

Endothelial Replacement and Angiogenesis in Arteriosclerotic Lesions of Allografts Are Contributed by Circulating Progenitor Cells

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Background—Endothelial regeneration and angiogenesis in the intima of the arterial wall are key events in the pathogenesis of transplantation arteriosclerosis. The traditional hypothesis that damaged endothelial cells are replaced by remaining cells of the donor vessel has been challenged by recent observations, but the cell origins of large arteries and microvessels are still not well established.

Methods and Results—Aortic segments were allografted between Balb/c and TIE2-LacZ (C57BL/6) mice expressing β -galactosidase (gal) in endothelial cells. β -gal⁺ cells in TIE2-LacZ vessels grafted to Balb/c mice completely disappeared, whereas the positive cells found in Balb/c aorta allografted into TIE2-LacZ mice 4 weeks after surgery indicated a host origin. En face analysis of allograft vessels displayed a unique distribution of β -gal⁺ cells on the surface at 3 days, 1 week, and 4 weeks. Interestingly, $35 \pm 19\%$ β -gal⁺ cells were found in arterial segments allografted into chimeric mice with TIE2-LacZ bone marrows. Furthermore, endothelial cells of microvessels within allografts had a β -gal⁺ staining in the media at 1 week and in the neointimal lesions and adventitia at 4 weeks. Allograft studies in chimeric mice demonstrated that β -gal⁺ cells of microvessels in transplant arteriosclerosis were derived from bone marrow progenitors.

Conclusions—We provide strong evidence that endothelial cells of neointimal lesions in allografts are derived from circulating progenitor cells and that bone marrow–derived progenitors are responsible for angiogenesis of the allograft, that is, the formation of microvessels in transplant arteriosclerosis. (*Circulation*. 2003;108:3122-3127.)

Key Words: transplantation ■ arteriosclerosis ■ angiogenesis ■ endothelium ■ lesion

Allograft-accelerated transplantation arteriosclerosis remains the leading cause of late graft failure and death in patients with solid organ transplantation.¹⁻³ It is believed that the pathogenesis of transplantation arteriosclerosis is initiated by alloimmune damage of endothelium followed by smooth muscle cell migration and proliferation within the intima.¹ According to this concept, neointimal endothelial cells originate from graft tissue and are therefore donor-derived, which is supported by several reports.⁴⁻⁷ However, this concept is challenged by recent findings that both endothelial and smooth muscle cells within arteriosclerotic lesions are derived from recipients.⁸⁻¹¹

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Accumulating evidence indicates the impact of angiogenesis in transplantation arteriosclerosis.^{12,13} Microvessels populate many atherosclerotic lesions of allografts and native atheroma in humans and in some lipid-driven models of atherosclerosis.^{14,15} Antiangiogenic treatment can limit experimental atherogenesis of allografts,¹⁶ suggesting that the density of microvessels in lesions is positively correlated with

the development of transplantation atherosclerosis. However, no data exist concerning the endothelial cell origins of these microvessels in atherosclerotic lesions.

Recently, it was demonstrated that adult bone marrow cells can differentiate into vascular endothelial cells,¹⁷ and both endothelial cells and smooth muscle cells could be differentiated from the same stem cells.¹⁸ Bone marrow–derived progenitor cells participate in the formation of microvessels or angiogenesis in ischemia-damaged tissues.^{17,19-21} This has aroused enthusiasm for whether bone marrow progenitor cells contribute to the replacement of endothelial cells in allograft vessels during the development of transplantation atherosclerotic lesions. The source of endothelial cells of allografts and microvessels within arteriosclerotic lesions is a fundamental issue in understanding the pathogenesis and therapy for this disease. Therefore, this study was designed to investigate whether the endothelium of allografted arteries and microvessels within arteriosclerotic lesions originate from donor vessels, recipient vessels, or bone marrow progenitor cells of the recipient. Using our animal models for transplantation atherosclerosis,²² we performed aortic allografts in transgenic

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Summary of Allografts in Mice

Group	Donor	Recipient	No.
1	BALB/c	C57BL/6J-TIE2/LacZ	10
2	C57BL/6J-TIE2/LacZ	BALB/c	10
3	BALB/c	C57BL/6J-TIE2/LacZ with C57BL/6J BM	12
4	BALB/c	C57BL/6J with C57BL/6J-TIE2/LacZ BM	12

BM indicates bone marrow.

mice expressing β -gal only in endothelial cells (TIE2-LacZ) and wild-type control mice. We demonstrated that circulating progenitor cells are responsible for the endothelial regeneration of both large arteries and microvessels, in which bone marrow progenitors are responsible for angiogenesis.

