ApoE-Deficient Mice Are a Model of Lipoprotein Oxidation in Atherogenesis
Demonstration of Oxidation-Specific Epitopes in Lesions and High Titers of Autoantibodies to Malondialdehyde-Lysine in Serum

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Abstract Apolipoprotein (apo) E-deficient transgenic mice develop marked hyperlipidemia and progressive atherosclerotic lesions. To explore whether oxidative modification of lipoproteins is involved in atherogenesis in this murine model, we performed extensive immunocytochemical studies. Atherosclerotic lesions ranging from early fatty streaks to very advanced plaques were examined from the aortic valve region and the thoracic and abdominal aorta. Using guinea pig antisera against malondialdehyde (MDA)-lysine and 4-hydroxynonenal-lysine, two epitopes generated during the oxidative modification of low-density lipoprotein (LDL), we demonstrated the presence of these "oxidation-specific epitopes" in atherosclerotic lesions. In early lesions, oxidation-specific epitopes were found predominantly in macrophage-rich areas, whereas diffuse extracellular staining predominated in necrotic areas of advanced lesions. We have previously shown that autoantibodies against MDA-lysine are present in the circulation of humans and rabbits and that the immunoglobulin fraction extracted from their lesions contains autoantibodies against several "oxidation-specific" epitopes. Sera from apoE-deficient mice also contained circulating autoantibodies to MDA-lysine, and both early and advanced lesions were rich in murine immunoglobulins. Titers of serum autoantibodies were significantly higher in apoE-deficient mice than in C57BL/6 mice. Autoantibodies in murine plasma recognized MDA-lysine epitopes in atherosclerotic lesions of rabbits, and the immunostaining was competitively inhibited by excess human MDA-LDL. Similar findings were obtained by competitive radioimmunoassay. Finally, a morphometric technique was developed and tested in these mice that allows a quantitative assessment of aortic atherosclerosis. These findings suggest that in apoE-deficient mice, lipoprotein oxidation is involved in atherogenesis and that these transgenic mice constitute an appropriate model with which to study the antiatherogenic effect of antioxidant intervention. (Arterioscler Thromb. 1994;14:605-616.)

Key Words • arteriosclerosis • apolipoprotein E • transgenic mice • oxidation • immunocytochemistry • autoantibodies • immunoglobulins • image analysis

The role of oxidatively modified lipoproteins in atherogenesis has been intensely studied in the past decade. All major vascular cell types are capable of oxidizing LDL, and several lines of evidence demonstrate the in vivo occurrence of oxidized lipoproteins in atherosclerotic lesions (for review, see References 1 through 3). These include immunocytochemical staining of lesions with oxidation-specific antibodies, the demonstration that low-density lipoprotein (LDL) gently extracted from lesions is partially oxidized, and the presence of autoantibodies to oxidized LDL in the circulation and in lesions. Evidence for the atherogenicity of oxidized lipoproteins is provided by the observation that antioxidants such as probucol, butylated hydroxytoluene (BHT), and others reduce lesion formation by mechanisms independent of lipid lowering. Numerous mechanisms have been suggested by which oxidized lipoproteins could enhance lesion formation. These include its rapid uptake by scavenger receptors of macrophages, its chemotactic and cytotoxic properties, and its ability to trigger cellular responses (eg, the expression of adhesion molecules, cytokines, and enzymes) in vascular cells (for review, see Reference 15). However, the factors determining lipoprotein oxidation in vivo as well as the contribution of the actual role of lipoprotein oxidation in lesion formation remain largely unknown. This lack of knowledge is partially due to the limitations of experimental models of atherosclerosis.

Recent advances in molecular biology and murine genetics have led to the definition of genes that determine the susceptibility to atherosclerosis and subsequently to the generation of transgenic murine strains that develop atherosclerotic lesions (for review, see Reference 23). Mice with genetic defects or overexpression of specific apolipoproteins, lipoprotein receptors, or enzymes involved in lipoprotein metabolism are potentially invaluable in studies of atherogenesis. In particular, murine models of atherosclerosis may allow the evaluation of the atherogenic relevance of individual factors involved in lipoprotein modification. To determine if oxidation of LDL occurs in murine models of atherosclerosis, we studied a broad spectrum of lesions from apolipoprotein (apo) E-deficient mice using anti-

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sera specific for malondialdehyde (MDA)-lysin and 4-hydroxynonenal (4-HNE)-lysin. We refer to these lipid-protein adducts as “oxidation-specific epitopes” because they occur as a consequence of lipid peroxidation and are found on oxidized but not native LDL.26,27 However, these antibodies are not specific for oxidized LDL but recognize their respective epitopes on other oxidized lipoproteins and proteins as well. The distribution of oxidation-specific epitopes was compared with that of macrophage foam cells.27 We also determined the titer and specificity of circulating autoantibodies to MDA-lysin in transgenic and control mice and analyzed the distribution of murine immunoglobulins in lesions. Finally, we developed a morphometric method to quantify atherosclerotic lesions in these mice.

