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Accelerated Atheromatous Lesions in Mouse Hearts Transplanted to Apolipoprotein-E-Deficient Recipients

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Arteriopathy, sometimes termed accelerated atherosclerosis, often impairs transplants. We employed apolipoprotein-E-deficient, hypercholesterolemic mice to determine how the hyperlipidemic environment affected transplanted hearts. Strain 129 hearts transplanted to C57BL/6 normal or C57BL/6 apolipoprotein-E-deficient recipients were evaluated by immunochemical and histological techniques. Analyses were possible both of differences in the coronary lesions that developed in a normolipidemic as compared with a hyperlipidemic environment and of the coronary atherosclerotic process in transplanted hearts compared with native hearts in the same hyperlipidemic environment. Aortas and coronary arteries of transplanted hearts in both recipient groups developed florid intimal thickening by 4 to 10 weeks, with marked lipid deposition, foamy macrophages, and infiltration of smooth muscle α-actin-positive cells in apolipoprotein-E-deficient mice. Lipid was layered against the internal elastic lamina as in human transplants. VCAM-1 was increased in various sites in both groups. Allotransplants to apolipoprotein-E-deficient recipients had more severe aortic and coronary lesions with characteristic T cell infiltration than native hearts. In this sense, transplants suffered from accelerated atherosclerosis. The character of coronary vascular changes in transplanted hearts was distinctly affected by their lipid environment, but their severity, in terms of luminal encroachment, was not markedly different. (Am J Pathol 1996, 149:91–99)

Interest in the atherosclerotic-like lesions, which often form in the arterial vessels of transplanted organs with considerable rapidity after transplantation, has increased recently not only because of their great importance to the patients concerned, especially heart transplant recipients, but also because of the possible relation of this process to atherosclerosis in general. Atherosclerosis is, of course, a complex process in which a number of factors are known to participate, but the central role of circulating lipids is now incontrovertible where the common form of atherosclerosis is concerned.[1] Lipid deposition is a prominent, although not ubiquitous, component of the chronic arteriopathic lesions seen in human heart transplants.[2] Accordingly, the importance of the lipid environment has attracted the attention of clinicians for some time as a possible target for treatment in patients suffering from atherosclerotic lesions in transplanted organs.[3] Nevertheless, the effect of this factor on the development and progress of these atherosclerotic changes has been difficult to sort out, and some studies, even in controlled animal experiments, have led to dissimilar conclusions regarding the nature of the contribution of high blood lipid levels to the process.[4–6]

Of the apolipoproteins, the E subclass (apoE) is considered a particularly important contributor to the clearance of lipoprotein particles from the plasma.[7] The production of mice deficient in apoE by genetic replacement through homologous recombination, or knockout, technology in several laboratories, has provided experimental subjects that have reinforced the impression of the importance of apoE to lipoprotein metabolism.[8–10] Not only do such mice manifest cholesterol levels in the range of 400 to 500 mg/dl on an ordinary

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animal chow diet, which is relatively low in lipid (approximately 4 to 5%), but these mice develop typical, lipid-laden atherosclerotic lesions in their arterial trees after only 2 months or so of life.[11]

We have recently devised an experimental system in which the accelerated atherosclerotic process associated with organ transplantation can be studied in the mouse in which highly defined immunogenetic conditions can be provided.[12] Heterotopically transplanted mouse hearts transferred between inbred mice of a variety of strains will survive with a suitable, brief course of immunosuppressive treatment and will continue to beat in a normal fashion while their coronary arterial vessels are developing complex inflammatory lesions that may encroach markedly upon their lumens. These lesions are initially composed of T lymphocytes, macrophages, and other inflammatory cells that adhere to the endothelial surfaces of affected vessels as well as proliferating components of donor vessel walls, especially smooth muscle cells, that participate increasingly in the course of time. Lesions do not form in the vessels of transplanted hearts if there is no immunogenetic difference between their donors and recipients, but a sufficient difference may consist of disparities in respect of antigens determined by genes of class I or class II of the major histocompatibility complex (MHC), or even of differences determined by genes outside the MHC altogether.[12] We have further found that the specific immune attack against the donor's vessels, which sets the process in the arterial wall into motion, can be confined exclusively to either the humoral[13] or the cellular portion of the response (unpublished observations).

