Flanking Recipient Vasculature, Not Circulating Progenitor Cells, Contributes to Endothelium and Smooth Muscle in Murine Allograft Vasculopathy

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Objective—The prevailing view assumes that circulating endothelial and smooth muscle progenitor cells participate in allograft vasculopathy (AV), although the seminal studies in the field were not designed to distinguish between circulating and migrating cells of recipient origin. We developed a double-transplantation technique to overcome this problem and reinvestigated the origin of endothelial cells (ECs) and smooth muscle cells (SMCs) in murine AV.

Methods and Results—Carotid artery segments from BALB/c mice were allografted to apolipoprotein E\(^{-/-}\) B6 mice with or without a “flanking” isograft interpositioned between the allograft and the recipient artery. Either recipient mice or interpositioned isografts expressed enhanced green fluorescent protein, and consequently, cells migrating into the allograft from the flanking vasculature could easily be tracked and distinguished from recruited circulating cells. Without immunosuppression, allograft donor cells vanished as expected, and AV developed by replacement and accumulation of ECs and SMCs of recipient origin. The double transplantation models revealed that all ECs and SMCs in AV had migrated into the allograft from the flanking vasculature without any contribution from putative progenitor cells in the blood.

Conclusion—Migrating cells from the flanking vasculature, not circulating progenitor cells, are the source of recipient-derived ECs and SMCs in murine AV. (Arterioscler Thromb Vasc Biol. 2011;31:808-813.)

Key Words: allograft vasculopathy ■ circulating progenitor cells ■ endothelial cells ■ migration ■ smooth muscle cells

Allograft vasculopathy (AV), also known as transplant arteriosclerosis, limits long-term function of organ transplants. It is caused by chronic immune-mediated injury to the vasculature of the transplanted organ, leading to intimal smooth muscle cell (SMC) accumulation, luminal narrowing, and eventually ischemic graft failure.\(^1\)

Establishing the origin of cells in AV is an important element in understanding its pathogenesis, and the recently reported involvement of recipient-derived endothelial cells (ECs) and SMCs has spurred much interest. In 2002, a sensational study reported that recipient-derived cardiomyocytes, SMCs, and ECs were common in sex-mismatched heart transplants,\(^2\) but conflicting results have been published.\(^3-7\) The presence of recipient-derived cells has, not surprisingly, been more consistent and of higher magnitude in experimental models of AV where no immunosuppressive drugs are administered to protect donor cells. In 2 seminal studies, Hu et al studied AV development in BALB/c arteries that were transplanted into hyperlipidemic apolipoprotein E\(^{-/-}\) (apoE\(^{-/-}\)) C57BL/6 (B6) mice and found lesional ECs and SMCs to be entirely of recipient origin.\(^8,9\) Similar results in wild-type mice and rats have been reported.\(^10,11\) The source of these cells, however, remains uncertain. One theory that has attracted particular interest is that circulating progenitor cells of bone marrow or other origin may home and differentiate into mature ECs and SMCs in the allograft.\(^1,11,12\) An alternative and much simpler hypothesis, however, which is in compliance with the majority of the reported data, including classic studies in the field, is that ECs and SMCs migrate into the allograft from the flanking recipient vasculature. No experiments so far have been designed to distinguish between these different cell sources.

In the present study, we devised a double-transplantation technique to address this issue and reinvestigated EC and SMC origin in a modified version of the murine AV model studied by Hu et al.\(^8,9\) We found that ECs and SMCs in AV originated entirely from the flanking vasculature with no contributions from circulating progenitor cells.

Methods

Transgenic Animals

Enhanced green fluorescent protein-positive (eGFP\(^{+}\)) apoE\(^{-/-}\) (H2b) (B6) mice (hemizygous for the eGFP transgene) were obtained by intercrossing eGFP\(^{+}\) (C57BL/6-Tg(ACTB-
EGFP1Os/J, The Jackson Laboratory, Bar Harbor, ME) and apoE⁻/⁻ mice (B6.129P2-Apoem11/Mm; Taconic M&B, Ry, Denmark), both backcrossed more than 10 times to the B6 strain. BALB/c (H2d) mice were obtained from Taconic M&B. All mice were fed normal chow. All procedures were approved by the Danish Animal Experiments Inspectorate.