Methods

Mice

Atherosclerotic lesions were studied in nine homozygous apoE-deficient mice generated by homologous recombination26 (third- and fourth-generation hybrids with a C57BL/6 x 129Oa background from the colony at The Rockefeller University). The aortas of two C57BL/6 and two Swiss Webster mice were included in the study as controls, although these inbred strains may have a different susceptibility to atherogenesis. To obtain a broad spectrum of lesions, apoE-deficient mice were studied between 2 and 7 months of age. Five of the transgenic animals had been on a diet containing 0.15% cholesterol: two 2-month-old mice for 5 weeks, two 5- to 6-month-old mice for 12 weeks, and one 7-month-old animal for 25 weeks. Four transgenic mice were fed regular mouse chow. Serum autoantibodies were titrated in 12 apoE-deficient and six C57BL/6 mice of the same age (5 to 6 months) and fed the same regular diet.

Tissue Preparation for Morphometric Determination of Atherosclerosis

Mice weighing between 20 and 36 g were killed, and the aortic tree was perfusion-fixed at a constant, near-physiological pressure via a cannula inserted in the right ventricle, allowing unrestricted efflux from an incision in the left atrium. Blood was removed by perfusion with phosphate-buffered saline (PBS) containing 20 μmol/L BHT and 2 μmol/L EDTA, pH 7.4. Perfusion was then continued for 20 minutes with formal-sucrose (4% paraformaldehyde, 5% sucrose, 20 μmol/L BHT, and 2 μmol/L EDTA, pH 7.4) to obtain an initial fixation. The aorta and its main branches were dissected from the aortic valve to the iliac bifurcation. The adventitia was removed as much as possible in situ to prevent errors resulting from Sudan staining of the vessels. Because the vessels are so thin, Sudan-stained adventitial tissue could be observed through the vessel and affect the quantification of atherosclerosis. The aortic tree was opened longitudinally with extremely fine Vanna microscissors (George Thieman Co) and pinned flat on a black wax surface in a dissecting pan with 0.2-mm-diameter stainless steel pins (Fine Science Tools). Each needle was placed at an outward angle so that it would cast minimal shadow on the aorta and so that both the needle itself and its shadow would fall entirely in either a lesioned or normal area of the aorta. After overnight fixation with formal-sucrose and a 12-hour rinse in PBS, images of the aorta were captured with a black-and-white video camera (COHU Electronic Inc) mounted on the C-mount of an Olympus SZ4055 stereo microscope. Alternately, a Sony DYC-960MD three-chip charge-coupled device color video camera was used. As an even illumination is essential to obtaining a relatively constant gray scale of the nonlesioned areas throughout the aorta, extensive preliminary measurements were made to optimize image capturing. Two 600-W quartz reflector lamps were used at equal distance and angle. Reflections from lesions were reduced by keeping the aorta submerged in PBS and by using a polarizing filter. To assure the most uniform illumination possible, before capturing the images the light intensity of a uniform black or white background along several horizontal and vertical lines across the visual field was measured with the image analysis system.

To increase the resolution, which is limited by the number of scanning lines of a video camera, each aorta was captured in at least four segments. Images were captured and analyzed by using a DT2851 frame grabber (Data Translation Inc) and Optimas Image Analysis software (Biocompatible computer and a separate Sony PVM-1343MD image monitor). Images were enhanced by adjusting the input look-up table to obtain a better differentiation between the lesioned and the normal aortic surface and occasionally by a digital sharpening filter. The pins and their shadows were retouched with the gray scale of the adjacent tissue, thus greatly reducing the error they cause in the surface determination. A threshold of gray scales was then selected for each lesion or group of lesions so that the shape of the highlighted threshold area on the processed image corresponded as closely as possible to that of the actual lesion as viewed through the microscope. The size of the lesion areas could then be determined by the software. The surface area of the aortic segment itself was determined using an autotracing feature. The choice of a black background greatly facilitated this autotracing. However, the dark background affected the gray scale of the normal areas of the aorta, particularly in the thinner abdominal aorta, and resulted in a slight gradient of darkness. Therefore, the analysis of each mouse aorta (35 to 45 mm long) had to be performed in multiple segments.

