Inhibition of Atherosclerosis in ApoE-Null Mice by Immunization with ApoB-100 Peptide Sequences

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***Objective*—**LDL oxidation is believed to play an important role in the development of atherosclerosis, and oxidized LDL particles have been shown to become targets for the immune system. Immunization of animals with oxidized LDL results in reduction of atherosclerosis, suggesting an atheroprotective effect of this immune response.

***Methods and Results*—**Using a polypeptide library covering the complete sequence of apoB-100, a large number of native and malondialdehyde-modified peptide sequences in apoB-100 that are recognized by antibodies in human plasma were identified. We report here that immunization with apoB-100 peptide sequences, against which high levels of IgG and IgM antibodies are present in healthy human controls, reduce atherosclerosis in apoE-null mice by about 60%. Immunizations with these peptides were also found to increase the collagen content of subvalvular lesions.

***Conclusions*—**These studies have identified peptide sequences in apoB-100 that induce immune responses, which inhibits atherosclerosis. This suggests a way of developing an immunization therapy for coronary heart disease. **(*Arterioscler Thromb Vasc Biol*. 2003;23:879-884.)**

Key Words: apolipoproteins ■ atherosclerosis ■ immunization ■ mice ■ peptides

Accumulation, aggregation, and modification of LDL particles in the arterial intima are believed to be among the most important initiating factors in atherosclerosis.[1,2] Oxidative modification of LDLs trapped in the vascular extracellular matrix is associated with generation of a number of highly reactive compounds, such as lysophosphatidylcholine, lipid peroxides, aldehydes, and oxysterols, that cause cell damage and local inflammation.[2,3] In general terms, the development of raised fibromuscular plaques can be said to represent a repair response to the vascular injury and oxidized lipids may be one factor causing such injury.[4] Several protective mechanisms exist to limit injury caused by oxidatively damaged LDL particles. One involves the removal of oxidized LDL by macrophage scavenger receptors.[5,6] Recent studies suggest a second protective mechanism involving specific immune responses against epitopes present in oxidized LDLs. These were initially identified in studies of hypercholesterolemic rabbits, in which immunization with oxidized LDL was found to reduce atherosclerosis by 40% to 60%.[7,8] Similar observations were subsequently also made in apoE-null and LDL receptor–null mice,[9–11] as well as in balloon-injured hypercholesterolemic rabbits.[12] In apoE-null mice, induction of hypercholesterolemia by a high-fat diet results in a dramatic increase in autoantibodies against oxidized LDLs. Circulating autoantibodies against oxidized LDLs are also abundant in humans and have been shown to correlate with severity of disease in cardiovascular patients.[13–16] These findings suggest the possibility of developing new treatments against atherosclerosis based on selective activation of atheroprotective immune responses against oxidized LDL antigens.

Oxidation of LDL is associated with formation of reactive aldehydes, such as malondialdehyde (MDA), that form covalent adducts with lysine and histidine residues in apoB.[17,18] These haptenized peptide sequences become targets for the immune system.[19,20] In other current experiments, a library of native and malondialdehyde-modified polypeptides covering the complete apoB-100 sequence was used to identify the immunogenic epitopes on oxidized LDL in humans.[21] The aim of the present study was to investigate whether immune responses against these structures are atheroprotective and represent a possible approach for development of an immune therapy or vaccine against coronary heart disease (CHD) in humans.

