Human-Derived Anti–Oxidized LDL Autoantibody Blocks Uptake of Oxidized LDL by Macrophages and Localizes to Atherosclerotic Lesions In Vivo

Peter X. Shaw, Sohvi Hörkkö, Sotirios Tsimikas, Mi-Kyung Chang, Wulf Palinski, Gregg J. Silverman, Pojen P. Chen, Joseph L. Witztum

Abstract—Autoantibodies to oxidation-specific epitopes of low density lipoprotein (LDL), such as malondialdehyde-modified LDL (MDA-LDL), occur in plasma and atherosclerotic lesions of humans and animals. Plasma titers of such antibodies are correlated with atherosclerosis in murine models, and several such autoantibodies have been cloned. However, human-derived monoclonal antibodies to epitopes of oxidized LDL (OxLDL) have not yet been reported. We constructed a phage display antibody library from a patient with high plasma anti–MDA-LDL titers and isolated 3 monoclonal IgG Fab antibodies, which specifically bound to MDA-LDL. One of these, IK17, also bound to intact OxLDL as well as to its lipid and protein moieties but not to those of native LDL. IK17 inhibited the uptake of OxLDL by macrophages and also bound to apoptotic cells and inhibited their phagocytosis by macrophages. IK17 strongly immunostained necrotic cores of human and rabbit atherosclerotic lesions. When 125I-IK17 was injected intravenously into LDL receptor–deficient mice, its specific uptake was greatly enriched in atherosclerotic plaques versus normal aortic tissue. Human autoantibodies to OxLDL have important biological properties that could influence the natural course of atherogenesis. (Arterioscler Thromb Vasc Biol. 2001;21:1333-1339.)

Key Words: atherosclerosis ■ imaging ■ antibodies ■ lipoproteins

Oxidation of LDL plays an important role in atherosclerosis.1 The oxidation of polyunsaturated fatty acids generates reactive breakdown products, such as malondialdehyde (MDA); these products subsequently interact with lysine residues of associated proteins to form Schiff base adducts, such as MDA-lysine.2,3 We have demonstrated that such “oxidation-specific” neoepitopes occur in vivo and are immunogenic.2,3 For example, autoantibody titers to MDA-LDL have been found in the plasma of apoE-deficient (apoE2/2) mice and have been correlated with the progression of atherosclerosis.4,5 Furthermore, immunization of LDLR–/– rabbits and mice with MDA-LDL led to an amelioration of the progression of atherosclerosis.5,7 We have recently cloned monoclonal antibodies to oxidized LDL (OxLDL) from apoE–/– mice5 and have shown that they not only bind to OxLDL but also block the uptake of OxLDL by macrophages.8

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To understand the potential role(s) that such autoantibodies to OxLDL play in atherosclerosis in humans, we prepared a human phage display combinatorial library to generate monoclonal autoantibodies to epitopes of OxLDL.9,10 By displaying the V_{H}/V_{L} (where V indicates the variable region, H indicates heavy chain, and L indicates light chain) antibody combinatorial library on the surface of a filamentous phage and panning against MDA-LDL, we selected human monoclonal Fab antibodies and characterized their immunologic properties and biological activities.

Methods

Cloning of Monoclonal Fab to MDA-LDL

A patient with coronary artery disease with high autoantibody titers to MDA-LDL was identified.4 Total RNA was isolated from his peripheral blood mononuclear cells by RNA STAT-60 (Tel-Test) and was used to synthesize cDNA with the use of a Superscript II cDNA synthesis kit (GIBCO-BRL). Reverse transcription–polymerase chain reactions were performed by using a cDNA template and 14 pairs of primers, including 4 pairs for the immunoglobulin light chain V_{k} genes and 5 pairs for V_{\lambda} genes (where k and \lambda are 2 types of light chain), as well as 5 pairs for heavy chain V_{H} genes, as described previously.10,11 The expected polymerase chain reaction products were pooled according to isotypes and cloned into the phage display vector, pComb3H, to generate 2 phage display libraries, V_{H}/V_{k} and V_{H}/V_{\lambda}. The resultant phagemid DNA was transformed into XL-1 blue Escherichia coli and rescued with a helper phage. After 5 successive rounds of panning against MDA-LDL, the phagemid DNA was prepared from infected bacteria and manipulated to express soluble Fab by removing gene III, which is fused to the C-terminus of the heavy chain gene and is essential for anchoring the Fab on the phage surface. Individual MDA-LDL