As mouse aortas are too fragile to sustain the removal of Sudan-positive adventitial tissue, which may interfere with the quantification of lesions, images were captured unstained. To identify early atherosclerotic lesions, the aortas were then stained with Sudan IV without removing the pins. Aortas were briefly rinsed in 70% ethanol, immersed for 6 minutes in a filtered solution containing 0.5% Sudan IV (Sigma Chemical Co), 35% ethanol, and 50% acetone for 6 minutes, and destained for 5 minutes in 80% ethanol. The Sudan-stained aortas were placed under a stereomicroscope and served as references for the quantification of the captured images described above. In contrast to the video camera, the human eye can generally differentiate between internal staining and adventitial staining visible through the aortic wall, and Sudan staining allowed the operator to better differentiate nonraised initial lesions from unequal vascular wall thickness.

Tissue Preparation for Immunocytochemistry

Aortas of apoE-deficient and control mice were perfusion-fixed with formal-sucrose as described above. The aortic tree was dissected in a more perforatory way, and without opening it longitudinally, to preserve as much as possible the antioxidative protection of the perfusate. Aortic segments (5 to 7 mm long) as well as the atrioventricular part of the heart containing the aortic valve were removed, and fixation was continued overnight in the presence of antioxidants. Tissues were then paraffin embedded, and 8-μm-thick serial sections were prepared.

Immunocytochemistry

Sections were rehydrated and immunostained with an avidi-biotin–alkaline phosphatase system (Vector Labs).26,28 Immunostaining for oxidation-specific epitopes was performed with two guinea pig antibodies against model epitopes generated during the oxidation of LDL: MAL-2, specific for MDA-lysin (dilution, 1:250), and HNE-7, specific for HNE-lysin (dilution, 1:350).27,28 Macrophages were detected with an absorbed rabbit antiserum against murine macrophages (dilution, 1:350) (AlA31240; Accurate Chemical & Scientific Corp) or with a rat monoclonal antibody against the monocytic/macrophage differentiation antigen MAC1 (dilution, 1:25) (MI/70.15.1;
FIG 1. En face preparation of an aortic tree of a 7-month-old apolipoprotein E-deficient mouse that had been fed a diet containing 0.15% cholesterol for 25 weeks. The aorta was perfusion-fixed and dissected as described in "Methods." A and B, Unstained segments of the thoracic and abdominal aorta, respectively, that were captured for image analysis and determination of the atherosclerotic surface area. C, Same aorta stained with Sudan IV and used as reference during analysis of the segmented images that were captured before staining. Extensive lesions are visible at the aortic valve (V), along the small curvature of the arch (A), throughout the brachiocephalic trunk (B), and at the branch point of the right subclavia (RS), the left common carotid (C), the left subclavta (LS), and some intercostal arteries (IC). Pronounced lesions are also found in the abdominal aorta, especially at the branch points of the mesenteric and renal arteries (M) and at the iliac bifurcation (I). Although the extent of atherosclerosis showed considerable variability from animal to animal and depended on age and diet, lesions were generally found at the same sites as in this figure. Lesions in this aorta, 27.7 mm²; total surface area, 106.3 mm². Scale at bottom of panels is in millimeters.

Serotec Ltd). Primary antibodies bound to the tissue were detected with appropriate biotinylated anti–guinea pig, anti-rabbit, or anti-rat immunoglobulins (Vector Labs). Endogenous mouse immunoglobulins present in lesions were detected by direct application of biotinylated antibodies against murine immunoglobulin (Ig) G and IgM (Vector Labs). Control slides were incubated with nonspecific antisera or in some cases without primary antibody. Altogether, approximately 50 different lesions stained with these antibodies were examined.

Immunostaining with the autoantibodies present in mouse sera was performed on atherosclerotic lesions of a Watanabe heritable hyperlipidemic (WHHL) rabbit aorta. Dilutions of mouse sera ranging from 1:100 to 1:1000 were used as primary antibody, and biotinylated anti-mouse IgG or combined anti-mouse IgG and anti-mouse IgM were used as secondary antibodies. Rabbit lesions were also stained with MDA2, a monoclonal antibody against MDA-lysine. The specificity of the autoantibodies to epitopes of oxidized LDL was determined by competitive immunostaining. Mouse sera with high titers of autoantibodies were preincubated with excess human MDA-LDL, and immunostaining was compared with that obtained with the same final dilution of serum without competitor. MDA-LDL was prepared as described.27

Determination of Autoantibody Titers and Specificity

Solid-phase radioimmunoassay (RIA) techniques were used to determine titers and specificity of antibodies.27 For the binding assay, 96-well polystyrene microtiter plates were coated with 50 µL human MDA-LDL (5 µg/mL) in PBS overnight at 4°C. Nonadherent antigen was aspirated, and remaining binding sites were blocked by incubation with 5% bovine serum albumin in PBS for 45 minutes at room temperature. The wells were then washed four times with PBS containing 0.02% NaN₃, 0.05% Tween 20, 0.1% bovine serum albumin, and 0.001% aprotinin using a microtiter plate washer model 1550 (Bio-Rad). Serial dilutions (50 µL) of sera from apoE-deficient and C57BL/6 mice were added per well and incubated overnight at 4°C. The amount of immunoglobulin bound was quantified with a goat anti-mouse IgG labeled at approximately 10 000 cpm/µg with ¹²⁵I using lactoperoxidase...
A B