Methods

Animals

Male apoE-null mice on C57BL/6 background were purchased from B&M (Ry, Denmark). The mice (n=10 per group) were given a first injection (100 μL/injection and mouse) with peptides conjugated to

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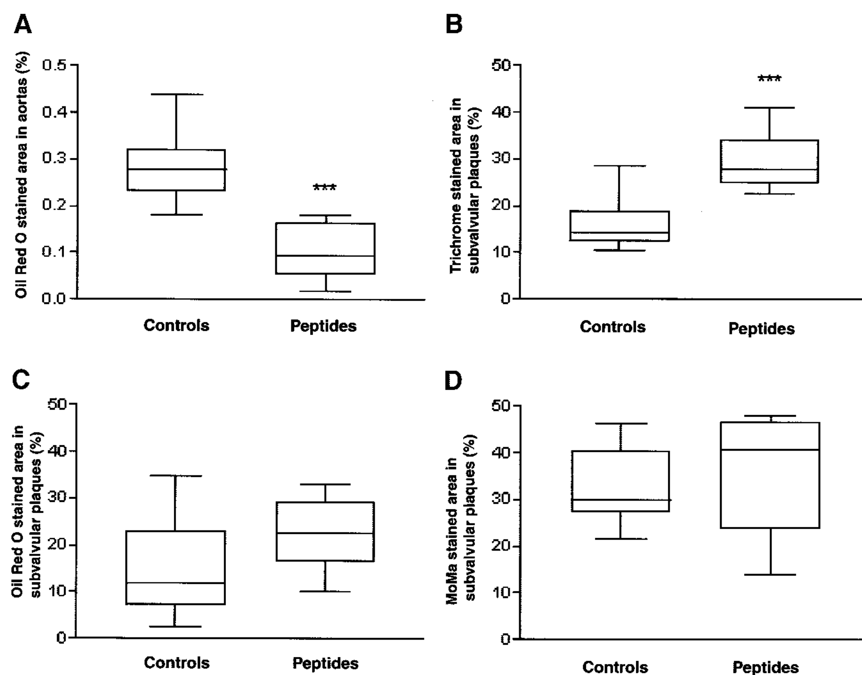

Figure 1. Stained plaque area in aortas from immunized apoE-null mice. The mice were immunized with carrier and adjuvant alone (controls) or with apoB-100 peptide sequences (peptides), respectively. Plaque areas in the descending aorta were assessed by Oil Red O staining of en face mounts of the descending aorta (a). Investigations of subvalvular plaques were performed using Trichrome staining for collagen (b), Oil Red O staining for lipids (c), and monocyte/macrophage immunostaining (d). Values represent stained area in percent of total area (a) or the plaque area (b–d) and are given as box plots, demonstrating median, 25th, and 75th percentiles, and with whiskers showing the highest and lowest values, n=8 to 10. ***$P<0.001$ vs controls.

the carrier or carrier alone at 6 weeks of age and a second injection 3 weeks later. The human apoB-100 peptide sequences used were amino acids 2131 to 2150 (IALDD AKINF NEKLS QLQTY; peptide 143) and amino acids 3136 to 3155 (KTTKQ SFDLS VKAQY KKNKH; peptide 210). The homology between the human and mouse sequences is 85% for peptide 143 and 90% for peptide 210 (Accession no P04114, human, and XP_137955, mouse). Each injection contained 50 μg of native peptides (25 μg of each) and 50 μg of the carrier cBSA (cationized BSA) dissolved in 0.083 mol/L sodium phosphate 0.9 mol/L NaCl pH 7.2, according to the manufacture's protocol (No. 77652, Pierce, Rockford, Ill) and with Alum (aluminum hydroxide, Pierce) as adjuvant. The selection of apoB-100 peptide sequences was based on studies demonstrating the presence of high IgG and IgM levels against these peptides sequences in man (c.f. Figure 1).[21] The mice were fed a cholesterol diet (0.15% cholesterol, 21% fat; AnalyCen Nordic, Linköping, Sweden) from the age of 10 weeks. At 25 weeks of age, the mice were killed by intraperitoneal injection of 300 μL distilled water, Hypnorm, and Dormicum (2:1:1) and exsanguinated by cardiac puncture, perfused with 0.15 mol/L PBS, pH 7.4 (Sigma, St Louis, Mo), and the aortic arch was collected and stored in the fixative Histochoice (Amresco, Solon, Ohio) at 4°C until processing. The descending aorta was dissected free of connective tissue and fat, cut longitudinally, and mounted en face lumen-side up on ovalbumin (Sigma)-coated slides[22] and stored in Histochoice. Serum was collected from cardiac punction and stored at −80°C until assayed. The Animal Care and Use Committee approved experimental protocol used in the study.

