Rabbit and Human Atherosclerotic Lesions Contain IgG That Recognizes Epitopes of Oxidized LDL

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Abstract Atherosclerotic lesions contain relatively large quantities of IgG. We have previously shown that both human and rabbit sera contain autoantibodies against epitopes of oxidized (Ox) low-density lipoprotein (LDL) and that LDL isolated from atherosclerotic lesions contains small amounts of tightly bound IgG. However, it is not known whether IgG isolated from atherosclerotic lesions recognizes epitopes present in native LDL or Ox-LDL. IgG was isolated from Watanabe heritable hyperlipidemic (WHHL) rabbit atherosclerotic lesions by sequential salt extractions, purified by fast protein liquid chromatography on protein G, and used in a solid-phase radioimmunoassay. IgG and immune complexes were also isolated from the saline extracts of human lesions by adsorption onto latex beads coated with anti-human IgG antibodies or protein A. IgG isolated from rabbit lesions showed significant titers against malondialdehyde (MDA)-modified LDL and LDL oxidized by copper ions for 4 and 18 hours but not against native LDL. On Western blots, lesion IgG stained MDA-LDL and fragments of Ox-LDL. Western blots of immune complexes isolated from human lesions revealed the presence in the isolated complexes of both apoprotein B and apoprotein B fragments, which reacted with antibodies to MDA-lysine. Furthermore, rabbit lesion IgG immunostained epitopes of Ox-LDL present in human atherosclerotic lesions. Immunostains obtained with rabbit lesion IgG were similar to those obtained with a monoclonal antibody specific for MDA-lysine. The results show that human and rabbit atherosclerotic lesions contain IgG that recognizes epitopes characteristic of Ox-LDL. These data suggest that immunologic processes may be an important component of the atherogenic process. (Atheroscler. Thromb. 1994;14:32-40.)

Key Words • atherosclerosis • immunity • inflammation • immunoglobulins • oxidized LDL

Atherosclerotic lesions are characterized by the accumulation of lipids in macrophages and other arterial cells, the proliferation of smooth muscle cells, and the accumulation of connective tissue components in the arterial extracellular space. Atherogenic processes may play an important role in the progression of atherosclerotic lesions. In addition, various forms of Ox-LDL can affect the gene expression of arterial cells and other cellular functions that may be partially responsible for the development of the complex pathological findings that are seen in advanced atherosclerotic lesions (reviewed in Reference 5). Atherosclerotic lesions contain many components that are typical of chronic inflammation, and several lines of evidence suggest that immunologic factors may play an important role in the progression of atherosclerotic lesions: (1) It has been shown by immunocytochemical and biochemical techniques that atherosclerotic lesions contain immunoglobulins and various complement components. (2) Human atherosclerotic lesions contain substantial numbers of T cells. (3) Arterial macrophages and smooth muscle cells express major histocompatibility complex (MHC) class II antigens, suggesting that these cells may be involved in the presentation of antigens to T cells. We and others have reported that Ox-LDL is immunogenic and that autoantibodies against Ox-LDL are present in human and rabbit sera. Serum autoantibodies can immunostain atherosclerotic lesions in a way that is almost indistinguishable from the immunostaining pattern obtained with antibodies against Ox-LDL. LDL isolated from human atherosclerotic lesions contains small quantities of tightly bound IgG that presumably recognizes some epitopes in lesion LDL. Various forms of modified LDL have been shown to be strongly immunogenic in experimental animals. Similarly, Ox-LDL is immunogenic, and autoantibodies to epitopes of Ox-LDL have been demonstrated in the sera of normal and Watanabe heritable hyperlipidemic (WHHL) rabbits and humans, as noted above. The presence of Ox-LDL in atherosclerotic lesions as well as immunoglobulins and complement components suggests that immune complexes with Ox-LDL may be present in the lesions. However, the specificity of the immunoglobulins in atherosclerotic lesions is unknown, and it has not been determined whether some of the lesion immunoglobulins recognize epitopes that are characteristic of Ox-LDL. The purpose of the present study was to isolate and purify IgG from atherosclerotic lesions of WHHL rabbits and humans, to see whether such lesion IgG recognized epitopes of Ox-LDL in vitro assays, and to observe whether these antibodies could specifically immunostain the oxidation-
specific epitopes that are known to be present in atherosclerotic lesions.23,27,20