FIG 2. Photomicrographs showing immunocytochemical staining using the avidin-biotin-alkaline phosphatase method of serial sections of the proximal aorta of a 6-month-old apolipoprotein E-deficient mouse fed 0.15% cholesterol for 12 weeks. Sections were cut perpendicular to the aortic root and show part of the valve leaflets and the sinus of Valsalva. Specific epitopes recognized by the primary antibody are indicated by the red color. A, Several early lesions primarily composed of macrophages stained with a rabbit antisera to mouse macrophages (dilution, 1:500). B, Section stained with HNE-7, a polyclonal antisera to 4-hydroxynonenal-lysine (1:350), which demonstrates the occurrence of this oxidation-specific epitope in these lesions. C and D, Higher-magnification phase-contrast images of the lesions at the left base of the atrial valve shown in A and B. E and F, Details of the macrophage clusters at the right base of the aortic valve. Although the hemodynamic patterns at these locations can be assumed to be fairly complex and nonrepresentative of aortic lesions, the morphological appearance of these early lesions is consistent with that of comparative-stage lesions in other areas not immediately adjacent to F, the valve leaflet, which is stained for macrophages. In all cases, staining with MAL-2, an antisera to malondialdehyde-lysine, was identical to that obtained with HNE-7, and both oxidation-specific epitopes were closely associated with macrophage/foam cells (original magnification ×69.5 [A and B]; ×222 [C, D, and E]; and ×173.8 [F]; bars=100 μm).

(Enzymobeads, Bio-Rad Laboratories). Plates were incubated for 4 hours at 4°C with 50 μL/well of the second antibody diluted to approximately 400 000 cpm/50 μL. As no preimmune sera were available a priori, antibody titers were defined as the reciprocal of the greatest dilution of the antisera that showed specific binding three times greater than binding to wells coated with bovine serum albumin but no antigen.

Competitive solid-phase RIA's were performed similarly, except that the antigen was plated at 1 μg/mL. A fixed and limiting dilution of the mouse serum (25 μL) was then added together with an equal volume of dilution buffer (3% bovine serum albumin, 0.02% NaN₃, 0.05% Tween 20, and 0.001% aprotinin in PBS) containing increasing amounts of human MDA-LDL. The results were calculated as B/B₀, ie, the amount of antibody bound to the plated antigen in the presence of competitor (B) divided by the binding in the absence of competitor (B₀).

Results

En face preparations of the aortic tree of male and female apoE-deficient mice aged 2 to 7 months revealed numerous atherosclerotic lesions. Although the extent of atherosclerosis showed considerable variation from animal to animal, lesion distribution throughout the aortic tree was remarkably constant in all animals studied, and predilection sites were easily identifiable. Fig 1 displays an aorta obtained from a 7-month-old
Fig 3. Photomicrographs showing oxidation-specific epitopes and macrophages in early atherosclerotic lesions. A, Branch point of an intercostal artery (arrow) of a 10-week-old apolipoprotein (apo) E-deficient mouse fed 0.15% cholesterol for 5 weeks that shows a patch of macrophages rich in malondialdehyde (MDA)-lysine epitopes (MAL-2, dilution, 1:250). B, A lesion in the aortic arch of the same animal. Very large lesions were often found in cholesterol-fed animals that fulfilled the morphological criteria of early lesions. Again, oxidation epitopes were almost exclusively macrophage associated (MAL-2, dilution, 1:250). Transitional lesion from a 5-month-old apoE-deficient mouse fed a cholesterol-enriched diet for 12 weeks that shows typical distribution of macrophages (rabbit anti-mouse macrophage antiserum, dilution, 1:350). D, MDA-lysine epitopes were predominantly but not exclusively found in macrophage-rich areas of transitional lesions (MAL-2, dilution, 1:250) (original magnification ×277.8 [A]; ×138.9 [B through D]; bars=100 μm).