Staining of the Descending Aorta

En face preparations of the descending aorta were washed in distilled water, dipped in 78% methanol, and stained for 40 minutes in 0.16% Oil-Red-O dissolved in 78% methanol/0.2 mol/L NaOH as previously described.[22] The cover slides were mounted with a water-soluble mounting media L-550A (Histolab, Göteborg, Sweden). Lipids are stained red, which makes the plaques bordeaux colored. Stained area (bordeaux colored) and total aortic areas were quantified blinded by microscopy and computer aided morphometry (Olympus Micro Image, Hamburg, Germany).

Analysis of Plaque Lipid, Macrophage, and Collagen Content

The aortic arch, including the area from the left subclavian artery into the ventricle, was embedded in OCT (optimal cutting temperature; Tissue-Tek, Zoeterwoulde, The Netherlands). Frozen sections of 10 μm were collected. The sections were dipped briefly in 60% isopropanol and stained in 0.24% Oil Red-O in 60% isopropanol for 20 minutes. Sections were briefly washed in 60% isopropanol, then washed in water and counter-stained with hematoxylin. Modified Masson's trichrome staining using Ponceau-acid fuchsin (Chroma-Gesellschaft, Schmid GmbH, Germany) and aniline blue (BDH, Dorset, England) was used to assess plaque collagen content.[23] Slides used for staining with rat anti-mouse MOMA-2 antibodies (monocyte/macrophage, BMA Biomedicals, Augst, Switzerland) diluted in 10% rat serum in PBS incubated at +4°C over night, were first fixed in ice-cold acetone for 10 minutes, washed in PBS for 5 minutes, and thereafter blocked with 10% mouse serum in PBS for 30 minutes and quickly dipped in PBS. Biotinylated rabbit anti-rat IgG (Cat. No. BA-4001, Vector Laboratories, Burlingame, Calif) was used as secondary antibody and DAB detection kit for color development (Vector). Omissions of the primary or secondary antibodies were used as controls. Stained area was quantified blinded by microscopy and computer aided morphometry (Olympus Micro Image).

Peptide ELISA

A mixture of either native or MDA-modified peptides 142 and 210 was used for coating (10 μg/mL of each in PBS pH 7.4) microtiter plates (Nunc MaxiSorp, Nunc, Roskilde, Denmark) in an overnight incubation at 4°C. MDA-modified peptides were prepared as described for LDL by Palinski et al.[24] Coated plates were washed with PBS with 0.05% Tween-20 and thereafter blocked with SuperBlock in Tris-buffered saline (TBS, Pierce) for 5 minutes at room temperature followed by an incubation of mouse serum diluted 1:50 in TBS-0.05% Tween-20 for 2 hours at room temperature and overnight at 4°C. After rinsing, depositions were detected by using biotinylated goat anti-mouse IgM or IgG antibodies (Jackson ImmunoResearch, West Grove, Pa) that were incubated for 2 hours at room temperature. The plates were washed and bound biotinylated antibodies were detected by alkaline phosphatase–conjugated streptavidin (Sigma). The color reaction was developed using phosphatase substrate kit (Pierce). The absorbency at 405 nm was measured after 1 hour of incubation at room temperature. Mean values were calculated after subtraction of background absorbance (n=3 per mouse).

