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Recombinant Human Antibodies Against Aldehyde-Modified Apolipoprotein B-100 Peptide Sequences Inhibit Atherosclerosis

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Background—Accumulation and oxidation of LDL are believed to be important initiating factors in atherosclerosis. Oxidized LDL is recognized by the immune system, and animal studies have suggested that these immune responses have a protective effect against atherosclerosis. Aldehyde-modified peptide sequences in apolipoprotein B-100 (apoB-100) are major targets for these immune responses.

Methods and Results—Human IgG1 antibodies against 2 malondialdehyde (MDA)-modified apoB-100 peptide sequences were produced through screening of a single-chain antibody-fragment library and subsequent cloning into a pcDNA3 vector. Three weekly doses of these antibodies were injected into male apoE−/− mice. Phosphate-buffered saline and human IgG1 antibodies against fluorescein isothiocyanate were used as controls. One of the IgG1 antibodies significantly and dose-dependently reduced the extent of atherosclerosis as well as the plaque content of oxidized LDL epitopes and macrophages. In cell culture studies, human monocytes were incubated with native LDL or oxidized LDL, in the presence of antibodies. The same antibody induced an increase in monocyte binding and uptake of oxidized LDL.

Conclusions—These findings suggest that antibodies are important mediators of atheroprotective immune responses directed to oxidized LDL. Thus, passive immunization against MDA-modified apoB-100 peptide sequences may represent a novel therapeutic approach for prevention and treatment of cardiovascular disease. (Circulation. 2004;110: 2047-2052.)

Key Words: atherosclerosis ■ antibodies ■ apolipoproteins ■ immune system ■ plaque

Atherosclerosis develops as a result of chronic arterial inflammation.1 Innate and adaptive immune responses against oxidized LDL (oxLDL) are believed to play important roles in this inflammatory process. The oxidation of aggregating LDL in the extracellular matrix of the artery wall leads to the formation of highly reactive lipid peroxides and aldehydes.2,3 The LDL protein apolipoprotein B-100 (apoB-100) is degraded, and aldehydes bind to free amino groups on the peptide fragments. This is associated with activation of an inflammatory response, including endothelial expression of adhesion molecules and infiltration of monocytes/macrophages and T cells.4 Macrophages express a family of scavenger receptors, which bind and ingest oxLDL particles.5 Continuous activation of such innate immune responses is believed to be a major cause of atherosclerotic plaque development.6

The presence of oxLDL also leads to the activation of more specific adaptive immune responses.7 T cells in atherosclerotic lesions have been shown to recognize epitopes on oxLDL when presented by macrophages in combination with major histocompatibility class II molecules.8 In atherosclerosis, the adaptive immune response has been suggested to provide atheroprotective effects. A number of studies have shown that immunization of hypercholesterolemic animals with native or oxLDL leads to a significant reduction of atherosclerosis development.9,10

Using a library of malondialdehyde (MDA)-modified polypeptides covering the complete amino acid sequence of human apoB-100, we have recently identified a large number of epitopes recognized by antibodies present in human plasma.11 The levels of several of these antibodies show an inverse association with plasma oxLDL, suggesting that antibodies are involved in the clearance of these particles. Immunization of apoE−/− mice with the corresponding human apoB peptides was found to result in reduced plaque formation and a stable plaque phenotype, as indicated by increased collagen content.12 This effect was associated with increased formation of IgG against the respective apoB-100 peptides. To further study the role of these IgG antibodies in the atheroprotective response and to test whether specific MDA—

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apoB-100 antibodies could be used for direct inhibition of atherosclerosis in apoE−/− mice, we produced recombinant human IgG1 that specifically recognizes 2 MDA-modified sequences in human apoB-100. Active immunization with these peptides has previously been shown to reduce atherosclerosis by ≈50% in mice.12

Methods

Generation of Human Recombinant Antibodies to Human MDA-Modified ApoB-100–Derived Peptides

Previous studies have shown high levels of IgG in coronary heart disease patients (P45) or high IgM and IgG levels in healthy controls (P210) against the MDA-modified peptides used.11 Single-chain antibody aptamer fragments with specificity for MDA-modified apoB-100–derived peptides P45 (IEIGLEQKGFPEFTEALFQ, amino acids 661 to 680) or P210 (KTTKQSFSDLVQAQYKKNH, amino acids 3136 to 3155) were selected from the single-chain fragment-variable (scFv) n-CoDeR library, essentially as described earlier.13 In brief, 3 consecutive rounds of selection were performed with 10 pmol of MDA-modified peptide bound to a solid phase. Competitors comprising unmodified peptide and an MDA-modified nonrealted peptide were included at a concentration of 4×10−7 mol/L in the last selection round to secure specificity against the MDA-modified peptides. Selected scFv were screened for specific binding to MDA-modified peptide in an automated system with an ELISA format with luminescence as the readout.14