Fig 4. Photomicrographs show immunocytochemistry of serial sections of the abdominal aorta of a 5-month-old normal chow-fed, apolipoprotein E-deficient mouse stained with (A) the antiserum MAL-2 (dilution, 1:250), (B) antisera against mouse immunoglobulin (Ig) G and IgM, and (C) the monoclonal anti-macrophage antibody MAC-1 (dilution, 1:20). An early lesion to the left, which is composed predominantly of macrophage/foam cells, is rich in immunoglobulins and oxidation-specific epitopes. A more advanced lesion to the right shows few intact macrophages and mostly diffuse extracellular staining in the core region. The distribution of murine immunoglobulins matches that of the malondialdehyde-lysine epitopes in this lesion (original magnification ×138.9; bars=100 μm).

apoE-deficient mouse fed a diet containing 0.15% cholesterol for 25 weeks. Lesions are typically found at the aortic root, in the vicinity of the aortic valve, and the small curvature of the arch. The orifices of the brachiocephalic artery, the left subclavian artery, and the common carotid arteries are among the earliest prediction sites. Such early lesions were found even in...
2-month-old animals fed cholesterol for only 5 weeks. In 5- to 7-month-old animals on both regular or cholesterol-enriched diets (n=6), most of the brachiocephalic trunk and the branch sites of the right carotid and subclavian arteries were covered by lesions. Lesions in the left carotid and subclavian arteries were also extensive. The branch points of the intercostal arteries constitute well-known predilection sites in other animal models of atherosclerosis. Whereas the distribution and gross appearance of lesions at the aortic root closely resembled that of other species, lesions in the vicinity of the branch sites of the intercostal arteries differed somewhat from those observed in WHHL rabbits.11 In WHHL rabbits, relatively uniform V-shaped lesions are usually found distal to the orifices of most intercostal arteries. In apoE-deficient mice, only a few of these sites showed lesions. Furthermore, the lesions in apoE-deficient mice often protruded to a remarkable degree into the vascular lumen. The orifices of the large abdominal arteries, in particular the mesenteric and renal arteries, also constituted predilection sites. Early lesions at these branch points were observed even in animals in which only 1% to 2% of the aortic surface was affected by lesions. Finally, all transgenic mice showed lesions at the iliac bifurcation, another typical site of lesion formation in the WHHL rabbit.

To verify if the extent of aortic lesions could be accurately determined in murine models of atherosclerosis, we modified a morphometric method that we developed for the quantitative assessment of lesions in WHHL rabbits. The image analysis equipment used and the experimental approach are described in "Methods." The measurements were made on unstained aortic segments (Fig 1A and 1B). Repeated determinations of segments of the same aorta revealed that the accuracy is surprisingly good. Although the small size of the vessel (diameter, approximately 0.7 mm and length, 35 to 45 mm) suggests that the dissection itself and the pins used to hold the opened aortas in place would significantly affect the determination, these two factors proved to be negligible. In particular, the effect of the pins was minimized by placing them entirely in lesioned or nonlesioned areas (but not at the interface between these areas) and retouching them in the captured video image with the gray scale of the adjacent tissue. The main source of error was the choice of the threshold values used to define lesioned areas. This was particularly true for early lesions. Although the Sudan-stained aorta viewed through a stereomicroscope was used for reference, an element of uncertainty was always involved in determining the margins of fatty streaks. In contrast, advanced lesions were generally raised and could be clearly delimited. It can therefore be assumed that the error of the method, reflected by the coefficient of variation of multiple determinations of the same segments, is inversely correlated with the extent of atherosclerosis. In an aorta in which 7.8 mm² of the aortic surface was covered by atherosclerosis (from a total surface area of 84.9 mm²), the coefficient of variation for multiple determinations of the lesioned area by the same investigator was 4.3%, ie, 0.33 mm² (n=6). Among the individual segments of the same aorta, the smallest coefficient of variation occurred in the arch, which contained the most extensive lesions. The method described eliminates some of the errors associated with traditional methods using manual border tracing and results in a highly reproducible measurement. However, it has to be emphasized that operator input in the selection of the threshold remains a source of error in this method. Ideally, comparative studies should therefore be performed by the same investigator.

Control C57BL/6 and Swiss Webster mice had no measurable atherosclerosis. The percentage of lesions in the aortas of apoE-deficient mice varied between 1% in a 2-month-old animal fed 0.15% cholesterol for 5 weeks and 26.1% in a 7-month-old animal fed 0.15% cholesterol for 25 weeks (27.7 mm² of lesions, 106.2 mm² of total surface area). In all cases, measurements of surface areas included 1 mm each of the brachiocephalic trunk and right carotid and subclavian arteries.

To study the occurrence of epitopes generated during the oxidative modification of LDL, segments containing the aortic valve and lesions from the thoracic and the abdominal aorta were immunostained using polyclonal guinea pig antisera MAL-2 and HNE-7 as primary antibodies against oxidation-specific epitopes.7 Lesions of other sites of the aorta (Figs 3 and 4) showed no lesions and no intimal staining with oxidation-specific epitopes. Lesions ranged from early fatty streaks, consisting primarily of macrophage clusters and lipid pools, to very advanced plaques with large necrotic cores, cholesterol crystals, and medial involvement.

