, more than 20 layers of cells formed neointimal lesions in mice, which contain an integrated endothelium on the surface and abundant cells and matrix protein deposition in the neointima (Figure 1e). Interestingly, atherosclerotic lesions with lipid deposition and foam cell formation were observed in 4-week vein grafts of apoE-deficient mice (Figure 1f), which is similar to lesions of vein graft atherosclerosis observed in humans.

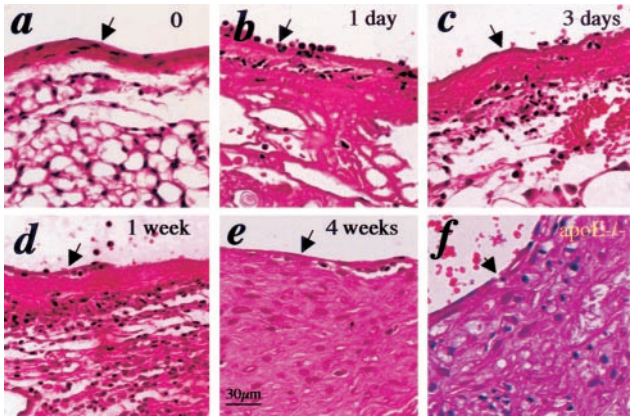

Figure 1. Morphology of neointimal and atherosclerotic lesions of vein grafts in mice. Under anesthesia, vena cava (a) of the mouse was removed and grafted into carotid arteries of wild-type (b through e) or apoE$^{-/-}$ (f) mice. Animals were euthanized at variety of times after surgery, and the grafted tissue fragments fixed in 4% phosphate-buffered (pH 7.2) formaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin-eosin (HE). Arrows indicate the surface of vessel intima. Note that a proportion of lesions was shown in e and f.

β-Gal$^+$ Cells on the Intimal Surface of Grafts

To determine whether β-gal$^+$ endothelial cells of vein segments are denuded after grafting, vessel grafts were harvested at a variety of time points. All vein segments and grafts were in situ perfusion-fixed, harvested, and re-fixed in vitro. After processing and X-gal staining, vessel segments of about 5 mm in length were opened lengthwise and laid flat with the endothelial surface up. The first model of vein grafts was performed by isografting the vena cava from TIE2-LacZ mice to wild-type animals. Endothelial cells of freshly harvested vena cava from TIE2-LacZ mice showed β-gal positivity (Figure 2a), whereas the intensity of blue color of β-gal$^+$ cells in vein grafts was decreased 1 day after surgery (Figure 2b). Surprisingly, no β-gal activity was detected on the surface of vein grafts at 3 days, 1 week, and 4 weeks (Figures 2c, 2d, and 2e). These results suggest that endothelial cells of vessel segments from the donor had died by 3 days after grafting.

There was evidence indicating the integrity of the endothelium of vein grafts after 1 to 4 weeks.[33] To confirm the presence of β-gal$^+$ cells that may be derived from the recipients, we performed the second model of vein grafts by isografting the vena cava from a wild-type mice to TIE2-

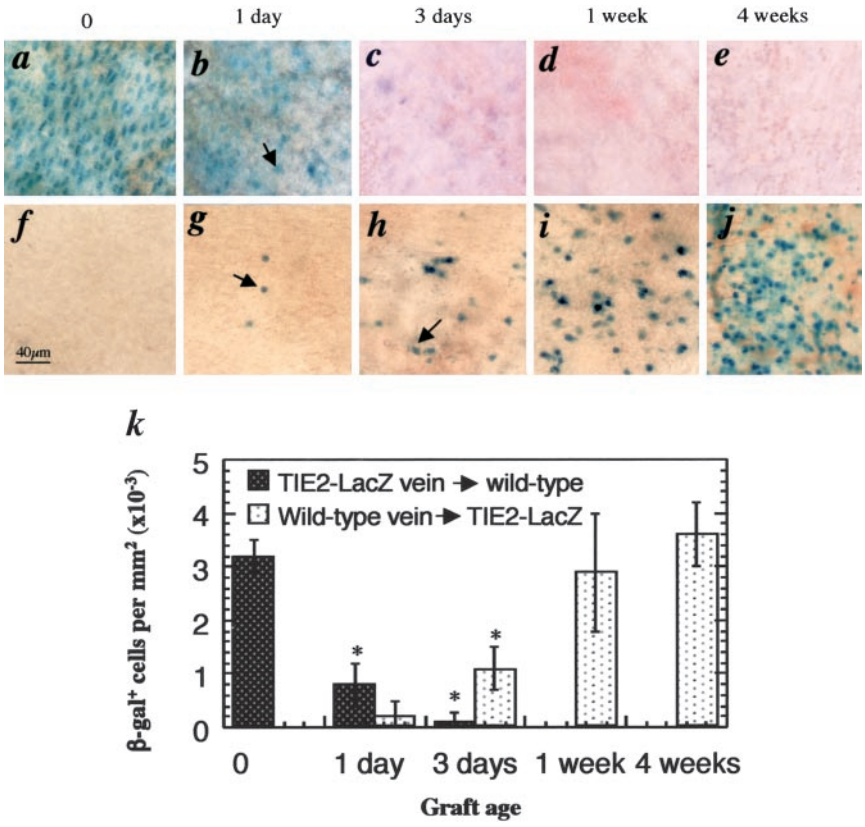

Figure 2. En face staining of β-gal$^+$ cells on vein grafts. a through e, Freshly harvested vena cava segments (a) from TIE2-LacZ mice were isografted into carotid arteries of wild-type mice (b through e). f through j, Freshly harvested vena cava segments (f) from wild-type mice were isografted into carotid arteries of TIE2-LacZ mice (g through j). Grafts were harvested at the time points as indicated and incubated with a substrate X-gal as described in the Materials and Methods. After processing, en face photographs were taken. Blue color indicates β-gal$^+$ cells. Arrows indicate examples of positive cells. The graph (k) summarizes data of mean±SD from 5 animals/group. *Significant difference from the group at day 0, $P<0.001$.

