Changes in immune responses to oxidized LDL epitopes during aging in hypercholesterolemic apoE(−/−) mice

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Dimayuga, Paul C., Xiaoning Zhao, Juliana Yano, and Kuang-Yuh Chyu. Changes in immune responses to oxidized LDL epitopes during aging in hypercholesterolemic apoE(−/−) mice. Am J Physiol Regul Integr Comp Physiol 291: R1644–R1650, 2006. First published July 6, 2006; doi:10.1152/ajpregu.00511.2005.—Atherosclerosis is a disease associated with aging and is subject to modulation by both the innate and adaptive immune system. The time course of age-dependent changes in immune regulation in the context of atherosclerosis has not been characterized. This study aims to describe alteration of the immune responses to oxidized LDL (oxLDL) during aging that is associated with changes in plaque size and phenotype in apoE(−/−) mice. Mice fed a Western diet were euthanized at 15–17, 36, or >52 wk of age. The descending aortas were stained for assessment of extent of atherosclerosis. Plaque lipid, macrophage, and collagen content were evaluated in aortic sinus lesions. The adaptive immune response to oxLDL was assessed using anti-malondialdehyde-oxidized LDL (MDA-LDL) and copper-oxidized LDL (Cu-oxLDL) IgG, and the innate immune response was assessed using anti-Cu-oxLDL and phosphorylcholine (PC) IgM. Aging was associated with a significant increase in plaque area and collagen content and a decrease in plaque macrophage and lipid content. MDA-LDL IgG significantly increased at 36 wk but was reduced in mice >52 wk. Cu-oxLDL IgG increased with age and IgG-apoB immune complexes were increased in the >52 wk group. Cu-oxLDL and PC IgM significantly increased with age. The expression of splenic cytokines such as IFN-γ, IL-4, and IL-10 increased with age. Our study shows a generalized increase in innate immune responses associated with progression of atherosclerosis and a less inflammatory and less lipid-containing plaque phenotype during aging. The adaptive immune response appeared to be less generalized, with a specific reduction in MDA-LDL IgG.

aththerosclerosis is a disease associated with aging and is modulated by the immune system. Autoantibodies to oxidized LDL (oxLDL) exist in animal models and humans. Experimental immunization using various LDL-related immunogens modulates the progression of the atherosclerotic disease, which points to a protective role for the antibody response to oxLDL elicited by immunization (1, 13, 23, 41). This response has predominantly been viewed as an adaptive response consisting of IgG isotypes. An autoantibody of the IgM class against oxLDL produced by a cell line cloned from apoE(−/−) mice has been identified and is identical to the T15 antibody, which recognizes phosphorylcholine (PC). (31) This indicates that molecular mimicry of oxLDL epitopes occurs naturally and is therefore part of innate immunity, because autoantibodies to PC are known to develop spontaneously (31, 32). Thus those findings also implicate the innate immune system in the development of atherosclerosis.

Although aging-related alteration in immune function has been studied in infectious disease and cancer, the temporal trend of this aging-related change in immune response has not been well characterized in the context of the interaction between aging and immune responses in atherosclerosis. In this study, we have described the progression of the atherosclerotic disease in apoE(−/−) mice with aging that is associated with altered immune responses to oxLDL. Our results showed shifts in immune responses concomitant with changes in the systemic and local indexes of atherosclerotic plaque development with age. The changes occurred in both cellular and antibody-mediated immune responses, with changes in specific antibody titers associated with various indexes of atherosclerosis.

METHODS

Animals. Male apoE(−/−) mice on the C57BL/6 background were purchased (Jackson Laboratory, Bar Harbor, ME) at 6 wk of age and fed a Western-type diet (0.15% cholesterol, 21% fat) throughout the study period. Three groups of apoE(−/−) mice were included in this study: 15- to 17-, 36-, and >52-wk-old mice. All materials were obtained from animals in protocols approved by the Institutional Animal Care and Use Committee. Mice were housed in a pathogen-free environment with sentinel mice periodically checked for infections. Blood was collected before euthanasia. The limited amount of serum available from each mouse prevented the performance of all assays on each sample. The aorta was collected for en face preparation, and the spleen was frozen in liquid nitrogen. The bases of hearts were embedded in OCT, and sections of the aortic sinuses were collected.

