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Donor versus Recipient: Neointimal Cell Origin in Allograft Vascular Disease

Anton I. Skaro, Robert S. Liwski, Paul Johnson, Jean-Francois Legare, Timothy D. G. Lee, and Gregory M. Hirsch

The most common and intractable pathological presentation of chronic rejection in heart transplants is allograft vasculopathy (AV) characterized by the formation of a diffuse, occlusive intimal lesion. To date there is no effective treatment or prevention of this condition, owing in part to an incomplete understanding of the mechanism underlying AV. It has been recently demonstrated that the neointimal lesion cells originate from the recipient, and derive at least in part from marrow-derived mesenchymal precursors. This is in direct conflict with the previously held hypothesis that lesion cells are derived from the adjacent vascular media in response to immune injury. A better understanding of this rejection process might provide new therapeutic strategies which more appropriately address the recruitment, proliferation, and differentiation of progenitor mesenchymal lesion cells derived from the recipient.

Introduction

Orthotopic heart transplantation is a well-established treatment modality for end-stage heart disease. Despite advances in immunosuppressive therapy and improved short-term graft survival, late graft failure has emerged as a significant cause of morbidity and mortality in clinical transplantation.¹ The leading cause of late heart and kidney graft loss is chronic rejection, for which there is no effective treatment. The failure of current management strategies is the result of an incomplete understanding of this rejection process.

Although there are organ-specific changes associated with chronic rejection, allograft vasculopathy (AV) has been described in all solid organ transplant patients suffering from chronic rejection.¹ AV is characterized by a diffuse, concentric intimal thickening with concomitant medial smooth muscle cell (SMC) loss. This leads to a progressive narrowing of the lumen, with eventual ischemic organ failure.² AV is central to graft failure following heart transplantation, and it has been suggested that it

contributes to irreversible organ damage in noncardiac transplants.²

The origin of the cells constituting the neointimal proliferative lesion has been the source of controversy for the past 3 decades.⁴ The generally accepted hypothesis has been that chronic alloresponses primarily target the graft endothelium, with subsequent leukocyte infiltration and production of cytokines, chemokines, and growth factors.⁵ In response, vascular SMCs in the media differentiate into a proliferative phenotype and transmigrate into the subendothelial compartment, resulting in occlusive lesion formation.⁶ According to this interpretation, the lesion consists of vascular SMCs derived exclusively from the graft and is therefore donor in origin.⁶ This hypothesis is currently being reevaluated based on recent data.

The Previous Hypothesis: Donor-Derived Lesion

Despite an increase in research interest in the field of chronic allograft rejection over the past decade, the pathophysiology of AV remains poorly under-

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stood. Ross⁷ proposed the response-to-injury hypothesis as the mechanism involved in native atherosclerosis, and it has been adopted to explain the pathophysiology of AV. This hypothesis states that endothelial injury mediated by modified lipoproteins and local shear stress is the initiating event in the development of atherosclerosis. In response to this injury, endothelial cells elaborate cytokines, chemokines, and adhesion molecules, which lead to leukocyte accumulation at the site of injury.⁷ Subsequently, the production of growth factors including platelet-derived growth factor, fibroblast growth factor, and insulin-like growth factor by activated endothelium and macrophages results in medial SMC migration to the intimal compartment and proliferation to form the atheromatous plaque.⁸ Despite many differences in the nature and distribution of these 2 disease processes, the predominant feature common to both AV and native atherosclerosis is the expression of α -actin by the cells populating the intimal proliferative lesions. Based on this expression of α -actin, it has been widely assumed that the neointimal cells are vascular SMCs and that they originate in the media of the vessel wall.^{2,7} This similarity between AV and native atherosclerosis prompted the adaptation of the response-to-injury hypothesis to explain the pathophysiology of AV.⁹

Early studies in animal models including both rat aortic and heterotopic heart allografts used light microscopy and immunocytochemistry to describe the basic features of allograft vascular disease. These results revealed that the intimal occlusive lesions consisted initially of macrophages, with a progressive increase over time in the number of α -actin positive SMCs.^{2,6} Concomitantly, there was a striking loss of cellularity within the media. Based on these observations, it was proposed that transmigration of medial SMCs to the intimal compartment accounted for both the medial SMC loss and intimal lesion formation.⁶