LacZ animals. As shown in Figures 2f through 2j, β-gal$^+$ cells appeared in vein grafts after grafting. As expected, no β-gal$^+$ staining on the surface of the vena cava from wild-type mouse was observed (Figure 2f), which in contrast appeared 24 hours after grafting into the carotid artery of a TIE2-LacZ mouse (Figure 2g). The numbers of β-gal$^+$ cells increased with the age of vein grafts between 3 days and 4 weeks and reached a monolayer on the surface of 4-weeks grafts (Figures 2h through 2j), suggesting that recipient cells replace the lost endothelium of grafts.

To quantify the β-gal$^+$ cell loss and regeneration, a group (n=5 per time point) of animals were used to isograft vein segments to carotid arteries between wild-type and TIE2-LacZ animals. Figure 2k shows statistical data indicating the number of β-gal$^+$ cells in vein segments. At day 1, the grafts were noted to have significant β-gal$^+$ cell loss (989±434 cells/mm^2) compared with those of the freshly harvested veins (3210±440 cells/mm2) ($P<0.01$). Cell density at day 3 was 1215±502 cells/mm^2 ($P<0.05$ when compared with day 0). β-Gal$^+$ cell numbers at day 7 were slightly below that seen in the ungrafted veins (2918±1130 versus 3210±442 cells/mm^2), but no significant difference was found. These data indicate that β-gal$^+$ cells of vein grafts were markedly lost at the early stage and that an equilibrium of cell number is reached by day 7 (Figure 3).

Although it was demonstrated that combination of the *TIE2* promoter with an intron fragment containing the enhancer allows LacZ gene expression specifically to vascular endothelial cells,[18] it was unknown whether β-gal$^+$ cells in vein grafts express endothelial marker proteins. In the present experiment, double immunostaining for von Willebrand factor and β-gal proteins was performed on en face grafts and cross sections. Data shown in Figure 3 indicate that β-gal$^+$ cells were, at least in part, positively stained for von Willebrand factor. As expected, almost all β-gal$^+$ cells in freshly harvested veins (normal vessel) showed von Willebrand factor$^+$, whereas no staining for negative controls was seen. Interestingly, all β-gal$^+$ cells in wild-type vein segments isografted into TIE2-LacZ mice had a positivity for von Willebrand factor. No β-gal$^+$ cells in TIE2-LacZ vein segments isografted into wild-type mice was seen, although von Willebrand factor was extensively stained positive (Figure 3).

Recipient Origins of Regenerated Endothelial Cells by Circulating Progenitors

As described above, our data clearly demonstrated the disappearance of β-gal$^+$ staining from donor vessels after vein segments from TIE2-LacZ mice were isografted into wild-type animals and the appearance when wild-type veins were grafted into TIE2-LacZ mice. These findings established the recipient origins of endothelial cells of vein grafts. The next step was to clarify whether recipient-derived endothelial cells originated from circulating blood or from carotid arteries where the vein grafts were anastomosed. β-Gal$^+$ endothelial cells of the carotid artery from TIE2-LacZ mice were evenly distributed on the surface (Figure 4a). If regenerated endothelial cells of vein grafts were the results of migration from the anastomosed artery, then β-gal$^+$ cells would be seen only in the early stages at the ends of the vein graft. We have analyzed the distribution pattern of β-gal$^+$ cells on the surface of all vein grafts, and found that β-gal$^+$ cells appeared equally on all areas of grafted vessels. Figure 4 shows representative