En face examination of aorta. Aortas were cleaned of connective tissue, cut longitudinally, and stained for lipids. Digital images of the stained aortas were captured, and percent stain area was measured using computer-assisted analysis (Optimas).

Aortic sinus plaque staining. Serial 6-μm-thick frozen sections were collected and examined for the appearance of at least two aortic valves to the disappearance of the aortic valve leaflets. Typically, three consecutive sections were collected on one slide, and a total of 25–30 slides was collected from one mouse. Every fifth slide was grouped for staining. For monocyte/macrophage staining, sections were fixed in ice-cold acetone for 5 min, and standard immunohistochemical protocol was used for macrophage/microglial antibody (MOMA-2; Serotec). Negative controls included nonimmune isotype antibody or omission of the primary antibody.

For oil red O staining, frozen sections were fixed in 4% paraformaldehyde at room temperature for 30 min. After being washed in water for 2 min, slides were stained with 0.24% oil red O in 60%
isopropanol for 20 min. After another wash in 60% isopropanol for 2 min, slides were counterstained with hematoxylin and fast green.

Collagen content in the plaque was identified using a standard Trichrome stain protocol. Collagen was identified as blue stain in tissue under a light microscope.

**Serum cholesterol.** Serum cholesterol levels were measured using a commercial kit (Sigma, St. Louis, MO).

**RT-PCR.** Total RNA was extracted from spleens using TRIzol (Invitrogen, Carlsbad, CA). Aliquots (1 μg) were then subjected to reverse transcription using oligo(dT) primers and the Superscript II system (Invitrogen). Equal aliquots were then used for PCR with previously described primers for murine β-actin, IFN-γ, IL-4 (21), IL-10 (34), and TGF-β (6) Cycling conditions were optimized for each primer, and PCR products were run on 1.2% agarose gel stained with ethidium bromide. Gels were digitally photographed, and densitometric analysis was performed. Values are expressed as ratios to β-actin.

**Oxidized LDL antibodies.** Antibodies to native LDL (nLDL) and malondialdehyde-oxidized LDL (MDA-LDL) were assayed using commercially available ELISA plates (catalog no. 1158; IMMCO Diagnostics, Buffalo, NY), and the manufacturer’s recommended protocol was followed. The system is protected from oxidation because the plates are sealed in an oxygen-free atmosphere. Sera were diluted 1:10, and dilutions of more than 1:10 yielded values too low to interpret. Copper-oxidized LDL (Cu-oxLDL) Ig titers were assayed using LDL oxidized overnight with CuSO4 (5 μM). Plates were coated with 10 μg/ml Cu-oxLDL as capture antigen. Sera were diluted 1:50, incubated on coated plates for 2 h at 4°C, washed, and detected with horseradish peroxidase (HRP)-labeled anti-mouse IgG (Pierce) or anti-mouse IgM with the ABTS system (SBA). Specific isotypes were determined using Cu-oxLDL-coated plates and HRP-labeled anti-mouse IgG1 and IgG2a. Values are expressed as optical density at 405 nm (OD405).

**Immune complex.** Presence of immune complex formation in serum was detected using a modified ELISA. Goat anti-ApoB antibody (Santa Cruz Biotechnology) was coated on an ELISA plate as capture antibody overnight at 4°C. Sera were diluted 1:50 and incubated in the ELISA plate for 2 h at room temperature. Wells were washed, and detection was performed using anti-mouse Ig conjugated with HRP, with ABTS as substrate. Reproducibility of the assay was assessed using three to four replicates and dilutions and determining the coefficient of variation, which ranged from 3 to 5.4%.

