Dramatic remodeling of advanced atherosclerotic plaques of the apolipoprotein E–deficient transplantation mouse

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Objective: Regression of atherosclerotic lesions is an important goal. No extensive experimental evidence shows that it can be achieved for advanced lesions. To study this, we developed a model to maintain a long-term change in the plasma lipoprotein environment of advanced arterial lesions of hyperlipidemic (apolipoprotein E [apoE]–deficient) mice.

Methods: The apoE-deficient mice (plasma total cholesterol of 1334 ± 219 [± SEM] mg/dL) on a typical Western diet for 38 weeks had advanced atherosclerotic lesions (ie, beyond the macrophage foam cell stage) throughout the arterial tree. Lesion-containing thoracic aortas were transplanted (replacing a segment of abdominal aorta) into either apoE-deficient or wild-type (WT) (total cholesterol of 86 ± 10 mg/dL) recipients. Grafts were harvested after 9 weeks.

Results: Compared with pretransplant lesions (area = 0.0892 ± 0.0179 mm²), lesion size tended to increase in apoE-deficient to apoE-deficient grafts (0.2411 ± 0.0636 mm²; P = .06), whereas a significant reduction was seen in apoE–deficient to WT grafts (0.0214 ± 0.0049 mm²; P < .001). Also, foam cells were absent in apoE-deficient to WT grafts, but abundant in pretransplant lesions and apoE-deficient to apoE-deficient grafts. Grafts were evaluated noninvasively in vivo with magnetic resonance imaging, and wall thickening was detected in the apoE-deficient to apoE-deficient group.

Conclusions: Nearly complete regression of advanced atherosclerotic lesions can be achieved with sustained normalization of the plasma lipoprotein profile. Syngeneic arterial transplantation in mice is a novel and valuable model system for atherosclerosis research; and magnetic resonance imaging can detect differences in characteristics in lesions undergoing regression. (J Vasc Surg 2001;34:541-7)
cell lesions\textsuperscript{6,8}; however, effects on the size of more advanced lesions were relatively modest (< 20%).\textsuperscript{6}

In this article we describe a mouse model for atherosclerosis research in which the lipoprotein environment of a lesion of any complexity can be changed rapidly and sustained indefinitely. This lipid-profile manipulation is achieved with microsurgical arterial transplantation techniques and allows effects on lesion characteristics to be studied over a longer period than currently possible with gene transfer approaches. This model demonstrates that advanced, complex plaques of apoE\textsuperscript{−/−} mice can undergo dramatic remodeling and regression after only 2 months of exposure to the normolipidemic plasma environment of the wild-type (WT) mouse. In addition, high-resolution magnetic resonance (MR) imaging noninvasively detected changes in lesion characteristics in vivo, thereby confirming the possibility of monitoring plaque characteristics that change over time in the intact animal.

**METHODS**

**Animals, diets, and experimental design.** The apoE\textsuperscript{−/−} and WT mice in the C57BL/6 background (Jackson Laboratories, Bar Harbor, Me) were used. This study was approved by the Institutional Animal Care and Use Committee, and the animal care complied with the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, Washington: National Academy Press, 1996.

In pilot studies, syngeneic aortic transplantation was performed among WT C57BL/6 mice to assess adverse sequelae, such as transplant arteriopathy or poor weight gain. No such sequelae were observed for up to 10 months. For the regression studies, apoE\textsuperscript{−/−} mice (n = 18) were weaned at 4 weeks of age onto the WD (21\% fat, 0.15\% cholesterol wt/wt\textsuperscript{3}; Dyets Inc, Bethlehem, Pa) and fed this diet for 9 months. These apoE\textsuperscript{−/−} mice were divided into two groups: one group of 12 served as donors of thoracic aortic segments, and one group of six was euthanized to obtain thoracic aortas for pretransplant (baseline) analyses. At the time of transplantation, all donors had severe hyperlipidemia (total cholesterol \[TC\] = 1334 ± 19 mg/dL, n = 9) and advanced atherosclerotic lesions (ie, beyond the macrophage foam cell stage; see later) diffusely distributed in the thoracic aorta. The recipients (28 weeks of age) of the aortic segments were either apoE\textsuperscript{−/−} (n = 4) or WT (n = 8) mice maintained on a standard chow diet (4.5\% fat) before and after transplantation. The apoE\textsuperscript{−/−} recipients served as a control for the transplantation procedure (eg, ischemia-reperfusion injury), with the pretransplant samples serving as the reference point of comparison to assess progression or regression.