Methods

Carrier-free sodium [131]iodide was purchased from American, Arlington Heights, Ill. Butylated hydroxytoluene (BHT) was from J.T. Baker Chemical Co, Phillipsburg, NJ. Aprotinin, elastatin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), tetramethoxyparpropane, high-molecular-weight markers, and protein A were from Sigma Chemical Co, St Louis, Mo. D-Phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) and benzamidine were from Calbiochem Behring Corp, La Jolla, Calif. Probucol was from Merrell Dow Pharmaceuticals, Indianapolis, Ind. Polyacrylamide gradient gels (4% to 12%), Tefogels were from Newex, Encinitas, Calif. Ninety-six-well polyvinyl chloride microtiter plates were from Dynatech Laboratories, Alexandria, Va. Tween 20, nitrocellulose membranes, Immunobead solid-phase particles, and Enzymobeads were from BioRad, Richmond, Calif. Affinity-purified goat anti-human IgG and goat anti-mouse IgG were from Co Biomedical, Malvern, Pa. Iodo-Gen was from Pierce Chemical Co, Rockford, Ill. X-Omat autoradiography film was purchased from Eastman Kodak, Rochester, NY. Biotinylated goat anti-rabbit and goat anti-human IgG antibodies and avidin-biotin-alkaline phosphatase reagents were from Vector Laboratories, Burlingame, Calif.

Collection of Arterial Specimens

Fresh human abdominal aortas and proximal iliac arteries were obtained from donors (n=2; ages, 35 and 41 years) of renal transplants immediately after surgery, washed with sterile saline, and placed on ice in cold extraction buffer containing antioxidants, protease inhibitors, and antibiotics29 (see below). The intima and inner media containing atherosclerotic lesions were dissected within 1 hour after removal of the arteries at 4°C and were used for isolation of immune complexes as described.30,29

Eighteen 1- to 2-year-old WHHL rabbits were anesthetized with intramuscular ketamine (35 mg/kg) and xylazine (5 mg/kg). Serum samples were taken from 10 of the animals. The animals were then exsanguinated by cannulating the right atrium. The whole aorta from the arch to the bifurcation was opened longitudinally and washed free of any adherent blood. The tubes were centrifuged at 100,000g for 30 minutes at 5°C in a Beckman L2 ultracentrifuge using a 0.14 mol/L NaCl/0.01 mol/L phosphate-buffered saline (PBS), pH 7.2, containing the following preservatives: 2.7 mmol/L EDTA, 2 mmol/L benzamidine, 1 mmol/L PMSF, 1 mmol/L leupeptin, 40 mmol/L elastatin, 10 mmol/L protobol, 1 mmol/L PPACK, 0.01% aprotinin, 0.008% chloramphenicol, and 0.008% gentamicin. The elution of soluble proteins with 0.14 mol/L NaCl/0.01 mol/L PBS, pH 5.0, containing the same inhibitors, antioxidants, and antibiotics indicated above. The extract was collected by low-speed centrifugation. The supernatants were combined. The extract was concentrated by low-speed centrifugation using a 1.5 mol/L NaCl-extracted IgG fraction. This latter IgG fraction was designated as the "1.5 mol/L NaCl-extracted IgG."

Isolation of Immune Complexes

Saline extracts of human atherosclerotic lesions were incubated with gentle mixing for 4 hours at 37°C in the presence of latex beads or micron-sized acrylic particles covalently coated with either protein A (Sigma), rabbit anti-human IgG, or rabbit anti-mouse IgG. To minimize nonspecific binding of proteins, the beads were preincubated for 30 minutes at 37°C in PBS containing 20 μmol/L BHT (pH 7.4) and 0.1% bovine serum albumin (BSA). After incubation with the arterial extracts, the beads were centrifuged at 1000g for 5 minutes and washed six times with PBS/20 μmol/L BHT. Beads were then suspended in 2x sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (see below), kept for 30 minutes at room temperature, and incubated in a boiling-water bath for 5 minutes. After a brief centrifugation, an aliquot of the supernatant was used for SDS-PAGE and immunoblotting.

