Short Communication

Vascular Smooth Muscle Cells of Recipient Origin Mediate Intimal Expansion after Aortic Allograft Transplantation in Mice

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Intimal expansion by vascular smooth muscle cells (SMCs) is a characteristic feature of graft vascular disease. Whether graft intimal SMCs arise from donor or recipient tissue is not well established but has important pathogenetic implications. We examined for the presence of male cells in the expanded intima of sex-mismatched mouse aortic allografts (C57BL/6-to-BALB/c) at 30 or 60 days after transplant by *in situ* hybridization using a Y-chromosome probe. Study groups included male-to-female allografts, female-to-male allografts, and female-to-female allografts in recipients previously engrafted with male bone marrow. Although intimal expansion developed in all allografts, male-to-female allografts lacked Y-chromosome-positive intimal cells. In contrast, such cells were abundant in female-to-male allografts and most of these cells co-labeled for smooth muscle α -actin by immunostain. Female-to-female allografts in recipients with male bone marrow showed a limited number of intimal Y-chromosome-positive cells. However, none of these clearly co-labeled for smooth muscle α -actin and their numbers declined throughout time, consistent with graft-infiltrating inflammatory cells. We conclude that intimal expansion of mouse aortic allografts is mediated by SMCs that originated from the recipient. There was little evidence of their derivation from the bone marrow, suggesting instead the adjacent host aorta as the primary source of intimal SMCs. (*Am J Pathol* 2001, 158:1943–1947)

vascular disease and evidence supports alloimmunity as an etiological factor in this process.¹ The source of the intimal SMCs has yet to be fully defined but its determination is fundamental to understanding the pathogenetic interactions that exist among the cell types involved. A conventionally accepted source of intimal SMCs is the donor arterial media, from which SMCs migrate across the internal elastic lamina, akin to what occurs after mechanical arterial injury.² This concept, however, has never been proved in the transplant setting and other possibilities warrant consideration. One such alternative source of SMCs is the walls of the recipient vasculature to which the graft is anchored by surgical anastomosis. In this case, the SMCs are of recipient origin and their movement across the plane of anastomosis may be directed by inflammatory cytokines expressed within the graft during episodes of alloimmune rejection.¹ A third and more speculative source of SMCs is the putative precursor cells of recipient bone marrow origin that follow a pattern of vascular SMC differentiation on activation. Such SMC-like cells have been isolated in studies of bone marrow cell culture³ and the paradigm of distant parenchymal repopulation by marrow-based progenitor cells has been demonstrated in the central nervous system of adult mice.⁴

We have previously described a mouse aortic allograft model of chronic vascular rejection and used it to assess immunocellular responses.⁵ Allograft intimal thickening develops in this model within 3 to 4 weeks of grafting with the principal histological features of human allograft coronary disease, including an accumulation of intimal SMCs in a proteoglycan-rich matrix. We chose this model to investigate the origin of allograft intimal SMCs by analyzing different combinations of sex-mismatched mouse aortic allografts, using a Y-chromosome probe to distinguish cells of male lineage. We found a vast predomi-

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Intimal expansion by vascular smooth muscle cells (SMCs) is a key feature in the development of allograft

nance of recipient-derived SMCs in the expanded allograft intima, with little evidence of their origin from the bone marrow, suggesting the adjacent recipient aorta as their primary source.

Materials and Methods

Animals

All mice were used in accordance with the guidelines of the Council on Animal Care of The University of Western Ontario, London, Canada. Male and female mice (age, 5 to 8 weeks; body weight, 25 to 30 g) of C57BL/6 (H-2^b) and BALB/c (H-2^d) strains (The Jackson Laboratory, Bar Harbor, ME) were used for aortic transplantation and bone marrow engraftment as specified.