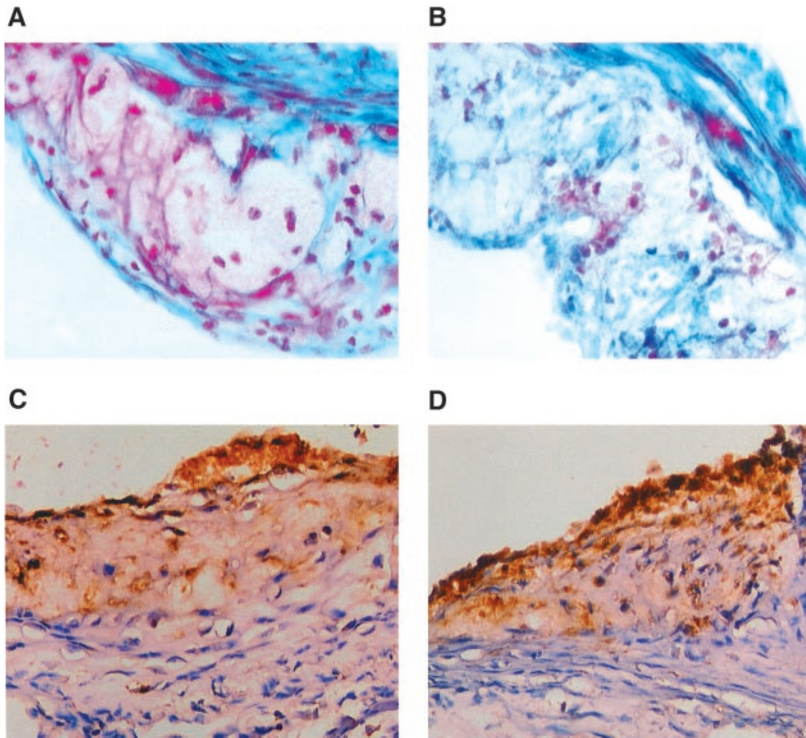

Figure 2. Collagen and macrophage staining in subvalvular plaques. Investigations of subvalvular plaques were performed using Trichrome staining for collagen using mice immunized with carrier and adjuvant alone (a) or with apoB-100 peptide sequences (b) and monocyte/macrophage immunostaining using mice immunized with carrier and adjuvant alone (c) or with apoB-100 peptide sequences (d).

Serum Cholesterol and Triglyceride

Total plasma cholesterol and plasma triglycerides were quantified with colorimetric assays, Infinity Cholesterol and Triglyceride (INT), respectively (Sigma). ApoB-containing lipoproteins were precipitated with $MgCl_2$ and dextran sulfate.[25] The method was optimized for mouse plasma: 91 mmol/L $MgCl_2$ and 1 mg/mL dextran sulfate (final concentrations) were added to plasma samples diluted 1:5 with PBS. The samples were incubated for 1 hour at 4°C, then centrifuged at 560*g* for 15 minutes at 10°C. HDL lipid content was measured in the supernatant.

Serum Amyloid A Determination

A commercially available ELISA kit (Biosource Int., Camarillo, California) was used for determine the level of serum amyloid A as recommended by the manufacturer.

Statistical Analysis

Data are presented as mean±standard deviation. Analysis of the data was performed using the Mann–Whitney two-tailed test. Statistical significance was considered at the level ≤0.05.

Results

ApoE-null mice were used to investigate the effect of immunization with selected apoB-100 peptide sequences. These mice have increased plasma cholesterol levels and spontaneously develop atherosclerotic plaques at 2 to 3 months of age. When given a high-fat diet, they become severely hypercholesterolemic, and the atherosclerotic disease progress more aggressive.

Determining the plaque area of the descending aorta using Oil Red O staining of en face mounts was used for assessment of atherosclerosis. Immunization with apoB-100 peptides resulted in reduction of atherosclerosis by about 60% compared with controls given carrier and adjuvant alone (Figure 1a).

Subvalvular plaques of animals immunized with apoB-100 peptides contained more collagen than control animals as assessed by a modified Masson's trichrome staining (Figures 1b, 2a, and 2b). Immunization did not influence the total area of subvalvular plaques (0.016±0.013 mm^2 versus 0.020±0.013 mm^2) or the presence of Oil Red O staining or macrophage immunoreactivity in subvalvular plaques (Figures 1c, 1d, 2c, and d), nor did it affect macrophage, collagen, and lipid content in plaques in the aortic arch (data not shown).