Methods

Mice and Artery Allograft Procedure

Artery transplantation was performed between BALB/c (H2^d) and C57BL/6 (H2^b) mice. TIE2-LacZ transgenic mice expressing β -gal under the control of the endothelial-specific protein TIE2 promoter²³ (The Jackson Laboratory) were crossed to C57BL/6 mice 6 generations in our laboratory before performing the experiments. Endothelium-specific enhancer in the first intron of the mouse *TIE2* gene was introduced. Combination of the *TIE2* promoter with an intron fragment containing this enhancer allows it to target reporter gene expression specifically and uniformly to virtually all vascular endothelial cells throughout embryogenesis and adulthood.²³ Three genotypes of LacZ^{-/-}, LacZ^{+/-}, and LacZ^{+/+} mice were identified by use of Jackson Laboratory PCR protocol.

The arterial graft procedure was similar to that described previously.²² Briefly, 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 artery was turned inside out over the cuff and ligated. The aortic segment was grafted between the two ends of the carotid artery by sleeving the ends of the aorta over the artery cuff and ligation. Total numbers of animals used in each group are summarized in the Table.

Histology

The grafts were harvested at 3 days and 1 and 4 weeks after surgery by cutting the transplanted segments from the native vessels at the cuff end, as described previously.²² Vessel samples were processed in routine histology. Sections (4 μ m) began at the center of the graft were stained with hematoxylin and eosin for histological evaluation.

En Face Preparation and β -Gal Staining

The procedure for en face preparation is similar to that described elsewhere.^{24,25} In short, aortic segments were harvested, and the samples were fixed with 2% formaldehyde and 0.2% glutaraldehyde at 4°C for 24 hours. Each vessel segment ($\approx 5 \times 5$ mm²) was prepared free from the adventitia and cut open. The procedure for X-gal staining was similar to that described previously.²⁶ Briefly, aortic segments were incubated with 1 mg/mL X-Gal (Sigma). Vessel segments were rinsed with 3% DMSO in PBS and mounted with the endothelium up on a glass slide (2.6 \times 7.5 cm). Nuclear counter staining was performed with the use of nuclear Fast Red (Sigma). Positive cells were enumerated under the microscope.

Bone Marrow Transplantation

The procedure used for creating chimeric mice was similar to that described previously.^{10,27} Briefly, femurs and tibias were removed, and marrow cavities were flushed. Single-cell suspensions were prepared by repeat pipetting. Six- to 8-week-old mice received a lethal dose of whole-body irradiation (900 rads) from a radiographic source. Irradiated recipients received 1×10^7 bone marrow cells by tail vein injection. The efficiency of bone marrow transplantation

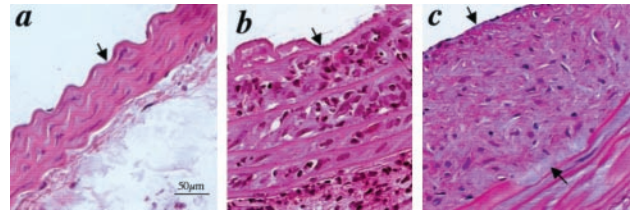


Figure 1. Lesion formation in aortic allografts in mice. Under anesthesia, common carotid arteries (a) of Balb/c mice were removed and grafted into carotid arteries of C57BL/6 (b and c) mice. Animals were killed 1 (b) or 6 (c) weeks after surgery; grafted tissue fragments were fixed in 4% phosphate-buffered (pH 7.2) formaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Arrows indicate intima or neointima.

was >95% as monitored by β -gal staining and Y-chromosome in situ hybridization for bone marrow sections of chimeric mice.^{10,27} Two types of chimeric mice were created, that is, wild-type mice with TIE2-LacZ bone marrow and TIE2-LacZ mice with wild-type bone marrow (Table). Aortic segments from Balb/c mice were transplanted to chimeric mice 4 weeks after bone marrow transfer.