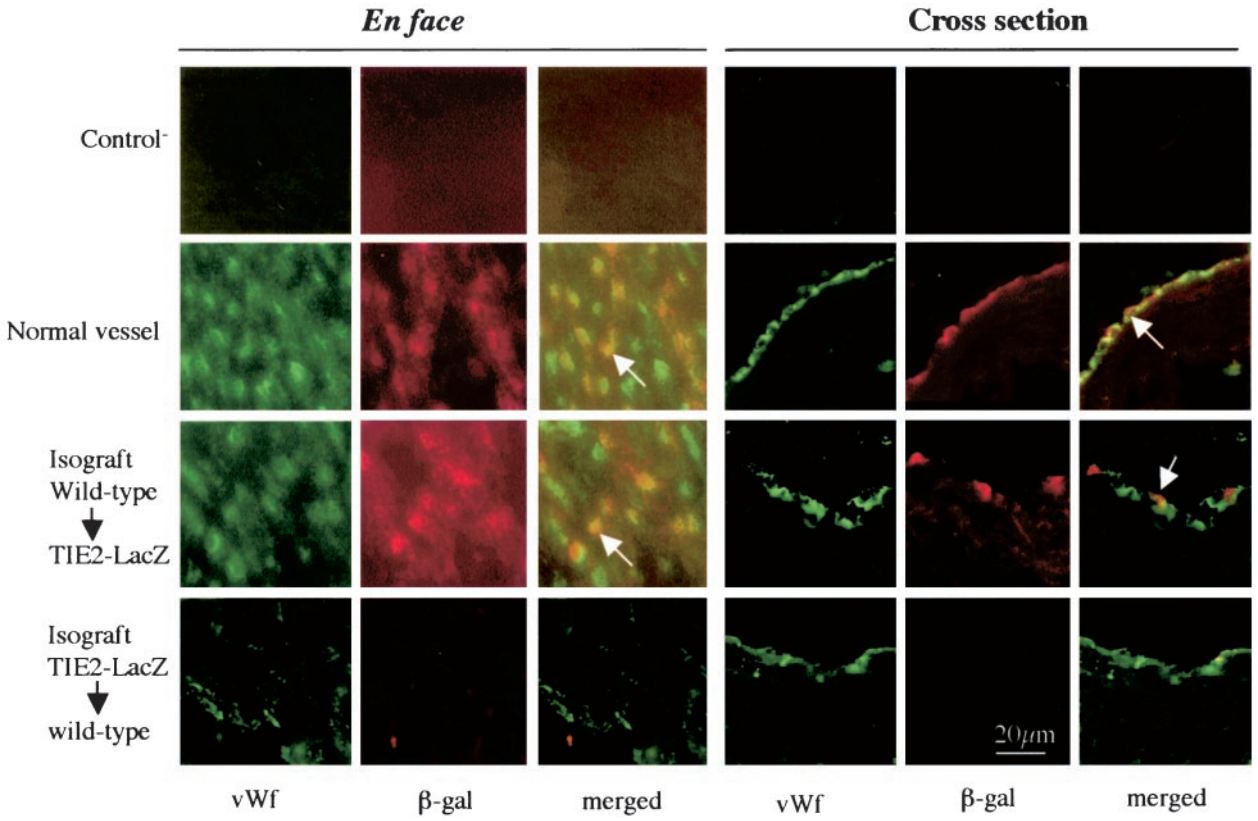

Figure 3. Double-immunofluorescent staining for β-gal and von Willebrand factor in the vessels. Freshly harvested veins (normal vessel) and 4-week allograft were prepared for en face or cross section, double labeled for β-gal, and von Willebrand factor was performed as described in the Materials and Methods. Arrows indicate examples of double-positive cells on the endothelium isografts.

data indicating that the number of β-gal$^+$ cells on the surface of vein grafts near the anastomosed artery is similar to that in the center of the graft 3 days postoperatively (Figures 4b and 4c). These findings suggest that regenerated endothelial cells possibly were derived from circulating blood rather than anastomosed artery.

Contribution of Bone Marrow Cells

As endothelial cells were identified as originating from circulating blood, therefore it would be important to determine the possible contribution of bone marrow cells. In the present study, chimeric mice were created by bone marrow transplantation after irradiation. To control the efficiency of bone marrow transfer, B6/ROSA26 mice expressing β-gal in all types of cells with the nucleus were used as bone marrow donors. Almost all bone marrow cells with nuclei from B6/ROSA26 chimeric mice were stained blue (β-gal$^+$; Figure 5a), whereas no β-gal activity was seen in bone marrow donated by wild-type mice (Figure 5b). Thus, the efficiency of bone marrow transplantation in our chimeric mice was high.

There is evidence that bone marrow cells can differentiate into endothelial cells.[34,35] To confirm whether the bone marrow cells of TIE2-LacZ mice can also differentiate into endothelial cells expressing β-gal, cells were cultivated in the presence of VEGF. This growth factor has been shown to effectively stimulate stem cells to differentiate into endothelial cells. Interestingly, a population of bone marrow cells from TIE2-LacZ mice showed β-gal positivity in response to VEGF (Figure 5c), and negative staining in the absence of VEGF (Figure 5d). To create chimeric animals, bone marrow cells from TIE2-LacZ mice were harvested and transferred to irradiated wild-type mice. The numbers of β-gal$^+$ cells in cultured bone marrow cells from TIE2-LacZ mice and chimeric mice carrying TIE2-LacZ marrows were similar (Figure 5e), further supporting the high efficiency of marrow transplantation.

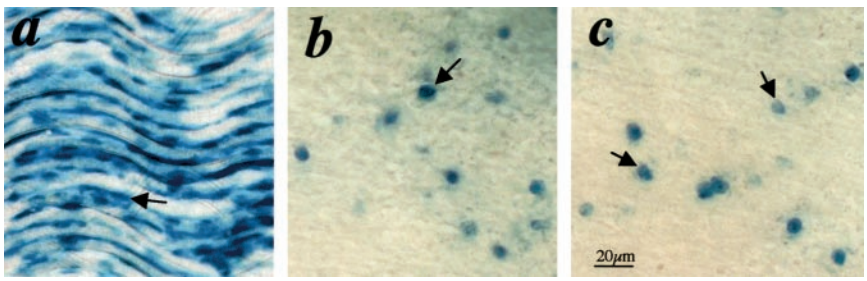

Figure 4. En face photographs showing distribution of regenerated β-gal$^+$ cells on vein grafts. a, Photograph of carotid artery from a TIE2-LacZ mouse served as a recipient. Wild-type vein segments were grafted into TIE2-LacZ mice and harvested 3 days postoperatively. After β-gal staining and en face preparation, positive endothelial cells of vein graft near the end (b) and the center (c) were compared. Arrows indicate examples of positive cells.

