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Smooth Muscle Cells in Transplant Atherosclerotic Lesions Are Originated From Recipients, but Not Bone Marrow Progenitor Cells

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Background—Smooth muscle cell (SMC) accumulation in the intima of vessels is a key event in the pathogenesis of transplant atherosclerosis. The traditional hypothesis that SMCs in the lesion are derived from the media of the donor vessel has been challenged by recent observations, but the cell origin is still not well established.

Methods and Results—Here, we use a simplified model of artery allografts in transgenic mice to clearly identify the source of SMCs in transplant atherosclerosis. Aortic segments donated by BALB/c mice allografted to ROSA26 (C57B/6) mice expressing β-galactosidase (gal) in all tissues showed that neointimal cells derived exclusively from host cells. It was also demonstrated that SMCs of neointimal and atherosclerotic lesions in vessels allografted to mice expressing β-gal only in SMCs (SM-LacZ) or to apoE-deficient/SM-LacZ mice originated from the recipient, and not donor vessels. Interestingly, bone marrow transplantation of SM-LacZ β-gal–expressing cells into aortic allograft recipients revealed completely negative β-gal staining of neointimal and atherosclerotic lesions. However, a population of β-gal–positive cells in lesions of allografts was observed in chimeric mice with ROSA26 β-gal–expressing marrow cells. When bone marrow cells from both ROSA26 and SM-LacZ mice were cultured and stimulated with platelet-derived growth factor-BB, α-actin and β-gal double-positive cells were found, suggesting that bone marrow cells have an ability to differentiate into SMCs.

Conclusions—Thus, we provide strong evidence that SMCs of neointimal and atherosclerotic lesions in allografts are derived from the recipients and that non–bone marrow–derived progenitor cells are a possible source of SMCs in atherosclerotic lesions. (*Circulation*. 2002;106:1834-1839.)

Key Words: transplantation ■ cells ■ muscle, smooth ■ atherosclerosis ■ genetics

Allograft-accelerated transplant atherosclerosis is the main limitation to long-term survival of patients with solid-organ transplantation. Characteristics of the lesions include mononuclear cell infiltration, smooth muscle cell (SMC) proliferation, and matrix protein deposition in the intima of the vessel wall.[1] The lesions eventually culminate in vascular stenosis and ischemic graft failure. Traditionally, it was believed that SMCs in the lesion were derived from the media of donor arteries in response to endothelial injury induced by alloimmune reactions.[2] Endothelial cell injury results in platelet activation and aggregation, with the release of platelet-derived growth factor (PDGF). This in turn stimulates SMCs in the media migrating through the internal elastic lamina into subendothelial spaces, where they proliferate and form neointimal lesions.[3] However, this hypothesis has never been proved by direct experimental evidence in vivo.

Recently, it was demonstrated that adult bone marrow cells can differentiate into vascular endothelial cells,[4] and both endothelial cells and SMCs could be differentiated from the same stem cells.[5] Han et al[6] found that bone marrow cells contribute to neointimal lesions in injured vessels of mice. This has aroused interest in whether the bone marrow progenitor cells contribute to the growth of SMCs observed in transplant atherosclerotic lesions. Shimizu et al[7] and Sata et al[8] demonstrated that ≈10% to 20% of α-actin–positive cells in the neointimal lesions of allografts were colocalized with β-gal–positively stained cells in a chimeric mouse expressing β-galactosidase (gal) in bone marrow cells. On the basis of this finding, the authors concluded that host bone marrow cells are a source of smooth muscle–like cells in transplant neointimal lesions. In fact, a large number of leukocytes infiltrate the vessel wall of allografts in the development of the disease, and they are in close contact with SMCs in the

lesion. This would make it difficult to distinguish whether the α-actin/X-gal double-stained cells in tissue sections are from 1 cell or 2 adjacent cells. Because the source of lesional SMCs is a fundamental issue in understanding the pathogenesis and therapy for this disease, the present study was designed to investigate whether SMCs of transplant atherosclerosis originate from donor vessels, recipient vessels, or bone marrow progenitor cells of the recipient. Using our animal models for transplant atherosclerosis,[9] we performed aortic allografts in 3 types of transgenic mice expressing β-gal, ie, (1) all tissues (ROSA26[10]), (2) only SMCs (SM-LacZ[11]), and (3) apolipoprotein E (apoE)-knockout mice[12] carrying LacZ genes in SMCs (SM-LacZ/apoE$^{-/-}$[13]).