Isolation of IgG

IgG fractions were isolated from the initial saline extract and the 1.5 mol/L NaCl extract with a fast protein liquid chromatography system (Pharmacia, Piscataway, NJ) equipped with a protein G–Superose column (Pharmacia). Samples were loaded in 20 mmol/L sodium phosphate (pH 7.1) and eluted with 0.1 mol/L glycine-HCl (pH 2.7). Eluted fractions were monitored by UV absorbance at 280 nm, and IgG fractions were collected using a fraction collector. Protein concentration of the isolated IgG fractions was determined according to Lowry et al.34 with rabbit IgG as a standard.

Radioimmunoassay (RIA)

Solid-phase RIAs of rabbit lesion IgG or rabbit serum were done as described.30,31 Briefly, 96-well microtiter plates were coated with native or modified LDL (5 μg/mL) in PBS containing 2.7 mmol/L EDTA and 20 μmol/L BHT and kept and the 1.5 mol/L NaCl extract with a fast protein liquid chromatography system (Pharmacia, Piscataway, NJ) equipped with a protein G–Superose column (Pharmacia). Samples were loaded in 20 mmol/L sodium phosphate (pH 7.1) and eluted with 0.1 mol/L glycine-HCl (pH 2.7). Eluted fractions were monitored by UV absorbance at 280 nm, and IgG fractions were collected using a fraction collector. Protein concentration of the isolated IgG fractions was determined according to Lowry et al.34 with rabbit IgG as a standard.

SDS-PAGE and Immunoblotting

SDS-PAGE was done as described32 using 4% to 12% tris(hydroxymethyl)aminomethane (Tris)/glycine gels (pH 8.3) preequilibrated with 0.1% SDS (Novex Mini gels). Before electrophoresis, the samples were heated in sample buffer for 3 minutes in a boiling-water bath. The sample buffer contained 0.063 mol/L Tris-HCl, 2% SDS, 10% glycerol, 10 μmol/L BHT, and 0.001% 2-mercaptoethanol (pH 6.8). A reducing agent was not included in the sample buffer. Proteins were then transferred to nitrocellulose membranes.27 After transfer, the remaining binding sites were blocked with 5% nonfat dried SW 41 swing-out rotor (Beckman Instruments, Palo Alto, Calif). A bottom pellet and a creamy top were discarded.27 The extract was concentrated by low-speed centrifugation using Amicon Centricron filters (Amicon, Danvers, Mass) and used for protein G chromatography. We designated this IgG fraction as the "saline-extracted IgG." After the initial saline extraction, the tissue was reextracted overnight at 4°C with similar volumes of 1.5 mol/L NaCl/0.05 mol/L PBS, pH 5.0, containing the same inhibitors, antioxidants, and antibodies indicated above. The extract was collected by low-speed centrifugation. The supernatants were combined and handled in the same way as the initial saline-extracted IgG fraction. This latter IgG fraction was designated as the "1.5 mol/L NaCl-extracted IgG."
milk proteins. The membranes were then incubated for 12 hours with primary antibodies in PBS containing 0.05% Tween 20 and 1% nonfat dried milk. The membranes were washed three times (30 minutes each) with the same buffer without antibodies and incubated for 12 hours in the presence of 125I-labeled secondary antibodies.28 LDL-labeled, affinity-purified goat anti-human IgG, goat anti-mouse IgG, and goat anti-rabbit IgG were used as secondary antibodies on Western blots. Antibodies were labeled by the lactoperoxidase method (Enzymobeads) to approximately 10,000 cpm/ng according to the manufacturer's instructions as described.25,26 The membranes were then washed four times over 2 hours in the same buffer without antibodies, dried, and subjected to autoradiography.