Study Design
The AV model (BALB/c segment transplanted into apoE⁻/⁻ mice) was described previously,13 with the exceptions that eGFP rather than β-galactosidase served as tracking marker, carotid arteries rather than aortas were used as allografts, and allografts were inserted by traditional end-to-end anastomoses rather than by cuff- ligation technique. No immunosuppressive drugs were administered.

First, to verify the important role of recipient-derived ECs and SMCs in AV lesion formation in this model, common carotid artery segments from BALB/c mice were allotransplanted orthotopically into eGFP⁺apoE⁻/⁻ B6 mice (n=4; Figure 1a). Mice were killed after 4 weeks.

Next, to locate the source of the recipient-derived ECs and SMCs, carotid artery segments were first transplanted from eGFP⁺apoE⁻/⁻ B6 mice into apoE⁻/⁻ B6 mice (n=6) or from apoE⁻/⁻ B6 mice into eGFP⁺apoE⁻/⁻ B6 mice (n=5) (isotransplantations except for the eGFP transgene). Four weeks later, mice were then underwent a second arterial transplantation, in which the isograft was transsected and a BALB/c allograft was inserted end-to-end (Figure 1b and 1c). Mice were killed 4 weeks after insertion of the allograft.

Transplanted Arterial Segments
The transplantation procedure was described previously.13 The anesthetized donor mouse (5 mg of pentobarbital IP) was killed by exsanguination, and the right common carotid artery was flushed and kept in saline at room temperature.Recipient mice were anesthetized with isoflurane (induction, 5%; maintenance, 1.5% to 2%) and buprenorphin (0.1 mg/kg SC), and the right common carotid artery was accessed through a midline incision and mobilized from the underlying media. Heparin was administered with isoflurane (induction, 5%; maintenance, 1.5% to 2%) and buprenorphin (0.1 mg/kg SC), and the right common carotid artery was accessed through a midline incision and mobilized from the underlying media. The AV model (BALB/c segment transplanted into apoE⁻/⁻ mice) was described previously,13 with the exceptions that eGFP rather than β-galactosidase served as tracking marker, carotid arteries rather than aortas were used as allografts, and allografts were inserted by traditional end-to-end anastomoses rather than by cuff- ligation technique. No immunosuppressive drugs were administered.

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Tissue Processing
Mice were killed by exsanguination under anesthesia (5 mg of pentobarbital IP), perfusion fixed with phosphate-buffered 4% formaldehyde (pH 7.2) at 100 mm Hg through the left ventricle for 5 minutes, and immersion fixed for 6 hours at room temperature. The right common carotid artery was removed, cryoprotected in sucrose solution (25% wt/vol for 24 hours + 50% wt/vol for 24 hours), embedded in O.C.T. compound (Sakura), and snap-frozen in liquid nitrogen-chilled methanol:acetone (1:1). Vessels were sectioned longitudinally at 4 μm thickness.