After 2 months, the recipients were euthanized and graft segments analyzed. Three days before euthanasia, a subset of mice randomly chosen was subjected to in vivo MR imaging so that changes could be explored noninvasively in the lesions in the grafts associated with the type of recipient (WT or apoE\textsuperscript{−/−}).

**Transplantation procedures.** Transplantation of a segment of donor thoracic aorta into a recipient infrarenal position was performed.\textsuperscript{9} Mice were anesthetized with pentobarbital sodium (60 mg/kg per body weight, Nembutal\textsuperscript{7}; Abbott Laboratories, North Chicago, Ill). Donor aortas were flushed with heparinized saline, and a 4-mm segment was taken from the midportion between the left subclavian artery and diaphragm. The infrarenal aorta of recipients was exposed, clamped proximally and distally, and transected. The donor aortic segment was then inserted as a tube graft by microsurgical anastomosis. (For details, see the appendix, online only.)

**Measurement of plasma lipids and lipoproteins.** TC was determined in plasma samples with a commercial kit (Boehringer-Mannheim, Mannheim, Germany).

**Histology, immunohistochemistry, and morphometry.** All specimens were analyzed by an investigator blinded to their source (pretransplant, apoE\textsuperscript{−/−} to WT isograft, or apoE\textsuperscript{−/−} to apoE\textsuperscript{−/−} isograft). Mice underwent perfusion fixation with 4\% paraformaldehyde in phosphate-buffered saline as described.\textsuperscript{10} Thoracic aortas (pretransplant group) or abdominal aortas containing the grafts (transplant group) were removed en bloc with the posterior bony structures, fixed in 4\% paraformaldehyde for 24 hours, decalcified overnight, sectioned transversely, and embedded in paraffin. Transplanted segments were identified by visualization of proximal and distal anastomotic nylon sutures. The segments of thoracic aorta at donor sites and engrafted infrarenal segments were divided at their midportion, and 5-\textmu \textit{m} thick sections were obtained for staining with hematoxylin and eosin (H&E) and a combined Masson’s trichrome elastic stains. Lesions were classified by histomorphologic criteria: type I, displaying scattered macrophage foam cells; type II, containing layers of foam cells and lipid-laden SMCs; type III, containing scattered collections of extracellular lipid and disrupted SMC architecture; type IV, having a larger, confluent, and more disruptive core of extracellular lipid; type V, containing thick layers of fibrous connective tissue; type Vb, largely calcified; type Vc, consisting primarily of connective tissue, with minimal accumulation of lipid or calcium; and type VI, displaying fissures, hematoma, and thrombus.\textsuperscript{7}

For immunohistochemistry, sequential sections were stained for \(\alpha\)-actin (alkaline-phosphatase–conjugated monoclonal anti-\(\alpha\)-smooth muscle actin; Sigma, St Louis, Mo; 1:100 final titer); macrophages (MOMA-2; rat antimonocyte-macrophages–monoclonal antibodies; Serotec, Kidlington, United Kingdom; 2 \(\mu\)g/mL); vascular cell adhesion molecule–1 (VCAM-1; rat antimonocyte endothelial cell molecule–1; VECAM-1; rat antimonocyte CD106/VCAM-1; Southern Biotechnology Associates, Birmingham, Ala; 10 \(\mu\)g/mL); intracellular adhesion molecule–1 (ICAM-1; rat antimonocyte ICAM-1; Seikagaku, Tokyo, Japan; 3 \(\mu\)g/mL); factor VIII–related antigen (to identify endothelial cells; rabbit antihuman von Willebrand's factor; Dako, Glostrup, Denmark; 0.57 \(\mu\)g/mL); and acylcoenzyme A cholesterol acyltransferase (ACAT; rabbit anti-ACAT peptide; gift of Drs Hongxing Wang [Mount Sinai School of Medicine,
New York, NY] and Ira Tabas [Columbia University, New York, NY]). The immunostaining protocol is described elsewhere. Negative controls were obtained by substitution of the primary antibody with an irrelevant antibody. When mouse monoclonal reagents were used, control sections were also stained with an immunoglobulin G isotype to assess specificity of the secondary antibody.