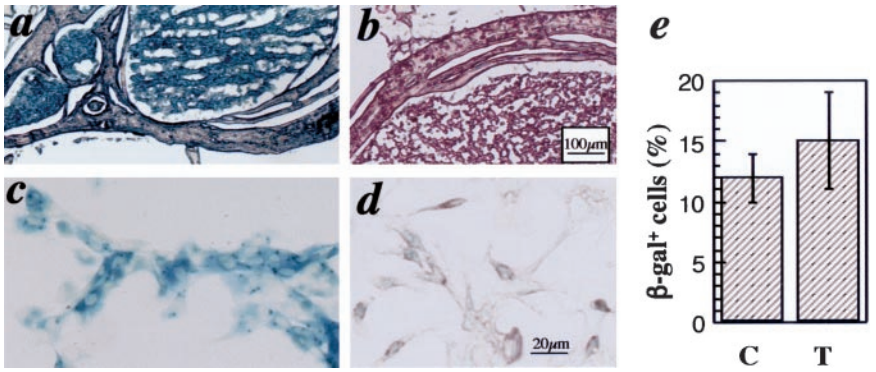

Figure 5. Creation of chimeric mice and culture of bone marrow cells. Femurs of the chimeric mouse with ROSA26 (a) or wild-type (b) bone marrow (BM) transplantation were harvested, sectioned, and stained for X-gal, in which positive cells were stained blue. Bone marrow cells from chimeric mice receiving TIE2-LacZ bone marrow were harvested and cultivated in RPMI 1640 supplemented with 20% FCS for 7 days in the medium in the presence (c) or absence (d) of VEGF and PDGF-BB (10 ng/mL). Cells were stained for β-gal. e, Statistical data of mean±SD (n=4), indicating no significant difference between chimeric (C) and TIE2-LacZ (T) mice.

Vena cava segments from both wild-type (Figure 6a) and chimeric mice having TIE2-LacZ bone marrows were β-gal negative (Figure 6b), indicating that bone marrow cells did not replace the endothelium of native vessels. When vein segments from wild-type mice were grafted into chimeric mice with bone marrow derived from TIE2-LacZ mice, β-gal activity was seen on the surface of vein grafts (Figure 6c) 1 week after surgery. Figure 6d shows a representative picture of β-gal$^+$ cells from a wild-type vein grafted into a TIE2-LacZ chimeric mouse that received wild-type bone marrow. No difference of β-gal$^+$ cells was observed between freshly harvested vena cava of TIE2-LacZ mice and chimeric mice that received wild-type bone marrow (Figures 6e and 6f), further supporting the notion that bone marrow cells did not