**PC antibodies.** Antibodies to PC were assessed using ELISA on serially diluted serum samples. Briefly, ELISA plates were coated overnight with 10 μg/ml PC-keyhole limpet hemocyanin (KLH). Diluted sera were then incubated in the washed and preblocked ELISA wells for 1 h at room temperature, washed, and detected using HRP-labeled anti-mouse IgM and the ABTS system. Specificity and reproducibility were assessed using a mouse monoclonal antibody to PC (T15 IgA; Sigma) and blocking with KLH or PC-KLH. Results were expressed as OD405. Cross-reactivity to oxLDL was determined using a competition assay. Wild-type (WT) C57Bl6/J male mice purchased from Jackson ImmunoResearch Laboratory were fed normal chow, and serum was collected at 20, 40, and >52 wk for comparison to the antibody titers of hypercholesterolemic apoE(-/-) mice. Tissue was harvested from 24-, 40-, and >52-wk-old mice.

**Total IgG and IgM.** Total IgG and IgM titers were determined using a commercially available assay (SBA) with goat anti-mouse immunoglobulin to capture (5 μg/ml) and goat anti-mouse IgG-HRP (SBA) or goat anti-mouse IgG-HRP (Pierce) for detection. Serum dilutions were 1:100 as recommended.

**Statistics.** Values are expressed as means (SD). Group comparisons were performed using ANOVA followed by Tukey-Kramer post hoc tests, unless otherwise indicated. Statistical significance was set at the 0.05 level.

**RESULTS**

**Atherosclerotic plaque extent and serum cholesterol.** Increasing age was associated with increasing extent of atherosclerosis in the descending aorta. Atherosclerotic lesions were detected at 15–17 wk (n = 5), increased markedly at 36 wk (n = 6), and increased more so at >52 wk of age (n = 8; Fig. 1A). A similar age-related increase was also observed in the aortic sinus plaque area (Fig. 1B). Serum cholesterol levels were comparable and did not differ significantly across the various age groups (782 ± 161, 776 ± 380, and 611 ± 99 mg/dl, respectively).

**Phenotypic composition of aortic sinus plaque.** The composition of aortic sinus plaques showed significant changes with a progressive decline in lipid content and macrophage (MOMA-2) immunoreactivity in the plaques with increasing age (Fig. 2, A and B). In contrast, collagen content increased with increasing age (Fig. 2C).

**Antibodies to MDA-LDL.** To determine the changes of the adaptive immune response to oxLDL with age, we assessed antibody titers to oxLDL epitopes. We observed a different temporal expression pattern of antibodies against MDA-LDL between C57Bl6 and hypercholesterolemic apoE(-/-) mice. Antibodies to MDA-LDL of the IgM isotype did not significantly change between the age groups in apoE(-/-) mice (n > 5), whereas MDA-LDL IgM in WT mice increased from 20 to 40 wk and then decreased significantly from 40 to >52 wk (n = 4 each; Fig. 3A). MDA-LDL IgG antibodies in apoE(-/-) mice significantly increased from 15 to 17 (n = 5) to 36 wk (n = 6) and then decreased significantly from 36 to >52 wk (n = 6). WT MDA-LDL IgG antibodies had a similar temporal profile (Fig. 3B). Antibodies to nLDL showed a pattern similar to that of MDA-LDL IgG (Fig. 3C). The results...
suggest that although MDA-LDL IgM antibodies are affected by hypercholesterolemia, MDA-LDL IgG and nLDL IgG are not, and the observed changes are likely due to the aging immune system.