**Immunocytochemistry**

Aortas of WHHL rabbits were perfusion-fixed with formal saline (4% paraformaldehyde, 5% sucrose, 50 μM/L BHT, and 1 mmol/L EDTA, pH 7.4) as described.27 Serial 8-μm-thick sections were rehydrated and immunostained with an avidin-biotin-alkaline phosphatase system (Vector Labs) as described.27 Immunostaining of these sections was performed either with dilutions of human serum or with a protein A-purified IgG fraction of human serum27 as the source of primary antibodies. Biotinylated anti-human IgG was used as the secondary antibody. In addition, arterial sections of atherosclerotic WHHL aortas were also stained with previously described monoclonal antibodies against oxidation-specific epitopes, i.e., MDA2, specific for malondialdehyde (MDA)-lysine; NAS9, specific for 4-hydroxynonenal-lysine; and OLF-3C10, which recognizes apoprotein fragments of copper-oxidized LDL (Cu-LDL).24 Controls included MB47, a monoclonal antibody against the receptor-binding domain of native apoprotein (apo) B-100; RAM-11, a macrophage-specific monoclonal antibody; anti-rabbit C3 serum (Organon Teknika-Cappel, Durham, NC); and nonspecific sera. Endogenous immunoglobulins in the rabbit serum were detected by direct application of biotinylated goat anti-rabbit IgG (Vector Labs) to the tissue sections.

Human aortic samples were immersion-fixed overnight at 4°C in formal sucrose containing antioxidants (see above) and embedded in paraffin. Serial sections of human atherosclerotic lesions were stained with the protein G–purified IgG fractions isolated from rabbit atherosclerotic lesions as described above. Other antibodies that were used to stain the human material included MDA2 (see above) and HAM-56, a monoclonal antibody against human macrophages (a generous gift from Dr A. Gown of the University of Washington, Seattle). The specificity of immunostaining with IgG extracted from rabbit lesions for MDA-lysine was determined by competitive immunostaining, for which the primary antibody (ie, the extracted IgG) was incubated for 30 minutes with an excess of human MDA-LDL or native LDL before immunostaining.

**Lipoprotein Modification**

LDL from healthy human donors was isolated by ultracentrifugation.29 Cu-LDL was made in the absence of cells by incubating LDL (100 μg/mL) at 37°C in Ham's F-10 for 4 or 18 hours in the presence of 10 μmol/L copper as described.29 In some experiments, Cu-LDL apo B was isolated after lipid extraction with chloroform/methanol (2:1 vol/vol). Malondialdehyde-modified LDL (MDA-LDL) was made on ice by repeated additions of freshly prepared MDA as described.29 Protein concentrations of the lipoproteins were determined according to Lowry et al.30 with BSA as a standard.

**Results**

Eighteen 1- to 2-year-old WHHL rabbits were killed and their aortas obtained at the time they were killed for titers of autoantibodies to various epitopes of Ox-LDL. Because Ox-LDL displays different epitopes as it undergoes progressive oxidation, we used a variety of models of Ox-LDL to present antigens that might occur in vivo. Thus, we tested the ability of autoantibodies to bind to MDA-LDL, to LDL oxidized by exposure to copper for 4 or 8 hours (ie, Cu-LDL), and to delipidated 4- or 8-hour Cu-LDL. Sera were studied at serial dilutions ranging from 1:2 to 1:4096. All 10 sera had titers against MDA-lysine ranging from 64 to >4096. Six sera had detectable titers against Cu-LDL oxidized for 4 or 8 hours (range, 4 to >4096). Eight sera had titers against delipidated Cu-LDL (range, 4 to >4096). While there was considerable variability in the titers against the different models of Ox-LDL tested, every animal had a serum titer >64 against at least one antigen, eight of 10 animals had a titer against two or more antigens, and four animals had high titers >4096 against all antigens tested. In addition, four animals displayed a titer against native LDL used as a control (range, 4 to 4096), probably due to oxidation of native LDL that may have occurred despite the presence of antioxidants.25,26

With the gentle elution technique used, only small amounts of IgG could be isolated from the intimal sections of the rabbit atherosclerotic lesions. After fast protein liquid chromatography on protein G, recoveries of the isolated saline-extracted and the 1.5 mol/L NaCl–extracted IgG fractions from WHHL rabbit atherosclerotic lesions were 50 and 37 ng/g lesion (wet weight), respectively. On SDS-PAGE, the isolated IgG fractions contained a single band that comigrated with IgG standards (data not shown). On solid-phase RIA, both isolated IgG fractions had significant titers against epitopes of Ox-LDL (Table). MDA-lysine in particular was an effective antigen. Reactivity was also seen with various forms of Cu-LDL, whereas no reactivity was seen with native human LDL. When Cu-LDL was delipidated (while plated on wells), the binding of lesion IgG was enhanced, particularly for the preparation of Cu-LDL oxidized for 4 hours.