Received December 18, 2000; accepted May 8, 2001.
From the Department of Medicine, University of California, San Diego, La Jolla, and the Department of Medicine (P.P.C.), University of California, Los Angeles.
Correspondence to Joseph L. Witztum, MD, Department of Medicine, University of California, San Diego, 9500 Gilman Dr, La Jolla, CA 92093-0682.
E-mail jwitztum@ucsd.edu
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binding clones were identified by ELISA and expressed on a larger scale, followed by affinity purification with the use of a column coupled with goat anti-human IgG(Fab) (Sigma Chemical Co) to Amino-Link Plus matrix (Pierce). Nucleotide sequences of selected clones were obtained by an automated sequence analyzer (ABI PRISM) and analyzed by using computer programs from the Genetics Computer Group in combination with the EMBL/GenBank database.

Antibody Characterization

The affinity-purified Fab antibodies were initially tested for direct binding to a variety of antigens by use of an ELISA.11 The binding affinities to MDA-LDL were determined by a competitive inhibition assay to calculate the dissociation constants \((K_d)\) of Fab antibodies according to the Klotz method (Friguet et al12). A molecular weight of 50,000 was used for apoB.

A chemiluminescent immun assay was used to determine antibody binding specificity.3 The purified Fab fragments (5 \(\mu g/mL\)) were added to MicroFluor (Dynex) microtiter plates coated with antigens (10 \(\mu g/mL\)) in the absence or presence of indicated concentrations of competitors. The amount of antibody bound was detected with alkaline phosphatase (AP)-labeled goat anti-human IgG (Fab specific), followed by 25 \(\mu L\) of 50% LumiPhos 530 (Lumigen) solution. The light emitted was measured in relative light units over 100 ms by using a Luminometer (Dynex Technologies). The competitors were prepared as described.3

Western blot was performed as previously described.3 The bound Fab was detected by AP-labeled goat anti-human IgG(Fab) and visualized by using the Bio-Rad AP-conjugate substrate development kit.

The ability of the antibodies to inhibit the binding or degradation of \(^{125}\)I-Cu-OxLDL by elicited murine peritoneal macrophages was determined as previously described.3 In both assays, the amount of OxLDL bound or degraded was expressed as a percentage of control.

Binding of Affinity-Purified Fab to Apoptotic Cells and Inhibition of Macrophage Uptake

FACScan

Apoptotic murine thymocytes were prepared as described13 and incubated with purified Fab or an isotype control, human IgG(Fab), and then incubated with fluorescence-conjugated F(ab')2 against human IgG(Fab) (Jackson Laboratory, Inc). Cells were stained with propidium iodide and analyzed by FACScan (Becton-Dickinson) with the use of CellQuest software as described.13

Phagocytosis Assay

Phagocytosis of apoptotic thymocytes was determined as described.13

Immunohistochemistry

Immunostaining was performed on lesions from human and rabbit arteries, most of which have been characterized with other antibodies.5,14,15 After paraffin embedding, 7-\(\mu m\) serial sections were prepared, rehydrated, and immunostained with affinity-purified Fab (100 \(\mu g/mL\)), which was biotinylated by using sulfo-NHS-Biotin (Pierce), followed by an avidin–biotin–alkaline phosphatase amplification step.5 Tissues were treated with 5 mmol/L levamisole (Sigma) and counterstained with methyl green.