The present experiments were performed in the light of our previous experience and were designed to observe the differences in coronary vascular changes that occur in hearts transplanted from donor mice of the 129 strain to normal C57BL/6 strain recipients as compared with C57BL/6 strain mice similar in every way to normal C57BL/6 animals save for their lack of apoE. Comparisons were also made of hearts transplanted to apoE-deficient mice with the native hearts of the same hyperlipidemic recipients. It was also of interest to determine whether the coronary lesions in hearts transplanted to hyperlipidemic recipients were more like those commonly observed in human recipients in terms of their inclusion of lipid components, whether the rejection process accelerated the atherosclerotic process and whether graft and "native" atherosclerosis differ.

Materials and Methods

Mice

129/J male mice were used throughout as donors and apoE-deficient strain (*Apoe*)[10] male or normal male C57BL/6 mice were used as recipients. We obtained these mice from The Jackson Laboratory, Bar Harbor, ME. They were fed a diet of ordinary chow and were maintained otherwise under standard laboratory conditions. All animals were 10 to 12 weeks old and weighed approximately 30 g at the time of transplantation. Twelve apoE recipients were studied, four at 4 to 7 weeks after transplantation and eight at 8 to 10 weeks. Twelve heart transplants to normal C57BL/6 recipients were also studied over the same time period.

Antibodies

Monoclonal antibodies against mouse CD4 (GK 1.5, a rat IgG2b) and CD8 (2.43, a rat IgG2b) were produced in our laboratory from cell clones acquired from American Type Culture Collection, Rockville, MD. These antibodies were used for immunosuppression (see below). Several additional monoclonals were used for immunopathological staining. M 1/70, a monoclonal prepared in rats against the mouse Mac-1 specificity (CD-11b) and Asm-1, a monoclonal prepared in mice and reactive to smooth muscle α-actin were both obtained from Boehringer-Mannheim Biochemica, Indianapolis, IN. The cell clone for YCD3–1, a rat IgG2b antibody directed to the mouse CD3 was obtained from K. Bottomly, Yale University. A monoclonal antibody directed to VCAM-1 (M/K-2.7) was produced in our laboratory, from a cell clone acquired from the American Type Culture Collection.

Operative Procedures and Immunosuppression

Our technique for heterotopic transplantation of the mouse heart has been described in detail.[14] Briefly, the procedure involved ligating the inferior and superior vena cavae and the pulmonary veins leading to the donor heart and anastomosing the donor aorta and pulmonary artery to the recipient abdominal aorta and vena cava respectively in end to side fashion. Survival for the first 2 days to enter the experiment with transplanted hearts manifesting excellent initial function was achieved in more than 95% of trials. We have found that the status of a transplanted heart in the mouse can be determined

satisfactorily by simple palpation of its cardiac impulse through the abdominal wall of the recipient. Grading of the cardiac impulse was on a scale of 0 to 4+. In general, fully viable cardiac allografts and isografts maintain stable cardiac impulses that rarely exceed 3+ after the first few days. Hearts transplanted between most inbred strains of mice that differ in respect of antigens determined by genes of the MHC are fully rejected by 9 to 12 days. Accordingly, our experimental system, in which atherosclerotic coronary lesions are allowed to develop, makes use of a brief course of immunosuppression with two monoclonal antibodies to T lymphocytes delivered entirely preoperatively. Our standard format is to administer both anti-CD4 and anti-CD8 monoclonal antibodies 6, 3, and 1 day before transplantation as intraperitoneal injections of 0.2 ml of combined ascites fluid containing each monoclonal.