To confirm the results of the solid-phase assay, isolated rabbit lesion IgG was also used as a primary antibody on Western blots of in vitro–modified human LDL. Lesion IgG heavily stained a preparation of MDA-LDL and also lightly stained a 190-kD fragment in a preparation of delipidated, 4-hour Cu-LDL. Lesion IgG did not stain intact, native apo B, but two fragments (150 and 190 kD) in the native LDL preparation stained with the lesion IgG (data not shown). We assume that these degradation fragments in the native LDL preparation arose as a result of minor oxidation that occurred despite the presence of EDTA. We have reported that monoclonal antibodies against either MDA-lysine or copper-oxidized apo B fragments frequently stain such fragmentation products of LDL on Western blots.29 Soluble immune complexes were also isolated from saline extracts of human atherosclerotic lesions by incubation with latex or acrylic beads that were covalently coated with either rabbit anti-human IgG, rabbit anti-mouse IgG (as a control bead that should not bind human IgG), or protein A. After extensive washing, the bound immune complexes were dissolved and subjected to SDS-PAGE, followed by blotting to a nitrocellulose membrane. Western blots with monoclonal antibodies specific for apo B (MB47) and MDA-lysine (MDA2) revealed the presence, in the isolated immune complexes, of intact apo B and apo B fragments, which also reacted with antibodies.
against MDA-lysine (Fig 1). Control incubations, in which rabbit anti-mouse beads were incubated with the saline extracts, were also performed. Solubilization of material bound to these beads did not show any staining with these antibodies (Fig 1). Beads coated with protein A (which would also be expected to bind IgG) were also effective in binding MB47- and MDA2-positive material, similar to the beads coated with anti-human IgG (Fig 1). In a separate experiment, the protein A beads were shown to be free of MB47 and MDA2 immunoreactivity (data not shown).

**Immunostaining of Rabbit and Human Arteries**

We have shown that human sera with significant titers of autoantibodies against MDA-lysine stain rabbit atherosclerotic lesions in a pattern like that seen with monoclonal antibodies generated against oxidation-specific epitopes. This is illustrated in serial sections of WHHL rabbit lesions immunostained with a protein A-purified IgG fraction isolated from human serum (Fig 2A), as well as with monoclonal antibodies MDA2 (against MDA-lysine, Fig 2B) and OLF4-3C10 (against oxidized apoprotein fragments of Cu-LDL, Fig 2C). Note the nearly identical staining patterns in these three panels. Furthermore, preincubation of the purified human IgG fraction with MDA-LDL markedly diminished but did not completely eliminate the staining shown in Fig 2A (data not shown). These areas are rich in macrophage/foam cells (Fig 2D). Native apo B is not found in the macrophage-rich portion of this lesion but mostly in areas with few macrophages (Fig 2E). Note that macrophage-rich areas containing the oxidation-specific epitopes also contain large amounts of rabbit IgG (Fig 2F) and complement C3 (Fig 2G). Higher magnifications of the staining patterns observed with human IgG and the antisemum against rabbit IgG are shown in Fig 3. Thus, the human IgG fraction clearly recognized oxidation-specific epitopes in rabbit atherosclerotic lesions.

To demonstrate that the rabbit IgG isolated from WHHL lesions can also react with oxidation-specific epitopes in the artery wall, we used this IgG to stain human lesions. (Note that rabbit lesions cannot be immunostained with rabbit IgG because of the extensive amount of IgG already present.) When immunocyto-