Antibody titers against the MDA-modified apoB-100 peptide sequences used for immunization (peptide 143 and 210) were determined at sacrifice. Immunization with apoB-100 peptides resulted in an increase in IgG antibody levels against MDA peptides (0.173±0.058 versus 0.097±0.020 absorbance units in controls; $P=0.012$). A similar increase was observed in IgG against the corresponding native peptide sequences (0.152±0.078 versus 0.092±0.087 absorbance units in controls), but this difference was not significant. There were no significant differences in IgM against native peptides or MDA peptides between immunized mice and controls (0.581±0.293 versus 0.395±0.268 absorbance units and 0.789±0.390 versus 0.690±0.299 absorbance units, respectively).

The plasma cholesterol level in control animals was 4.57±1.33 mg/mL at the time of sacrifice and the HDL

Lipids, Body Weight and SAA in Immunized Apo E Null Mice at the Age of 25 Weeks

	Peptides ($n = 10$)	Controls ($n = 10$)
Plasma cholesterol, mg/mL	4.38±1.58	4.57±1.33
HDL cholesterol, mg/mL	0.23±0.04	0.18±0.12
Triglycerides, mg/mL	0.76±0.13	0.80±0.12
Body weight, g	36.2±4.2	38.4±3.0
SAA, μg/mL	227±67	252±94

cholesterol level 0.18±0.12 mg/mL. Immunizations with apoB-100 peptides did not influence plasma and HDL cholesterol levels (Table). Serum amyloid A levels did not differ between the groups, indicating that there was no major difference in general inflammatory activity (Table).

As part of these studies a separate group of mice (n=10) were immunized in the same way but with a mixture of 5 human apoB-100 peptide sequences (peptide 10: amino acid 136 to 155, ALLVP PETEE AKQVL FLDTV; peptide 45: amino acids 661 to 680, IEIGL EGKGF EPTLE ALFGK; peptide 154: amino acids 2296 to 2315, NLIGD FEVAE KINAF RAKVH; peptide 199: amino acids 2971 to 2990, GHSVL TAKGM ALFGE GKAEF; and peptide 240: amino acids 3586 to 3605, FPDLG QEVAL NANTK NQKIR). The peptides were selected because high IgG levels against these sequences were identified in pooled plasma from CHD patients.[21] Immunization with this peptide mixture did not inhibit the development of Oil Red O–stained plaques in the aorta (0.338±0.075 versus 0.287±0.075% stained area in controls, P=0.052). Mice immunized with these peptides had increased IgG antibody levels against the respective native peptides (0.135±0.086 versus 0.034±0.019 absorbance units in controls, P=0.008). There was no difference in IgG levels against MDA-modified peptides, IgM against native or MDA-modified peptides, subvalvular plaque areas, macrophage immunostaining, Masson's trichrome staining, or plasma lipids (data not shown). During the time these studies were performed, the amino acid sequence of mouse apoB-100 became available (accession no XP_137955). Comparison between the mouse and human sequences (accession no P04114) demonstrated a complete lack of homology for peptides 154, 199, and 240. Although only 3 of 5 peptides in this group were nonrelevant, the results still suggest that apoB-100 sequence homology is important for the atheroprotective effects of immunization described above.

Discussion

Our studies demonstrate the possibility of inhibiting development of atherosclerosis by activation of atheroprotective immune responses against apoB-100 peptide sequences. The existence of atheroprotective immune response has previously been suggested by studies demonstrating that treatment with cyclosporin accelerates atherosclerosis in hypercholesterolemic rabbits[26] and mice[27] and by the observation of increased atherosclerosis in major histocompatability complex class I–deficient C57BL/6 mice fed a high-fat diet.[28] B cell reconstitution inhibits development of atherosclerosis in splenectomized apoE-null mice,[29,30] as well as neointima formation after carotid injury in RAG-1 mice.[31] The latter studies suggest that humoral immune responses are particularly important for atheroprotection, a notion that is further supported by studies demonstrating that repeated injections of immunoglobulins reduce atherosclerosis in apoE-null mice.[32]