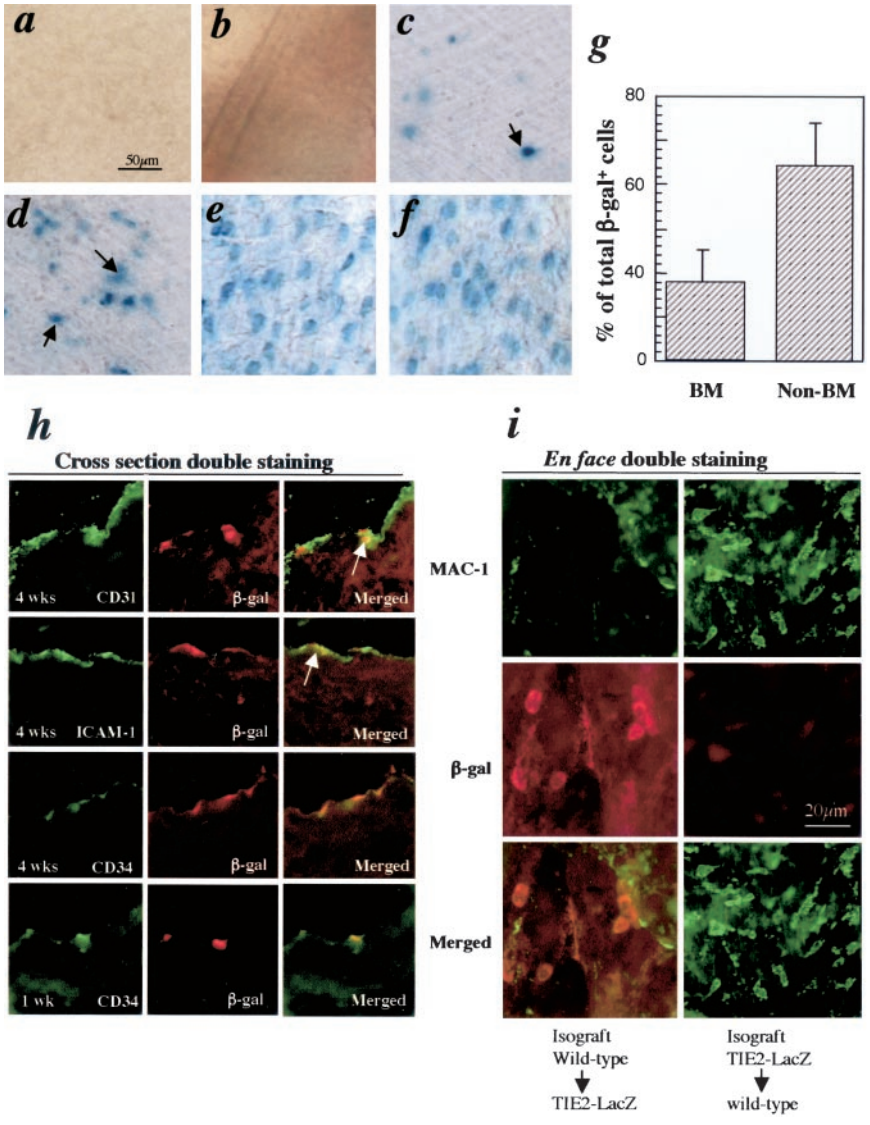

Figure 6. Bone marrow (BM) and non-BM origins of endothelial cells. Wild-types were isografted into the carotid arteries of chimeric mice (c; wild-type/TIE2-LacZ BM) (d; TIE2-LacZ/wild-type BM). Vein grafts were harvested 1 week after grafting, stained with X-gal and processed for en face photography. a, Picture of vena cava of a wild-type mouse as a recipient. b, Picture of vena cava of a chimeric mouse (wild-type with TIE2-LacZ bone marrow) 8 weeks after bone marrow transplantation. e, Picture of vena cava of a TIE2-LacZ mouse as a bone marrow donor. f, Picture of vena cava of a chimeric mouse (TIE2-LacZ with wild-type bone marrow) 8 weeks after bone marrow transplantation. g, Summary of data of mean±SD from 4 animals per group. Group one consisted of isografts donated by wild-type animals, which were grafted into wild-type mice with TIE2-LacZ bone marrow (BM). Group two consisted of isografts from wild-type animals to TIE2-LacZ mice with wild-type bone marrow (non-BM). Grafts were harvested 1 week after surgery and developed with X-gal. β-Gal$^+$ cells on the surface of the grafts were counted separately. Percentage of non- and bone marrow–derived β-gal$^+$ cells was calculated against total positive cells of two groups serving as 100%. h, Double immunostaining for β-gal and cell markers (CD31, CD34, and ICAM-1) in cross sections of 4-week isografts from wild-type animals to chimeric mice (wild-type with TIE2-LacZ bone marrow). Arrows indicate examples of double-positive cells. i, En face double immunostaining for β-gal and macrophage marker MAC-1 in 1-week isografts from wild-type animals to TIE2-LacZ mice (left) or from TIE2-LacZ mice to wild-type animals (right). Note no double positive cells seen.