For computerized morphometry, two sections from each lesion were analyzed. Cross-sectional areas measured were luminal area, area bounded by internal elastic lamina, and area bounded by external elastic lamina. Lesion area was calculated as internal elastic lamina area minus luminal area and represents the average of two values expressed as square millimeter per section. Macrophage content was determined by measuring the area of the lesion positively stained with MOMA-2.

**Fig 1.** Histologic characteristics of atherosclerotic lesions in apoE−/− mouse aortas before and after transplantation. Thoracic aortic segments that contained advanced atherosclerotic lesions were transplanted into either WT (normocholesterolemic) or apoE−/− (hypercholesterolemic) syngeneic recipients. **A,** Representative histologic cross sections of aortas before (pretransplant) and 2 months after transplantation (top row, H&E staining; bottom row, MOMA-2 immunostaining; original magnification, ×40). Pretransplant lesions of apoE−/− mice have thin cap, are rich in macrophage foam cells, and contain extracellular lipid core. A larger, more advanced lesion is seen in section taken from graft in apoE−/− recipient. In contrast to these two lesions, lesion in the graft in WT recipient is remarkable for lack of foam cell–containing intima and thinner medial layer, indicating both regression and remodeling compared with pretransplant state. **B,** Morphometric analysis of neointima area and macrophage content (assessed by area of MOMA-2 immunostaining) in lesions from pretransplant thoracic aortic segments and grafts in apoE−/− and WT recipients. Data are displayed as mean ± SEM.

group, underwent MR imaging, as described previously. In vivo MR microscopy of the abdominal aorta was performed with a 9.4-T, 89-mm vertical bore system, operating at a proton frequency of 400 MHz, and equipped with a 50-mm internal diameter gradient insert. Eight transverse slices, 1 mm thick, with a center-to-center gap of 2 mm, were acquired from the abdominal aorta with proton density-weighted sequence with a time of repetition/time of echo of 2000/13 ms, respectively. A 256 × 256-matrix spin-echo sequence was used for two-dimensional axial imaging with a pixel size of 117 × 117 × 1000 µm³. The total imaging time was 17 minutes for each proton density-weighted sequence. The MR images were correlated
with histopathologic cross sections by means of anatomic landmarks.

**Statistical analysis.** Numerical data are expressed as means ± SEM and were analyzed with the unpaired, 2-tailed Student *t* test, assuming unequal variance. *P* values less than .05 were considered significant.

**RESULTS**

Heterotopic, syngeneic aortic transplantation was first evaluated in WT mice of the inbred, C57BL/6 strain. Approximately 80% survival was obtained, as reported originally with this procedure (in that report, allogeneic transplants were used to study transplant vasculopathy). Recipient mice were euthanized 2, 3, 6, and 10 months after transplantation (n = 2, for each time point). In contrast to the original report, as expected, a histologic examination of the syngeneic grafts showed no evidence of transplant arteriopathy, such as lymphocytic infiltration, and all recipients grew normally and appeared healthy.

After weaning, 18 apoE–/– mice on the C57BL/6 background were fed a WD for 9 months, to allow the formation of advanced, complex atherosclerotic lesions (ie, lesions containing foam cells, SMCs, extracellular matrix, and lipid deposits). To assess the size and stage of lesions before transplantation, we euthanized six mice and graded their thoracic aorta lesions according to histologic composition and structure. The average grade was 3.5 (a grade ≤ 2 represents fatty streaks and foam cell lesions, ie, “early” lesions), consistent with the complexity of the lesions (Fig 1, A). Segments of thoracic aorta containing such lesions were taken from the remaining mice and transplanted to the infrarenal aorta of either apoE–/– (n = 4) or WT (n = 4) recipients.