In early lesions of the aortic valve region (Fig 2) and of other sites of the aorta (Figs 3 and 4), MDA-lysine and 4-HNE–lysine epitopes were found in a distribution similar to that observed in the WHHL rabbit.5,6 These epitopes predominantly colocalized with macrophage foam cells, which were identified by using a rabbit antiserum or a rat monoclonal antibody against murine macrophages. In cholesterol-fed apoE-deficient mice, lesions were observed that matched advanced plaques in relative size but were composed almost exclusively of foam cells and lipid and thus met the morphological criteria of early lesions (Fig 3B). In these lesions, too, staining for oxidation-specific epitopes overlapped with that of macrophages. Early lesions in areas characterized by unusual hemodynamic patterns, such as the aortic valve (Fig 2) and branch sites of intercostal arteries (Fig 3A), also showed similar staining patterns. Transitional lesions displayed predominantly macrophage-associated oxidation epitopes, with very little diffuse extracellular staining in the cap region (Figs 3C, 3D, and 3C). In contrast, comparatively few oxidation-specific epitopes were found in advanced lesions (Fig 4A, right lesion, and Fig 6A). Pockets of intact macrophages and oxidation epitopes were found in the shoulder regions of these lesions. The necrotic cores showed predominantly diffuse, extracellular staining for both macrophage epitopes (Fig 5D) and oxidation epitopes (data not shown). Aortic sections of control mice showed no lesions and no intimal staining with oxidation-specific antibodies (Fig 7), whereas adventitial staining was frequent. These findings are entirely consistent with other animal models of atherosclerosis.5,6 However, the nature of the adventitial staining is uncertain, as oxidative artifacts during the dissection of the aorta cannot be completely ruled out (whereas the media and intima are clearly protected by the antioxidants in the perfusates). Controls in which the primary antibody was omitted or with irrelevant primary antibodies were devoid of staining (Fig 6C).
Photomicrographs of higher magnifications of selected lesions show distribution of macrophages and murine immunoglobulins. A and B, Details of the early lesion on the left side of Fig 4. A is stained with the rabbit antiserum to macrophages and B, with combined antiserum against murine immunoglobulin (Ig) G and IgM. Immunoglobulins were consistently found in very early stages of lesions (B) and appeared to be predominantly concentrated in the immediate vicinity of macrophages (A). In contrast, most of the oxidation-specific staining in early lesions collocalized with macrophages (see Figs 2, 3, and 7). C, Transitional lesion stained with the rabbit anti-mouse macrophage antiserum. In transitional and advanced lesions intact macrophage/foam cells (and oxidation epitopes; data not shown) were predominantly found in the shoulder area of lesions. D and E, Details of the advanced lesion on the right side of Fig 4. D, Staining for both macrophages and oxidation-specific epitopes in the core region was predominantly diffuse and extracellular (D was also stained with the antiserum against murine macrophages). The diffuse staining in the necrotic core probably reflects decayed macrophages. Immunoglobulins were prevalent in necrotic areas, both in diffuse distribution (E, stained with the combined anti-mouse IgG and IgM antiserum) and accumulated in the vicinity of cholesterol crystals (F, showing a detail of a serial section of the very advanced lesion of Fig 6, stained with the combined anti-mouse IgG and IgM antiserum) (original magnification ×695 [A, B, D, E, and F]; ×277.8 [C]; bars=20 μm [A, B, D, E, and F]; bar=100 μm [C]).

Circulating autoantibodies recognizing MDA-lysine epitopes are prevalent in humans and several other species, including rabbits, and immunoglobulins are a well-known component of human atherosclerotic lesions. We have recently shown that a fraction of the immunoglobulins extracted from human and rabbit lesions recognizes oxidized LDL and is present in lesions in the form of immune complexes with oxidized LDL.7-10 Mouse lesions also contained significant amounts of immunoglobulins (Figs 4B and 6B). At higher magnification (Fig 5A and 5B) the murine immunoglobulins present in fatty streaks seemed to occur predominantly extracellularly, but dense accumulations were seen in the immediate vicinity of macrophages. In advanced lesions, immunoglobulins often occurred throughout the lesions, and their distribution no longer matched that of oxidation epitopes (Fig 6A and 6B). Higher magnifications showed diffuse staining patterns (Fig 5E) and sometimes dense accumulations of immunoglobulins adjacent to cholesterol crystals in the necrotic core (Fig 5F). The specificity of the immunoglobulins present in murine lesions remains to be determined. However, the extraordinarily high titers of circulating autoantibodies recognizing MDA-lysine in mice and the demonstration of antibodies against oxidation-specific epitopes and immune complexes between these antibodies and oxidized LDL in human and WHHL lesions suggest...
that at least a part of the immunoglobulins present in murine lesions may be directed against oxidized lipoproteins.