Methods

Mice and Artery Allograft Procedure

Artery transplantation was performed between BALB/c (H2^d) and ROSA26 LacZ transgenic mice, which were purchased from Jackson Laboratories. The ROSA26 mouse is a β-gal–transgenic mouse produced by random retroviral LacZ gene insertion into embryonic stem cells; there are 3 transcription start sites, involving unknown housekeeping gene promoters.[10] ROSA26 mice express β-gal activity throughout embryonic life, with variable levels of expression in adult tissues. Transgenic SM-LacZ mice expressing β-gal under the control of the smooth muscle–specific protein SM22 promoter have been described.[11] ApoE$^{-/-}$ mice (Jackson Laboratories) were crossed with SM-LacZ mice, and heterozygous offspring were mated to produce apoE-deficient mice expressing β-gal in arterial SMCs, as described previously.[13] All transgenic mice are C57BL/6 (H2^b) strain.

The arterial graft procedure was similar to that described previously.[9] Briefly, the aorta was harvested from the donor. For the recipient, the right common carotid artery was mobilized at the distal end toward the proximal and cut in the middle, and a cuff was placed at the end. The aortic segment was grafted between the 2 ends of the carotid artery by sleeving the ends of the aorta over the artery cuff.

Histology and Immunohistology

The grafts were harvested at 2, 4, and 6 weeks after surgery (6 to 8 mice per group). Bones of the femurs and tibias were harvested for either section or bone marrow cell preparations. The grafts were processed in routine histology and embedded in paraffin. The procedure for immunohistological staining used in the present study was similar to that described previously.[14,15]

Bone Marrow Transplantation

Donor mice were killed, and their femurs and tibias were removed aseptically. Marrow cavities were flushed, and single-cell suspensions were prepared. Cells were washed 2 times in HBSS and resuspended at 3×10^7 cells/mL before transplantation. Six- to 8-week-old mice received a lethal dose of whole-body irradiation (900 rads). Irradiated recipients received 1×10^7 bone marrow cells in 0.3 mL RPMI 1640 by tail vein injection. Aortic transplantation was performed 4 weeks after bone marrow transfer.

Reverse Transcription–Polymerase Chain Reaction

The procedure used for RT-PCR was similar to that described elsewhere.[5] Total RNA was prepared with RNA RT-PCR Miniprep Kit (Stratagene). The following primers were used: LacZ (5′-ATCAGGGCTGAACAGCTA-3′ and 5′-GCGAACAGC-GCACGGCGT-3′); GAPDH (5′-CGGAGTCAACGGATTTGG-TCGTAT-3′ and 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′).

In Situ Hybridization

Bone marrow smears were air-dried, fixed in methanol/acetic acid, washed in 2×SSC buffer for 10 minutes at 37°C, and dehydrated in
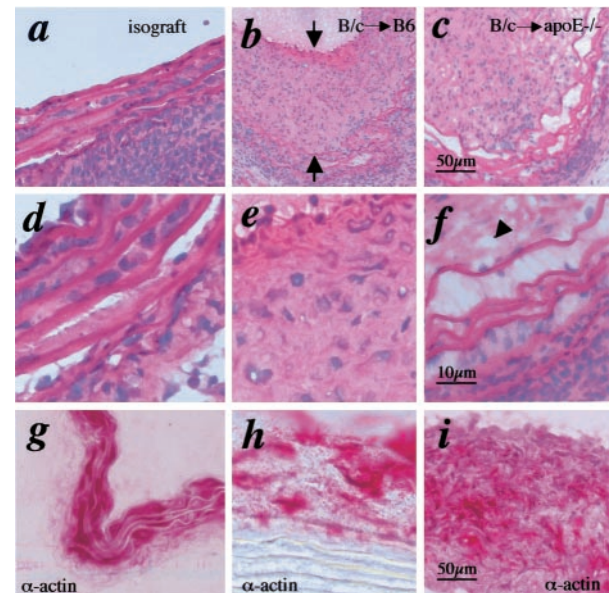
Figure 1. Lesion formation in aortic allografts in mice. a through f, Hematoxylin-eosin–stained sections of mouse isograft artery and artery allografts. Under anesthesia, common carotid arteries (a) of B/c mice were removed and grafted into carotid arteries of B/c (a and d), B6 (b and e), or apoE$^{-/-}$ (c and f) mice. Animals were killed 6 weeks after surgery, and grafted tissue fragments were sectioned and stained with hematoxylin-eosin. Arrows indicate neointima. g through i, α-Actin staining of isograft (g), allografts to B6 (h), or apoE$^{-/-}$ (i) mice.