Aortic Grafting and Experimental Design

Aortic segment transplantation was performed by end-to-end anastomosis in the infrarenal aorta as previously described.⁵ C57BL/6-to-BALB/c allografts were studied without immunosuppressive medication. Study groups included male-to-female allografts ($n = 6$); female-to-male allografts ($n = 6$); female-to-female allografts in recipients previously engrafted with male bone marrow ($n = 10$); and male-to-male or female-to-female allografts (sex-matched control; $n = 2$ each). The aortic graft, spleen, and testis/ovary were removed at 30 or 60 days after transplant as specified, fixed in 4% formaldehyde, embedded in paraffin, and sectioned 4- μ m thick for standard histological staining, *in situ* hybridization, and immunohistochemical analysis. In addition, we performed sex-mismatched BALB/c-to-BALB/c aortic grafts (male-to-female; syngeneic control; $n = 2$) for standard histological assessment alone.

Bone Marrow Transfer

Female BALB/c mice were engrafted with male BALB/c bone marrow cells as described.⁶ Recipient mice were pretreated by total body irradiation at 650 Rad and then received 1×10^7 bone marrow cells in 0.3 ml of RPMI 1640 via tail vein injection within 6 hours of irradiation. No wasting or mortality was encountered in the reconstituted mice during 3 weeks of observation after irradiation. This was followed by aortic transplantation with the marrow-reconstituted mice serving as recipients. *In situ* hybridization for the Y chromosome was used to confirm male lineage repopulation of the recipient spleens sampled individually at the time of aortic graft retrieval.

In Situ Hybridization

The 145SC5 probe is a 1.5-kb cDNA fragment cloned into the *Eco*RI site of Bluescript vector and was obtained from Dr. Y. Nishioka (McGill University, Montreal, Canada).⁷ Digoxigenin-dUTP was incorporated by random primed labeling using a commercial kit (DIG-High Prime; Boehringer Mannheim, Laval, Quebec, CA). For *in situ* hybrid-

ization, tissue sections were dewaxed in xylene and endogenous peroxidase was quenched in 3% H₂O₂. The tissues were digested with 3 mg/ml pepsin in 0.1 N HCl for 6 minutes at 37°C, and then incubated for 20 minutes at 100°C with prehybridization buffer containing 50% deionized formamide, 5 $\times$ standard saline citrate, 2% blocking reagent, 0.1% *N*-lauroylsarcosine, 0.02% sodium dodecyl sulfate, and 100 ng/ml salmon sperm DNA. Hybridization was performed at 37°C for 16 hours in hybridization buffer consisting of cDNA probe diluted 1:4 in prehybridization buffer. The slides were washed in a series of graded salt solutions (2 $\times$ and 0.5 $\times$ standard saline citrate twice each) and anti-digoxigenin sheep Fab conjugated with alkaline phosphatase (Boehringer Mannheim) was applied. A blue-black reaction was produced with nitroblue tetrazolium/bromochloroindolyl phosphate and nuclear fast-red was used as counterstain.

Immunostaining and Co-Labeling

A standard avidin-biotin immunoperoxidase technique⁸ was used with an Elite Vectastain kit (Vector Laboratories, Burlingame, CA). Primary monoclonal antibodies were reactive to smooth muscle α -actin (clone 1A4; DAKO, Carpinteria, CA), CD3 (clone CD3-12; Novocastra, Newcastle, UK) and Mac-3 (clone M3/84; PharMingen, Mississauga, Ontario, CA). These were chosen for their demonstrated effectiveness in formaldehyde-fixed mouse tissues. Negative controls were performed by substituting nonimmune serum for primary antibody. In the case of smooth muscle α -actin, isotype control IgG2a was also used in place of clone 1A4. Diaminobenzidine was used as chromogen (brown). To co-label by immunostain after *in situ* hybridization, tissue sections were microwaved with 10 mmol/L sodium citrate, pH 6.0, for 10 minutes for antigen retrieval. The sections were immunostained for smooth muscle α -actin as above, except for substituting the use of doubly dilute diaminobenzidine for color development.

Results

Sex-mismatched C57BL/6-to-BALB/c aortic allografts showed diffuse intimal thickening at 30 days after transplant, indistinguishable from sex-matched allograft controls. Conversely, sex-mismatched syngeneic controls (BALB/c-to-BALB/c) remained free of intimal lesions. Thus, donor-recipient sex mismatch in itself neither precipitated, nor prevented, intimal lesion development.