More recently, through the use of electron microscopy (EM), investigators have made attempts to determine the phenotypic characteristics of the vessel wall cells of aortic allografts undergoing chronic rejection. Of particular interest are the observations that early changes in endothelial integrity, likely the result of both immune-dependent and

Summary:

- Cellular and molecular mechanisms of allograft vasculopathy remain unclear
- Allograft vasculopathy is characterized by medial smooth muscle cell loss and intimal hyperplasia
- Medial smooth muscle cell loss occurs via cytolytic cell induced apoptosis and not transmigration to the subendothelial space
- Recipient-derived smooth muscle-like cells form the intimal proliferative lesion of allograft vasculopathy in animal models but this requires further confirmation in human transplantation
- Neointimal lesion cells consist of mesenchymal progenitor cells which express α -smooth muscle actin and are at least in part derived from the bone marrow of the host

immune-independent mechanisms, are accompanied by damage to the internal elastic lamina (IEL).¹⁰ EM analysis of the media of these allografts as early as 1 week following transplantation showed evidence of a shift in the inner layer of SMCs toward a synthetic phenotype as revealed by a scarcity of myofilaments and more prominent rough endoplasmic reticulum and Golgi apparatus. Bojakowski et al.¹⁰ speculated that migration of these modified putative SMCs through fine holes in the IEL into the intimal compartment is the mechanism responsible for lesion formation. Although this work appears to support the hypothesis of transmigration of medial SMCs to the intimal compartment in AV, it is inconclusive in that it infers transmigration from the development of breaks in the IEL and the appearance of a putative synthetic SMC phenotype, with the occasional cell purported to be migrating. The inability to directly confirm by immune EM the exact nature of these putative modified SMCs limits the interpretation of these data. Furthermore, this type of static analysis is unable to determine the source of the smooth muscle-like cells that are recruited to the subendothelial space. It is, for example, possible that marrow-derived mesenchymal progenitor cells are homing to the site of injury in response to various chemokines and

ALLOGRAFT VASCULOPATHY

Immune-mediated injury of vessels within transplanted organs leading to vascular remodeling including medial smooth muscle cell loss and neointimal lesion formation.