graded ethanol. Smeared cells were denatured by immersion in 70% formamide/2×SSC at 73°C for 4 minutes. We used the probe pY353B as described by Bishop et al[16] that hybridizes specifically to a series of repetitive sequences on the Y chromosome. Positive hybridizations identifying Y-bearing cells were recognized by a small, discrete area of brown precipitate in the nucleus.

Bone Marrow Cell Culture

Harvested bone marrow cells (1×10^6/mL) were plated on 8-well slide chambers in RPMI 1640 supplemented with 10% FCS and incubated at 37°C in 5% CO_2 for 3 hours. Adhered cells were fixed and stained for β-gal and/or α-actin. Another portion of cultured cells was incubated at 37°C in the presence of PDGF-BB (50 ng/mL; Sigma) for 2.5 days, fixed, and stained for β-gal and/or α-actin.
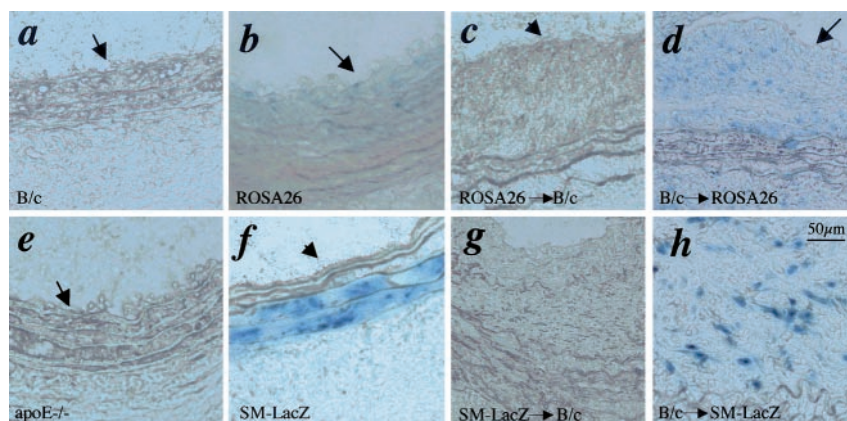
β-Gal Staining

The procedure for determining β-gal activity in sections or cultured cells was similar to that described by Sanes et al.[17] Briefly, sections or cultured cells were incubated at 37°C for 18 hours in PBS supplemented with 1 mg/mL X-Gal (Sigma).[18] For double-staining of β-gal and SMC α-actin, we performed SMC α-actin staining before β-gal staining.

Results

Characterization of Transplant Atherosclerosis

When isografts in B/c mice were performed, no neointimal lesions in grafts were found after 6 weeks (Figure 1, a and d). Strong positive staining for α-actin in the isograft was observed, indicating the existence of SMCs in the media (Figure 1g). Figure 1, b and e, shows increased neointimal lesions in aortic allografts that contain a large number of α-actin–positive SMCs, but not in the media (Figure 1h). This suggests that the disappearance of medial SMCs is a result of

Figure 2. Recipient origins of SMCs in neointima of allografts. Native vessels (a, b, e, and f) or allografts (c, d, g, and h) were collected 6 weeks after transplantation. Blue shows β-gal staining. a, B/c native aorta. b, Native ROSA26 aorta. c, ROSA 26 donor aorta transplanted into a B/c wild-type mouse. d, B/c aortic allografts in ROSA26 hosts. e, ApoE$^{-/-}$ native aorta. f, SM-LacZ native aorta. g, SM-LacZ donor aorta transplanted into a B/c wild-type mouse. h, B/c aortic allografts in SM-LacZ hosts. Arrows indicate surface of intima.

apoptosis induced by alloimmune reactions.[19] Some evidence indicates a contribution of hyperlipidemia to atherogenesis; therefore, apoE-deficient mice (B6) with blood cholesterol levels of 300 to 500 mg/dL were used as graft recipients.[12] Atherosclerotic lesions in allografts in apoE$^{-/-}$ mice were significantly larger than those in wild-type controls 6 weeks after transplantation (Figure 1c). Three allografts out of 6 animals were completely occluded, indicating that hyperlipidemia enhances the lesion development. Furthermore, transplant atherosclerotic lesions in apoE$^{-/-}$ mice contain lipid droplet–like structures (Figure 1, c and f) and α-actin–positive SMCs (Figure 1i).