In Vivo Aortic and Plaque Uptake of \(^{125}\)I-IK17

The IK17 Fab was iodinated with Enzymobeads (Bio-Rad)16 and showed binding properties similar to those of nonlabeled antibody. Eighteen-month-old LDLR\(^{-}\)mice (n = 10, weight 31.6 ± 3.4 g, total plasma cholesterol 1112 ± 251 mg/dL) on a 1.25% cholesterol–enriched diet for 6 months were injected through the tail vein with 10 \(\mu g\) of 125I-Fab and euthanized at 4, 8, and 24 hours after injection. Pharmacokinetics, blood analysis, and tissue uptake were determined as described.16,17 The aorta was then dissected, counted for radioactivity in a gamma-counter spectrometer, and stained with Sudan IV. Individual plaques and visually normal areas were then dissected free and counted separately as described.16,17 Autoradiography of the aortas was obtained with Kodak Biomax high-speed film after 2 weeks of exposure at 4°C.

Results

Sequencing Analysis of Anti–MDA-LDL Clones

Of 120 randomly chosen clones from the V\(\alpha\)/V\(\kappa\) library that were selected on the basis of MDA-LDL binding, 3 were found to be strong binders to MDA-LDL, namely, IK3, IK17, and IK103. No specific binders were isolated in equivalent numbers from the V\(\kappa\)/V\(\lambda\) library. As outlined in the Table, the V\(\alpha\) genes of IK103 and IK3 were likely derived from B cells in the same clonally related set, inasmuch as they are both encoded by rearrangements of the V\(\alpha\)6 (6-01/DP-74) gene to D\(\alpha\)6-19 and to J\(\alpha\)4b, with only 2 nucleotide differences in their encoding sequences. However, these V\(\alpha\)n regions are each paired with distinct V\(\kappa\) regions; IK3 V\(\kappa\)1 is encoded by the V\(\kappa\)1 gene, HK137+, rearranged to J\(\kappa\)4, whereas IK103 is a rearrangement of a V\(\kappa\)3 gene, V\(\kappa\)g/L6, to J\(\kappa\)4b, with only 2 nucleotide differences in their encoding sequences. In both assays, the amount of OxLDL bound or degraded was expressed as a percentage of control in the absence of any competitor.
ranged to Jk2, and this Vκ segment is also highly expressed in adult repertoires. By comparison, the closest known homologous antibody genes encoding these antibodies have 91.3% to 97.8% homology, suggesting the presence of somatic hypermutations that are commonly created in their germinal center reactions during antigenic in vivo selection. These findings, with comparable levels of hypermutation of both heavy and light chains, are consistent with features previously described for IgG-expressing B-cell clones rescued by conventional cellular methods.

Characterization of MDA-LDL–Binding Fabs
All 3 affinity-purified antibodies bound strongly to MDA-LDL and Cu-OxLDL but showed no significant binding to unrelated antigens, including tetanus toxoid, chicken ovalbumin, type VI collagen, and calf thymus single-stranded DNA (data not shown). Figure 1 shows that the binding of each antibody to MDA-LDL is inhibited 50% by 1 mg/mL of soluble MDA-LDL. Calculated according to Klotz plots, the dissociation constants (Kd's) for IK3, IK17, and IK103 were 2.9×10⁻⁸, 3.7×10⁻⁸, and 2.1×10⁻⁸ mol/L, respectively. Because IK17 was a more productive Fab secretor, it was used for further studies.

Binding Property of IK17
The ability of various competitors to compete for IK17 binding to MDA-LDL was determined (Figure 2A). MDA-LDL and Cu-OxLDL were effective competitors, whereas native LDL was not. Surprisingly, neither MDA-modified BSA nor MDA-polylysine nor MDA-murine IgG (data not shown) competed even at high concentrations. 4-Hydroxynonenal–modified LDL, another prominent epitope of OxLDL, did not compete even at 200 μg/mL (data not shown). Whereas native HDL did not compete, MDA-modified HDL did show 20% to 40% competition when added at concentrations of 100 to 200 μg/mL (data not shown).

In a parallel experiment, the ability of various competitors to compete with IK17 for binding to Cu-OxLDL was also tested (Figure 2C). Again, Cu-OxLDL and MDA-LDL were effective competitors, whereas MDA-modified proteins and native LDL failed to compete. In addition, the lipids and protein separated from Cu-OxLDL inhibited the binding of IK17 to MDA-LDL, whereas those of native LDL did not (Figure 2B).