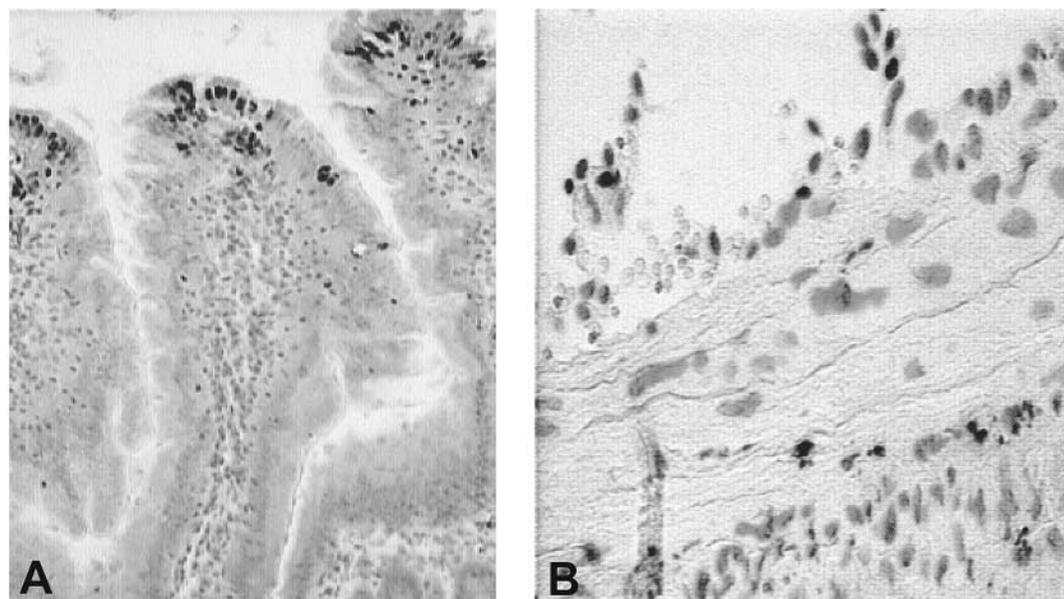


Figure 1. Detection of nuclear DNA fragmentation indicative of apoptosis was assessed by in situ terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick-end labeling staining of formalin-fixed and paraffin-embedded tissue in positive control small intestine (A) and wild-type aortic allograft 20 days following transplantation (B). Figure derived from work previously reported in Legare JF, Issekutz T, Lee TD, Hirsch G. CD8⁺ T lymphocytes mediate destruction of the vascular media in a model of chronic rejection. *Am J Pathol* 2000;157:859-65.

growth factors and are being misidentified in transit from the adventitia to the neointima.

The Current Hypothesis: Recipient-Derived Lesion

The response-to-injury hypothesis in native atherosclerosis would suggest that lesion formation is the direct result of migration of medial SMCs into the intimal compartment.⁷ However, Han et al.,¹¹ using a mouse vascular mechanical injury model of native atherosclerosis, showed that circulating bone marrow cells contribute to neointimal formation. They suggested that the neointimal lesion of native atherosclerosis is, at least in part, marrow derived, thus weakening support for the SMC transmigration hypothesis.¹¹ Han et al. repopulated the marrow of lethally irradiated female mice with male bone marrow cells and then subjected these animals to 2 types of mechanical vessel injury. Four weeks later, they found that the arterial lumen was obliterated by predominantly α -actin staining cells, approximately half of which were of male origin as indicated by in situ hybridization with a Y-chromosome-specific probe.¹¹

There is accumulating evidence that SMC migration does not account for the neointimal lesion cells characteristic of AV. In a rat aortic allograft model, Plissonnier et al.,¹² using putative strain-specific polyclonal antibodies, suggested that lesions of chronic rejection contain recipient-derived endothelium and smooth muscle-like cells. However, we and others have been unable to confirm this finding using the polyclonal antibody, and questions of strain specificity have arisen. In light of this, we targeted the development of medial SMC loss, which is a characteristic of AV. If we could show that medial SMCs were destroyed in situ during the process of chronic rejection, this would provide at least indirect evidence that the transmigration hypothesis is incorrect. The currently accepted interpretation suggests that medial SMC dropout is the result of a depopulation of the media in favor of intimal recruitment.⁶ We sought to determine the mechanisms involved in this loss of SMCs from the media and eventually the relationship to the propagation of the intimal lesion.

Using reverse transcriptase polymerase chain reaction (PCR), we observed an up-regulation of

NEOINTIMA

Proliferative lesion of allograft vasculopathy consisting of smooth muscle-like cells within the subendothelial space.

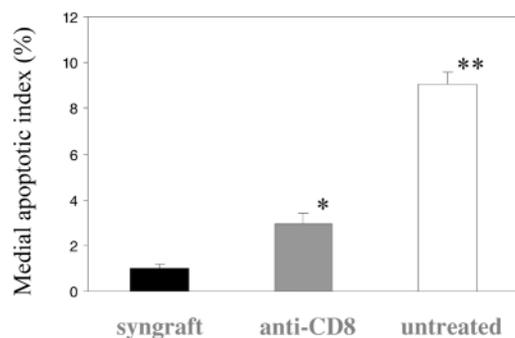


Figure 2. Medial smooth muscle cell apoptotic index was assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay at 20 days after transplantation in syngeneic controls (syngraft), CD8⁺ T cell-depleted allograft animals (anti-CD8), and untreated allograft animals (untreated). * $P < 0.02$ between syngeneic and CD8⁺ T cell-depleted animals. ** $P < 0.001$ between untreated allograft animals. Five animals per group were used. Reprinted with permission from the American Society for Investigative Pathology. Legare JF, Issekutz T, Lee TD, Hirsch G. CD8⁺ T lymphocytes mediate destruction of the vascular media in a model of chronic rejection. *Am J Pathol* 2000;157:859-65.

