The Role of Interleukin 12 in the Development of Atherosclerosis in ApoE-Deficient Mice

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Abstract—The cytokine profile of atherosclerotic aortas from apoE-deficient mice was assessed by reverse transcriptase-polymerase chain reaction. The results clearly showed that the expression of mRNA for IL-12p40 was evident in aortas from 3-month-old apoE-deficient mice. The mRNA for IL-10 was detected in aorta from these mice at the age of 6 months, indicating that expression of IL-12 is earlier than that of IL-10 in these animals. Concurrent with IL-12p40, the mRNA for the T-cell cytokine IFN-γ, but not IL-4, was detected in aortas of mice at young and old ages. Both in situ hybridization and immunostaining further demonstrated the localization of IL-12 in macrophages of atherosclerotic lesions. Immunohistochemistry also demonstrated the expression of costimulatory molecules B7–1 and B7–2 in macrophages, suggesting that activation of T lymphocytes by macrophages may occur via surface antigens in lesions. When the immunoglobulin isotype of the antioxidized LDL antibodies in sera of apoE-deficient mice was determined, it revealed that both IgM and IgG were present. Furthermore, IgG2a is predominant and comprises ≈50% of the antioxidized LDL IgG in sera from young mice (3 months), but decreased to lower levels (35%) in older mice (6 months). Daily administration of IL-12 led to an increase in serum levels of antioxidized LDL antibodies and accelerated atherosclerosis in young apoE-deficient mice compared with control mice injected with PBS alone. Taken together, these data suggest that IL-12 plays an active role in regulating the immune response during the early phase of atherosclerosis in apoE-deficient mice. (Arterioscler Thromb Vasc Biol. 1999;19:734-742.)

Key Words: interleukin 12 ■ atherosclerosis ■ oxidized LDL

Atherosclerosis is a chronic pathological process which eventually leads to the occlusion of large arteries. Although the pathogenesis of this disease is not yet fully understood, histological assessment has shown that monocytes/macrophages and T lymphocytes are present in atherosclerotic lesions, implicating the involvement of the immune system in atherogenesis. This notion is further supported by a number of observations. For example, the deposits of complement factors and immunoglobulins are localized in atherosclerotic lesions. Autoantibodies specific for oxidized LDL are present in both sera and lesions of humans and experimental animals developing atherosclerosis, and both MHC class I and II molecules are expressed on endothelial cells and smooth muscle cells of lesions. Furthermore, immunosuppression of rabbits and mice, or MHC class I-deficient mice, developed severe atherosclerosis after feeding with a high-cholesterol diet. Nevertheless, how the local immune reaction is initiated and propagated to affect the progression of atherosclerotic lesions remains unclear.

Recently, a study by Uyemura et al demonstrated that IL-12 and IL-10, which are 2 principal cytokines influencing the differential development of T lymphocytes to the Th1 and Th2 phenotypes, are expressed in macrophages of human atherosclerotic lesions. Because the expression of IFN-γ, but not IL-4, was preferentially detected in lesions, it was suggested that the Th1 cell is the predominant T lymphocyte present in atherosclerotic plaques. However, the interplays between different cytokines as well as the interactions between macrophages and T cells in relation to the development of atherosclerosis is not yet well-characterized. In an attempt to understand the mechanisms by which the immune response is triggered during the development of atherosclerosis, we used apoE-deficient mice, which spontaneously develop atherosclerosis with features similar to those seen in humans, as the animal model to investigate the potential function of IL-12 in atherogenesis. In addition to its primary role in initiation of cell-mediated immunity, IL-12 has been shown to influence the humoral immune response by affecting the immunoglobulin isotype switch to IgG2a in mice. To examine whether IL-12 exerts an effect on the humoral immune response in apoE-deficient mice, we also assessed the subclass distribution of the antioxidized LDL IgG in these mice, which will provide important evidence to support the pathological relevance of IL-12 in this disease.

Because 1 major function of the macrophage is to serve as an antigen-presenting cell (APC) to T cells, it is conceivable that macrophages can regulate T cell activation via interac-

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tions between cell surface molecules. It is known that one of the early events occurring in the subendothelial space prone to the formation of atherosclerotic lesions is the oxidation of LDL. The oxidized LDL is subsequently taken up by macrophages via the scavenger receptors to form the lipid-laden foam cells. A recent study by Stenman et al. has shown that T-cell clones isolated from human atherosclerotic lesions are activated by oxidized LDL in the presence of autologous APCs. It is envisaged that the antigenic peptides derived from oxidized LDL processed in macrophages may be recognized by T cells in atherosclerotic lesions via TCR/MHC complex engagement. In addition to the signal generated through the binding of the MHC/antigen peptide complex to the T-cell receptor, the costimulatory signal delivered by the interaction of the CD28 molecule on T cells and its counter ligand B7 molecules on APCs has been shown to be required for the optimal activation of T lymphocytes. In view of the importance of B7 molecules in the activation of T cells by APCs, the expression of the B7 molecules, B7–1 and B7–2, on macrophages of atherosclerotic lesions was also examined.