Histological Techniques

Transplanted hearts were removed at selected times from their recipients, frozen in OCT compound (Ames Co., Division of Miles Laboratories, Elkhart, IN), and stored at −20°C. Tissue blocks containing the hearts were placed upon the freezing stage of the microtome. Sections were cut at 4 μm, acetone or formalin fixed, and routinely stained with oil red O or Weigert's stain for elastic fibers. Frozen sections were also stained by the immunoperoxidase technique employing the monoclonal antibodies to CD3, CD11b, smooth muscle α-actin, and VCAM-1, noted above. Biotinylated secondary antibodies directed against rat and mouse IgG were obtained from Vector Laboratories (Burlingame, CA) or PharMingen (San Diego, CA). Stained sections were developed in a solution of 3-amino-9-ethyl carbazole (Aldrich Chemical Co., Milwaukee, WI), post-fixed in 4% formaldehyde, counterstained with hematoxylin, and mounted in Gelvatol (Monsanto Co., Springfield, MA), as described.[12] The severity of the obstructive arterial lesions in the coronary systems of transplanted hearts was evaluated on frozen sections stained with the elastic tissue stain according to a previously described grading system.[12,15] This provided a useful initial impression of the severity of luminal encroachment of coronary vessels in the various hearts under study, but the numbers of animals available for direct comparison at given times after transplantation were insufficient for a full and formal quantitative determination of this particular variable in the fashion we have employed previously in other experiments.[12,13]

Results

The following findings are based on analyses of allogeneic hearts surviving in apoE-deficient or normal recipients for from 4 to 10 weeks as mentioned above. Thus, the apoE recipients were 15 to 24 weeks old by the time these observations were made.

Native Hearts and Aortas of ApoE-Deficient Mice

In confirmation of previous reports,[10,11] the earliest lesions in apoE-deficient mice were seen at the base of the aortic valves often extending up the proximal aorta (Figure 1A). The coronary arteries showed no lesions, lipid deposition, or cellular infiltration (Figures 1C and 2C). Marked thickening of the aortic intima was present with infiltration of spindle-shaped, smooth muscle α-actin$^+$ cells and CD11b$^+$ mononuclear cells with abundant cytoplasm, indicative of macrophages. These macrophages contained oil red O$^+$ lipid, which was also present in the extracellular matrix. T cells infiltrated the adventitia in areas underlying cellular plaques to a variable degree (1 to 2+) and rare CD3$^+$ cells were present in the intima and media. Focal loss of smooth muscle from the inner media, well shown with α-actin stains, occurred focally underlying plaques in native aortas. In these hearts, one interesting finding was that VCAM-1 was strikingly positive (4+) in the subendothelial spindle cells of atherosclerotic lesions in the aortic root; the media was also strongly positive in these sites whereas the endothelium was minimally positive (1+) and the foam cells were negative (see Figure 4A). VCAM-1 was occasionally detectable in the endothelium and media of the large coronary arteries and small intramyocardial vessels.

Transplanted Hearts and Aortas in ApoE-Deficient Mice

The aortas of transplanted hearts in apoE-deficient recipients generally developed florid intimal thickening in association with lipid deposition (oil red O$^+$) and smooth muscle (α-actin$^+$) and macrophage (CD11b$^+$) infiltration (Figures 1, B and D, and 3, A and B). Lipid was often distributed in two layers in the donor aortas, just beneath the endothelium and in macrophages against the internal elastica (Figure 1E). The latter was more prominent in transplanted than in native aortas, similar to the pattern classically described in human transplants.[2,16] Cholesterol crystals were evident in the intima of transplanted