Immunohistochemistry
ECs were identified by staining for von Willebrand Factor (vWF) using polyclonal rabbit anti-human vWF (Dako, 1:200) after blocking of nonspecific binding with normal goat serum (Jackson Immunoresearch) and followed by Texas Red–conjugated goat anti-rabbit secondary antibody (Jackson Immunoresearch, 1:400). IgG antibodies from nonimmunized rabbits (Rabbit IgG, Dako) at the same concentration served as negative control. SMCs were identified by staining for smooth muscle α-actin (SMA) using mouse monoclonal anti-SMA (clone 1A4, Dako, 1:50) after blocking of endogenous mouse immunoglobulins with donkey anti-mouse Fab fragments (Jackson Immunoresearch, 1:10) and formaldehyde fixation, and followed by Texas Red–conjugated donkey anti-mouse secondary antibody (Jackson Immunoresearch, 1:400). An irrelevant monoclonal antibody (clone DAK-G05, Dako, 1:50) of the same isotype and concentration was used as negative control. The Mac2 epitope was stained as a marker for plaque macrophages by using rat anti-mouse Mac2 antibody (CL8942AP, Cedarlane Labs, 1:50) followed by Texas Red–conjugated goat anti-rat secondary antibody (Jackson Immunoresearch, 1:200). An irrelevant rat monoclonal antibody (R2a00, Caltag, Trichem, 1:50) was used as negative control. All antibodies were diluted in 1% normal serum in Tris-buffered saline. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (Sigma). Sections were mounted in Slowfade Light Antifade (Invitrogen). eGFP was detected by its natural fluorescence, and the validity of eGFP detection was confirmed against positive (eGFP⁺apoE⁻/⁻) and negative (apoE⁻/⁻) control tissues (Supplemental Figure I, available online at http://atvb.ahajournals.org).

Histological Analysis
Serial sections of allografts were analyzed in an Olympus Cell-R epifluorescence microscope system followed by software deconvolution. Briefly, widefield z-axis image stacks (31 layers spanning 10 μm) were acquired using the motorized focus and deconvoluted using a blind 3D deconvolution algorithm (Autoquant Deblur 9.3, Autoquant Imaging). ECs and SMCs were analyzed for colocalization with the eGFP tracking marker in a single optical section through the center of the tissue section. ECs were recognized as nucleated cells with intracellular location of vWF and SMCs as cells with the expected subplasmalemmal distribution of SMA. Differential interference contrast (DIC) microscopy was used to resolve cellular borders and morphology. The number of analyzed cells was counted to estimate statistical power.

Results
BALB/c allografts transplanted into apoE⁻/⁻ B6 mice developed AV in both single- and double-transplantation experiments (Figure 2). AV lesions were characterized by abundant SMC-rich neointima covered by ECs as described below. The underlying media was almost devoid of SMCs, corresponding to the described disappearance of graft SMCs by cytotoxic T cell–mediated apoptosis in this type of model.14

Recipient Origin of ECs and SMCs
To determine whether neointimal ECs and SMCs in AV were of donor or recipient origin, BALB/c allografts were trans-
planted into eGFP<sup>+</sup>apoE<sup>−/−</sup> B6 mice (n=4) (Figure 1a), and the origin of SMCs and ECs was studied after 4 weeks by high-resolution microscopy. Consistent with previous reports,<sup>8,9,11</sup> BALB/c allografts were repopulated by recipient ECs and SMCs. We found 99.8% eGFP<sup>+</sup>SMαA<sup>+</sup> cells (among 2219 SMαA<sup>+</sup> cells analyzed) and 80.5% eGFP<sup>+</sup>vWF<sup>+</sup> cells (among 206 vWF<sup>+</sup> cells analyzed) in AV lesions (Figure 3). These fractions of eGFP<sup>+</sup> cells were similar to those found previously in atherosclerotic plaques of eGFP<sup>+</sup>apoE<sup>−/−</sup> B6 mice, indicating that virtually all cells in AV lesions were of recipient origin.<sup>13,15,16</sup>