The scFv antibody fragments identified as being specific for the MDA-modified variants of the peptides were then transferred from the scFv format to a full-length human IgG1 format through cloning into a modified pcDNA3 vector.15 The different complementary determining region sequences of these are presented in Table 1. The cloned sequences were then transfected into NS0 cells with Lipofectamine 2000 reagent (Invitrogen), and transfectants were selected by using G418 sulfate (Invitrogen) as described.15 Human IgG1 was purified from spent cultivation medium on a MabSelect protein A column (Amersham Bioscience). The purity of the preparations exceeded 98%, as determined by polyacrylamide gel electrophoresis analysis, and contained between 1 and 12 endotoxin units/mL, as tested by a limulus amoebocyte lysate test (QCL-1000, BioWhittaker). The specificity of the purified IgGs for MDA-modified LDL was demonstrated with a luminescence-based ELISA, in which the determination of apoB-containing lipoproteins were precipitated with copper (0.5 mg/dose in the first study; 0.25, 0.5, and 2.0 in the second) of the human IgG1 antibodies directed to MDA-modified apoB-100 peptides (see earlier sections). As controls, phosphate-buffered saline (PBS) or nonspecific human IgG1 antibodies directed to fluorescein isothiocyanate (FITC) were used. The injections were repeated 2 times at 1-week intervals.

All mice were humanely killed at 25 weeks of age by exsanguination through cardiac puncture under anesthesia with 300 μL distilled water, fentanyl/fluanisone, and midazolam (2:1:1, vol/vol/vol), administered intraperitoneally. After whole-body perfusion with PBS followed by Histochoice (Amresco), the heart and the aortic arch were dissected out and stored in Histochoice at 4°C until processing. The descending aorta was dissected free of external fat and connective tissue, cut longitudinally, and mounted en face, lumen side up, on ovalbumin- (Sigma) coated slides (termed flat preparation).16 The Animal Care and Use Committee approved the experimental protocol used in this study.

Analysis of Lipid, Macrophage, and oxLDL Epitopes in Plaques

Staining and quantification of plaque area in flat preparations of ascending aorta and subvalvular plaque macrophage content were done as previously described.12 A protocol similar to that for macrophage staining was used for detection of oxLDL epitopes in the plaques with IEL-E3 (100 μg/mL) as the primary antibody and a biotinylated mouse anti-human IgG1 antibody (25 μg/mL; ImmunKemi F&D AB) diluted in PBS as the secondary antibody.

Serum Cholesterol and Triglyceride

Total plasma cholesterol and plasma triglycerides were quantified by colorimetric assays (Infinity cholesterol and triglyceride, respectively; Sigma). ApoB-containing lipoproteins were precipitated with MgCl2 and dextran sulfate as previously described.12

Preparation of Unlabeled and 125I-Native LDL or oxLDL

LDL was isolated from blood by sequential preparative ultracentrifugation in a narrow density range (1.034 to 1.054 kg/L). Copper-mediated oxidation was achieved by incubating freshly prepared LDL in PBS with a sterile solution of CuCl2 at a final concentration of 10 μmol/L. The extent of LDL modification was assessed electrophoretically. Native LDL and oxLDL were labeled by the iodine monochloride method. The endotoxin levels in both preparations were <0.015 endotoxin units/mL.
**TABLE 2. Analysis of Clones With Biacore**

<table>
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<th>Antibody</th>
<th>On Rate (k&lt;sub&gt;p&lt;/sub&gt;)</th>
<th>Off Rate (k&lt;sub&gt;n&lt;/sub&gt;)</th>
<th>Equilibrium (K&lt;sub&gt;eq&lt;/sub&gt;)</th>
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<td>IgG1 IEI-E3</td>
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<td>5×10&lt;sup&gt;-4&lt;/sup&gt;</td>
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**Isolation and Culture of Monocytes**

Human monocytes were isolated from buffy coats from different donors by the Ficoll-Hypaque procedure, plated at a density of 4×10<sup>6</sup> cells/mL into 12-well plates (1 mL/well), and cultured in RPMI 1640 medium (Gibco, Life Technologies) supplemented with 2 mmol/L N-acetyl-L-alanyl-L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 1% nonessential amino acids, 2% sodium pyruvate, and 20 mmol/L HEPES without serum at 37°C in 5% CO<sub>2</sub>. The experiments were performed within 24 hours after plating of monocytes.