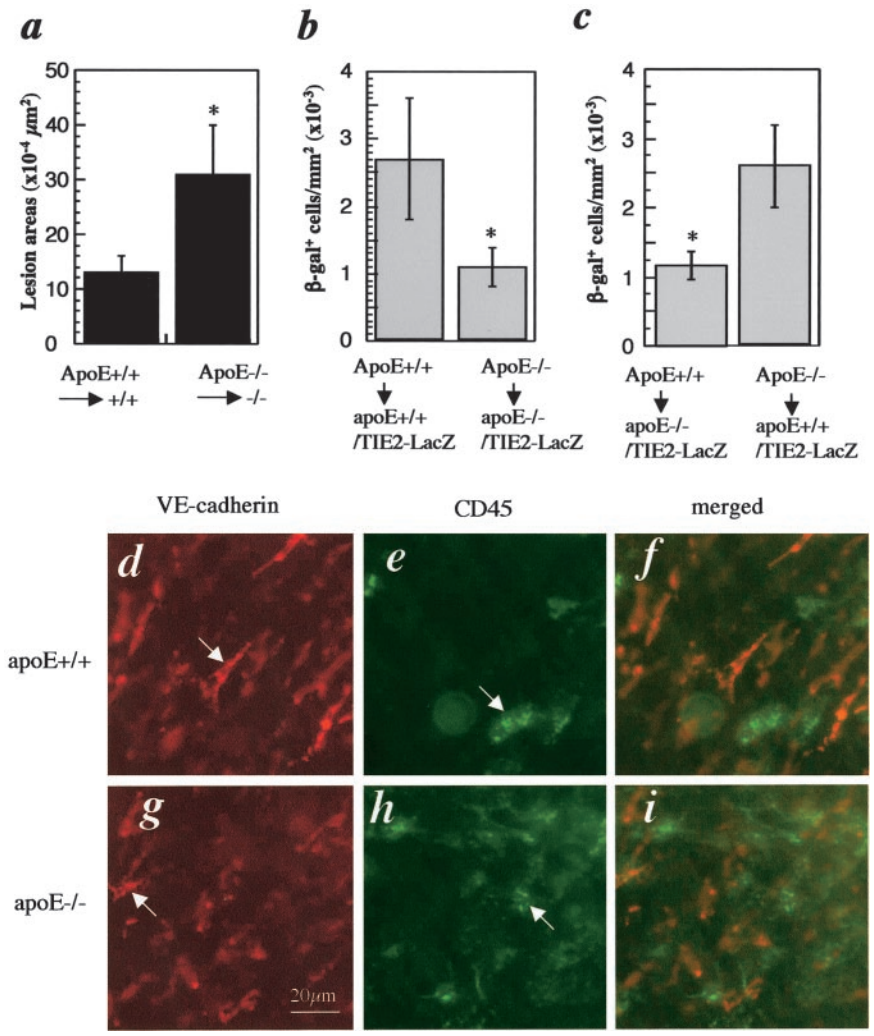

Figure 7. Diminished endothelial regeneration of vein grafts in apoE$^{-/-}$ mice. Four genotypes of mice (TIE2-LacZ/apoE$^{-/-}$, TIE2-LacZ/apoE$^{+/+}$, apoE$^{+/+}$, and apoE$^{-/-}$) were used for these experiments. a, Areas of neointimal (wild-type) and atherosclerotic (apoE$^{-/-}$) lesions 4 weeks after grafting. b, Data of β-gal$^+$ cells on the surface of vein segments donated by apoE$^{+/+}$ or apoE$^{-/-}$ mice, which were isografted into carotid arteries of TIE2-LacZ/apoE$^{+/+}$ and TIE2-LacZ/apoE$^{-/-}$ mice, respectively. c, Data of β-gal$^+$ cells on the surface of vein segments donated by apoE$^{+/+}$ or apoE$^{-/-}$ mice, which were isografted into carotid arteries of TIE2-LacZ/apoE$^{-/-}$ and TIE2-LacZ/apoE$^{+/+}$ mice, respectively. Grafts were harvested 1 week postoperatively, processed for en face, and stained with X-gal. β-Gal$^+$ cells on the surface of vein grafts were enumerated under the microscope. Data are mean±SD from 5 animals per group. *Significant difference between two groups, $P<0.01$. d through i, En face double staining for VE-cadherin and CD45 in vein grafts 4 weeks after surgery. Arrows indicate examples of positive cells.

repair the endothelium of normal vessels within 8 weeks after marrow transplantation. Figure 6g summarizes data from six animals per group indicating that about one third (38%) of regenerated β-gal$^+$ endothelial cells were derived from bone marrow cells.

To characterize bone marrow–derived β-gal$^+$ cells in vein grafts, double immunostaining for β-gal and other cell markers was explored. When wild-type vein segments were isografted into chimeric mice (wild-type with TIE2-LacZ bone marrow), β-gal$^+$ cells in the sections of 4-week grafts were strongly stained by CD31 and ICAM-1 antibodies, respectively, indicating that bone marrow-derived β-gal$^+$ cells in grafts express endothelial cell markers (Figure 6h). Some β-gal$^+$ cells in the grafts also showed a CD34$^+$ staining, although the level of CD34$^+$ staining was different between 1- and 4-week grafts (Figure 6h). Furthermore, en face double immunostaining for MAC-1 and β-gal in 1-week grafts indicated that MAC-1$^+$ macrophages had no β-gal activity (Figure 6i).

Diminished Regeneration of β-Gal$^+$ Endothelial Cells in ApoE$^{-/-}$ Mice

Previously, we provided evidence of accelerated atherosclerotic lesions in vein grafts of apoE$^{-/-}$ mice,[17] which might be due to dysfunction of endothelial progenitor cells. To test this hypothesis, TIE2-LacZ and apoE$^{-/-}$ mice were crossed, and apoE-deficient mice expressing LacZ genes in endothelial cells (TIE2-LacZ/apoE$^{-/-}$ mice) were generated. When vein grafts were performed in these mice, atherosclerotic lesions in the vein grafts of TIE2-LacZ/apoE$^{-/-}$ mice were significantly increased 4 weeks after grafting (Figure 7a). Interestingly, the numbers of β-gal$^+$ cells on the surface of the isograft in apoE$^{-/-}$/TIE2-LacZ mice were markedly diminished at 7 days (apoE$^{-/-}$/TIE2-LacZ versus apoE$^{+/+}$/TIE2-LacZ; 1098±298 versus 2720±902 cells/mm^2) ($P<0.05$; Figure 7b).

The mechanism of lower rate of cell regeneration in TIE2-LacZ/apoE$^{-/-}$ mice was investigated by undertaking vein isografts between TIE2-LacZ/apoE$^{-/-}$ and TIE2-LacZ/apoE$^{+/+}$ mice. When apoE$^{+/+}$ vein segments were grafted into carotid arteries of TIE2-LacZ/apoE$^{-/-}$ mice, a lower number of β-gal$^+$ cells in the vein graft were seen (Figure 7c). However, β-gal$^+$ cells of apoE$^{-/-}$ veins grafted into TIE2-LacZ/apoE$^{+/+}$ mice showed a similar number of positive cells in the grafts. These data indicate that the alteration of endothelial progenitor cells in apoE$^{-/-}$ mice may be responsible for the decreased numbers of β-gal$^+$ cells, and that the donor vessel per se from apoE$^{-/-}$ mice had little effect.