To confirm whether the epitopes for IK17 were covalently bound to the protein moiety of Cu-OxLDL, we performed a Western blot of native LDL, native HDL, OxLDL, and oxidized HDL after SDS-PAGE under reduced conditions. IK17 bound extensively to the protein moiety (apoB) of Cu-OxLDL, MDA-LDL, and protein moieties of MDA-HDL but not to the protein moieties of either native LDL or HDL (data not shown).
the binding and degradation of $^{125}$I-Cu-OxLDL to macrophages to a similar extent as did IK17.

**IK17 Binds to Apoptotic Cells and Inhibits Their Phagocytosis by Macrophages**

We have previously demonstrated that apoptotic cells display oxidation-specific epitopes on their surface. As shown in Figure 4A, IK17 bound to apoptotic thymocytes compared with control human IgG(Fab), reflected by the right shift on fluorescence. Figure 4B demonstrates a plot of propidium iodide staining versus forward scatter. Cells in region 1 have undergone a minimal degree of apoptosis, whereas those in region 2 are apoptotic. Figure 4C demonstrates that IK17 bound almost exclusively to cells in region 2. Essentially no binding occurred to cells of region 1 (data not shown).

We also tested the ability of IK17 to inhibit the uptake of apoptotic thymocytes to determine whether epitopes of IK17 on apoptotic cells are ligands for phagocytic clearance by macrophages. The percentage of macrophages that phagocytosed labeled thymocytes was calculated by analyzing calcein-AM fluorescence in the gated region that includes macrophages but excludes thymocytes. As shown in Figure 4D, 24.1% of the macrophages contained apoptotic thymocytes in the absence of antibody. As shown in Figure 4E, in the presence of a control nonspecific human Fab, a similar number of macrophages phagocytosed thymocytes (23.82%), whereas IK17 reduced the phagocytosis to 13.8% (Figure 4F), a 43.6% inhibition of apoptotic cell uptake.

**Immunohistochemistry**

Staining of atherosclerotic lesions in human and rabbit arteries indicated that the epitopes recognized by IK17 occurred mostly in the necrotic core. Macrophage-rich early lesions and shoulder areas of transitional lesions generally
showed very little IK17 staining. Only a few early lesions in arteries showed weak cellular staining (Figure 5A). In contrast, strong IK17 staining was found in necrotic areas of advanced lesions of human coronary artery (B) and in the core of classic atheromas in human brain artery (C). D, IK-17 staining in necrotic areas of Watanabe heritable hyperlipidemic rabbit aorta. E, Large macrophage- and lipid-rich lesion from a balloon-catheterized cholesterol-fed New Zealand White rabbit stained with IK17. F, Adjacent section stained with EO3, which is identical to EO6 in its variable regions and staining patterns, as seen with most of our other oxidation-specific epitopes. Bars=100 µm.

Figure 5. IK17 immunostains atherosclerotic lesions. Epitopes recognized by biotinylated-IK17 are indicated by red color; the nuclei are counterstained with methyl green. A, Section of human coronary lesion stained with IK17. B and C, IK17 staining necrotic areas of advanced lesions of human coronary artery (B) and in the core of classic atheromas in human brain artery (C). D, IK-17 staining in necrotic areas of Watanabe heritable hyperlipidemic rabbit aorta. E, Large macrophage- and lipid-rich lesion from a balloon-catheterized cholesterol-fed New Zealand White rabbit stained with IK17. F, Adjacent section stained with EO3, which is identical to EO6 in its variable regions and staining patterns, as seen with most of our other oxidation-specific epitopes. Bars=100 µm.