IN SITU HYBRIDIZATION

Technique used to detect particular chromosomes or genetic sequences (i.e., DNA and RNA) using specific probes.

mRNA coding for cytotoxic T lymphocyte (CTL) mediators of apoptosis including perforin, granzyme B, and Fas ligand in aortic allografts undergoing chronic rejection.¹³ This suggested to us that CD8⁺ CTL might be playing an important role in the events occurring within the media of vessels undergoing chronic rejection. Using in situ terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick-end labeling, we were able to demonstrate marked medial SMC apoptosis by detecting nuclear DNA fragmentation (Fig. 1).¹⁴ Moreover, in a CD8⁺ CTL-depleted environment through monoclonal antibody depletion, there was significant medial SMC sparing as indicated by a reduction in medial apoptosis (Fig. 2) and a concurrent preservation of α -actin staining cells (Fig. 3).^{14,15} Taken together, these data provide convincing evidence that the profound thinning and loss of cellularity within the media of allograft vessels is the result of cytolytic cell-induced apoptosis^{14,15} and not depopulation secondary to SMC transmigration as originally proposed.⁶

Given the evidence that medial SMC loss was not due to transmigration and in light of the findings of Plissonnier et al.¹² and Han et al.,¹¹ we sought to determine the origin of the neointimal cells. We de-

veloped strain-specific primers for the variable regions of the rat major histocompatibility complex (MHC) class 1 (RT1A) allele in order to distinguish between brown Norway (BN) and Lewis (Lew) DNA (Fig. 4).¹⁶ We used the well-established rat aortic interposition graft model between fully histoincompatible inbred strains (BN and Lew), which consistently produces the hallmark characteristics of AV.² Aortic allograft tissue was harvested 8 weeks following transplantation and using a combination of chemical (ethylene diamine tetra-acetic acid [EDTA]) treatment and microdissection, the neointima was isolated without contamination by the subjacent media.¹⁶

In nonimmunosuppressed animals, PCR analysis was performed using the strain-specific primers and DNA extracted from the isolated neointimal tissue. In this strain combination (BN to Lew and Lew to BN), only recipient DNA was detected within the neointima of the aortic allograft tissue (Fig. 5).¹⁶ Arguably, given the profound host-derived inflammatory infiltrate, it is not surprising that we see amplification with recipient-specific primers. However, the presence of robust α -actin staining within the neointimal tissue, coupled with the absence of any donor-derived amplification (despite the sensitive nature of this PCR technique), rules out the possibility of a donor-derived component.¹⁶ Similarly, Hillebrands et al.¹⁷ recently showed recipient origin of both endothelial cells and smooth muscle-like cells in the neointima of rat aortic allografts in nonimmunosuppressed animals using a single-cell PCR technique.

To make this observation more widely applicable to the clinical situation, we repeated this experiment in animals that were treated with 5 mg/kg/d cyclosporin A following transplantation. Again, PCR analysis (data not shown) revealed that amplification was observed only with recipient-specific primers. This confirmed that the neointimal cells are of recipient origin in the presence of immunosuppression.¹⁶

Source of Recipient Cells

Although it seems evident that the neointimal cells are of recipient origin, the nature of the cells that form this occlusive lesion and their mechanism of entry into the subendothelial compartment re-