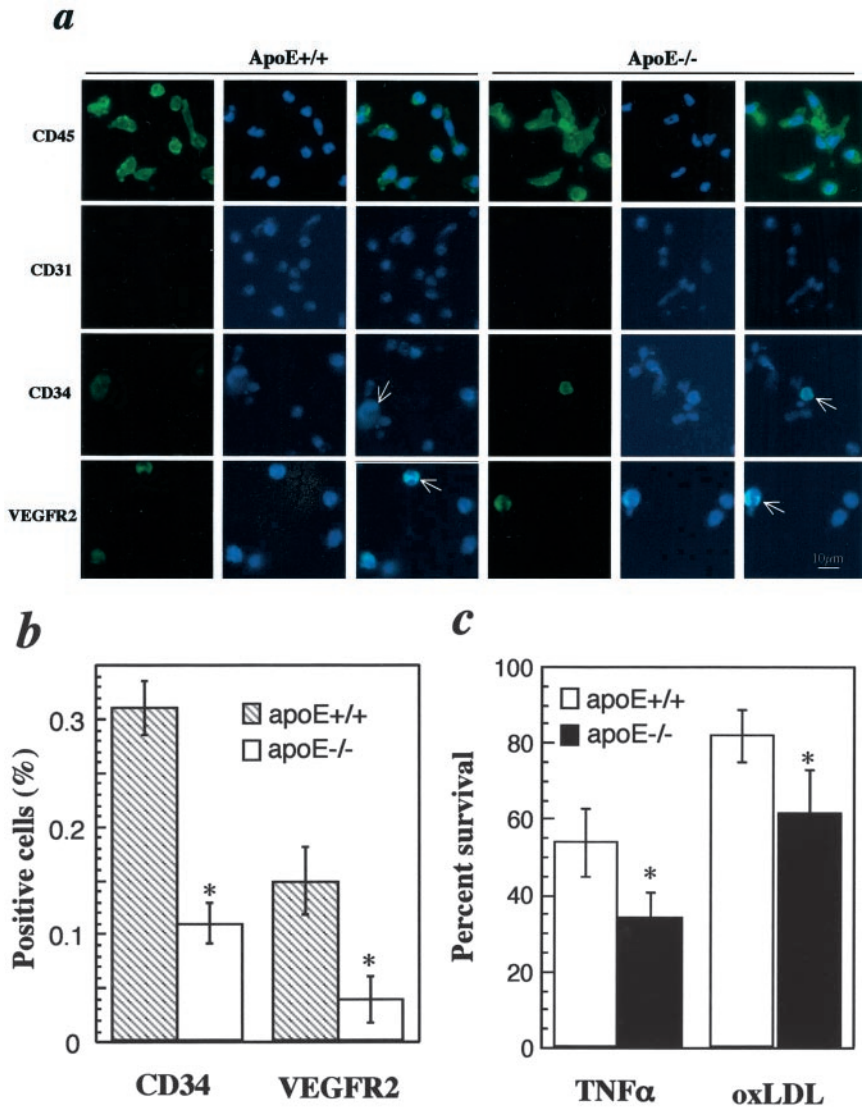

Figure 8. Comparison of blood endothelial progenitor cells between apoE$^{-/-}$ and apoE$^{+/+}$ mice. a, Immunostaining of blood mononuclear cells. Attached blood mononuclear cells were labeled with rat monoclonal antibodies and visualized with anti-rat Ig-FITC. Nucleus staining was performed using Hoechst 33258. b, Statistical data of mean±SD (n=5). *Significant difference from apoE$^{+/+}$ mice. c, Progenitor cell survival in response to TNF-α and oxidized-LDL. Bone marrow cells from apoE$^{-/-}$/TIE2-LacZ and apoE$^{+/+}$/TIE2-LacZ mice were harvested and cultivated for 7 days in the medium in the presence of VEGF (10 ng/mL). Cells were further incubated at 37°C for 24 hours in the presence or absence of oxidized-LDL (200 μg/mL) or TNF-α (100 ng/mL). Cells were washed, fixed, and stained for β-gal. Data are mean±SD of three experiments. *Significant difference between apoE$^{-/-}$/TIE2-LacZ and apoE$^{+/+}$/TIE2-LacZ cells.

To further characterize regenerated endothelial cells of vein grafts in apoE$^{-/-}$ mice, VE-cadherin, an endothelial marker, and CD45, a leukocyte marker, were double labeled on en face segments of 4-week grafts. The intensity of fluorescence staining for VE-cadherin in apoE$^{-/-}$ mice was much weaker than that of wild-type controls, whereas CD45^{+} cells were abundant on the surface of the graft in apoE$^{-/-}$ mice (Figures 7d through 7i). These results support the notion of decreased endothelial cell regeneration in, and increased leukocyte adhesion to, vein grafts of apoE$^{-/-}$ mice. Furthermore, VE-cadherin^{+} cells in vein grafts were CD45 negative, confirming the nature of endothelial cells.

The mechanism of decreased endothelial regeneration in vein grafts were further emphasized by in vitro studies, for which blood mononuclear cells were cultivated from both apoE$^{-/-}$ and apoE$^{+/+}$ mice, and stained for progenitor markers. As expected, almost all cells expressed CD45 proteins, whereas no CD31^{+} cells were detectable (Figure 8a). CD34^{+} and VEGFR2^{+} cells were found in the blood of both apoE$^{-/-}$ and apoE$^{+/+}$ animals, but significantly lower in apoE-deficient mice (Figures 8a and 8b). Theoretically, the reduction of blood VEGFR2^{+} cells in apoE$^{-/-}$ mice could be due to either decreased proliferation or increased cell death of bone marrow progenitor cells or both. Therefore, we compared the endothelial progenitor cells in bone marrows between apoE$^{-/-}$ and apoE$^{+/+}$ mice. After stimulation with VEGF, the number of β-gal^{+} cells from apoE$^{-/-}$/TIE2-LacZ and apoE$^{+/+}$/TIE2-LacZ were similar (12.5±2.2% versus 13.2±3.2%), indicating a similar ability of cell proliferation/differentiation. However, these cells were more sensitive to apoptotic stimuli, when they were incubated with TNF-α and oxidized-LDL (Figure 8c). These results suggest that decreased numbers of endothelial progenitors in blood are largely caused by short life span of the cells due to increased apoptosis.