High levels of IgG and IgM against both apoB-100 peptide sequences used in the present study have been demonstrated in pooled control plasma (c.f. Figure 1).[21] Antibodies against one of these peptides (peptide 210; amino acids 3136 to 3155) were also analyzed in clinical studies. IgM levels against this peptide sequence were significantly related to carotid intima media thickness. This association provides strong support for a role of apoB-100 autoantibodies in development of carotid disease but does not explain the nature of this association. Antibodies could (1) contribute to plaque development, (2) be a marker of disease severity without a functional role, or (3) have a protective role and being produced in relation to

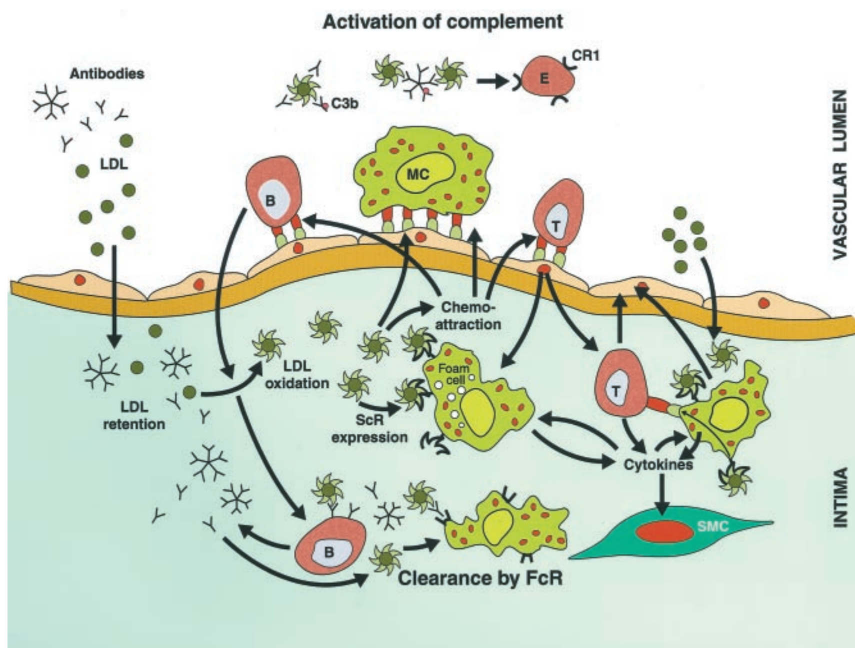

Figure 3. Possible mechanisms for removal of oxidized LDL. Circulating immune complexes containing oxidized LDL and antibodies activate complement resulting in binding of fragments of complement factor 3 (C3b). This leads to a clearance of the immune complexes from the circulation by erythrocytes through complement receptor 1 (CR1). LDL particles that have entered the arterial intima may become trapped and more extensively oxidized. This generates proinflammatory lipids and expression of adhesion molecules on the endothelium and secretion of chemokines. These factors cause adhesion and entry of monocytes (MC), T lymphocytes (T), and possibly also B lymphocytes (B). Monocytes differentiate into macrophages, upregulate scavenger receptors (ScR) that internalize oxidized LDL, and become foam cells. This leads to presentation of fragments of oxidized LDL to antigen-specific T cells that produce proinflammatory cytokines. Cytokines, such as interferon-γ, tumor necrosis factor-α, and interleukin-1, in turn stimulate endothelial cells to express adhesion molecules and smooth muscle cells (SMCs) to migrate into the intima. Activated T lymphocytes can also stimulate B lymphocytes to produce antibodies. Immune complexes containing these antibodies and oxidized LDL can bind to antibody receptors (FcR) on macrophages and thereby be eliminated from the intima.

disease severity. Taken together, the findings of the present two studies favor the latter possibility.