**Recipient-Derived ECs and SMCs Originate Entirely From the Flanking Vasculature**

To determine whether recipient-derived ECs and SMCs entered the allografts through the circulation (by homing and differentiation of putative circulating progenitor cells) or migrated from the contiguous recipient vasculature, we performed the double-transplantation experiments shown in Figure 1b and 1c. First, to track cell migration from the flanking vasculature, we inserted BALB/c allografts into eGFP-positive isografts that previously had been transplanted into apoE<sup>−/−</sup> B6 mice (n=6) (Figure 1b). A longitudinal section from one of the transplanted mice is shown in Figure 2. We found 99.3% eGFP<sup>+</sup> SMαA<sup>+</sup> cells (among 2452 SMαA<sup>+</sup> cells analyzed) and 77.9% eGFP<sup>+</sup>vWF<sup>+</sup> cells (among 657 vWF<sup>+</sup> cells analyzed) in AV lesions from these mice, indicating that the great majority of SMCs and ECs, if not all, entered the lesions by direct migration from the flanking eGFP<sup>+</sup> arterial segments (Figure 4). A number of migrating cells from the flanking vasculature did not stain for either SMαA or vWF. These cells may be phenotypically modulated SMCs that have lost detectable SMαA expression or, possibly, adventitial fibroblasts.

To track putative ECs and SMCs derived from circulating progenitor cells, we inserted BALB/c allografts into eGFP-negative isografts that were previously transplanted into eGFP<sup>+</sup>apoE<sup>−/−</sup> B6 mice (n=5) (Figure 1c). In this situation, all recipient cells except for those of the flanking vasculature were eGFP<sup>+</sup>. As expected, the AV lesions contained many eGFP<sup>+</sup> cells, the majority of which were demonstrated to be macrophages by the murine macrophage marker Mac2 (Figure 5), but not a single eGFP<sup>+</sup> SMαA<sup>+</sup> or eGFP<sup>+</sup>vWF<sup>+</sup> cell (among 3162 SMαA<sup>+</sup> and 672 vWF<sup>+</sup> cells analyzed, respectively) was found (Figure 6). In rare instances, SMαA and eGFP signal overlapped slightly in sections, but this never took on the morphology of an eGFP<sup>+</sup> SMC (see Supplementary Figure II), which was easily identified in positive controls by its green fluorescent cytoplasm and subplasmalemmal SMαA staining (Figure 4). Because it was observed only in cases where multiple cell nuclei were closely apposed, we
find it reasonable to assume that it reflects the fact that even with three-dimensional deconvolution microscopy, z-axis resolution is limited, and therefore, partly overlying SMCs and bone marrow–derived cells cannot always be completely resolved.

**Discussion**

The present study describes a novel double-transplantation technique to track cell migration from the flanking recipient artery into allografts. We applied this method to a modified version of the AV model studied previously by Hu et al in which graft cells are fully eradicated and AV develops entirely through the recruitment of cells from the recipient.8,9 In conflict with a widespread theory, we found that all recipient-derived ECs and SMCs migrated into allografts from the flanking recipient vasculature with no contributions from putative circulating progenitor cells.

**Vessel Transplant AV Models**

Vessel transplantation AV models, such as the one used in the present study, have a number of dissimilarities to AV developing in human or animal organ transplants. The use of immune suppression with organ transplantation protects the donor arterial media and endothelium and sustains the local vessel wall as the main source of SMCs and ECs in AV with minor, but possibly functionally important, contributions from the recipient.2–7 In vessel transplant models without immune suppression, however, the involvement of donor-derived cells is completely blocked and only recipient-derived cells are allowed to participate. Such models should therefore not be considered as comprehensive models for AV but as tools to investigate selected aspects of AV pathogenesis. They have been central for establishing the hypothesis that circulating progenitor cells contribute to AV,1,17 and it is therefore not merely academic to understand whether results obtained in such models have been interpreted correctly.

**Cellular Repopulation Through Local Proliferation and Migration**

Many rodent studies during the last decade have shown that recipient cells repopulate allografted vessels when no immune suppression is administered.8,9,10,11,18,19 Hillebrands et al,18 Shimizu et al,11 and Hu et al8,9 hypothesized that the majority of these recipient-derived cells originate from circulating progenitor cells. This idea was inferred mainly from the absence of observable inward migration fronts of ECs and SMCs at selected early time points after allografting8,9,18 or other suggestive observations,11 but no previous study has been designed to directly test the hypothesis. Our study is
The ApoE−/− Mouse Model

Our strategy was to choose the mouse model of AV for which the most robust recruitment of recipient-derived cells to allografts has been reported for our exploration of recipient cell source. This is provided by the apoE−/− model by Hu et al, in which the lack of apoE leads to more aggressive AV with an increased flux of recipient-derived SMCs into allografts compared with wild-type models.8

Although the lack of apoE in other experimental settings has been suggested to affect the number of circulating endothelial progenitor cells,24 it is not plausible that the lack of apoE in our model could have biased our examination of the recipient cell source. For this to occur, apoE should act as a molecular switch that when absent totally abolishes progenitor cell seeding to allografts, and indeed nothing in the literature indicates this to be the case.