**125<sup>I</sup>-Native LDL and 125<sup>I</sup>-oxLDL Uptake Assay**

Monocytes were incubated in the absence or presence of labeled native LDL (40 μg/mL) or oxLDL (50 μg/mL), alone or combined with the antibodies (100 μg/mL). Thereafter, the cells were washed with PBS and scraped into 0.5 mol/L NaOH for uptake measurement. The radioactivity was determined in an LKB 1271 automatic gamma counter.

**Native LDL and oxLDL Binding Assay**

Monocytes were incubated in the absence or presence of antibodies and unlabeled native LDL or oxLDL, alone or in combination. LDL binding studies at 4°C were performed as previously described. Radioactivity of released 125<sup>I</sup>-native LDL or 125<sup>I</sup>-oxLDL from monocytes was measured in a gamma counter.

**Statistical Analysis**

Data are presented as mean±SD. Analysis of the data was performed with the Mann-Whitney 2-tailed test. Statistical significance was considered at a level of P<0.05.

**Results**

A total of 4 scFv with specificity for the MDA-modified apoB-100 peptide composed of amino acids 661 to 680 (IEI-A8, IEI-D8, IEI-E3, IEI-G8) and 2 for the peptide consisting of amino acids 3136 to 3155 (KTT-B8, KTT-D6) were identified and chosen to be transformed to the human IgG1 format after the selection and screening process. The affinity of the antibodies to human MDA-modified apoB-100 was compared with the Biacore technique (Table 2), and specificity was assessed by using a number of different MDA-modified antigens (Figure 1A). The IEI-E3 antibody had a lower affinity but was relatively more specific to the MDA-IEI peptide compared with a high-affinity binder such as IEI-G8 (Table 2 and Figure 1A). None of the scFv recognized the respective unmodified apoB-100 peptide (data not shown). Furthermore, the scFv bound to MDA-modified but not to native LDL (Figure 1B). Also, after the specificities had been transferred to the IgG1 format, the selectivity for MDA-modified LDL was evident (Figure 1C), demonstrating a desired target specificity of the antibodies.
of mouse anti-human IgG1 and human IgG1 (r = −0.56, P<0.001) was observed.

To verify the inhibitory effect of the IEI-E3 antibody on the development of atherosclerosis, we then performed a dose-response study. The design was identical to that of the initial study with the exception that human IgG1 against FITC (FITC-8) was also used as a specificity control, in addition to PBS. In mice treated with IEI-E3 antibodies, atherosclerosis was reduced by 2% in the 0.25-mg group (P=NS), by 25% in the 0.5-mg group (P=NS), and by 41% in the 2.0-mg group (P=0.035) compared with the corresponding FITC-8 antibody–treated groups (Figure 2). There was also a 33% reduction (P=0.02) of macrophage immunostaining in atherosclerotic plaques in mice treated with 2 mg IEI-E3 antibody compared with the matching FITC-8 antibody group (Figure 3A, 3B, and 3E).

Immunohistochemical staining with IEI-E3 as the primary antibody demonstrated the presence of the IEI-E3 epitope predominantly close to the lumen (Figure 3C and 3D). Blocking experiments by preincubation of the IEI-E3 antibody with human oxLDL and native LDL confirmed that the staining was specific for oxLDL (data not shown). There was a 20% reduction (P=0.04) in IEI-E3 immunostaining in plaques of mice treated with 2 mg IEI-E3 antibody compared with the FITC-8 controls (Figure 3F). However, no human IgG1 was detected in the atherosclerotic plaques at the time of euthanization (data not shown).

We also studied how the antibodies influenced the metabolism of oxLDL by analyzing the binding and uptake of oxLDL in cultured human monocytes/macrophages. Addition of IEI-E3 antibodies resulted in an increase in the binding (P=0.001) and uptake (P=0.006) of oxLDL compared with FITC-8. Similar observations were also made after incubation with IEI-D8 (P=0.004 and P=0.001, respectively) and KTT-B8 (P=0.004 and P=0.001, respectively) antibodies, whereas there was no effect of the antibodies on the binding and uptake of native LDL (Figure 4A and 4B).