β -Gal Staining for Sections

Eight-micrometer sections were prepared and fixed in 2% formaldehyde and 0.2% glutaraldehyde in PBS for 5 minutes and stained similarly as described for aortic segments. Sections were counterstained with Hoechst 33258 (1 μ g/mL) or Fast Red after β -gal staining.

Immunofluorescent Staining

The procedure used for immunofluorescent staining was similar to that described previously.²⁸ Briefly, frozen sections were labeled with rabbit anti-von Willebrand factor antibodies and visualized with swine anti-rabbit 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.

Statistical Analysis

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

Results

Characterization of Transplantation Arteriosclerosis

Aortic transplantation was carried out between BALB/c (B/c; H2^d) and C57BL/6 (B6; H2^b) mice through the use of a cuff technique, as described previously.²² Freshly harvested aorta comprised a monolayer of endothelium and 4 or 5 layers of smooth muscle cells and elastica laminae (Figure 1a). Interestingly, a large number of cells infiltrated into the media of allografts, which made up the vessel wall, increasing almost 2-fold (Figure 1b). Figure 1c shows data indicating increased neointimal lesions in aortic allografts that contain a large number of cells.

Recipient Origins of Allograft Endothelial Cells

To determine the source of endothelial cells in allografts, Balb/c and TIE2-LacZ transgenic mice as recipients or donors were used for aortic transplantation, and the allografts were analyzed by en face staining for β -gal enzymatic activity through the use of X-gal substrate. Strong β -gal

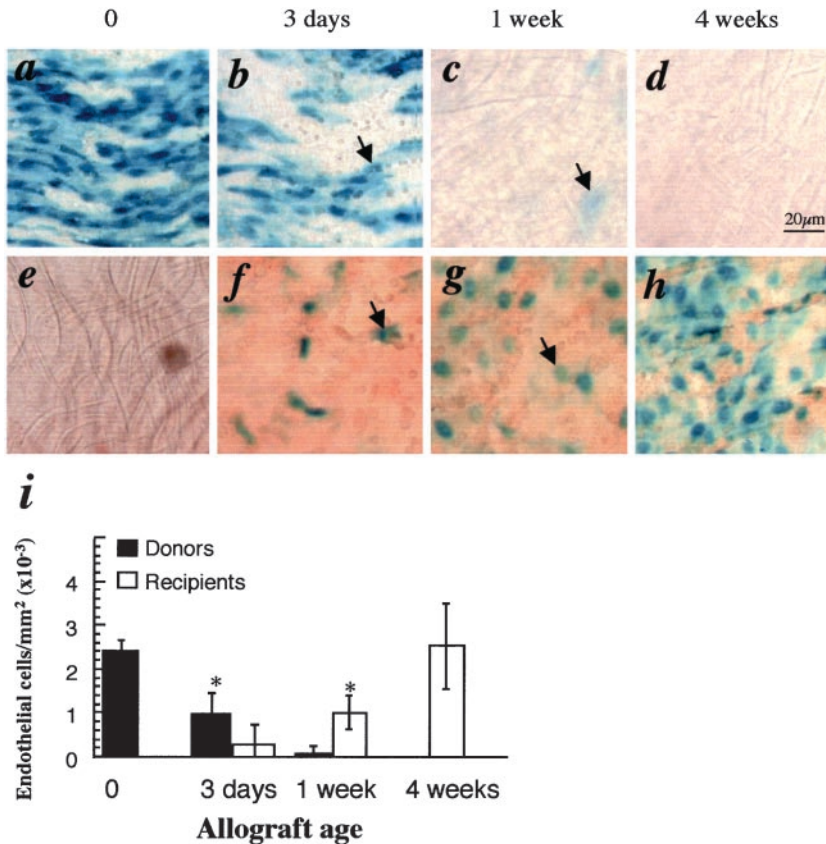


Figure 2. En face staining of endothelial cells on aortic allografts. a through d, Freshly harvested aorta segments from TIE2-LacZ mice (a) were allografted into carotid arteries of Balb/c mice (b through d). e through h, Freshly harvested aortic segment from a Balb/c mouse (e) was isografted into carotid arteries of TIE2-LacZ mice (f through h). Grafts were harvested at time points indicated and incubated with a substrate X-gal as described in Methods. After processing, en face photographs were taken. Blue indicates β -gal+ cells. Arrows indicate examples of positive cells. Graph (i) summarizes data of mean \pm SD from 5 animals per group. *Significant difference from group at day 0, $P < 0.05$.