Immunization with apoB-100 peptides resulted in an increase of specific IgG antibodies. This may suggest that a T-cell–dependent switch to synthesis of IgG antibodies against epitopes in oxidized LDL is involved in atheroprotective immune responses. The importance of T-cell–dependent antibody response in protection against atherosclerosis has previously also been shown in apoE-null mice immunized with homologous MDA-LDL.[11] However, interestingly, there was no association of IgG levels against peptide 210 and carotid intima media thickness in the clinical studies.[21] These findings suggest the existence of complex interactions between immune response to apoB-100 peptides and the atherosclerotic vascular wall.

Our finding that immunization with apoB-100 peptide sequences reduces atherosclerosis is also in line with several previous studies demonstrating that immunization with oxidized LDL inhibits development of atherosclerosis.[7–12] Indeed, the apoB-100 peptide sequences used in the present study are likely to be similar or identical to the structures in oxidized LDL responsible for activation of atheroprotective immune responses. Interestingly, immunization with peptide mixture containing mainly nonhomologous peptide sequences did not inhibit atherosclerosis.

ApoE-null mice express mainly apoB-48, and only about 30% of apoB-containing lipoproteins in apoE-null mice carry apoB-100.[33,34] The apoB-48 protein is truncated at amino acid 2153.[35,36] Peptide 143 corresponds to amino acids 2131 to 2150 of apoB-100 and is thus part of apoB-48.

Native peptide sequences were found to induce atheroprotective immune responses in the present study. In accordance, immunization with native as well as oxidized LDL has been shown to inhibit atherosclerosis.[8,9] One possible explanation to this phenomena is that oxidation of both native apoB-100 peptides and native LDL may occur during the immunization procedure. This possibility is supported by the observation that immunization with native apoB-100 peptides results in increased IgG antibodies recognizing the MDA-modified peptides as well as native LDL is associated with increased levels of antibodies against oxidized LDL.[8,9] Another possibility is that protective effect of immunization with human native peptides is mediated by immune responses against native mouse apoB peptide sequences.

The mechanisms through which these atheroprotective immune responses operate remain to be fully elucidated. One possibility is that antibodies facilitate removal of oxidatively damaged LDL particles by macrophage Fc receptors (Figure 3). Macrophage scavenger receptors only recognize LDL with extensive oxidative damage.[2] Recent studies have identified the existence of circulating oxidized LDL.[37,38] These particles have only minimal oxidative damage and are not recognized by scavenger receptors. Binding of antibodies to these circulating oxidized LDL particles would help to remove them from the circulation before they accumulate in the vascular tissue (Figure 3).[39] The finding that the decrease in antibodies against apoB-100 peptide sequences that occurs with age in humans is associated with an increase in the plasma level of oxidized LDL support this notion.[21]

Immunization with native apoB-100 peptides reduced en face–stained fatty lesions in the aorta but not the size of more advanced plaques in the aortic origin. This may suggest that the protective effect is primarily targeted on early lesions. However, previous studies in apoE-null mice fed a Western type diet have also shown that en face Oil Red O stains of the aorta are highly correlated with the aortic plaque size in serial cross sections but not with the extent of lesions present in the aortic origin,[22,40] indicating that partly different mechanisms may be involved.

It is also possible that atheroprotection is dependent on cell-mediated immune responses. Induction of severe hypercholesterolemia in apoE-null mice by changing from normal chow to high-fat diet leads to a switch in cellular immune responses from Th1 to Th2.[41]

Total lesion area detected in the descending aorta was relatively low in these mice. The use of male mice represents one possible explanation for this because females have larger and more advanced atherosclerotic lesions.[42]

Previous studies have demonstrated atheroprotective immune responses against oxidized LDL. In the present studies we have identified the molecular target for some of these atheroprotective immune responses. In contrast with oxidized LDL, these apoB-100 peptide sequences represent reproducible constituents for a possible development of a vaccine against atherosclerosis.

Acknowledgments

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