In situ hybridization was performed on aortic allograft sections using a digoxigenin-labeled cDNA probe for a repetitive sequence in the mouse Y chromosome.⁷ This method produced a positive signal over the cell nucleus in control sections of male mouse spleen or testis (Figure 1A, inset), with absence of signal in female mouse spleen or ovary. In male-to-female aortic allografts, no hybridization signal was detected in the thickened intima, indicating a lack of donor-type cells in this location (Figure 1A). Also, no hybridization signal was detected in the media of these allografts. The latter finding is consistent with the

Figure 1. The intimal cells in sex-mismatched mouse aortic allografts were assessed by *in situ* hybridization with a digoxigenin-labeled Y-chromosome probe. **Inset in A** shows probe hybridization (blue-black signal) over the nuclei of sperm-producing cells in a control section of mouse testis. Hybridization signals were not detected in the expanded intima of male-to-female allografts (**A**) but were abundant in the case of female-to-male allografts (**B**). *In situ* hybridization (**C**) followed by immunostaining for smooth muscle α -actin (cytoplasmic brown signal; **D**) in female-to-male allografts demonstrated the presence of co-labeled intimal cells (three examples in matching circles in **C** and **D**). Nuclear fast-red counterstain in all panels; immunoperoxidase stain in **D**. Scale bar, 50 μ m.

known inflammatory damage of the allograft media within the first 2 weeks of grafting in this model, with the consequent replacement of medial SMCs by loose cellular debris and matrix.⁵

A different hybridization pattern was observed in female-to-male aortic allografts. There were abundant positive signals in the thickened intima, identifying the vast majority of the intimal cells (Figure 1B). Positive signals were additionally noted in portions of the media and the adventitia. Whereas the latter medial and adventitial cells could be taken to represent residual inflammatory cells of the host, such an explanation was unlikely in the well-established, thickened intima, where previous data have indicated a clear predominance of vascular SMCs.⁵ We suspected, therefore, that many of the recipient-type intimal cells identified in the female-to-male allografts were of vascular SMC lineage, rather than inflammatory.

To evaluate this, we used co-labeling with *in situ* hybridization for the Y chromosome followed by immunostaining for cytoplasmic smooth muscle α -actin. The re-

sults of a representative section of female-to-male aortic allograft before and after the application of the second label are shown in Figure 1, C and D, respectively. Multiple intimal Y-chromosome-positive cells were strongly immunoreactive for smooth muscle α -actin, substantiating their identity as recipient-derived vascular SMCs. Conversely, immunostaining for inflammatory cell subsets in subjacent sections showed only a small minority of intimal cells to be positive for CD3 or Mac-3 (T cells or macrophages, respectively, data not shown).

To address the novel possibility that allograft intimal SMCs may develop from a precursor fraction of recipient bone marrow, we examined aortic allografts from female C57BL/6 donors placed into female BALB/c recipients engrafted previously with male BALB/c bone marrow cells. In these mice, native bone marrow had first been ablated by total body irradiation. The marrow-reconstituted mice thus preserved their inbred BALB/c haplotype but were chimeric for having male lineage cells of bone marrow origin. *In situ* hybridization for the Y chromosome

Figure 2. Mouse aortic transplantation from a female C57BL/6 donor to a female BALB/c recipient previously engrafted with male BALB/c bone marrow cells. In the recipient spleen (A), Y chromosome *in situ* hybridization revealed male lineage extramedullary hematopoiesis in the red pulp and male lineage repopulation of the periarteriolar lymphoid sheath, sparing the female central arteriole (circular void in hybridization signals; near top right corner of A). In contrast, hybridization signals were sparse over the allograft intima (B). Nuclear fast-red counterstain. Scale bar, 50 μ m.

in spleen sections of these mice at aortic graft retrieval (~ 2 months after reconstitution) demonstrated a robust repopulation of the periarteriolar lymphoid sheath by male lineage cells (Figure 2A). Aortic allografts in these chimeric recipients developed intimal thickening indistinguishable from that in nonchimeric recipients. *In situ* hybridization for the Y chromosome in the thickened allograft intima of these chimeric recipients showed male lineage cells although in low abundance (Figure 2B). Moreover, the scattered Y-chromosome-positive cells present could not be clearly identified as vascular SMCs by co-labeling and their numbers diminished from 30 to 60 days after transplant, in keeping with a subsiding inflammatory cell response. The co-labeling procedure did highlight plentiful, singly positive, intimal cells expressing smooth muscle α -actin. The data thus suggest that few, if any, of the allograft intimal SMCs were derived from recipient bone marrow.