**Fig 2.** Immunohistochemical analysis of apoE–/– mouse atherosclerotic lesions before and after transplantation. Lesional sections from: top row, pretransplant lesion from thoracic aorta. Note abundant immunostaining for functional foam cell marker, ACAT (brown), adhesion molecules (also brown), and presence of SMC-marker α-actin (red) within lesion. Middle row, Graft from WT recipient. Compared with top and bottom rows, note thinner intima and media, and reduced immunostaining for α-actin, ACAT, and VCAM-1, but not endothelial ICAM-1. Bottom row, Graft from apoE–/– recipient. Note similarities to pretransplant lesion, with presence of multiple cholesterol crystals and gross disruption of tissue architecture from continued progression. Original magnification, ×100.
8) recipients. Recipient mice were fed standard chow diets, which maintained plasma lipoproteins at either normal (WT mice; TC: 86 ± 10 mg/dL) or moderately hyperlipidemic (apoE–/– mice; TC: 558 ± 26 mg/dL) levels. (One apoE–/– and two WT recipients died in the early postoperative period; the mortality rate was 25%).

The engrafted segments were removed for analysis 2 months after transplantation. Lesion size and the area of immunostaining for specific proteins were quantified. Remodeling (ie, a change in lesion composition) and regression (ie, a change in lesion size) were obvious in grafts transplanted into WT recipients (Fig 1, A, top row). Compared with the pretransplant lesion area of 0.0892 ± 0.0179 mm² (Fig 1, B), lesions significantly ($P = .003$) progressed in the setting of hyperlipidemia to a cross-sectional area of 0.2411 ± 0.0636 mm², in the apoE–/– to apoE–/– grafts. In contrast, the lesion from the WT recipient is notable for the decreased areas of α-actin immunostaining of intima and media. The results for the media probably indicate a change in SMC phenotype that is part of the wide-scale remodeling process that must occur during the 2 months the lesions were in the normolipidemic environment.

Fig 1, B. Note the remarkable decrease ($P < .001$) from the pretransplant value of 0.0253 ± 0.0055 mm² (22% of lesion area) to 0.0002 ± 0.0001 mm² (1% of lesion area) in the apoE–/– to WT grafts. In contrast, apoE–/– to apoE–/– grafts had increased MOMA-2 staining, to 0.0510 ± 0.0157 mm²; however, the percentage of lesion area positively stained (21%) remained similar to the pretransplant value (22%).

For further assessment of lesion remodeling, immunostaining was performed for α-actin, a marker for SMC; ACAT, the intracellular enzyme that converts cholesterol to cholesteryl ester (ie, a functional marker of foam cells); and the adhesion molecules, ICAM-1 and VCAM-1. Representative results are shown in Fig 2. The presence of the SMC marker α-actin in the expanded neointima confirmed the complexity of the lesions in the pretransplant aorta and apoE–/– to apoE–/– graft (ie, that they had progressed past the macrophage foam cell stage). In contrast, the lesion from the WT recipient is notable for the decreased areas of α-actin immunostaining of intima and media. The results for the media probably indicate a change in SMC phenotype that is part of the wide-scale remodeling process that must occur during the 2 months the lesions were in the normolipidemic environment.
ACAT immunostaining (Fig 2) was extensive in the pretransplant and apoE−/− to apoE−/− lesions (top and bottom rows, respectively), but absent from the apoE−/− to WT lesion (middle row), which was consistent with the results for MOMA-2. Note the overlapping immunostaining patterns of ACAT and α-actin, implying that foam cells could be of macrophage or SMC origin.

Immunostaining for two principal adhesion molecules, ICAM-1 and VCAM-1, was assessed after confirmation with factor-VIII–related antigen immunostaining that intact endothelium was present in each sample (data not shown). As shown in the representative sections displayed in Fig 2 (panels 3 and 4), expression of ICAM-1 was comparable in the sections of all groups. In contrast to the results for ICAM-1, despite no obvious difference in VCAM-1 expression between pretransplant and apoE−/− to apoE−/− lesions, VCAM-1 was absent in the sections from the apoE−/− to WT grafts (Fig 2).