**Titers of Rabbit IgG Isolated From Atherosclerotic Lesions**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution</th>
<th>MDA-LDL</th>
<th>Cu^2+-LDL, After 4 Hours</th>
<th>Cu^2+-LDL, After 18 Hours</th>
<th>Cu^2+-Apo B, After 4 Hours</th>
<th>Cu^2+-Apo B, After 18 Hours</th>
<th>Native LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline extract</td>
<td>10 000</td>
<td>29</td>
<td>0</td>
<td>0</td>
<td>195</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1 000</td>
<td>98</td>
<td>0</td>
<td>0</td>
<td>129</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>521</td>
<td>0</td>
<td>0</td>
<td>114</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1619</td>
<td>0</td>
<td>0</td>
<td>267</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1.5 mol/L NaCl extract</td>
<td>10 000</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>102</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1 000</td>
<td>185</td>
<td>0</td>
<td>0</td>
<td>191</td>
<td>173</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>100</td>
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<td>0</td>
<td>117</td>
<td>159</td>
<td>545</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2378</td>
<td>1591</td>
<td>2774</td>
<td>664</td>
<td>5370</td>
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</tbody>
</table>

Wells of microtiter plates were coated with the indicated antigen (5 μg/mL), and the remaining binding sites were blocked with 5% bovine serum albumin. Indicated dilutions of protein G-purified rabbit lesion IgG were then added, and the amount of bound IgG was determined by use of a radiolabeled goat anti-rabbit IgG. "Saline extract" refers to the initial 0.14 mol/L NaCl buffer extraction of intima/media sections, while "1.5 mol/L NaCl extract" refers to the second extraction performed with a higher salt concentration. The following antigens were used: MDA-LDL, malondialdehyde-modified human low-density lipoprotein (approximately 84% of lysines modified); Cu^2+-LDL after 4 hours; Cu^2+-LDL after 18 hours; human LDL exposed to Cu^2+ for 18 hours; Cu^2+-Apo B after 4 hours; delipidated apoprotein B of 4-hour Cu^2+-oxidized LDL; Cu^2+-Apo B after 18 hours, delipidated apoprotein B of 18-hour Cu^2+-oxidized LDL. Native LDL was plated in the presence of antioxidants to prevent oxidation as described. Values are expressed as counts per minute per well of bound secondary antibody and represent values after subtraction of the amount of secondary antibody bound to wells that were identical except that they lacked antigen. Each dilution was assayed in duplicate for each antigen.
chemistry of human atherosclerotic lesions was performed with the IgG isolated from rabbit lesions, the same staining pattern was observed as when the lesions were stained with the induced antibodies to MDA-lysine (Fig 4). Their specificity was further confirmed by demonstrating that preincubation of rabbit IgG with excess amounts of human MDA-LDL significantly inhibited the intimal staining (Fig 4E). This demonstrated that MDA-lysine was a significant epitope to which the lesion IgG was directed. Higher magnifications of the staining obtained with rabbit lesion IgG revealed patterns characteristic of those observed with induced antibodies against oxidation-specific epitopes. In areas rich in macrophage/foam cells, most of the staining was cell associated (showing both punctuate and circular patterns), although some diffuse and clearly extracellular staining was also present (Fig 4G). Very advanced lesions showed predominantly diffuse extracellular staining, especially in acellular areas of the necrotic core (Fig 4H), similar to the patterns we previously obtained with our oxidation-specific monoclonal antibodies.\(^7\)
FIG 2. Facing page. Comparative immunocytochemistry of serial sections of the thoracic aorta of a 14-month-old Watanabe heritable hyperlipidemic rabbit. The aorta was perfusion-fixed in the presence of EDTA and butylated hydroxytoluene as described in “Methods,” embedded in paraffin, and stained by the avidin-biotin-alkaline phosphatase method with a red substrate. Nuclei were counterstained with methyl green. A, Section was stained with a 1:20 dilution of the IgG fraction isolated from human serum with a high titer of autoantibodies against malondialdehyde (MDA)-lysine. B, Section was stained with a 1:50 dilution of MDA-L2, a mouse monoclonal antibody specific for MDA-lysine epitopes. Specificity of the autoantibodies in the human IgG fraction was further demonstrated by the fact that incubation of the IgG fraction with excess MDA-LDL before immunocytochemistry markedly diminished but did not completely eliminate specific staining within the atherosclerotic intima (data not shown). Other induced antibodies against oxidation-specific epitopes yielded similar staining patterns: C, Section was stained with a 1:1000 dilution of OLF4-3C10, a monoclonal antibody generated against delipidated apoprotein fragments of LDL oxidized with Cu\(^{2+}\) ions. Both autoantibodies and induced oxidation-specific antibodies recognized epitopes predominantly located in macrophage-rich areas of these transitional lesions, as indicated by the section in D, which was stained with a 1:1000 dilution of RAM-11, a monoclonal antibody against rabbit macrophages. By contrast, the distribution of native apoprotein is primarily found in areas that contain few macrophages and in the vicinity of the lamina elastica interna, as shown in E, stained with a 1:500 dilution of MB47, a monoclonal antibody against the receptor-binding domain of native apoprotein B-100. Epitopes recognized by the autoantibodies and the induced antibodies against oxidized LDL were generally found in areas that are rich in rabbit immunoglobulins and complement. F, Section was stained with a 1:50 dilution of a biotinylated antiserum against rabbit IgG and the section in G with a 1:100 dilution of antiserum against rabbit C3. H Is a control section in which the primary antibody was omitted. L indicates lumen; IEL, internal elastic lamina. Bars=100 \(\mu\)m.