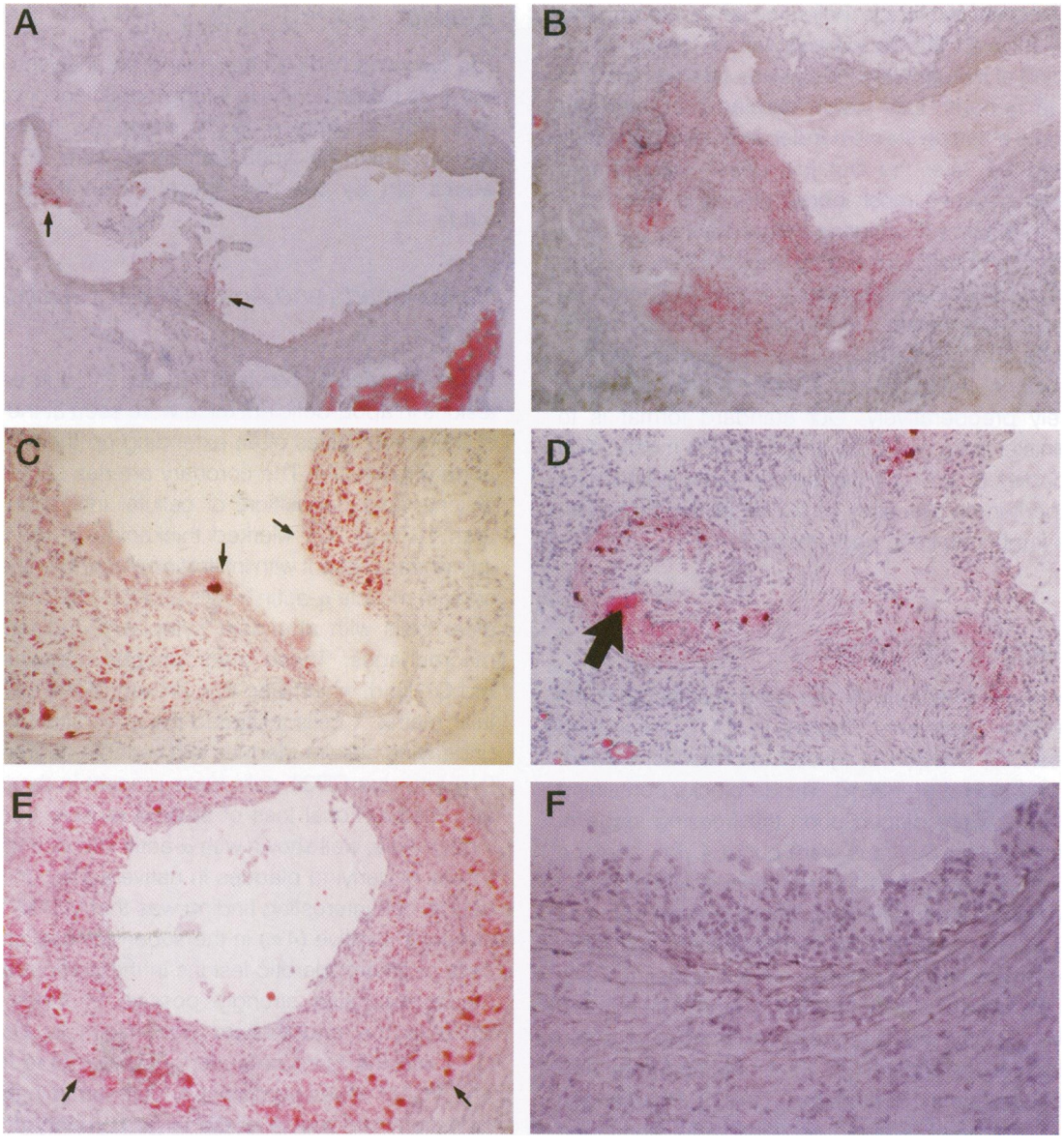

Figure 1. *Oil red O stains for lipids in native* (A *and* C) *and allograft* (B, D, *and* E) *aortas in apoE-deficient recipients. Deposits of lipid, which stain red, are present focally in the intima at the root of the aorta around the aortic valves of the native aortas* (arrows *in* A). B: *Allograft aorta from the same apoE recipient as in* A, *which had been in place for 29 days. The severity of the lipid deposits exceeds that of the native heart, even though the allograft had been exposed to a hyperlipidemic environment for a shorter time.* C: *Even with the severe aortic involvement, the native coronaries are spared, as shown in a section through the coronary ostium* (arrows) *from another recipient.* D: *In contrast, marked lipid deposition extends into the allograft coronary artery* (arrow). E: *Allograft aorta from another apoE-deficient recipient shows deposition of lipid along the internal elastic lamina, a pattern typical of allograft arteriopathy in humans.* F: *Allograft aorta from a heart transplanted into a C57BL/6 normolipidemic recipient. No lipid is detected in the lesion.*

aorta and coronary arteries. High grade stenosis was frequently seen (grade 2 to 3).