Notably, the accelerated lesion development observed in the apoE−/− murine AV model is fully consistent with an important role of migrating SMCs from the flanking vasculature. Neointima formation is substantially increased in apoE−/− compared with wild-type B6 mice after mechanical vascular injury, an effect that appears to be mediated by both hypercholesterolemia and the loss of the direct inhibitory effect of apoE on migration and proliferation of local medial SMCs.26–29

Chimerism of the Transplanted Human Heart

Detection of the Y chromosome in gender-mismatched (female-to-male) heart transplants has provided a unique opportunity to distinguish donor cells from recipient cells, but the technique is challenging, and potential pitfalls have been highlighted in recent publications.16,25,30–32 Allografts are always invaded by immune cells of recipient origin, and reliable detection of neighboring nonimmune cells, such as ECs and SMCs, is difficult and requires optimal tissue processing, appropriate cell markers, and single-cell-resolution microscopy.

We will not attempt to extrapolate from our AV model to the more complex biology of a transplanted organ, but our experiments challenge the support that rodent vessel transplantation studies in the past have lent to the idea that blood-derived ECs and SMCs participate in human AV. Furthermore, our data remind us that recipient origin of some ECs and SMCs in human AV is not proof that these cells originate from circulating progenitor cells. How far resident cells may migrate into human transplanted organs is uncertain, but it has been suggested that the entire allografted heart may be repopulated from the recipient atrial remnant.33

Limitations

Our study was not designed to investigate whether the migrating recipient cells were mature, proliferating ECs and SMCs. We believe this to be a very likely mechanism but cannot exclude the potential involvement of adventitial myofibroblasts or putative local stem cells in the arterial wall.32

Conclusion

Migrating vascular cells rather than circulating progenitor cells were the source of recipient-derived ECs and SMCs in AV.
a murine model of AV. These results challenge the proposed importance of circulating progenitor cells in the development of AV in experimental models and humans.

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Disclosures
None.

References
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Supplementary Figure I

Positive (eGFP⁺apoE⁻/⁻) and negative (apoE⁻/⁻) control tissues were used for validity of eGFP detection. Carotid artery segment from an eGFP⁺apoE⁻/⁻ mouse (a) and an apoE⁻/⁻ mouse (b). The figures are adjusted for autofluorescence by brightness and contrast adjustments with fixed settings. Green indicates eGFP; blue, nuclei; gray, DIC. Scale bars=50 µm.
Supplementary Figure II

Rare examples of overlapping red and green fluorescence in images and their interpretation. The images show BALB/c carotids that were inserted end-to-end into a non-fluorescent isograft in an eGFP⁺apoE⁻ B6 mouse. Arrow in (a) marks dots of red fluorescence (indicating SMαA) that appears to co-localize to eGFP⁺ cells. The arrows in (d) mark distinct spots with co-localizing red and green (yellow) fluorescence. In neither case, however, does the appearance resemble an eGFP⁺ SMC, which can easily be studied in positive controls and is characterized by green fluorescent cytoplasm and subplasmalemmal SMαA staining. Slight spatial overlap between SMαA⁺ and eGFP⁺ cells or spots of autofluorescent pigments are likely explanations. Green indicates eGFP; red, SMαA⁺; blue, nuclei; a+d, merged color channels; b+e, green channel; c+f, red channel; grey, DIC; L, lumen. Scale bars=25 µm.