As circulating autoantibodies against oxidized LDL are prevalent in other species, we screened the plasma of 12 apoE-deficient and 6 C57BL/6 mice of similar age for autoantibodies to MDA-lysine by using solid-phase RIAs. Fig 8A shows the binding curves of these sera. Autoantibody titers in apoE-deficient mice were the highest we have observed in any species (greater than 65 536 in 10 of 12 animals), whereas C57BL/6 mice showed titers comparable to those found in other species (greater than 4096 in only 2 of 6 animals). Competitive RIAs confirmed the specificity of autoantibodies in the sera of apoE-deficient mice. Human LDL extensively modified in vitro by MDA was an effective competitor and yielded competition curves similar to those obtained with autoantibodies isolated from humans and rabbits (Fig 8B).

To determine whether circulating murine autoantibodies are capable of binding to oxidation-specific epitopes in atherosclerotic lesions, sera of two apoE-deficient mice were used as "primary antibody" in immunocytochemistry of aortic lesions of WHHL rabbits. Mouse serum used as primary antibody yielded identical staining patterns as MDA2 (Fig 9A), an induced murine monoclonal antibody against MDA-lysine (Fig 9B). Staining with mouse serum was effectively competed for by excess human MDA-modified LDL (Fig 9C) but was not inhibited by competition with native LDL (data not shown). These results were similar to those previously obtained with human autoantibodies.

Discussion

Several transgenic mouse models that develop atherosclerotic lesions may be suitable to study the in vivo relevance of factors involved in lipoprotein oxidation. However, only limited information is available as to the nature of the lesions in these transgenic animals. The present study demonstrated by immunocytochemical methods that epitopes generated during lipoprotein oxidation are present in atherosclerotic lesions of apoE-deficient mice. These mice showed extensive aortic lesions, predominantly at characteristic predilection sites. MDA-lysine and 4-HNE-lysine adducts, characteristic of oxidized lipoproteins, were found in lesions ranging from early fatty streaks to advanced plaques. Their distribution was analogous to that found in atherosclerotic lesions of WHHL rabbits. In particular, these oxidation-specific epitopes colocalized with macrophage foam cells in early and transitional lesions and showed diffuse extracellular distribution in necrotic areas of advanced lesions. These data suggest that oxidation of lipoproteins occurs in the intima of apoE-deficient mice and contributes to the pathogenesis of atherosclerotic lesions. However, intervention studies
We also demonstrated that apoE-deficient mice had significantly higher titers of circulating autoantibodies to MDA-lysine than C57BL/6 mice. It is well established that slight modifications of the lysine residues of LDL render it highly immunogenic.

Oxidized lipoproteins present in atherosclerotic lesions may therefore have induced formation of these antibodies. Both humans and rabbits demonstrate circulating autoantibodies that recognize several different epitopes of oxidized LDL.

Since these autoantibodies recognize MDA-lysine on other proteins as well, it is possible that MDA adducts with other proteins, e.g., MDA-collagen, might also have been the antigen.

The autoantibody titers in the transgenic animals were greater than in any other species tested to date and reached levels commonly seen in immunized animals. Although genetic differences between the apoE-deficient animals (C57BL/6 x 129ola hybrids) and the control animals (C57BL/6) cannot be ruled out as the cause of the higher titers, it is likely that the presence of such high titers of autoantibodies in apoE-deficient animals provides indirect evidence for the occurrence of lipoprotein oxidation in lesions. The high titer also raises the question of the potential role of such autoantibodies in the atherosclerosis observed in apoE-deficient mice.

The involvement of immune mechanisms in atherosclerosis is increasingly recognized. Human lesions contain immunoglobulins and immunocompetent cells, such as macrophages and several subclasses of T lymphocytes. The expression of interleukin-2 receptors on T lymphocytes, the presence of secreted interferon-γ, and the presence of C5b-9 complement complex all indicate activation of the cellular and humoral immune systems in lesions. However, it is unknown a priori whether these immune responses constitute a protective mechanism, whether they enhance lesion formation, or whether their role changes from beneficial to detrimental at certain stages of lesion formation.

Circulating human and rabbit autoantibodies are capable of binding to MDA-lysine epitopes in lesions, and atherosclerotic lesions of WHHL rabbits and humans contain immune complexes between these autoantibodies and oxidized LDL. Similar immune complexes between oxidized lipoproteins and autoantibodies may occur in murine lesions. Formation of such immune complexes may lead to enhanced uptake by macrophages, either via Fc receptors or phagocytosis. This may enhance the removal of oxidized lipoproteins, which have many toxic effects on the arterial wall, and thus complement the normal scavenger function of mononuclear phagocytes. On the other hand, increased uptake of immune complexes containing oxidized lipoproteins could contribute to increased foam cell formation and thus enhance the progression of lesions. The formation of immune complexes is not the only mechanism that links oxidized lipoproteins to immune processes. Oxidized LDL has been shown to be chemotactic not only for monocytes but also for T lymphocytes (which may increase the number of intimal lymphocytes) and to activate T cells in the presence of monocytes.