Smooth muscle α-actin$^+$ cells were typically concentrated in a band just under the endothelium, a site where T cells were also present (Figure 3, A and D). CD3$^+$ T cells infiltrated the intima in all lesions and ranged in intensity from moderate to marked (2 to 3+). Adventitial T cells were also present underlying plaques and these focally invaded the media. Patchy loss of smooth muscle, well shown with α-actin stains, occurred commonly in the lesions and was more extensive than in the native hearts. Mac-1$^+$ cells were abundant in the intima (under the endothelium, against the internal elastica) as well as in the adventitia, and these cells occasionally infiltrated the media as well (Figure 3B). VCAM-1 expression in transplanted hearts was found in lesions involving the donor aorta (strongly positive subendocardial spindle cells and medial smooth muscle cells, weakly positive or negative endothelium; Figure 4B).

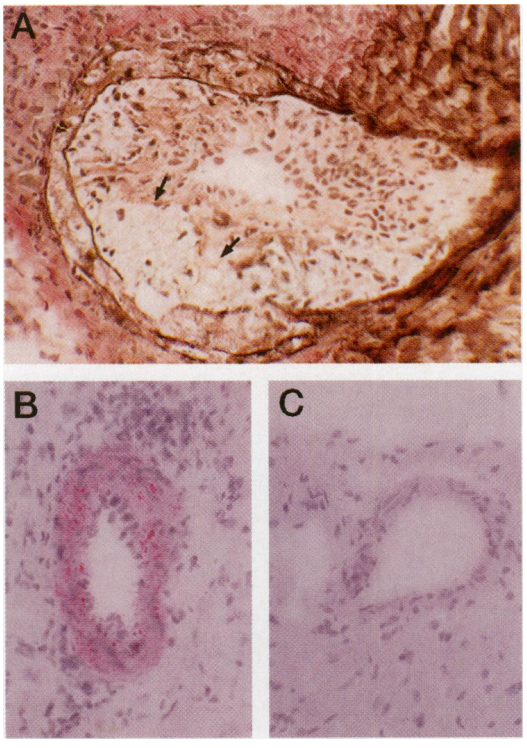

Figure 2. *Coronary arteries from apoE-deficient recipients* (A *to* D). *Severe stenosis (grade 3, >50%) and foamy macrophages* (arrows) *are present in a typical allograft coronary* (A; *elastic tissue stain*). *Intramyocardial arteries show lipid deposition in the media of the allograft coronaries* (B), *even without obvious intima inflammation. No lipid is seen in the native heart* (C).

In contrast to the native heart, the coronary arteries of allogeneic transplanted hearts had strongly positive endothelial staining for VCAM-1 (3+), even in uninvolved segments. Spindle cells in the media and intima were also positive in atherosclerotic lesions (Figure 2B). The intramyocardial capillaries were positive.

In comparison with the native hearts of apoE-deficient subjects, the lesions in hearts transplanted to them were more extensive and severe. For example, in an animal studied 4 weeks after transplantation, the lesions were circumferential and widespread in the allogeneic aorta. In contrast, the native aorta and the aortic valves from the same animal showed only early focal lesions (Figure 1, A and B). Larger coronary arteries in transplanted hearts, which were unaffected in native hearts, showed marked lipid deposition in the intimal lesions (Figure 1, C and D). In addition, smaller coronary arteries showed lipid accumulation in the media, even when no intimal or medial inflammation was evident (Figures 2, B and C). The lesions in transplanted hearts also manifested more abundant T cell infiltration and more prominent smooth muscle loss than similar lesions in native hearts (Figures 3, C and D).