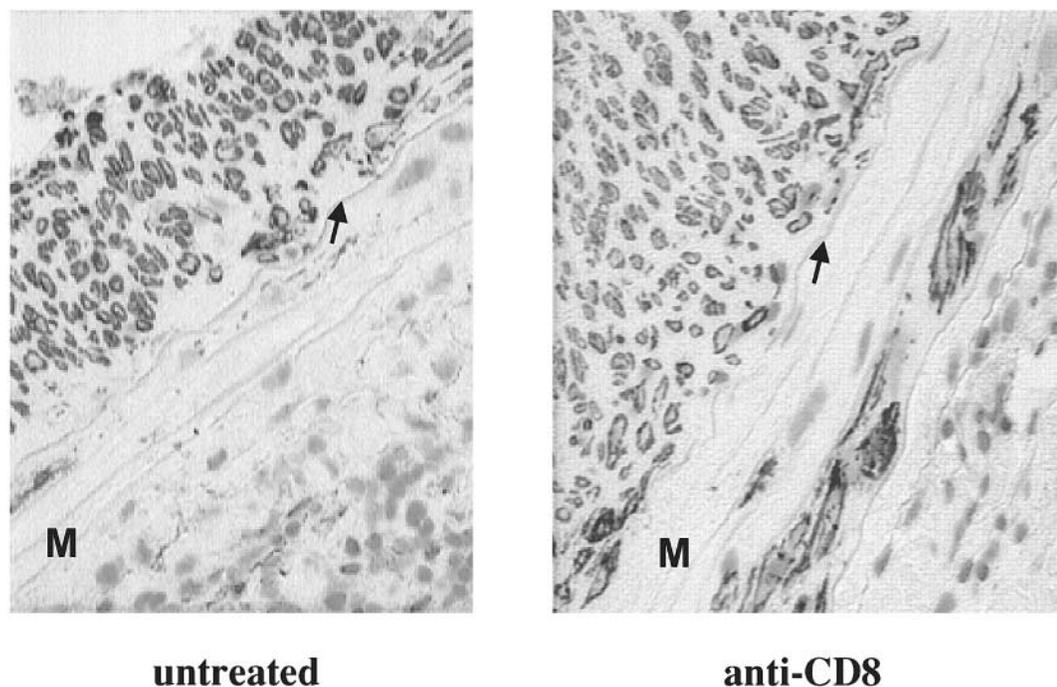


Figure 3. Peroxidase immunocytochemistry of aortic allograft tissue at 60 days after transplantation in CD8⁺ T cell-depleted animals (A) and untreated wild-type animals (B) using anti-smooth muscle α -actin monoclonal antibody (mAB) (clone HHF35). Note the preservation of medial α -actin-positive cells with anti-CD8 monoclonal antibody treatment. M = media. The arrow indicates the internal elastic lamina. Reprinted with permission from the American Society for Investigative Pathology. Legare JF, Issekutz T, Lee TD, Hirsch G. CD8⁺ T lymphocytes mediate destruction of the vascular media in a model of chronic rejection. *Am J Pathol* 2000;157:859-65.

POLYMERASE CHAIN REACTION

Technique used to detect specific DNA sequences via amplification of DNA even when they are present at extremely low levels in a complex mixture.

main poorly understood. The cellular characteristics of the neointimal cells suggest that they are of mesenchymal origin.¹⁸ These cells may enter the intimal compartment either by linear transmigration from the adjacent host media or by recruitment of circulating progenitor cells.^{11,16,17} It seems intuitive that primary adherence within the vessel lumen is less likely than recruitment via the microcirculation where velocity and flow parameters are more compatible with durable cell-to-cell interactions, facilitating firm adhesion and subsequent diapedesis. Using the rat aortic allograft model, Aziz et al.¹⁹ observed that AV was abrogated by transiently freezing the recipient aorta adjacent to the donor segments with liquid nitrogen. In contrast, AV was observed when donor segments were frozen prior to transplantation.¹⁹ Although the authors concluded that recipient medial SMCs must populate the neointima by linear transmigration, these results must be interpreted with caution given the un-

known impact of freezing tissue on the complex mechanism culminating in the development of AV.

More recently, Saiura et al.²⁰ showed that circulating progenitor SMCs contribute to cardiac allograft vasculopathy in a mouse heterotopic heart transplant model. Moreover, it is important to emphasize that recipient cells form the intimal proliferative lesions within whole-organ (heart) allografts as well as arterial allografts, making this observation more widely applicable to the human condition.