Discussion

The integrity of the vessel wall endothelium is essential for preventing the formation of thrombosis and subsequent atherosclerosis. It is known that endothelial cells of vein grafts are damaged at early stage and regenerate thereafter.[4,8] It was believed that the remaining endothelial cells of vein grafts replace dead cells to cover the denuded surface of the

vessel.[36,37] In the present study, we provide the first evidence that the regenerated endothelial cells of vein grafts are originated from recipient circulating blood, and not the remaining endothelial cells of donor vessels. We also demonstrate that about one-third of endothelial cells of vein grafts are derived from bone marrow progenitor cells. These data establish that circulating progenitor endothelial cells cover the surface of neointimal and atherosclerotic lesions of vein grafts. Thus, our findings are crucial for understanding the pathogenesis of vein graft atherosclerosis, and in establishing new strategies for therapeutic intervention in this disease.

One of the most important observations in this study was the complete loss of the endothelium in vein grafts within 3 days after surgery, which was not yet fully replaced by circulating endothelial cells. This indicated that certain surface areas of the vein graft lack a monolayer of endothelial cells for an initial period of time. Such exposure of the subendothelial matrix proteins to blood can be a risk for thrombosis formation. Supporting this notion is the fact that between 3% and 12% of saphenous vein grafts in patients occlude in the first week due to the formation of a large thrombosis,[1,38,39] which can be explained by our findings of the presence of denuded endothelium in vein grafts. If the original endothelium of vein segments could be better preserved at an early stage, reduced thrombosis or atherosclerotic lesions in vein grafts would be seen.

What is the initial factor or stimulus resulting in endothelium denudation of grafted veins? Surgical or traumatic and ischemic injury to the vein segments may be partially responsible for endothelial damage in the vein grafts, but we posit that mechanical stress plays a key role in initiating endothelial apoptosis via signal transduction pathways leading to p53 activation.[7] In grafted veins, mechanical forces on the vessel segment suddenly increase more than 10-fold (arterial versus venous blood pressure), which provides a strong stimulus to vascular endothelial cells. We previously demonstrated that acutely elevated blood pressure and mechanical stress activate p38MAPK-p53 signal pathways, which leads cell apoptosis in vivo and in vitro.[4,40,41] Other reports have established that the limitation of mechanical stretch by an external stent on vein segments significantly reduces cell apoptosis in grafted vessels,[42] ie, better preserved endothelium in vein grafts, which results in a marked reduction of atherosclerotic lesions.[42,43] Thus, altered mechanical stress could be largely responsible for the early damage of endothelial cells in vein grafts.

Recent data from Dzau's group demonstrated that ex vivo treatment of vein segments with E2F decoy oligonucleotide significantly inhibits neointimal lesions of vein grafts in animal models[44] and humans.[45] This treatment sufficiently inhibits smooth muscle cell proliferation, because about half of the neointimal smooth muscle cells in vein grafts are derived from the donor vessel.[27] They also found that E2F decoy treatment did not inhibit endothelial cell proliferation in vein grafts,[8] for which our findings would give a better explanation, ie, that ex vivo treatment of vessel segments has no effect on endothelial cells derived from circulating blood. On the other hand, their data provide indirect support to our results for the endothelial origins of vein grafts.

Accumulating evidence indicates the existence of two types of circulating endothelial cells, ie, bone marrow–derived progenitors and vascular (progenitor) endothelial cells.[46,47] Bone marrow–derived endothelial progenitors are largely responsible for generating endothelial cells of microvessels or neovascularization in ischemic or damaged tissues.[35,48] The present data provide the first evidence that more than 60% of regenerated endothelial cells on the surface of large vessels (vein grafts) are originated from non–bone marrow progenitor cells, indicating that vascular progenitor cells in blood are main sources. However, further studies would be needed to clarify where non–bone marrow progenitor cells are derived from, and how they are released into the blood.

Recent evidence indicates that smooth muscle progenitors exist in circulating blood,[49] and that $VEGFR2^+$ cells derived from embryonic stem cells can differentiate into both endothelial and smooth muscle cells.[50] Using the ROSA26 and SM-LacZ transgenic mice expressing LacZ gene in either all types of nuclear cells or only in smooth muscle cells, we previously demonstrated that about 60% of smooth muscle cells were derived from donor vessels and 40% from circulating blood.[27] There was no evidence that bone marrow progenitor cells contribute to the accumulation of smooth muscle cells in lesions using chimeric mouse models. The present results indicate that about one-third of regenerated endothelial cells were derived from bone marrow cells. Thus, bone marrow progenitors can partially contribute to the formation of the endothelium, but not smooth muscle cells in vein graft atherosclerosis.

It is established that endothelial dysfunction is a crucial event in the development of atherosclerosis, which can be induced by hyperlipidemia.[51–53] In the mouse model, atherosclerotic lesions of vein grafts in $apoE^{-/-}$ mice were accelerated, because of delayed regeneration of endothelial cells. Data from cross-isografting between $apoE^{-/-}$ and $apoE^{+/+}$ mice indicate an alteration of blood endothelial progenitor cells, but not vein segments per se. This decreased ability of endothelial progenitors to regenerate the endothelium in $apoE^{-/-}$ mice may be due to lower concentrations of endothelial progenitors, because $CD34^+$ and $VEGFR2^+$ cells were significantly decreased compared with wild-type controls. Concomitantly, it was demonstrated that the number of circulating endothelial progenitor cells were decreased in patients with coronary heart disease, which was inversely correlated with cardiovascular risk.[10,54] Therefore, enhancement of blood endothelial progenitor cell concentrations by drugs or cell therapy should be considered as therapeutic interventions for vein graft disease.

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