Comparison of Allografts in ApoE-Deficient Recipients with Those in Normal C57BL/6 Recipients

The lesions in vessels of hearts transplanted to C57BL/6 normolipidemic recipients were oil red O negative and lacked foamy macrophages (Figure 1F).[12] The degree of stenosis that had developed in these vessels appeared to be similar to that in allotransplants to apoE-deficient animals, although, as stated above, the numbers of animals available for valid comparison were insufficient to permit a formal statistical evaluation of this relationship (Figure 2A). In nontransplanted hearts (129 strain), VCAM-1 expression was focally detectable (0 to 1+) in the coronary artery endothelium and the endocardium. No VCAM-1 was evident in the arterial media, myocytes, or capillaries. Allografts in C57BL/6 mice showed equivalent VCAM-1 compared with the allografts in apoE-deficient recipients, including very strong aortic endothelial VCAM-1 (Figure 4C).

Discussion

The main objectives of our experiments were to determine how a high lipid environment influences the character of transplant-related arteriopathy and to compare the evolution of atherosclerotic changes in the native hearts of hyperlipidemic subjects with that which occurs in allogeneic hearts transplanted to these same subjects. It was also considered of interest to see whether the changes that occur in transplants to hyperlipidemic mice resemble more closely those seen in cardiac transplant patients, as the coronaries of transplanted human hearts typically contain more lipid than is seen in hearts transplanted to normolipidemic mice.

Our results indicate that hearts transplanted to apoE-deficient mice develop lipid deposits in the transplanted aortic root and in the coronary arterial intima, which have the same distribution as those seen in their human counterparts. In human allotransplants, lipid is characteristically deposited in foamy macrophages along the internal elastic lamina.[2,17] The prominent myointimal cells, which are smooth muscle actin$^+$, were also evident in the mouse where they were often disposed along the intima in a subendothelial band forming a fibrous cap over a core of lipid debris. Thus, these lesions in the mouse reproduce with fidelity those seen in human allotransplants.

Comparison of native with allotransplanted hearts and aortas permitted an assessment of the effect of