Discussion

The present studies definitively establish that the SMCs in the expanded intima of mouse aortic allografts arise from the recipient. This is important proof of principle that remodeling of the allograft vessel may be orchestrated, at least in part, by recipient-derived SMCs. Unequivocal identification of this phenomenon was likely facilitated by two key aspects of the model we used. First, in mouse aortic allografts, a substantial loss of donor medial SMCs occurs early after inflammatory cell infiltration, biasing the response in favor of recipient SMCs. Secondly, the unmitigated rejection in the model poses a powerful and sustained inducement to recipient SMCs across the anastomotic site to respond to the mitogenic and chemotactic signals of the cytokines released.¹ On speculation, donor and recipient SMCs might coexist in the intimal lesion in a particular setting, depending on how successfully these or other factors are manipulated.

A contribution of recipient SMCs to allograft intimal expansion has been suggested in the rat aortic allograft model. Inward migration of recipient SMCs was implicated by the pattern of intimal lesion formation after cryoablation of either the donor or the recipient side of the graft anastomosis.⁹ However, no marker was used in this study to distinguish donor from recipient cells. In a different study, fluorescence cell scanning of allograft intimal cell suspensions detected only the recipient serotype, although the extent to which SMCs were represented in suspension was unknown.¹⁰ In this study, as in a separate study in mice,¹¹ immunostaining for major histocompatibility class II molecules failed to disclose the donor or recipient origin of allograft intimal SMCs because of inadequate antigen expression. Thus, our data uniquely provide direct *in situ* evidence of the recipient origin of allograft intimal SMCs, incorporating the use of co-labeling for simultaneous determination of cell origin and cell type.

Pertinent information in humans is limited to a few studies involving one to two patients each. Two cases of coronary disease in female-to-male human heart transplants have been studied by fluorescence *in situ* hybridization for the Y chromosome.¹² Hybridization signals were found over inflammatory cells but not over coronary medial or intimal SMCs, suggesting the donor origin of the latter. In a different report on a single patient, polymerase chain reaction for three polymorphic gene loci was used to compare the genotype of cells in coronary atherectomy fragments of a transplanted heart with that of biopsied donor myocardium and that of recipient blood leukocytes.¹³ Cells of recipient origin contributed as much as 10% of the genetic material associated with the coronary lesion but the data did not allow recipient blood contamination at atherectomy to be distinguished from pathogenic involvement of recipient-type cells in coronary lesion formation. In humans, therefore, the limited amount of data precludes a firm conclusion.

Our findings did not provide support for the contention that allograft intimal SMCs may be derived from recipient bone marrow. The Y chromosome probe may underestimate the presence of male lineage cells because of positioning of the nuclei partly or completely off the plane of section, and therefore it is conceivable that a small number of marrow-derived cells were missed. However, our data are clearly contrary to any major contribution of such cells to the composition of the mature intimal lesion. It is also theoretically possible that the relevant SMC precursor population was selectively lost during marrow transfer, however we note that progenitor cells are typically more resistant than differentiated ones. Moreover, an aliquot of the marrow transfer inoculum was placed in culture in medium enriched for vascular SMC growth as we previously used.¹⁴ We observed in these marrow cultures the emergence of cells with typical SMC morphology replete with suitable immunoreactivity for markers such as smooth muscle α -actin and calponin (data not shown). Therefore, in the final analysis, we believe that marrow-derived SMCs are not materially responsible for the expanded intima of mouse aortic allografts.

In summary, we have shown by *in situ* hybridization for the Y chromosome in sex-mismatched mouse aortic allografts that the expanded intima, which is characteristic of allograft vascular disease, is comprised of abundant vascular SMCs of recipient origin. These cells seem to have arisen from the adjacent portions of the recipient aorta, in contrast to a significant contribution from a recipient bone marrow source.

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