activity was observed throughout the surface of aorta from TIE2-LacZ (Figure 2a) transgenic mice, whereas native aorta (Figure 2e) from Balb/c mice showed no staining. However, in the vessels of TIE2-LacZ (Figure 2, b through d) transgenic mice allografted into the carotid arteries of Balb/c mice, β -gal activities were decreased by 3 days, barely detectable at 1 week, and gone completely by 4 weeks after grafting, indicating that endothelial cells of donor vessels were dead. Interestingly, there were many β -gal-positive cells on the surface of Balb/c vessels allografted into TIE2-LacZ transgenic mice (Figure 2, f through h). Figure 2i shows statistical data indicating the number of endothelial cells in allograft vessels. At days 3 and 7, the grafts were noted to have significant β -gal+ cell loss compared with those of the freshly harvested aortas ($P < 0.05$). Endothelial cell numbers at 4 weeks were similar to those seen in the ungrafted vessels.

Endothelial Cells Regenerated by Circulating Progenitors

It was necessary to clarify whether recipient-derived endothelial cells originated from circulating blood or from carotid arteries where allografts were anastomosed. β -gal+ endothelial cells of the carotid artery from TIE2-LacZ mice were evenly distributed on the surface of the vessel along the direction of blood flow from left to right (Figure 3a). If regenerated endothelial cells of aortic grafts were the results of migration from the anastomosed artery, then β -gal+ cells would be seen in the early stages at the ends of the aortic graft. Figure 3 shows representative data indicating that the number of β -gal+ cells on the surface of aortic grafts near the

anastomosed artery was similar to that in the center of the graft 3 days after surgery (b versus c).

To clarify whether the bone marrow cells of TIE2-LacZ mice contributed to regeneration of endothelium, we created chimeric mice, having bone marrow cells donated by TIE2-LacZ animals, which resulted in a β -gal+ cells on the aortic surface (Figure 4a). When Balb/c aorta was allografted into carotid artery of chimeric mice with bone marrow derived from TIE2-LacZ mice, β -gal activity was seen on the surface of allografts (Figure 4c) 4 weeks after surgery. Figure 4b showed a representative picture of β -gal+ cells of a Balb/c vessel allografted into a TIE2-LacZ chimeric mouse that received wild-type bone marrow. Figure 4d summarizes data from 5 animals per group, indicating that about one third ($35 \pm 19\%$) of regenerated endothelial cells were derived from

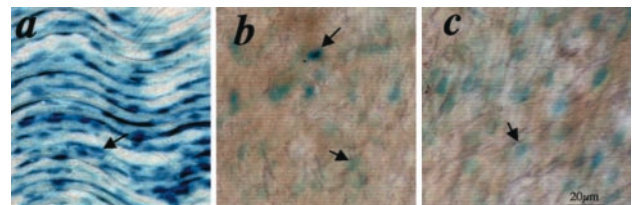


Figure 3. En face photographs show distribution of regenerated endothelial cells on allografts. a, Photograph of aortic segment from TIE2-LacZ mouse that served as recipient. Balb/c vein segments were grafted into TIE2-LacZ mice and harvested 1 week after surgery. After β -gal staining and en face preparation, positive endothelial cells of allograft near the end (b) and the center (c) were compared. Arrows indicate examples of positive cells.