Recipient Origins of Lesional SMCs

To determine the source of neointimal SMCs in allografts, B/c, B6, ROSA26, and SM-LacZ transgenic mice as recipients or donors were used for aortic transplantation, and the allograft lesions were analyzed for β-gal enzymatic activity with X-gal substrate. Native aortas (Figure 2, a and e) from B/c or apoE$^{-/-}$ mice showed no staining, whereas strong β-gal activity was observed throughout the media of RO-SA26 (Figure 2b) and SM-LacZ (Figure 2f) transgenic mice. However, in the vessels of ROSA26 (Figure 2c) and SM-LacZ (Figure 2g) transgenic mice allografted into the carotid arteries of B/c mice, no β-gal activity was detected, indicating that neointimal cells were not derived from donor vessels. Even in the media of these allografts, β-gal–positive cells were rarely found (Figure 2, c and g) because of disappearance of donor medial cells in allografts.[19,20] Interestingly, many β-gal–positive cells in neointimal lesions of B/c vessels allografted into either ROSA26 (Figure 2d) or SM-LacZ (Figure 2h) transgenic mice. The Table summarizes data of numbers of animals with β-gal–positively stained cells, suggesting a recipient origin of SMCs in the lesions.

No Evidence for Bone Marrow Origins of Lesional SMCs

When aortic segments from B/c mice were grafted into these irradiated mice (B6/ROSA26 BM) that had only β-gal activity in bone marrow cells or marrow-derived cells, then β-gal–positive cells in lesions were observed (Figure 3a). However, no β-gal activity was found in lesions of vessels allografted into B6/SM-lacZ chimeric mice (Figure 3b; 8 animals per group). We also performed double staining for

α-actin and β-gal in sections of allografts from B6/ROSA26 chimeric mice with bone marrow β-gal positivity. Data shown in Figure 3, c and d, indicate the presence of double-positive-like cells, ie, closely colocalized blue and red cells. Semiquantitative analysis revealed 5% to 10% double-positive-like cells of total α-actin–positive cells in allograft lesions from B6/ROSA26 chimeric mice. Double staining of α-actin and β-gal for sections of allografts from SM-LacZ mice as a recipient revealed that almost all β-gal–stained cells are α-actin–positive (Figure 3e).