We tested whether in vivo MR could distinguish between the grafts in the two types of recipient mice. Fig 3 shows representative gross, histologic, and MR images of apoE−/− to WT grafts (top row) and apoE−/− to apoE−/− grafts (bottom row). The MR images were obtained 1 day before the mice underwent euthanasia. Note that the difference in wall thickness between the two lesions, apparent by gross or histologic examination, is paralleled by a corresponding change in the wall thickness detected with MR imaging.

**DISCUSSION**

From the results obtained in these experiments, we conclude that (1) regression and remodeling of atherosclerotic lesions that progressed past the macrophage foam cell stage in the apoE−/− mouse model can be achieved by sustained normalization of the plasma lipoprotein profile; (2) magnetic imaging resonance can detect differences in characteristics in lesions undergoing regression; and (3) systemic imaging resonance can detect differences in macrophage characteristics over time noninvasively, without the intercurrent sacrificing of animals for monitoring. The capability of MR to image lesions in the abdominal aorta of living apoE−/− mice has been shown.4,11 Although not an end point in the current study, the MR images obtained suggest MR may be sufficiently sensitive to serially monitor over time the progression or regression of lesions noninvasively in mouse models of atherosclerosis after metabolic, genetic, and pharmacologic interventions.

In summary, this transplantation model has demonstrated that advanced atherosclerotic lesions in apoE−/− mice have properties more dynamic than predicted, according to studies in human beings and other animals. It should now be possible with this model to undertake a number of studies aimed at discovering or better defining systemic antiatherosclerosis factors and their mechanisms.

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APPENDIX (METHODS)

MOUSE HETEROTOPIC AORTIC TRANSPLANTATION

The transplantation model used in the current study was adapted from the original description by Koulack et al.1

Procedures are carried out with the aid of an operative microscope (Carl Zeiss, Thornwood, NY), with 20 to 40× magnifications.

**Donor operation.** A midline laparotomy is made and extended through bilateral parasternal thoracotomies. The sternum is elevated, exposing the mediastinum. Heparin solution (3 mL, 100 U/mL; Hep-Lock, Elkins-Sinn, Cherry Hill, NJ) is injected into the left ventricle to flush the aorta. A 4-mm long segment of thoracic aorta (from its midportion, between left subclavian artery and diaphragm) is harvested. Periaortic fat is excised, and intercostal arteries are transected at a distance (1-2 mm) from their take off. This maneuver leads to retraction and contraction of the arterial stumps, preventing bleeding and avoiding the use of cautery or sutures for hemostasis.

**Recipient operation.** After adequate anesthesia, the animal’s paws are fixed to the table, and a midline abdominal incision is made. The intestines are retracted with moist sponges, exposing the retroperitoneum. The midportion of the infrarenal aorta is dissected free from the vena cava and surrounding tissues and isolated between minivessel clamps (Accurate Surgical and Scientific Instruments, Westbury, NY). Hemostasis can be accomplished with a Malls Bipolar Coagulator (Codman & Schurtleff, Randolph, Mass). The abdominal aorta is transected between the clamps, and a 1-mm long segment is excised. This excision combined with retraction of the aortic stumps allows appropriate space for insertion of the graft without tension or kinking. The donor segment (4 mm in length) is sutured as an interposition graft. Proximal and distal end-to-end anastomoses are constructed with interrupted sutures of 11-0 nylon monofilament (US Surgical, Norwalk, Conn).

Animals are kept on a warming pad until fully recovered from anesthesia. They are monitored clinically each hour for the first 4 hours and then once daily. Postoperative analgesia is provided with acetaminophen (Tylenol), orally, diluted in the drinking water.

Complications of this procedure include thrombosis of the aorta, evisceration, and bleeding. Aortic thrombosis consistently results in lower extremity paralysis, usually followed by rapid deterioration of general condition and death within 24 to 48 hours. Signs of distress, such as paralysis of lower extremities, labored respiration, decreased mobility, or avoidance of food, prompt immediate euthanasia.

The perioperative mortality rate for this procedure is expected to be at least 20%.1 In the current study, the mortality rate was 25%. Fashioning of anastomosis is challenging because of the small diameter of the mouse abdominal aorta (< 0.8 mm). Even under adequate magnification, the surgery requires highly skilled maneuvers.

**REFERENCE**