IgM antibodies to Cu-oxLDL epitopes. The innate antibody response to oxLDL epitopes was assessed using IgM immune responses to Cu-oxLDL and PC. Cu-oxLDL IgM and PC IgM antibody titers in apoE(−/−) mice gradually increased with age, doubling at >52 wk (n = 6) compared with 15–17 wk (n = 5 for 15–17 and 36 wk; P < 0.05; Fig. 4, A and B). Cu-oxLDL IgM and PC IgM increased dramatically at 40 wk in the WT mice, more than double the levels at 20 wk (P < 0.05), and leveled-off (n = 4 each age group; Fig. 4, A and B). Anti-PC IgM was determined to cross-react with oxLDL by a competition assay using pooled sera from the mice (see Fig. 6A). Sera from each of the three different age groups were used to assess changes in serum apoB-IgM immune complexes. There was a progressive increase in serum apoB-IgM immune complex in apoE(−/−) mice, which was doubled at >52 wk (n = 6 each for 15–17 and >52 wk, n = 4 for 36 wk; Fig. 4C).

No differences were observed in the apoB-IgM immune complex in WT mice (n = 6 for 20 wk, n = 4 for 40 and >52 wk; Fig. 4C).

IgG antibodies to Cu-oxLDL epitopes. Cu-oxLDL IgG titers also increased progressively with age in the apoE(−/−) mice (n = 5 each for 15–17 and 36 wk, n = 6 for >52 wk; Fig. 5A). This increase was not isotype specific, because both IgG1 and IgG2a increased to a similar degree (n = 4 for 15–17 wk, n = 5 for 36 wk, n = 6 for >52 wk; Fig. 5B). WT Cu-oxLDL IgG titers were again increased more than twofold at 40 wk (n = 4) compared with 20 wk (n = 3; P < 0.05) and remained so at >52 wk (n = 4; Fig. 5A). There was no change in the immune complexes of apoE(−/−) mice from 15–17 (n = 6) to 36 wk (n = 4). However, there was a sixfold increase from 36 to >52 wk (n = 6; P < 0.05; Fig. 5C). No changes were observed in WT serum immune complex at any time point (n = 6 for 20 wk, n = 4 each for 40 and >52 wk; Fig. 5C).

Total immunoglobulin. There was a very slight but statistically significant increase in total IgG and IgM levels in apoE(−/−) mice at >52 wk (n = 4 for 15–17 wk, n = 5 for 36 wk, n = 6 for >52 wk; Fig. 6A). This change was not observed in the WT mice (n = 4 each; Fig. 6, B and C).

Splenic cytokine profile. Using RT-PCR, we further determined the cytokine expression pattern from spleens in apoE(−/−) mice in relation to aging (Fig. 7 and Table 1). The expression of IFN-γ, IL-4, and IL-10 were low in 15-
17-wk-old mice, gradually increased in mice at 36 wk of age, and significantly increased in mice at 52 wk of age. TGF-β mRNA expression had a slight increase at the oldest time point tested (n = 3 each). Splenic cytokine expression in WT mice also increased at 40 wk and was maintained to 52 wk (Table 2).

DISCUSSION

Aging is a major risk factor for the development of diseases such as cancer or atherosclerosis. Although a dysfunctional immune response has been shown to be an important underlying mechanism for atherosclerosis (3, 15, 40), how different indexes of immune responses to atherosclerosis change with aging has not been systematically described. A previous report (24) tracked the changes in MDA-LDL antibody titers and the development of atherosclerosis in LDL receptor-deficient [LDLR(−/−)] mice but did not proceed beyond 36–40 wk of age. We therefore initiated a longitudinal study of the indexes of atherosclerosis during aging as they relate to immune functions in hypercholesterolemic apoE(−/−) mice.

This study shows that advanced age is associated with changes in aortic atherosclerotic plaque composition characterized by decreased intraplaque lipid and macrophage, and increased collagen content, suggesting a more stable plaque phenotype. These changes occurred without any significant changes in circulating cholesterol levels. Our findings are consistent with a previous report (25) that the atherosclerotic lesions in aortic sinus became less cellular and were covered with fibrous cap as hypercholesterolemic apoE(−/−) mice aged. Convincing experimental models of spontaneous plaque rupture in mouse have been difficult, if not impossible, to obtain (9, 26). This could be due to the observed increase of collagen content and reduction of lipid and macrophage content in the plaques as mice aged, and the clinical observation that the vulnerability of atherosclerotic plaques to rupture is not a linear function of the plaque burden (8, 29, 30).