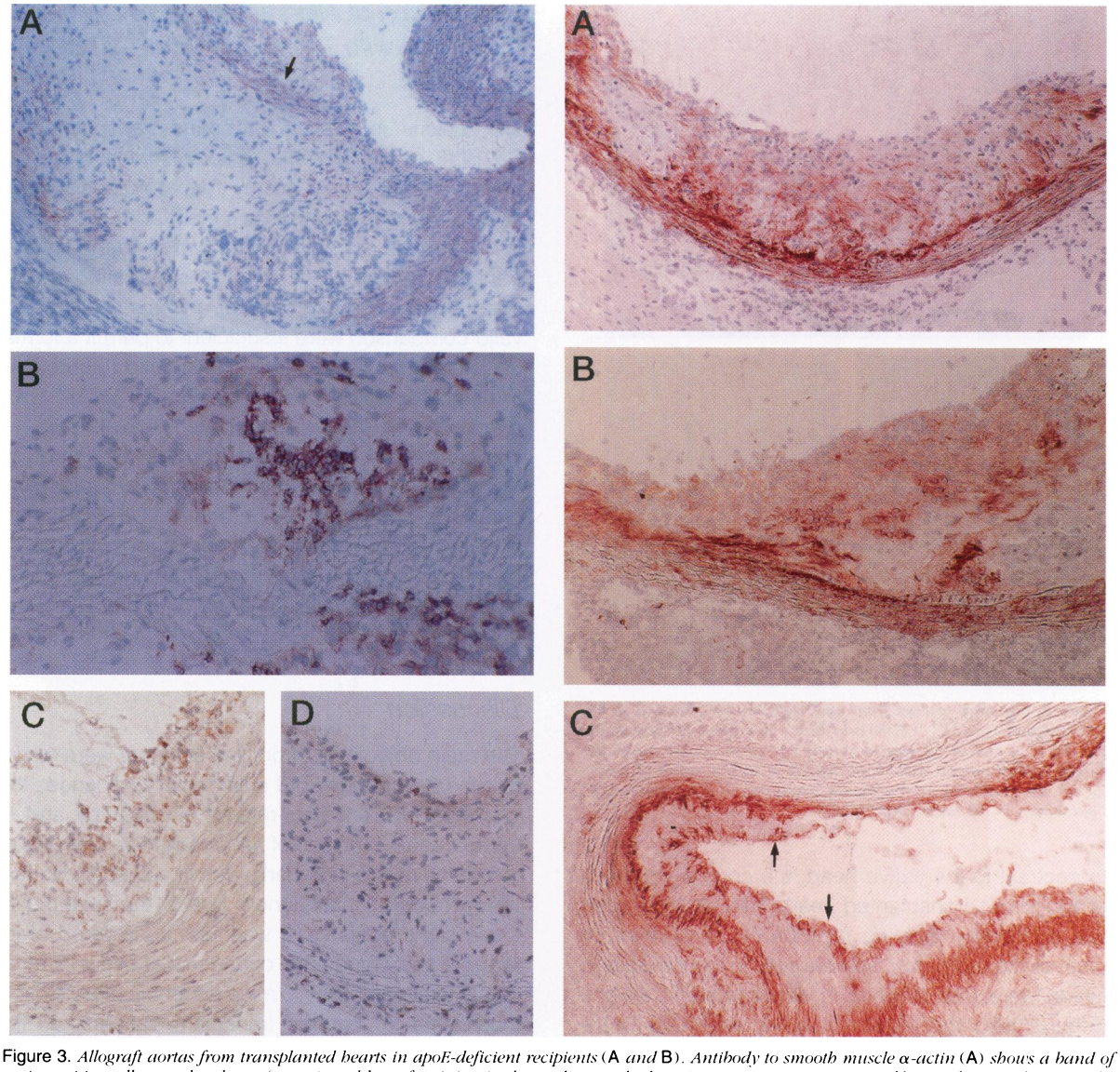

Figure 3. *Allograft aortas from transplanted hearts in apoE-deficient recipients* (A *and* B). *Antibody to smooth muscle α-actin* (A) *shows a band of actin-positive cells over the plaque* (arrow) *and loss of staining in the media. Antibody to Mac-1 stains an intense infiltrate of macrophages in the intima and focally in the subendothelium and adventitia* (B). *Aortas stained with antibody to CD3* (C *and* D). *More T cells infiltrate the intima in the allograft* (C), *although occasional T cells are in the atherosclerotic native aorta from the apoE recipient.*

Figure 4. *VCAM-1 stains in native* (A) *and allograft* (B) *aortas in apoE-deficient mice and an allograft aorta in a normolipidemic recipient* (C). *Prominent intimal and medial staining is evident, as well as increased endothelial staining, especially prominent in* C (arrows).

chronic graft rejection on the rate and extent of the atherosclerosis. In the aortas of apoE-deficient mice fed a standard lab chow diet, the sequence of lesions has been carefully described.[10,11] Beginning at approximately 8 weeks, macrophage adhesion is evident, and this progresses to foam cell accumulation by approximately 10 weeks. Intermediate lesions, with spindle and foam cells, are seen from 10 to 15 weeks, and fibrous plaques, with necrotic cores and fibrous caps with smooth muscle actin$^+$ cells are seen after 20 weeks. ApoE-deficient mice, fed a standard chow diet, do not develop atherosclerotic lesions in their coronary arteries in the first 20 weeks of life and, indeed, none were detected in this study in the mice up to 24 weeks old. In contrast, coronary vessels in the allografts acquired extensive lipid deposition in the proximal aorta in the earliest samples, after only 4 weeks in this hyperlipidemic environment. Full-blown fibrous plaques were found in samples taken from 4 to 8 weeks and the main coronary branches of the allografts showed prominent lipid deposition (whereas none was seen in the native coronaries). Thus, aortic and coronary artery lesions develop faster and are more extensive in transplanted than in native hearts, clearly justifying the term accelerated atherosclerosis.

