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Immunologic targets in the etiology of allograft vasculopathy: Endothelium versus media

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Abstract

The respective roles of the endothelium and the media as allo-immune targets in the generation of allograft vasculopathy (AV) have yet to be clearly defined. Although endothelial damage has been implicated in the progression of AV, evidence from mechanical vascular injury models suggests that medial injury may play a more dominant role. The overall objective of this research was to determine the relative importance of the endothelium versus the media as a target for immune injury and induction of AV.

To investigate this we developed a novel model which involved the creation of chimeric aortic segments. To accomplish this we removed aortic segments from C3H/HeJ (C3H) mice and stripped them of endothelium by a short pulse with EDTA. The stripped C3H grafts were implanted into immunodeficient C57BL/6 (B6) RAG1$^{-/-}$ mice for a period of 21 days. As the immunodeficient mice did not mount an allo-immune response to the grafts, the endothelium was renewed by normal repair mechanisms. The new endothelium was recipient in origin, resulting in a chimeric graft with C3H media and B6 endothelium. We confirmed complete denudement by immunocytochemistry for endothelial specific markers, as well as by transmission and scanning electron microscopy. Replacement of endothelium with recipient endothelial cells was confirmed by immunocytochemistry, electron microscopy and by using a green fluorescent protein mouse transplant combination. Subsequent re-transplantation of the chimeric grafts into either B6 or C3H recipients demonstrated that an allogeneic media is more important than an allogeneic endothelium in inducing robust AV.

Keywords: Allograft vasculopathy; Allograft arteriosclerosis; Chronic rejection

1. Introduction

Acute rejection of cardiac transplants can be controlled by potent immunosuppressive drugs, such as cyclosporine or tacrolimus, generally used in combination with agents such as azathioprine and mycophenolate mofetil. Unfortunately this therapy does not prevent late failure of cardiac transplants and, as such, late graft failure has emerged as a major problem in clinical heart transplantation [1]. The most common and refractory aspect of late cardiac graft failure is allograft vasculopathy (AV) [2].

AV is characterized by a progressive neointimal lesion in the coronary arteries that becomes occlusive over time [2]. This occlusive neointimal lesion consists of α-actin positive smooth muscle-like cells and infiltrating leukocytes [3]. These characteristics are present in both human cardiac allografts and animal models such as aortic allografts [2,4]. The nature of the neointimal lesion and the role that the various vascular elements play in its generation are still controversial. It was originally proposed that the neointimal lesion is populated by donor SMC that transmigrate from the donor graft media and subsequently proliferate within the subendothelial space [5,6]. The stimuli for this was suggested to be growth factors, such as basic fibroblast growth factor (bFGF;[7]) and platelet-derived growth factor (PDGF; [8]) released by platelets and damaged endothelial cells [9–11].

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More recent evidence has demonstrated that, in rodent models of AV, including models using calcineurin inhibition immunosuppression, the cells comprising the neointimal lesion are of recipient origin, and not donor origin ([12–18]. The evidence from human studies has been less consistent, primarily because of the technical problems associated with hybridization to the Y antigen which forms the basis of most of the studies. Differences between rodent and human also may reflect the existence of pre-existing sub-endothelial inflammatory cells in the human donor coronary arteries before heart transplantation that are not seen in the strictly controlled rodent models [19]. Taken together, the human studies suggest a chimeric intimal lesion with both donor and recipient elements.

The transmigration hypothesis was based on the observation of a significant SMC loss in the media during the progression of AV. Current evidence [3,20] suggests that this SMC loss is due to SMC apoptosis rather than migration. What remains unclear is the role of this SMC loss in the induction of AV. We have posited a hypothesis that the vascular media is a significant immunological target in the development allograft vasculopathy and that medial cell loss is a major factor in the induction of the neointimal lesion. We base this partly on our understanding of the lesion being a form of vessel repair and partly on mechanical injury studies that have implicated medial cell damage in the induction of vasculopathy.

2. Hypothesis

Based on the evidence of SMC loss in AV and the obvious importance of medial damage to vasculopathy in mechanical injury models, we developed a hypothesis that the media is a significant immunological target in the development of allograft vasculopathy and, as such, that immune mediated medial damage plays an important role in lesion formation in AV. We further hypothesized that the endothelium is a less significant immunological target than previously thought and that, as in mechanical injury models, immune injury to the endothelium alone would not initiate a robust neointimal lesion.