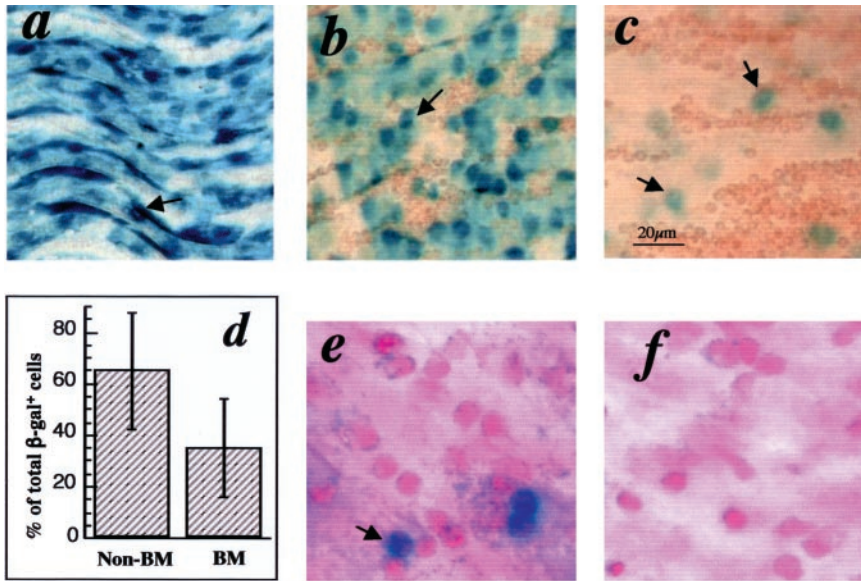


Figure 4. Bone marrow (BM) and non-BM origins of endothelial cells. Balb/c aortic sections were allografted into carotid arteries of chimeric mice (c, wild-type/TIE2-LacZ BM) (b, TIE2-LacZ/wild-type BM). Allografts were harvested 4 weeks after grafting, stained with X-gal, and processed for en face photography. a, Picture of aorta from TIE2-LacZ mouse as BM donor. d summarizes data of means from 5 animals per group. β -Gal-positive cells on the surface of 5 grafts from each group were enumerated under the microscope ($\times 400$ field). Three fields from each graft were included. Total positive cells from both groups serve as 100% for calculation of percentage. e, Picture of allograft to chimeric mouse (wild-type/TIE2-LacZ BM) with counterstaining using nuclear Fast Red; f, allograft to wild-type mouse.

bone marrow cells. Nuclear counterstaining for en face allograft to a chimeric mouse with TIE2-LacZ bone marrow confirmed that a proportion of cells were β -gal⁺ (Figure 4e) but not allograft to a wild-type mouse (Figure 4f).

Endothelial Origins of Microvessels Within Arteriosclerotic Lesions

When Balb/c aortas (Figure 5a) were allografted into the carotid arteries of TIE2-LacZ (Figure 5, b through d) transgenic mice, many β -gal⁺ cells were observed in the media (Figure 5b), neointima (Figure 5c), and adventitia (Figure 5d) 1 week (b) or 4 weeks (c and d) after surgery. In contrast, β -gal activities in TIE2-LacZ aortas (Figure 5e) allografted into Balb/c mice were rarely detectable at 1 week and completely disappeared 4 weeks after grafting, indicating that endothelial cells of microvessels within allografts were derived from recipients.

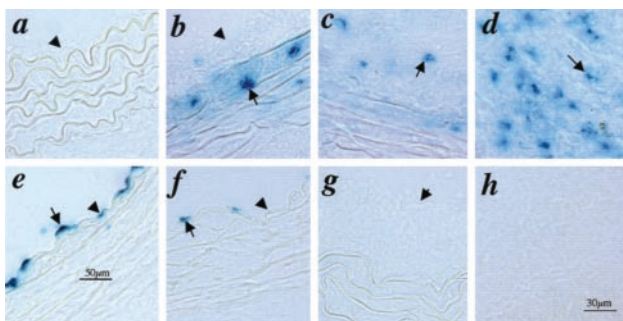


Figure 5. Donor origins of endothelial cells of microvessels within aortic allografts. a through d, Freshly harvested aortic segments from a Balb/c mouse (a) were isografted into carotid arteries of TIE2-LacZ mice (b through d). e through h, Freshly harvested aortic segments from TIE2-LacZ mice (e) were allografted into carotid arteries of Balb/c mice (f through h). Allografts were harvested at time 0 (a and e), 1 week (b and f), or 4 weeks (c, d, g, and h); sectioned; and incubated with a substrate X-gal as described in Methods. Blue indicates β -gal⁺ cells. Arrows indicate examples of positive cells. Arrowheads indicate surface of intima.