Because of the known link between atherosclerosis and antibody response to oxLDL (24), we sought to determine whether the change in plaque phenotype and increase in size were associated with changes in antibody responses to oxLDL epitopes. The temporal courses of antibody response to MDA-LDL or Cu-oxLDL are distinctly different. IgG antibody titers to MDA-LDL increased in the 36-wk-old mice but clearly decreased in the older mice (>52 wk), whereas Cu-oxLDL IgG and IgM antibody titers increased gradually between 15 and 17 wk and 36 wk, and significantly from 36 to >52 wk. The increase in MDA-LDL titers at 36 wk of age is in agreement with a previous report (24) characterizing MDA-LDL antibody response.

Fig. 4. Copper-oxidized LDL (Cu-oxLDL) IgM (A) and phosphorylcholine (PC)-IgM (B) in apoE(−/−) mice gradually increased over time, reaching statistical significance at >52 wk. In the WT mice, the increase was more abrupt at the 40-wk time point and leveled off. Circulating apoB-IgM immune complexes in apoE(−/−) mice gradually increased over time, doubling by >52 wk (C). No differences were observed in WT mice. *P < 0.05 vs. 15–17 wk. †P < 0.05 vs. 20 wk.

Fig. 5. Cu-oxLDL IgG titers in the apoE(−/−) mice gradually increased from the 15- to 17-wk-old group to the 36-wk-old group and significantly increased in the >52-wk-old group (A). The change in Cu-oxLDL titers was not isotype specific, because both IgG1 and IgG2a increased in a similar manner (B). Cu-oxLDL IgG in WT mice significantly increased at 40 wk and leveled off at >52 wk (A). Circulating apoB-IgG immune complexes (IC) in apoE(−/−) mice were not changed between 15 and 17 and 36 wk but were significantly increased at >52 wk (C). No differences in circulating apoB-IgG IC were observed in the WT mice (C). *P < 0.01; **P < 0.05 vs. 15–17 wk. †P < 0.05 vs. 36 wk. ‡P < 0.01; §P < 0.05 vs. 20 wk.
response in LDLR(−/−) mice. The changes in MDA-LDL IgG titers appear to be related to aging rather than to atherosclerotic disease progression, since nondiseased WT mice had a very similar profile to that of apoE(−/−) mice. Interestingly, although MDA-LDL IgM changes occurred in WT mice, this was not observed in apoE(−/−) mice.

The innate immune antibody response in atherosclerosis has gained interest with the characterization of an IgM antibody against oxLDL shown to be identical to T15, which is a PC-reactive antibody, identifying PC as a recognizable epitope on oxidatively modified LDL (31). PC is a known autoantigen involved in educating the host innate immune response to produce antibodies involved in the first response to infectious challenges (16, 32). We therefore used PC as an antigen to assess innate antibody response to oxLDL in our study. PC-IgM titers had age-related kinetics similar to Cu-oxLDL IgM. Cross-reactivity between PC IgM and Cu-oxLDL was confirmed by competition assay. The importance of the molecular mimicry observed between Cu-oxLDL and PC is underscored by the observed cross-reactivity between antibodies to PC-bearing dental plaque bacteria and oxLDL epitopes (28).

The IgM response to Cu-oxLDL and PC-bearing epitopes in the WT mice has different kinetics compared with apoE(−/−) mice during the aging process. Although a pattern of progressive increase in IgM antibody titers was observed in the apoE(−/−) mice, the increase in the WT mice was rather abrupt between 20 and 40 wk of age and then subsequently leveled off, not increasing further (Fig. 4, A and B). One possible explanation to account for this difference is that the apoE(−/−) mice were under constant immunologic pressure from hypercholesterolemia, and hence the immune response continued to mount with aging. It is noteworthy that detectable circulating apoB-IgM immune complex paralleled the progressive increase in IgM titers in apoE(−/−) mice. There were no changes in detectable apoB-IgM immune complex titers in WT mice.