In support of these findings, there is an accumulating body of evidence that the adult bone marrow harbors somatic stem cells, which are pluripotent and are able to differentiate into various lineages including both endothelium and vascular SMCs.²¹ Moreover, implanted synthetic vascular grafts in humans have been shown to be seeded by host endothelium.²² In an elegant study, Shimizu et al.²³ directly evaluated the source of recipient cells that constitute the intimal proliferative lesion. They

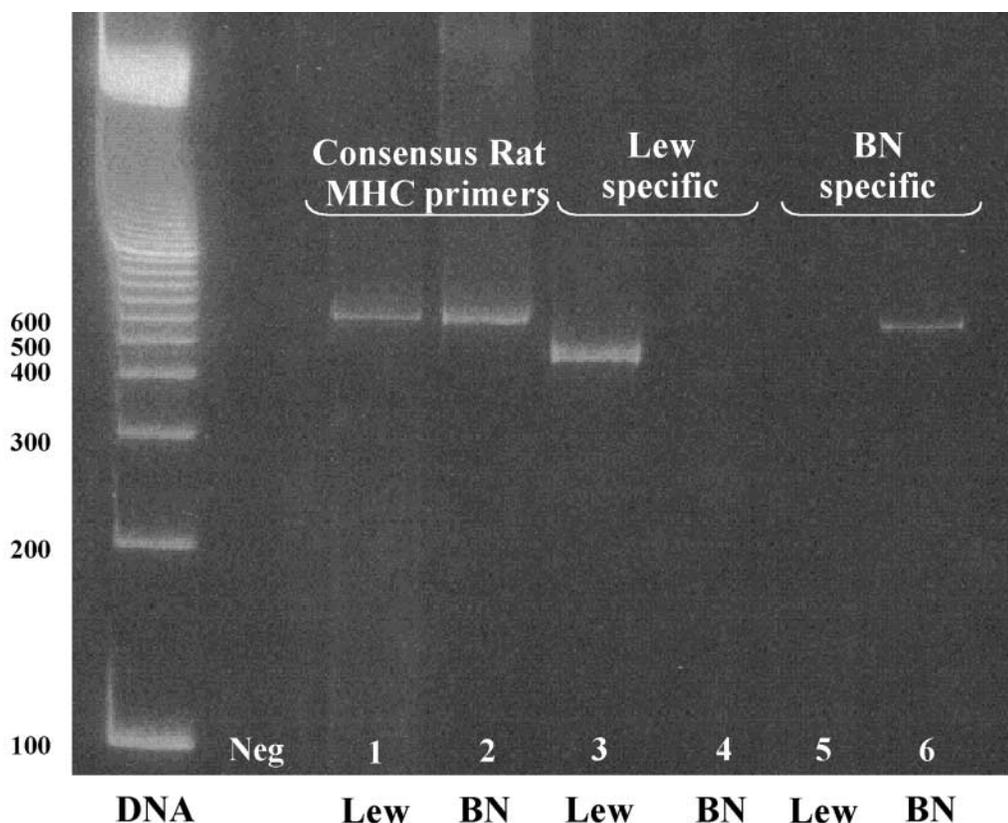


Figure 4. Rat strain differentiation based on strain-specific polymerase chain reaction (PCR) for the major histocompatibility complex (MHC) class I (RT1.A) allele. Genomic DNA purified from brown Norway (BN) and Lewis (Lew) hepatocytes (as indicated below the lanes) was subjected to PCR using the consensus rat MHC primers (lanes 1 and 2) and Lew-specific (*Lew^w/Rat*) (lanes 3 and 4) and BN-specific (*BN^w/Rat*) (lanes 5 and 6) primer sets as indicated above the lanes. Adapted from Johnson P, Carpenter M, Hirsch G, Lee T. Recipient cells from the intimal proliferative lesion in the rat aortic model of allograft arteriosclerosis. *Am J Transplant* 2002;2(3):207-14.

performed fully histoincompatible BALB/c aortic transplantation into irradiated C57BL/6 recipients of syngeneic ROSA26 bone marrow cells, which exhibit β -galactosidase enzymatic activity. Analysis of these aortic allografts yielded a significant proportion of X-gal/ α -actin double-positive cells in the graft intima, indicating that the smooth muscle-like cells of the neointima can derive from bone marrow cells.²³