We have shown that lesions of apoE-deficient mice also contain significant quantities of murine immunoglobulins. The specificity of these immunoglobulins is at present unknown, and it is questionable whether sufficient amounts could be extracted from atherosclerotic lesions of mice to fully characterize them. The assumption that some of the immunoglobulins found in murine lesions are directed against epitopes generated during lipoprotein oxidation is based on the fact that antibodies against oxidation-specific epitopes have been isolated from lesions of other species. The presence of murine immunoglobulins in very early lesions, consisting primarily of macrophage-derived foam cells and lipid accumulations and rich in oxidation-specific epitopes, would support this hypothesis. In early lesions, immunoglobulins often seemed con-
centrated near macrophages, and in the necrotic core of advanced lesions staining was particularly intense near cholesterol crystals. In this context, it may also be relevant that cholesterol crystals may induce complement activation and that oxidized cholesterol derivatives may also be immunogenic.

The high titers of autoantibodies binding to oxidized LDL in apoE-deficient mice may reflect the extent of hyperlipidemia and lesions in these animals. Because MDA-lysine could occur as the result of lipid peroxidation from any source, it is conceivable that the antigen initiating the immune response occurred at sites other than the atherosclerotic lesion. However, in a small group of patients, higher titers of circulating autoantibodies against MDA-LDL were correlated with increased progression of carotid arteriosclerosis. Elevated autoantibody titers were also found in preeclamptic women, who have decidua vascular pathology reminiscent of atherosclerosis. However, the titer of circulating autoantibodies is likely to reflect a large number of variables that affect antibody concentrations, including immune-response genes, the rate of generation, the degree of tissue binding, and the rate of antibody consumption. Neither the titer of circulating autoantibodies nor the presence in lesions of immune complexes between autoantibodies and oxidized lipoproteins per se proves their role in atherogenesis. Further studies will have to address this issue.

A substantial body of evidence indicates that lipoprotein oxidation constitutes an important element in atherogenesis and that antioxidants may reduce lesion formation. The presence of oxidation-specific epitopes and the high titers of circulating autoantibodies to MDA-lysine in apoE-deficient mice suggest that lipoprotein oxidation may also be involved in atherogenesis in this new model. The morphology of murine lesions presents many similarities to other accepted models of atherosclerosis. Nevertheless, the pathophysiological mechanism triggering lesion formation may be atypical in apoE-deficient mice as a result of the altered lipoprotein metabolism. ApoE is one of the primary cholesterol transport proteins. It is bound to the surface of most lipoprotein particles and mediates receptor-dependent clearance of lipoproteins from plasma. Plasma cholesterol levels in cholesterol-fed apoE-deficient mice may exceed 2000 mg/dL, and it is therefore likely that atherosclerosis is primarily induced by the impairment of the plasma clearance of apoE-containing lipoproteins in the liver via binding of apoE to the apoB/E receptor and the LDL receptor-related protein. However, apoE is also found in atherosclerotic lesions. Macrophages are capable of synthesizing and secreting apoE in vitro, and Northern blot analysis and polymerase chain reaction studies show that apoE message is expressed within the artery wall. Combined immunocytochemistry and in situ hybridization show that macrophages synthesize and secrete apoE in lesions and probably account for most of the apoE found in the atherosclerotic artery. Furthermore, apoE is implicated in cell proliferation and differentiation as well as in the modulation of immune processes. It is therefore conceivable that the inability of vascular macrophages in apoE-deficient animals to secrete apoE may also enhance atherogenesis.

Whatever the mechanisms responsible, the data presented suggest that oxidation of lipoproteins is involved in the atherogenic process, and it will be important to determine if antioxidant therapy inhibits atherosclerosis in the apoE-deficient mice, as it does in WHHL rabbits. The morphometric method described here allows the determination of the extent of aortic surface covered by atherosclerotic lesions and should therefore be useful in assessing the ability of antioxidant intervention to inhibit atherogenesis.
Apoe-deficient mice may constitute a suitable model to study the factors involved in the oxidative modification of LDL and the antiatherogenic effect of antioxidants. Furthermore, in view of the well-characterized immune system of mice, transgenic mice also provide a promising model to study the role of immune processes in atherogenesis, and in particular, the role of humoral- and cell-mediated responses to oxidative modification of LDL.

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ApoE-deficient mice are a model of lipoprotein oxidation in atherogenesis. Demonstration of oxidation-specific epitopes in lesions and high titers of autoantibodies to malondialdehyde-lysine in serum.

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