Bone Marrow Cells Are Sources of Microvessels Within Allografts

When Balb/c aorta (Figure 6a) was allografted into the carotid artery of chimeric mice with bone marrow derived from TIE2-LacZ mice, β -gal activity was seen within neointimal lesions of allografts (Figure 6b) 4 weeks after surgery. No β -gal activity was detected in Balb/c aorta allografted into TIE2-LacZ mice having wild-type bone marrow (Figure 6d), although the aorta from the chimeric animal showed positive staining (Figure 6c), indicating that all endothelial cells of microvessels were derived from bone marrow cells. To further confirm the bone marrow origins of endothelial cells, we detected microvessels within neointimal lesions using en face techniques. β -Gal activity was not detected in Balb/c aorta allografted into TIE2-LacZ mice having wild-type bone

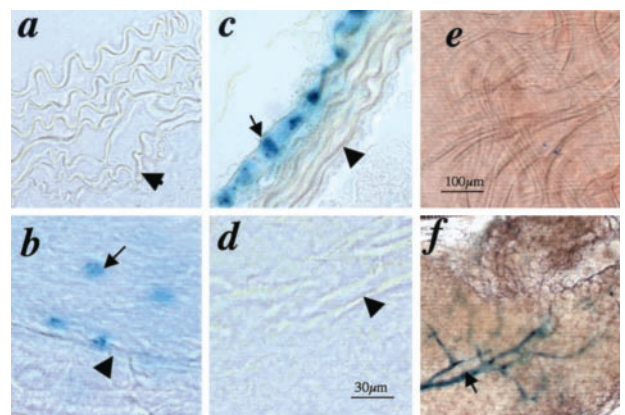


Figure 6. Bone marrow (BM) and non-BM origins of endothelial cells. Balb/c aortic segments (a) were allografted into carotid arteries of chimeric mice (b, wild-type/TIE2-LacZ BM) (d, TIE2-LacZ/wild-type BM). Allografts were harvested 4 weeks after grafting, sectioned, and stained with X-gal. c, Aorta from TIE2-LacZ/wild-type BM chimeric mouse. e and f, Pictures of en face preparation of aortic sections (e) allografted into chimeric mouse (wild-type/TIE2-LacZ BM). Allografts were harvested 1 week (f) after grafting, stained with X-gal, and examined directly with transmission microscope.

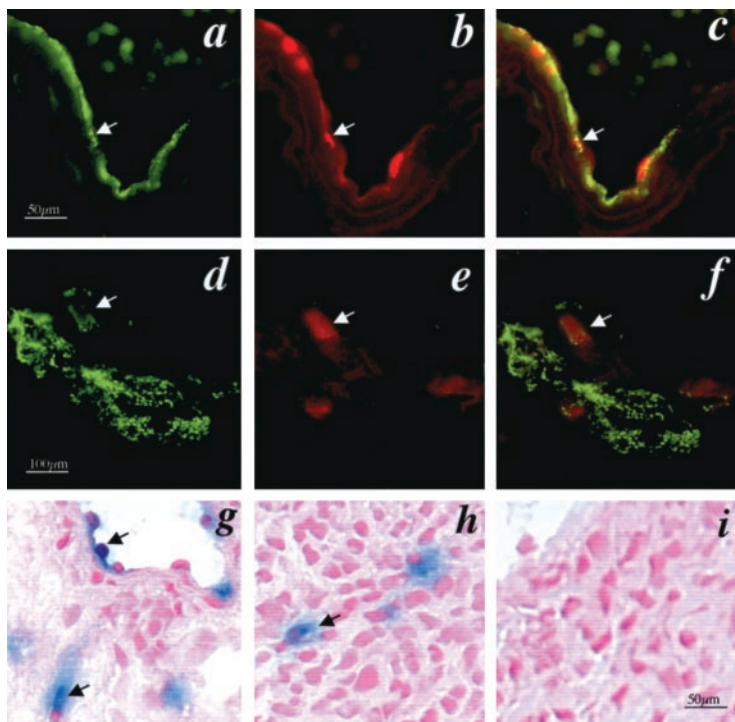


Figure 7. Double immunofluorescent staining for β -gal and von Willebrand factor in vessels. Freshly harvested aorta (a through c) and 4-week allograft (d through f) were sectioned and double-labeled for β -gal and von Willebrand factor, as described in Methods. Arrows indicate examples of double-positive cells on endothelium (a through c) and microvessels (d through f) of allografts. g through i, Sections of normal vessel (g), 4-week allograft to a chimeric mouse (wild-type/TIE2-LacZ BM) (h), and allograft to a wild-type mouse (i) developed with X-gal and counterstained with Fast Red.

marrow (Figure 6e); microvessels were found in Balb/c aorta allografted into wild-type mice having TIE2-LacZ bone marrow 1 week (Figure 6f) after surgery.