Shimizu et al.²³ also investigated the hypothesis of adjacent native aorta as a source of intimal lesion cells by irradiating host ROSA26 mice prior to the transfer of wild-type bone marrow cells. In this instance, no X-gal-positive cells were found traversing from the adjacent native aorta into the graft neointima. This provides strong evidence that the

smooth muscle-like cells of the intimal lesion are not derived from contiguous extension from the host media but rather from a circulating pool of mesenchymal progenitor cells that are at least in part derived from the host bone marrow.²³ Given that this phenomenon was not complete, it cannot be ruled out that other nonmarrow sources may contribute to the circulating population of vascular wall precursors.

Recently, Hillebrands et al.²⁴ suggested that marrow-derived cells are not the major source of circulating progenitors responsible for cell replacement in transplant arteriosclerosis. Shimizu et al.²³ provided data that led to the same conclusion. The exact anatomical origin of the remaining lesion cells is uncertain; however, other blood-borne mesenchy-

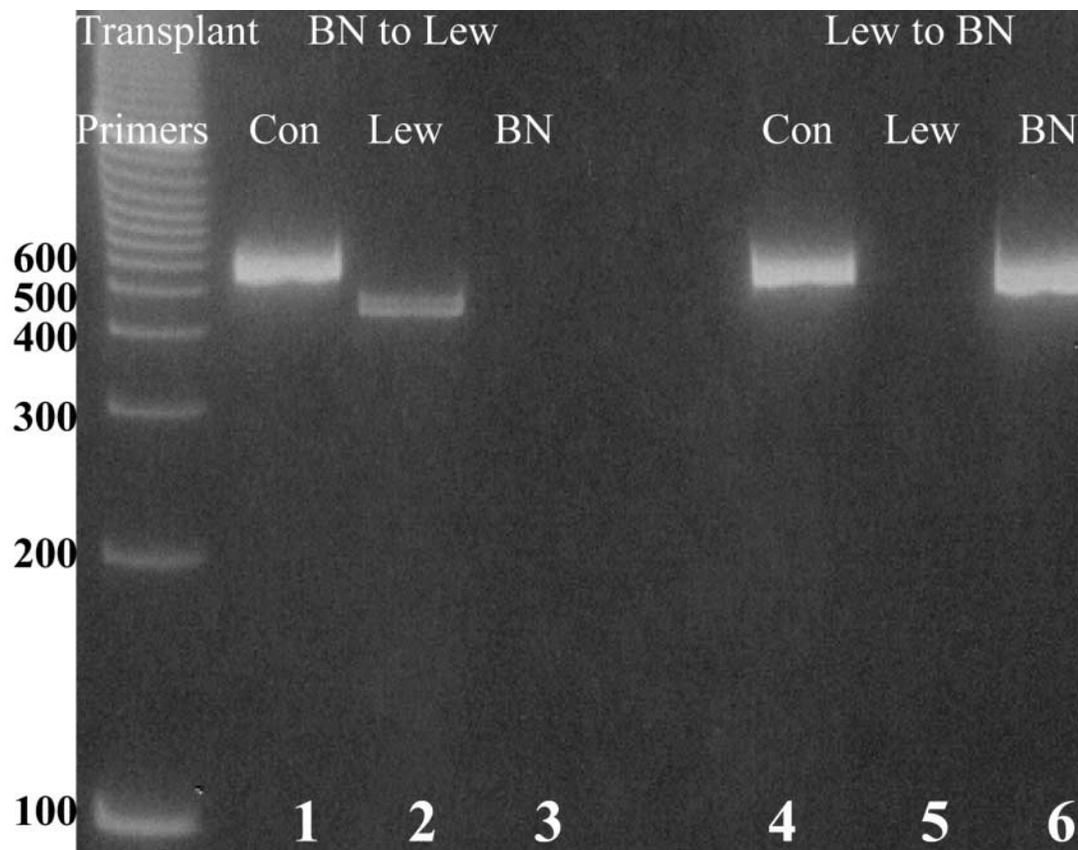


Figure 5. Determination of neointimal cell origin using rat strain-specific polymerase chain reaction (PCR). PCR analysis was performed using DNA extracted from isolated neointimal tissue from aortic transplant segments. Primer sets (consensus [Con] [Rat/Rat], Lewis [Lew] specific [Lew/Rat], and brown Norway [BN] specific [BN/Rat]) and transplant strain combination (donor to recipient) are indicated above the lanes. Adapted from Johnson P, Carpenter M, Hirsch G, Lee T. Recipient cells from the intimal proliferative lesion in the rat aortic model of allograft arteriosclerosis. *Am J Transplant* 2002;2(3):207-14.

APOPTOSIS

Programmed cell death resulting from activation of an enzymatic cascade intrinsic to the cell. It is characterized by preservation of the plasma membrane, membrane blebbing, condensation of chromatin, and fragmentation of nuclear DNA, distinguishing it from necrotic cell death.

mal precursors are a likely source. Circulating precursors have indeed been identified in other physiological and pathological processes.²¹ Although the existence of marrow-derived mesenchymal progenitors is incontrovertible, their contribution and functional relevance in AV is still undetermined.

AV in Humans

Initial evaluation of human cardiac allografts by Bieber et al.²⁵ suggested that the neointimal cells were of donor origin. Similarly, Hruban et al.²⁶ studied 2 human hearts using *in situ* hybridization for the Y chromosome and came to a similar conclusion. However, others have identified a mixed