To test this hypothesis we developed chimeric aortic grafts in which the endothelium and the media were from different, fully disparate, strains. Transplants were designed such that the recipient animals were syngeneic to the endothelium but allogeneic to the media or the reverse, thus limiting immune injury to the endothelial, or the medial component.

3. Materials and methods

3.1. Mice

All mice were purchased from Jackson Laboratories, (Bar Harbor, ME). Aortas were removed from 8–10 week old male C3H/HeJ (C3H; H-2^k) mice for preparation of denuded aortic segments for grafting. Re-epithelialization occurred in 8–10 week old male C57BL/6 (B6; H-$2^{b)}$ immunodeficient B612957-$Rag1^{tm1mom}$, referred to as $RAG1^{-/-}$ mice, that lack both T cells and B cells or the transgenic C57BL/6-Tg(ACTB-EGFP)1Osb/J, referred to as GFP mice. Transplant recipients included 8-10 week old male wild type C57BL/6 or wild type C3H/HeJ mice. Mice were housed at the Carleton Animal Care Facility, at Sir Charles Tupper Medical Building, in a pathogen free environment. Food and water were given ad libitum.

3.2. Removal of the endothelium from the aortic segments

Freshly isolated aortic segments were flushed intralumenally with room temperature (RT) normal saline for 1 min, using a 1 ml syringe with a 25G needle. This was followed by flushing with 1.3×10^{-4} M EDTA in Ca^{++} and Mg^{++} free HBSS for 1 min.

Flushed aortic segments were then incubated in 1.3×10^{-4} M EDTA at RT for 2 min.

After 2 min incubation the segments were flushed again with EDTA and incubated for a further 2 min. This yielded the 4 min incubation time point. Incubations for 6 min, 8 min or 10 min involved repeats of the last incubation and flushing once, twice or three times respectively. The final step was to flush the incubated segments with saline for 1 min before immediate transplant into recipients.

3.3. Histology

Aortic grafts were fixed in buffered formalin and processed for histological examination using standard protocols. Sections (5 μm) were cut transversely so that the layers of the aortic wall could be analyzed. Sections were stained with hematoxylin and eosin (H&E).

Digital Images of the H&E stained tissues were captured using Zeiss Axiovert 200 and AxioCam camera (Carl Zeiss, Thornwood, NY). Intimal and medial areas of the aortic grafts were outlined using Adobe Photoshop® (Microsoft). The intimal areas were quantified using a digital image analysis program (Scion Image software, Scion Corp., Frederick, MD).

3.4. Immunocytochemistry for PECAM-1 staining

Preparation of aortic segments was accomplished as previously described [4]. Briefly, aortic segments were placed in OCT: 20% sucrose, frozen in liquid nitrogen and stored at −80 °C until use. Cross sections (6 μm) of the aortic segments were cut on a cryostat and mounted on Fisherbrand SuperFrost/Plus positively-charged microscope slides. Sections were fixed in acetone. Endogenous peroxidase activity was blocked using 2% hydrogen peroxide in 0.2 M PBS. Avidin/biotin blocking was performed using the Vector® avidin/biotin blocking kit following the manufacturer's instructions (Vector Labs Inc. Burlingame, CA). The sections were incubated with the primary monoclonal antibody, biotin-conjugated rat anti-PECAM-1; (MEC 13.3; PharMingen, San Diego, CA). Sections were washed in PBS and incubated with a peroxidase avidin/biotin complex (Vector Labs, Inc.). Staining was visualized with diaminobenzidine (DAB) in 0.01%

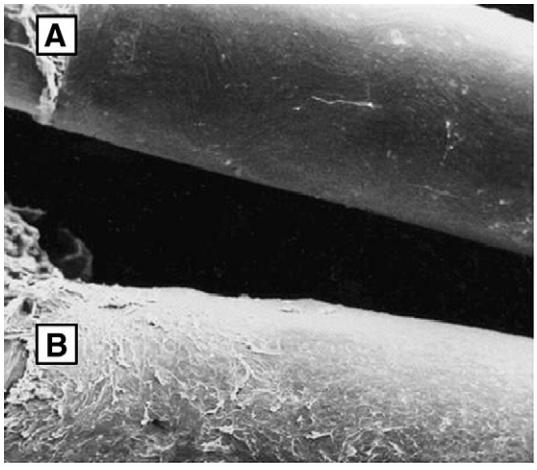

Fig. 1. SEM analysis confirms EDTA treatment completely denudes the intimal endothelial cells. Scanning micrograph comparing everted aortas from EDTA treated (A) and normal untreated (B) specimens. Completely denuded internal elastic lamina is visible in the treated everted aorta.