Discussion

It has been shown that atherosclerotic lesions contain immunoglobulins and complement components that are not present in normal arterial tissue. In previous studies we have demonstrated that even minor modifications of native LDL render it immunogenic and that humoral antibodies can be elicited that are specific for the modifications to apo B. Oxidation of LDL also leads to major alterations in apo B, including the derivatization of lysine residues with aldehydes, that presumably result from oxidatively induced decomposition of unsaturated fatty acids. Autologous Ox-LDL has been shown to be immunogenic in guinea pigs and mice, and antisera and monoclonal antibodies specific for epitopes present on Ox-LDL, such as MDA-lysine or 4-hydroxynonenal-lysine, have been generated. We have termed these “oxidation-specific epitopes” and have demonstrated that they are present in atherosclerotic lesions from rabbits and humans. Autoantibodies to these epitopes have also been demonstrated in sera of rabbits and humans. Because it was known that large amounts of IgG are present in atherosclerotic lesions, in the present study we characterized the IgG isolated from atherosclerotic lesions and found that some lesion IgG can recognize oxidation-specific epitopes present in Ox-LDL. Clearly, some of these epitopes were present on LDL (Fig 1 and Reference 29). However, it should be appreciated that the epitopes recognized by the lesion IgG could be present on other lesion proteins as well, since the generation of oxidation-specific epitopes may be a common by-product of the oxidation of tissue proteins. In this respect it is important to note that at least some lesion IgG was present in the form of soluble immune complexes that contained both apo B and...
Fig 4. Immunocytochemistry of advanced atherosclerotic lesions of two human aortas with purified IgG fractions extracted from atherosclerotic lesions of Watanabe heritable hyperlipidemic rabbits. The aortas were immersion-fixed in the presence of EDTA and butylated hydroxytoluene, embedded in paraffin, and immunostained as described in the legend to Fig 3. A, Section was stained with a 1:10 dilution of the purified IgG fraction (prepared from the saline extract) as the primary antibody. B, Section was stained with a 1:10 dilution of the IgG fraction prepared from the 1.5 mol/L saline extract. The staining pattern was very similar to that noted in C, stained with MDA2, the malondialdehyde (MDA)-lysine-specific monoclonal antibody, and was most intense in areas rich in macrophage/foam cells, as shown in D, stained with a 1:1000 dilution of HAM 56, a monoclonal antibody against human macrophages. Preincubation of the saline-extracted IgG fraction with excess human MDA-low-density lipoprotein effectively prevented intimal staining (E, phase contrast), whereas the adventitial staining was reduced but not completely eliminated (F). Higher-magnification view of the macrophage-rich area of the same lesion (G) reveals the predominance of cell-associated staining, whereas higher magnification of an area of the necrotic core of a different aortic lesion (H) shows diffuse staining, particularly on the boundaries of what presumably were lipid deposits and cholesterol crystals that were ethanol-extracted during the paraffin embedding and immunostaining procedures. Bars=100 µm in A through F; bars=10 µm in G and H.
epitopes characteristic of Ox-LDL, indicating that oxidized, lesion LDL components are accessible to circulating antibodies. In the present study, the high-salt (1.5 mol/L NaCl)–extracted IgG fraction was somewhat more active toward oxidized epitopes than the normal saline–extracted IgG fraction, probably due to a higher efficiency of the high-ionic-strength solution to liberate IgG and LDL deposited in the lesions. However, the fact that the saline-extracted IgG fraction also recognized oxidized epitopes is not surprising, since at least part of the IgG was in the form of soluble apo B (LDL)–IgG immune complexes and the saline extraction procedure has been shown to remove approximately 50% of the saline-extractable LDL from atherosclerotic tissue. Because only small amounts of immunoglobulins could be recovered from atherosclerotic lesions, we could not evaluate the presence or specificity of other immunoglobulin classes present in the lesions.