Finally, neointimal cells in allografts were also identified to express endothelial marker von Willebrand factor, both on the endothelial surface and microvessels of vessels (Figure 7, a through f), indicating a maturation of these endothelial cells. In addition, nuclear counterstaining of the sections from freshly harvested vessel (Figure 7g) and allografts (Figure 7h) to TIE2-LacZ mice showed β -gal+ cells localized on the surface and the microvessels of the adventitia (g) and the lesions (h) but not allograft to a wild-type mouse (i).

Discussion

The conventional hypothesis that endothelial cells in vascular allografts are replaced by remaining endothelial cells of donor vessels has been challenged by recent findings demonstrating the contribution of recipients.⁸⁻¹¹ It is known that endothelial cells of vascular allografts are damaged at the early stage by alloimmune reactions and regenerated thereafter. In the present study, we provide solid evidence that the regenerated endothelial cells of arterial allografts originate from recipient circulating blood but not remaining endothelial cells of donor vessels. We also demonstrate that about one third of endothelial cells covering the surface of allografted vessels were derived from bone marrow progenitor cells. These data establish that circulating progenitor cells are sources of neointimal lesions of allografts. Thus, our findings are crucial in understanding the pathogenesis of transplantation atherosclerosis and in establishing new strategies for therapeutic intervention in this disease.

It was realized that thrombosis formation on the surface of the vessel wall in transplantation is a crucial event in the

pathogenesis of transplantation arteriosclerosis.²⁹ In the present study, we observed a massive loss of the endothelium in allografts between 3 and 7 days after surgery, which is not yet fully replaced by circulating endothelial cells. This indicates that certain surface areas of the vascular 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. If the attachment of circulating progenitor cells could be enhanced at an early stage, the failure of transplantation would be reduced due to inhibition of thrombosis or neointimal lesions in allografts.

Hillebrands et al³⁰ reported that <3% of endothelial cells on allograft vessels were derived from bone marrow. The present data provide the evidence that >30% of regenerated endothelial cells on the surface of large vessels (allografts) are originated from bone marrow progenitor cells, indicating the impact of bone marrow progenitors as a source of endothelial cells. This difference between the two studies could be due to the different techniques, for example, section versus en face analyses. We found that it was very difficult to quantify the positive endothelial cells on sections when small numbers of cells exist. Our en face technique allows us to visualize all cells on the surface of the vessel wall and thus to obtain more reliable data.

Another important observation in the present study is the cell origin of microvessels within the media and neointimal lesions of allografts. In the absence of atherosclerosis, normal vessel walls have a microvasculature that is confined to the adventitia.^{14,15} In the arteriosclerotic lesions of allografted arteries, abundant microvessels were observed.¹⁶ We found that microvessels appeared in allografts earlier than neointimal formation and became abundant in neointimal lesions. Although the function of microvessels in lesions remains to be studied, angiogenesis within the intima is a necessary

event for the development of lesions in much the same way that a tumor progresses.^{31–33} There is a potential clinical significance of these microvessels in atherosclerotic lesions, since they might be involved in plaque rupture caused by the higher density of these microvessels.^{33,34} We provide the first evidence that endothelial cells of microvessels within allograft vessels are derived from bone marrow progenitor cells, which could be valuable information for directing therapeutic intervention in transplantation arteriosclerosis.

Evidence indicates the existence of two types of circulating endothelial cells, that is, bone marrow–derived and vascular-derived progenitors cells,^{19,35} which is supported indirectly by our data. Bone marrow progenitors are responsible for angiogenesis in the vessel wall of allografts, whereas non–bone marrow progenitors are the main sources for endothelial regeneration of large vessels. Endothelial cells from both sources having β -gal activity were positively stained by anti–von Willebrand factor (Figure 7) and anti–VCAM-1 antibodies (data not shown) in allografts, suggesting the presence of endothelial functions of both types of cells. If the attachment of non–bone marrow progenitors to the surface of the damaged large vessel can be enhanced by either drugs or gene transfer, and if angiogenesis by bone marrow progenitors can be inhibited, reduced size in arteriosclerotic lesions could be achieved.

Acknowledgments

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