origin of donor and recipient cells in human cardiac transplants.⁴ These early studies evaluating the origin of the neointima within human organ transplantation are few and severely limited. Many of these studies have used tissues derived from transplants between males and females and have relied on differences related to sex chromosomes between the graft and recipient. The major limitation of this approach is the inability to identify both donor- and recipient-derived elements simultaneously if indeed they are present. This type of analysis allows for the identification of only the donor or recipient cells but not both, and as a result we are left to assume the presence of the opposite cell type based on the absence of a signal or marker.

Most recently, examination of human renal allografts undergoing chronic rejection using in situ hybridization of a Y-chromosome-specific probe coupled with immunocytochemical analysis has yielded more direct results.²⁷ Using this combined technique, Grimm et al.²⁷ showed that recipient mesenchymal cells form the neointimal proliferative lesions of renal allografts undergoing chronic rejection. Similarly, Quaini et al.²⁸ showed significant chimerism within transplanted human hearts that was caused by the migration of primitive cells originating from the recipient. These cells were found in increased numbers relative to control hearts and were able to differentiate into all cardiac elements including vascular SMCs, endothelium, and cardiac myocytes.²⁸ These findings are consistent with the emerging data from animal models of chronic rejection; however, there remains a degree of uncertainty with respect to the issue of neointimal cell origin in human AV. This phenomenon will need to be confirmed in other human solid organ transplants, including heart, using more sensitive techniques to confirm the nature of the lesion in the setting of clinical transplantation.

Conclusion

In conclusion, evidence from sensitive and specific techniques including strain-specific PCR, in situ hybridization, and immunocytochemistry indicate that the smooth muscle-like cells in lesions of experimental AV are of recipient origin.^{16,17,20,23,27} Furthermore, the evidence also suggests that the source of these recipient cells can be accounted for, at least in part by circulating mesenchymal cells derived from the bone marrow of the recipient.^{20,23} With the current available evidence, we are unable to determine whether these circulating precursor cells directly adhere via the lumen of the vessel or are recruited to the subendothelial space via the adventitial microvasculature. Future experimentation should focus on the chemokines and growth factors that are active in the recruitment and proliferation of the neointima. Finally, these observations await confirmation in human AV. If confirmed, these findings provide new insight into the pathogenesis of AV and may provide new therapeutic strategies that more appropriately address the recruitment, proliferation, and differenti-

ation of putative progenitor SMCs derived from the recipient.

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