Increasing evidence suggests that many characteristics of an inflammatory reaction are present in lesions, possibly at very early stages, but almost certainly during the progression of the lesion. As part of this process, there is an early involvement of immune mechanisms. Active immunologic processes could influence the progression of atherosclerotic lesions in several ways. Human atherosclerotic lesions contain both CD4+ (helper) and CD8+ (cytotoxic) T cells. Lesion macrophages and smooth muscle cells have been shown to express MHC class II proteins, and these cells may present antigens to CD4+ T cells, thereby causing their immunomobilization. This could lead to the production of interferon gamma and interleukin-2 by CD4+ T cells, creating another pathway leading to activation of lesion cells, including macrophages. In addition to initiation of localized cell-mediated immunity, IgG-LDL immune complexes could be taken up directly by lesion macrophages via Fc receptor–mediated uptake, which could contribute to the formation of lipid-filled, macrophage-derived foam cells. In particular, arterial LDL, which contains oxidation-specific epitopes, is likely to be present in part in an aggregated state. Antibody-coated aggregates are a particularly effective ligand for Fc receptor–mediated uptake and subsequent cholesteryl ester accumulation.

IgG deposited in atherosclerotic lesions may also induce complement activation in situ. The presence in atherosclerotic lesions of complement activation complex C5b-9 strongly suggests that at least some complement activation occurs in vivo in lesions. Activated complement could lead to cellular injury, increased procoagulant activity, and chemotactic products that may recruit yet more monocyte/macrophages into the lesion area. It is not known whether the IgG fractions isolated from atherosclerotic lesions are produced in the lesion area or elsewhere in the body, since only very few if any antibody-producing B cells are detectable in atherosclerotic lesions, but B cells are present in the adjacent adventitia, at least in coronary artery specimens. Although the exact relationship between serum autoantibodies and IgG fractions isolated from atherosclerotic lesions is not known, it should be noted that immunostaining of rabbit atherosclerotic lesions with human serum autoantibodies produces a staining pattern that is very similar if not identical to that obtained with antibodies against Ox-LDL (Figs 2 and 3). Similarly, rabbit lesion IgG produced comparable staining of human lesions (Fig 4). Thus, it is likely that the lesion Ig was derived at least in part from circulating IgG. The relationship between the titer of serum autoantibodies and the oxidation of LDL within lesions is unknown, but the recent demonstration in a Finnish population that the serum titer of autoantibodies to MDA-lysine is a good predictor of the progression of carotid atherosclerosis suggests that the antibody response might be a reflection of underlying disease activity, at least in some populations. Whether or not such an immune response accelerates or possibly hinders the atherogenic process is unknown. When the rate of oxidation of LDL is low, it is possible that an immune response might lead to an accelerated macropage uptake of Ox-LDL and thereby play a protective role. When the rate of oxidation is enhanced, however, the immune response might play a pathogenetic role, leading to accelerated macropage uptake that overwhelms the capacity of the macrophage to handle the ingested Ox-LDL. Furthermore, immune complexes may play a significant role in the activation of memory T cells that can initiate the transcription and secretion of interferon gamma, interleukin 2, and cytokines stimulating factors, which in turn can have profound local consequences. Our present results demonstrating that human and rabbit atherosclerotic lesions contain IgG that can recognize epitopes typical of Ox-LDL adds to the growing body of evidence that immunologic processes may play an important role in determining the natural history of atherosclerotic lesions.

Acknowledgments
This work was supported by National Heart, Lung, and Blood Institute grant HL-14197 (Specialized Center of Research on Arteriosclerosis), the Sam and Rose Stein Institute for Research on Aging, and the Emil Aaltonen Foundation (Finland).

References


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