Cu-oxLDL IgG titers increased in a manner similar to the IgM titers. As in the IgM titers, apoE(−/−) mice progressed to high Cu-oxLDL IgG titers over time, whereas WT mice again abruptly increased titers and leveled off. The spontaneous increase in Cu-oxLDL IgG titers in apoE(−/−) mice was not associated with a shift in isotype response (Fig. 5B). The dramatic increase in detectable apoB-IgG immune complexes in the >52-wk-old group occurred concomitantly with increased Cu-oxLDL IgG titers and decreased intraplaque lipid and macrophage, suggesting increased clearance of Cu-oxLDL epitopes, which contain apoB. This may represent a pathway for atheroprotective effects of increased antibody response to oxLDL.
Cu-oxLDL. The increase in antibody levels in the apoE(−/−) mice occurred also, because the total IgG and IgM levels slightly increased. Although statistically significant, it is unclear whether the very slight changes are physiologically relevant.

Caligiuri et al. (5) reported that sex and age affect splenic T-cell proliferative response in vitro. The cultured splenocytes from old male apoE(−/−) mice (48 wk old) developed a stronger proliferative response against Cu-oxLDL compared with those from young male mice (16 wk old), yet there was minimal difference in the response against MDA-LDL between splenocytes from young and old mice. This observation is in line with our data that antibody response against Cu-oxLDL increased with age and had a different temporal course compared with the antibody response against MDA-LDL.

It has been reported that splenic T or B lymphocyte populations do not differ significantly with age in apoE(−/−) mice. Although the number of CD4 subset of T lymphocytes slightly increased with age, the CD8 subset of T lymphocytes decreased with age (5). We have further extended these observations and report that there was a general increase in splenic cytokine expression with age. This finding is in line with previous studies of aging WT mice (17, 18, 38). It is unclear whether the increase in cytokine expression is the result of activation by a specific oxLDL epitope.

The physiological meaning of the increased antibody titers against LDL with age is unclear at present. It is also unknown whether it is a response or a contributor to the progressively increased atherosclerotic burden. The increase in nondiseased mice would argue for either a protective effect or a bystander role. Experimental evidence has shown that augmentation of antibody response to LDL by immunization reduces atherosclerotic plaque size (1, 7, 11, 14), and splenectomy reduced antibody response to LDL with concomitant increase in plaque burden (4); hence, it is reasonable to speculate that such an increase of antibody response to LDL with age could be atheroprotective but occurred too late (7). Further experiments are needed to test this hypothesis.

Our results have several clinical implications. Numerous studies have been published to establish a relationship between circulating oxLDL antibodies and atherosclerotic disease; however, the results have been conflicting. Some studies showed a higher oxLDL antibody titer in patients with clinical disease (2, 10, 22, 27, 39), whereas others showed inverse relationship between oxLDL antibody titers and measured atherosclerotic end points (12, 19) or no relationship at all (20, 35, 36). It is difficult to account for the reported discrepancies, but they may be due to differences in experimental design (progressive study vs. case control study), oxLDL antibody and its isotype measured (MDA-LDL vs. Cu-oxLDL, IgG vs. IgM), or atherosclerotic end points measured (clinical myocardial infarction vs. angiographic coronary artery disease vs. carotid intimal-medial thickening vs. healthy subjects). Age of the studied subjects is another important factor, because these studies often enrolled patients with a wide range of age. Our observation that the measured atherosclerotic indexes related to LDL varied with age supports such a view and is consistent with a recent longitudinal human study (33).

In conclusion, our study delineates the natural temporal course of changes in indexes of atherosclerosis and its related changes in the immune responses, which are important in understanding the dynamics of immune response against LDL or its related epitopes and the evolution of atherosclerosis with age.

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**REFERENCES**