In Vivo Localization of 125 I-IK17 in Atherosclerotic Lesions
To show that IK17 specifically bound to atherosclerotic tissue in vivo, we injected 125 I-IK17 into LDLR−/− mice and calculated its uptake in the entire aorta and in plaques versus normal tissue. After intravenous injection, the blood clearance of 125 I-IK17 was biexponential, with an α half-time of 24 minutes, a β half-time of 411 minutes, and a total half-time of elimination of 327 minutes (data not shown). During the time course of aortic 125 I-IK17 uptake over 24 hours, the entire aortic uptake (expressed as the percent injected dose per gram aortic tissue) was 1.05 ± 0.40, 0.73 ± 0.39, and 0.24 ± 0.03 at 4, 8, and 24 hours, respectively. However, the specific uptake, eg, the ratio of the uptake in plaque tissue compared with normal aortic tissue, increased from 1.6 at 4 hours to 3.2 at 24 hours, suggesting specific accumulation of 125 I-IK17 in atherosclerotic lesions. Figure 6 shows a Sudan IV–stained aorta (left) and the corresponding autoradiograph (right) from an antibody MDA2,5 which is an oxidation-specific antibody that binds to MDA-LDL. However, immunostaining with IK17 showed only weak and scattered staining (data not shown). Thus, IK17 appears to give a pattern of immunostaining of atherosclerotic lesions that is different from that of other oxidation-specific antibodies that we have studied.
LDLR−/− mouse that was euthanized 8 hours after injection of 10 μCi 125I-IK17, which confirms the presence of 125I-IK17 exclusively within atherosclerotic lesions that are accurately reflected in their entirety.

Discussion

To understand the potential role(s) that autoantibodies to OxLDL play in atherogenesis in humans, we isolated several human monoclonal IgG Fab antibodies that bound to epitopes of OxLDL, initially focusing on MDA-LDL, a model epitope of OxLDL. To our knowledge, these are the first human monoclonal antibodies against oxidation-specific epitopes of OxLDL to be characterized. All 3 Fab antibodies showed distinct antigen-binding specificity to MDA-LDL compared with other unrelated antigens. To isolate these antibodies, we used a phage display combinatorial library technique that enables rapid enrichment of desired Fab clones by using antigen-coated surfaces.10 This technique has been used to isolate monoclonal Fab antibodies from immunized animals and to study pathogenic autoantibodies in various human autoimmune disorders.23–25 Although it is not feasible to directly compare hybridomas and combinatorial libraries, several studies have demonstrated that repertoires cloned from phage display libraries have immunologic properties identical to those of donors’ sera and are capable of competing for binding of the infectious antigens.24,26 For example, Silverman’s laboratory (Roben et al25) studied the Fab antibody repertoires from 2 clinically active lupus patients and from the healthy identical twin of 1 of these patients. Anti-DNA autoantibody repertoires were associated only with the lupus patients but not with the healthy twin. Furthermore, from heavy chain shuffling experiments, they demonstrated that the in vitro anti-DNA binding activity required restrictive pairing of a heavy chain with a Vα light chain similar to those anti-DNA antibodies in circulation.25

IK17, a strong binder to MDA-LDL, was selected for detailed characterization. IK17 displayed a number of unique properties that are of considerable interest. First, it bound to a common epitope on MDA-LDL, MDA-HDL, and Cu-OxLDL but did not bind to MDA-modified BSA, polylysine, or murine IgG. It also bound to the isolated lipid and protein of OxLDL, suggesting that it recognized MDA-modified lipid and MDA-modified lipid-protein adducts that presumably resulted from Schiff base formation. This is in contrast to MDA2, a previously cloned murine monoclonal that recognizes MDA-lysine on a variety of modified proteins. IK17 is also unique in our experience in that it binds so effectively to both MDA-LDL and OxLDL. However, the exact chemical nature of the IK17 epitope common to MDA-LDL, OxLDL, and oxidized HDL is not yet defined. It should be noted that the presence of this epitope in all these compounds but not in other MDA-modified proteins makes the epitope recognized by IK17 unique in our experience and suggests that IK17 may be a highly useful agent to specifically detect each of these modified lipoproteins in vitro and in vivo.