To confirm the contribution of bone marrow cells as a possible source of SMCs in allograft atherosclerosis, aortic segments of B/c mice were grafted into SM-LacZ/apoE$^{-/-}$ (Figure 3f) or apoE$^{-/-}$ chimeric mice with SM-LacZ bone marrow (Figure 3g). Similarly, β-gal–positive SMCs were abundant in atherosclerotic lesions of vessels allografted to SM-LacZ/apoE$^{-/-}$ mice (Figure 3f), but were lacking in lesions of apoE$^{-/-}$ chimeric mice with SM-LacZ bone marrow (Figure 3g; Table). Furthermore, LacZ mRNA in allografts from apoE$^{-/-}$ chimeric mice with SM-LacZ bone marrow was not detectable (Figure 3h), indicating that bone marrow cells may not be a source of SMCs in allografts.
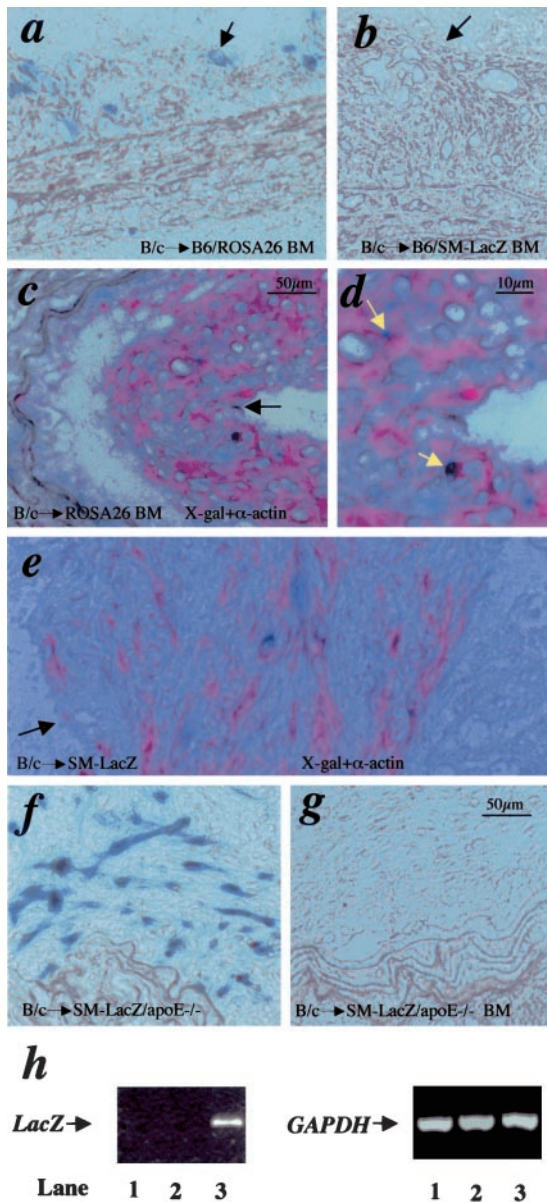
Bone Marrow Cells

To monitor the efficiency of bone marrow transplantation, bone marrow cells and bones were collected when allografts were harvested. More than 95% of male bone marrow cells from sex-mismatched chimeric mice were positively stained for the Y chromosome, as identified by in situ hybridization (Figure 4a), whereas no staining was observed in the female bone marrow cells (Figure 4b).

Summary of Data on X-gal–Positively Stained Cells

Mice	Recipient	Donor
ROSA26	7/7	0/7
SM-LacZ	8/8	0/8
ApoE$^{-/-}$SM-LacZ	6/6	0/6
B6/ROSA26 BM	8/8	…
B6/SM-LacZ BM	0/8	…
B6/SM-LacZ/apoE$^{-/-}$ BM	0/8	…

Data are numbers of animals showing β-gal–positively stained cells vs total animals per group. BM indicates bone marrow.

Figure 3. Non–bone marrow origin of SMCs in neointimal and atherosclerotic lesions. Bone marrow (BM) cells of ROSA26 (a, c, and d) or SM-LacZ (b) mice were transplanted into irradiated B6 wild-type recipient mice 4 weeks before aortic transplantation of B/c aortas. Sections were stained for β-gal (a through d) or for both β-gal and α-actin (c and d). e, β-Gal and α-actin double-positive staining for sections of B/c allograft to SM-LacZ mice. β-Gal staining for sections of B/c allografts to SM-LacZ/apoE$^{-/-}$ (f) or to apoE$^{-/-}$ mice with SM-LacZ/apoE$^{-/-}$ bone marrows (g). Black arrows indicate surface of intima, yellows indicate double-positive-like cells. h, RT-PCR data for LacZ genes. Lane 1, allograft from apoE$^{-/-}$/SM-LacZ bone marrow mice; lane 2, negative control omitted reverse transcriptase; lane 3, B/c aorta allografted to apoE$^{-/-}$/SM-LacZ mice as a positive control.

Furthermore, no β-gal activity was seen in bone marrow donated by B6 mice (Figure 4, c and e), whereas almost all bone marrow cells with nuclei from B6/ROSA26 chimeric mice were stained blue (Figure 4, d and f). Thus, the efficiency of bone marrow transplantation in our chimeric mice was high.