**Discussion**

oxLDL particles contain MDA-modified peptide fragments derived from degradation of apoB-100.2 Autoantibodies against several such MDA-modified apoB-100 peptides have been found in humans.11 The present studies show that human IgG1 generated against one of these MDA peptide sequences reduces atherosclerosis in apoE−/− mice and that this is associated with reduced accumulation of the corresponding oxLDL-associated epitope and of macrophages in atherosclerotic plaques.

These observations are consistent with earlier studies demonstrating that immunization with oxLDL inhibits the development of atherosclerosis in mice and rabbits.9,10 Activation of this protective immunity is associated with a marked increase in oxLDL-specific IgG. We have recently identified a large number of MDA-modified sequences in apoB-100 that are recognized by antibodies present in human plasma.11 Immunization of apoE−/− mice with some of these peptide sequences resulted in inhibition of atherosclerosis to a similar extent as that observed after immunization with oxLDL and was also associated with an increase in peptide-specific IgG.12

The present findings suggest that specific antibodies constitute an important component of atheroprotective immunity but do not exclude the involvement of cell-mediated immunity. Support for the existence of atheroprotective humoral immunity also comes from studies in apoE−/− mice demonstrating inhibi-
tion of atherosclerosis by repeated injections of polyclonal IgG and by B-cell rescue of splenectomized mice.18,19

The mouse model of atherosclerosis used in this study has some limitations when it comes to analyzing the effect of human antibodies against human oxLDL epitopes. Homology to the corresponding mouse apoB-100 sequences is not complete (95%), and the sequence recognized by the KTT antibodies is not expressed in the majority of mouse LDL particles in apoE<sup>−/−</sup> mice, because most of these are carrying apoB-48.20,21 Moreover, the protective effect of human antibodies may be inhibited by expression of mouse antibodies against human IgG1, which were found to be present in all IgG-treated mice at the time of euthanization. These circumstances are likely to limit the effectiveness of the antibody treatments in mice by inducing clearance of the human IgG1.

Autoantibodies specific for the same epitopes as IEI and KTT antibodies are present in humans. IgM levels against these epitopes show significant correlations with plasma levels of oxLDL and carotid artery intima-media thickness, suggesting that they are involved in the disease process.11 IgG against the same epitopes is present only at lower levels. The mechanisms through which IgG1 directed to aldehyde-modified apoB-100 peptides sequences inhibits atherosclerosis in mice remains to be clarified. Low numbers of the MDA–apoB-100 epitope in plaques treated with the corresponding IgG1 suggest that these antibodies inhibit uptake of oxLDL in

Figure 3. Staining of macrophages and oxLDL epitopes in subvalvular plaques of apoE<sup>−/−</sup> mice. Staining of macrophages in groups injected with (A) FITC-8 and (B) IEI-E3 antibodies, respectively. IEI-E3 epitope staining in plaques from same groups, (C) FITC-8 and (D) IEI-E3, respectively. Values represent percentage of stained area per total subvalvular plaque area, (E) macrophage staining, and (F) epitope staining *P<0.05 vs FITC-8. Abbreviations are as defined in text.
plagues and/or facilitate the removal of oxLDL from the circulation or plaques. The decrease in plaque macrophage immunoreactivity observed with the highest IEI-E3 dose indicates reduced inflammation, which in turn could slow disease progression. There is also some support for a removal effect of oxLDL from clinical studies demonstrating an inverse relation between antibody levels and oxLDL in plasma.11

The IEI-E3 antibody effectively enhanced binding and uptake of oxLDL in cultured human monocytes/macrophages but did not affect the binding and uptake of native LDL. This mechanism represents a possible removal pathway of oxLDL, either by Kupffer cells in the liver or by macrophages in peripheral tissues. In contrast, Hörkö et al22 have shown that IgM directed to oxLDL phospholipids, but not IgM directed to MDA-LDL, inhibits oxLDL uptake by macrophages. Taken together, these observations suggest that IgG mediates uptake of oxLDL through binding to Fc receptors, whereas IgM may lack this effect.

The ability to induce an atheroprotective immunity by active or passive immunization against oxLDL epitopes has been clearly established in experimental animals. In this study, the antibodies found to inhibit atherosclerosis were human IgG1 specific for MDA-modified apoB-100 sequences. However, it still remains to be determined whether a similar atheroprotective immunity can be induced in humans. If this is shown to be the case, it would represent a possible novel therapeutic approach for prevention and treatment of cardiovascular disease.

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