Second, IK17 blocked the binding and degradation of OxLDL by macrophages, suggesting that the epitope(s) recognized by IK17 represents one class of ligands on OxLDL that mediates its binding and uptake by macrophage scavenger receptors. We have shown that oxidized phospholipids also constitute ligands of OxLDL mediating macrophage uptake2,7 and that a group of autoantibodies from apoE−/− mice (eg, EO6 or EO3) that bound to POVP [palmitoyl-2-(5-oxovaleryl)-3-sn-phosphorylcholine] and OxLDL was also able to inhibit the uptake of OxLDL by elicited macrophages.8 MDA-modified phospholipids could conceivably occur as well. An atherogenic role for macrophage uptake of OxLDL is supported by studies with apoE−/− mice that have been crossed into mice with gene deletions of SRA28 or CD36.29 Because IK17, like EO3 and EO6, can inhibit macrophage uptake of OxLDL, these data suggest that these autoantibodies could play a “protective” role against atherogenesis.

Third, apoptotic cells, which are known to be under oxidative stress, express oxidatively modified moieties on their surface that mediate macrophage recognition and phagocytosis.30,31 We have previously demonstrated that oxidation-specific monoclonal antibodies from apoE−/− mice, originally identified by their ability to bind to OxLDL or MDA-LDL, also bind to apoptotic cells and inhibit their phagocytosis by macrophages.8 We now demonstrate that IK17 also specifically binds to the surface of apoptotic cells, but not to normal cells, and significantly inhibits their phagocytosis by macrophages. If this were to occur in vivo, it is unclear what the consequences would be. Clearance of apoptotic cells appears to occur by multiple pathways, and we speculate that such antibodies alone might have a minimal effect. By analogy, it has recently been reported that no excess accumulation of apoptotic cells occurs in scavenger receptor class A–deficient animals, even through in vitro studies suggest that scavenger receptor class A plays a significant role in the murine macrophage uptake of apoptotic cells.72 Nevertheless, these data suggest that oxidatively modified moieties may be a general mechanism that marks the surface of OxLDLs, apoptotic cells, and/or necrotic cells for phagocytosis. Indeed, the presence of such cell-surface neo–self-determinants may play an important role in the maintenance of such oxidation-specific autoantibodies.33,34

Fourth, we demonstrate that IK17 preferentially, though not exclusively, immunostained advanced atherosclerotic lesions, particularly the necrotic core area. Presumably, these are MDA-modified lipids and/or proteins. Whatever their exact nature, these structures are greatly enhanced during lesion progression. The fact that IK17 specifically localized to these sites suggests that such antibodies could play an important role in vivo. The qualitatively distinct pattern of staining with IK17 appears to be different from that observed with other antibodies that we have isolated that are specific to epitopes of OxLDL.125I-IK17 localizes to atherosclerotic lesions of LDLR−/− mice, suggesting that it specifically binds to oxidation-specific epitopes in the lesions. We have previously shown that labeled murine MDA2, a prototype antibody to MDA-LDL, was found to accurately detect and image atherosclerotic lesions in live rabbits, as well as to detect and quantify lesion progression and regression, ie, depletion of MDA-LDL, in mice.16,17 Because IK17 is a human autoantibody, it may be useful in a similar manner for human subjects, and experiments are currently under way to determine its clinical utility for this purpose. Theoretically, IK17 could also be used therapeutically to block the uptake of OxLDL by macrophages or, conceivably, even to deliver molecules of therapeutic interest to lesions.
Acknowledgments

These studies were supported by Postdoctoral Research Fellowships from the American Heart Association, Western Affiliate (P.X.S. and S.H.), and from the California Tobacco Related Disease Research Project (M.-K.C.); by a startup grant from the Stein Institute of Research on Aging (P.X.S.); by National Heart, Lung, and Blood Institute (NHLBI) grants HL-56989 (Specialized Center of Research in Molecular Medicine and Atherosclerosis) and HL-57505 (J.L.W.); by National Institutes of Health grants RO1 AI-40305 (G.J.S.) and MAMDC Molecular Biology Core (AR-40770); by an NHLBI Mentored Clinical Scientist Development Award (HL-07444 to S.T.); and by a New Investigator Award from California Tobacco Related Disease Research Project (HT0106 to S.T.).

References

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_Arterioscler Thromb Vasc Biol._ 2001;21:1333-1339
doi: 10.1161/hq0801.093587

_Arteriosclerosis, Thrombosis, and Vascular Biology_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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