We were particularly interested in the expression of VCAM-1, a molecule of activated endothelium, as it is detected *in vivo* in hyperlipidemic rabbits in precursor lesions of atherosclerosis.[18,19] VCAM-1 serves as a ligand for $\alpha 4\beta 1$ integrin on monocytes and activated T cells and exerts a co-stimulatory function.[20] We found VCAM-1 focally in coronary endothelium of some normolipidemic mice, a finding not detected in a previous report.[21] This presumably represents variable *in situ* activation from some unknown cause. Cytokines, such as interferon-γ and interleukin-4, induce the expression of VCAM-1 in vascular endothelium and smooth muscle cells *in vitro*.[19,22] Increased vascular VCAM-1 occurs in acute rejection in the rat and rabbit[23,24] as well as in cardiac allografts in mice.[21] Endothelial VCAM-1 is also increased in acute rejection in human hearts, kidneys, and livers.[25–27] Administration of anti-VCAM-1 monoclonal antibodies inhibits acute rejection of mouse cardiac allografts.[21,28]

Studies of chronic rejection and VCAM-1 are limited but suggest its participation in chronic allograft arteriopathy.[26,29] In these experiments, we found a marked increase in VCAM-1 in the evolving chronic aortic lesions in both native and allotransplanted hearts in apoE-deficient mice, similar to that recently reported in normolipidemic mice.[30] This was most prominent in the smooth muscle of the media and the intimal spindle cells (believed to be derived from the media). Curiously, the endothelium of the aorta stained less intensely than the coronary arteries of transplanted hearts, which had markedly increased VCAM-1 compared with the native hearts. It is interesting that the transplants in normolipidemic animals showed equivalent vascular VCAM-1 expression, as oxidized low density lipoproteins are known to stimulate macrophage cytokines that induce endothelial VCAM-1 production.[31,32] It is clear that sufficient mediators arise in the chronic rejection process to induce endothelial and smooth muscle cell VCAM-1 independent of hyperlipidemia. In chronic vascular rejection, VCAM-1 probably plays a role in the intimal localization of monocytes and T cells. Additional studies with antagonists of the VCAM-1/VLA-4 interaction in chronic rejection should be of interest.

An unexpected finding was the presence of lipid in the media of small intramyocardial arteries of hearts transplanted to apoE-deficient mice. No lipid was detected in similar vessels in the native heart nor in hearts transplanted into mice with intact apoE. The lipid accumulated even without endothelial inflammation or intimal thickening, suggesting that it may be an early marker of endothelial injury, perhaps as a consequence of increased endothelial permeability. Even though no inflammatory reaction was evident this phenomenon is likely to be immunologically mediated, as the native hearts exposed to the same lipid environment did not manifest it. The same mechanism is probably responsible for the acceleration of the atherosclerosis by chronic rejection, namely, altered endothelial permeability to lipids due to injury. Increased endothelial VCAM-1 staining also was evident in the intramyocardial arteries preceding the intimal thickening, analogous to the sequence of atherosclerosis described in the rabbit.[19]

Our observations clearly establish that the lipid environment prevailing in a heart transplant recipient can have an important influence on the character of the arteriopathy that develops in the foreign heart. Comparisons of transplanted hearts with native hearts in hypercholesterolemic recipients show that the immunological effects of chronic rejection combine with the effects of the lipid environment to produce markedly accelerated atherosclerotic lesions in the transplanted heart as contrasted with the native heart.

The important issue of whether hyperlipidemia worsens graft atherosclerosis was not formally settled by the present experiments. Studies in rabbits have shown that hyperlipidemia increased the frequency[6] and worsened the stenosis[5] of arterial lesions in cardiac allotransplants. In contrast, a careful morphometric study in rats showed no worsening of the stenosis by hyperlipidemia.[4] This disparity may be related to the degree of hyperlipidemia, the timing of the measurements, the immunosuppression, species differences, or the small numbers of subjects studied. We have not included, so far, a formal evaluation of any quantitative differences that may be present in the severity of the arteriopathic lesions that occur in apoE-deficient recipients as compared with normal C57BL/6 mice in terms of the degree of luminal encroachment that is found at various times after transplantation, because in our opinion, a definitive conclusion regarding a difference between these groups, which appears to be small at most, would require much larger numbers of animals to establish. The lesions produced in apoE-deficient recipients are decidedly different, however, from those that occur in the absence of hypercholesterolemia, especially in their rich lipid content. These lesions could be classified as more severe in that their rich lipid content may make them more subject to ulceration and other mechanical alterations that, in turn, could lead to important consequences for vascular patency.

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