To clarify whether the bone marrow cells of SM-LacZ mice can differentiate into smooth muscle–like cells, bone marrow cells were cultivated in the presence of PDGF-BB. All cells from ROSA26 (Figure 5a) stained positively for β-gal, but B6 bone marrow cells did not (Figure 5b). Interestingly, a population of bone marrow cells from SM-LacZ mice showed β-gal positivity in response to PDGF-BB (Figure 5c) and negative staining in the absence of PDGF-BB (Figure 5d). Concomitantly, α-actin staining revealed that a population of bone marrow cells was positive in response to PDGF-BB (Figure 5e), but not in cells without PDGF-BB stimulation (Figure 5f). Double staining for α-actin and β-gal in PDGF-BB–stimulated bone marrow cells derived from ROSA26 (Figure 5g) and SM-LacZ (Figure 5h) mice confirmed that bone marrow cells could differentiate to SMCs. To identify whether bone marrow transfer results in a depletion of certain populations of stem or progenitor cells, bone marrow cells were simultaneously cultivated from SM-LacZ and chimeric mice and stained for α-actin and β-gal. Data shown in Figure 6 indicate a similar proportion of double-positive cells from bone marrow of SM-LacZ mice and chimeric animals with SM-LacZ bone marrow. These results suggest no selective depletion of smooth muscle progenitor cells in chimeric mice.

Discussion

The conventional hypothesis that neointimal SMCs in vascular allografts migrate from the media of donor vessels has been challenged by recent findings demonstrating the contribution of recipients.[7,21,22] These studies indirectly identified a source of SMCs in neointimal lesions by using double staining for α-actin and Y-chromosome or β-gal activity. In the present report, we provide direct evidence that neointimal SMCs of arterial allografts originated from recipients, but not donor vessels, as identified by directly SMC SM22-driven β-gal expression. In addition, it has been demonstrated that hyperlipidemia is essential for the formation of atheromatous lesions. Using apoE$^{-/-}$ mice combined with SMC SM22-driven β-gal expression,[11] we provide the first evidence that SMCs of transplant atherosclerotic lesions in hyperlipidemic apoE$^{-/-}$/SM-LacZ mice originated from the recipients. Taken together, these findings establish the recipient origins of SMCs in both neointimal and atherosclerotic lesions of allografts.

A recent report by Shimizu et al[7] showed that some smooth muscle–like cells in the lesions of transplant arteriosclerosis originate from bone marrow–derived cells. Accordingly, we performed a similar experiment using the same strain of transgenic mouse, ROSA26. A similar result of α-actin and β-gal double-positive-like cells was obtained, ie, that ≈5% of α-actin–positive cells also seem to be β-gal–positive in chimeric mice with β-gal–expressing bone marrow cells. Very recently, Sata et al[8] performed a similar experiment with chimeric mice carrying green fluorescent protein (GFP)-labeled bone marrow cells and demonstrated a portion of neointimal cells showing GFP and α-actin double positivity. However, because of overlapping staining for α-actin caused by the high density of SMCs in the neointima, it was difficult to identify individual α-actin–positive cells. Between these
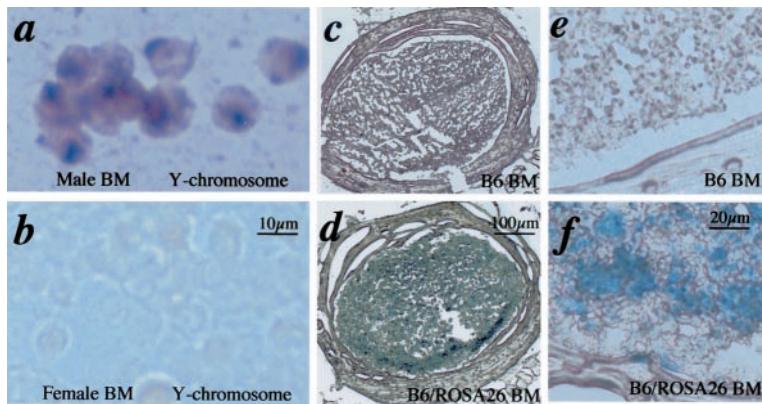
Figure 4. High transfer efficiency of bone marrow (BM). Bone marrow cells harvested from chimeric mice received sex-mismatched marrow cells, prepared for smears and stained with in situ hybridization for Y chromosome (a and b). Femurs of chimeric mouse with B6 (c and e) or B6/ROSA26 BM (d and f) bone marrow transplantation after irradiation were harvested, sectioned, and stained for β-gal, in which positive cells are blue.

SMCs, there were infiltrated β-gal–positive or GFP-positive leukocytes. Possibly, the small population (5% to 10%) of double–positive cells observed by us, Shimizu et al,[7] and Sata et al[8] was artificial. Double-positive cells may be adjacent regions of SMCs and leukocytes that were too close to be separately recognized in sections (Figure 1i). Supporting this issue is the fact that a proportion of double-positive cells (yellow) were seen in sections of graft vessels labeled for α-actin (red) and MAC-1–positive macrophages (green), which are known to be separately present in SMCs and macrophages (Figure 2 of Mayr et al[23]). An alternative approach, eg, higher-resolution imaging or immunoelectron microscopy, would be more suitable for study of this aspect. Conversely, our findings using chimeric mice demonstrated that neointimal SMCs were not derived from bone marrow cells, because of the complete absence of β-gal activity in the

lesions of allografts in mice expressing smooth muscle β-gal in bone marrow cells.

Where do lesional SMCs of allografts originate? One possibility is that medial SMCs of recipient anastomosed arteries migrate into the graft. We have harvested allografts in SM-LacZ mice at different time points, eg, 2 and 4 weeks, and cut sections longitudinally, but did not find any difference in SMC accumulation between the end and middle portions of allografts (data not shown). In addition, we used cuff techniques to anastomose the ends of the graft to the recipient, in which no media SMCs contact each other between the donor and recipient.[9] There is also evidence that neointimal SMCs are clonal and phenotypically distinct from medial SMCs.[24] Thus, our study does not support the notion of direct migration from recipient vessels across the anastomosis to the graft. Another possibility is that circulating SMC progenitor cells are a source of neointimal SMCs, as postulated by Saiura et al.[25] Although our data do not support bone marrow progenitor cells as a source of lesional SMCs, SMC progenitors might be present in other organs, eg, pericytes in the microvasculature.[26,27] These microvessels are able to generate new vessels after injury or stimulation, indicating a potential for angiogenesis. In addition, liver and spleen might also be a source of SMC progenitor cells, because evidence indicates that adult-derived stem cells from the liver could become myocytes in the heart.[28] To investigate this hypothesis, further studies will be needed to confirm the presence of SMCs in the circulation and to determine their origins. We
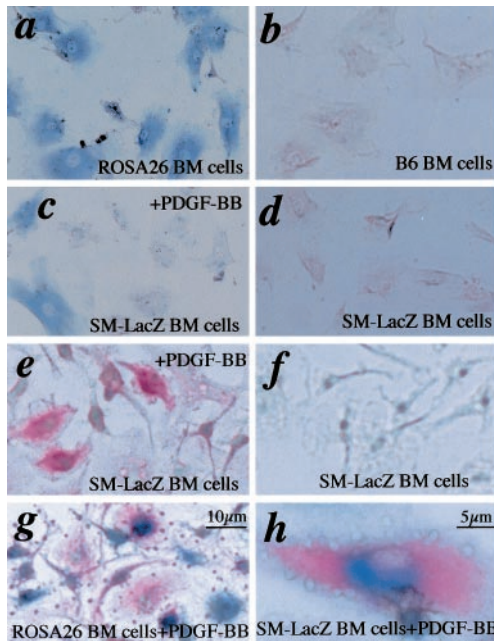
Figure 5. Bone marrow (BM) cells can differentiate to SMCs. Bone marrow cells from chimeric mice were harvested. Cells were either harvested (b, d, and f) or continuously cultivated for 2.5 days in serum-free medium in presence of human PDGF-BB (50 ng/mL; a, c, e, g, and h). Cells were stained for β-gal (a through d), α-actin (e and f), or both (g and h). Note β-gal stained blue and α-actin red.
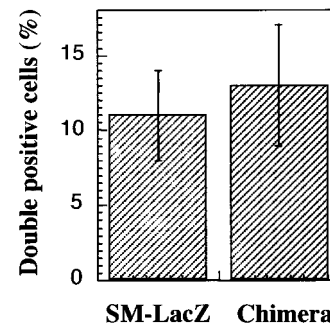
Figure 6. Comparison of α-actin and β-gal double-positive cells between chimeric and SM-LacZ mice. Bone marrow cells from SM-LacZ and chimeric mice were harvested and cultivated in presence of human PDGF-BB. Cells were stained for β-gal and α-actin. Data are mean$\pm$SD of 3 experiments.

believe that data from these studies will result in valuable clinical implications for treatment of this disease.

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