In the present report, we demonstrate that IL-12 is expressed in aortas of young mice as early as 3 months, suggesting that IL-12 may play an active role in the initial phase of atherosclerosis. The detection of the costimulatory molecules B7–1 and B7–2 on macrophages further supports the regulatory role of macrophages in activation of T lymphocytes in plaques. The observation that IgG2a is the predominant subclass of antioxidized LDL IgG in the sera of these mice suggests that IL-12 plays a role in the modulation of the humoral response. The pathophysiological link between IL-12 and the development of atherosclerosis was further supported by the observation that daily injections of IL-12 into mice for a month augmented the progression of atherosclerosis and the production of antioxidized LDL antibody in these mice.

Materials and Methods

Animals

Homozygous apoE-deficient mice on C57BL/6J background (C57BL/6J-ApoE<sup>-/-</sup>) were purchased from the Jackson Laboratory (Bar Harbor, Me). The C57BL/6 mice were from the National Animal Center of Taiwan. The mice fed with normal chow diet were from the Animal Center of Taiwan. The C57BL/6J-Apoe<sup>–/–</sup> mice were from the Jackson Laboratory (Bar Harbor, Me). The C57BL/6 mice were from the National Animal Center of Taiwan. The mice fed with normal chow diet were purchased from the Jackson Laboratory (Bar Harbor, Me).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA from each tissue specimen was isolated according to the method described by Chomczynski and Sacchi. The quality and purity of the RNA was determined by absorbance at 260 nm and the ratio of 260 nm to 280 nm. The integrity of the RNA was monitored by ethidium bromide staining of the 28S and 18S ribosomal RNAs analyzed by electrophoresis on 1% agarose gel. The cDNA was synthesized from 2 µg of total RNA by reverse transcription using 0.2 µg random hexamers (Promega) and 200 U Moloney Murine Leukemia Virus reverse transcriptase (Gibco Life Technologies) in the presence of 0.4 mM/L of each deoxynucleotide triphosphate, 10 mM/L dithiothreitol, and 10 U RNasin (Promega) in a final volume of 20 µL. After 1 hour incubation at 37°C, the reaction was terminated by heating at 95°C for 5 minutes, immediate cooling on ice, followed by dilution with DEPC-H2O to 100 µL. Aliquots of the synthesized cDNAs were then used for PCR. The primers used for analysis of β-actin were 5'-TGGAACTGTGACATCATG-3' (sense), and 5'-AACCGAGCTCAGTAAACGTTCC-3' (antisense); for IL-12p40, 5'-CAGAAAGCTTACCATCCGTGGTGTG-3' (sense), and 5'-TCCGGAGTATTTTCTGTGAGTGATG-3' (antisense); for IL-10, 5'-CCAGTTTTACCTGTGAGTGAAG-3' (sense), and 5'-TGGCTCATGTTGCTGACGACACAC-3' (antisense); for IFN-γ, 5'-AGGCGTGACTGAATCAGGATTG-3' (sense), and 5'-GTCACAGTCTTACGCTTATAGG-3' (antisense); and for IL-4, 5'-CGAAGAACACCCACAGAGTGAATGGA-3' (sense), and 5'-GACTCTTTATGTCGACAGC-3' (antisense).

Histological Staining

Histology was examined on paraffin sections with hematoxylin and eosin (H&E) staining. Sections were stained with Mayer's hematoxylin solution (Sigma) for 3 minutes, washed, and stained with eosin Y (Sigma) for 10 minutes. Slides were viewed by light microscopy. For lipid staining, the cryosections were fixed with 5% acetic acid in 95% ethanol for 10 minutes, then stained with 1% Sudan III (Sigma) for 10 minutes. Slides were viewed by light microscopy. For lipid staining, the cryosections were fixed with 5% acetic acid in 95% ethanol for 10 minutes, then stained with 1% Sudan III (Sigma) for 10 minutes.

In Situ Hybridization

IL12p40 cDNA, which contains 394 bp, was subcloned into pCRII vector (Invitrogen). The antisense and sense IL12p40 RNAs were then synthesized by T7 RNA polymerase and SP6 RNA polymerase, respectively, and labeled with digoxigenin (DIG)-UTP according to manufacturer’s instructions (Boehringer Mannheim Biochemicals). Paraffin sections were treated with 0.25% acetic anhydride in 0.1 M/L triethanolamine and 0.9% NaCl for 10 minutes. Sections were then washed with 2× SSC (1× SSC consists of 150 mM/L NaCl, 15 mM/L Na-citrate, pH 7.0), dehydrated with increasing concentrations of ethanol, and air dried for 30 minutes. Before hybridization, sections were prehybridized in a humid chamber with 100 µL prehybridization solution containing 5× SSC, 5× Denhardt’s solution, 50% deionized formamide, 250 µg/ml yeast RNA, 250 µg denatured salmon sperm DNA, and 40 µg/ml EDTA for 3 hours at 50°C. Hybridization was performed at 42°C for 16 to 24 hours in a solution.
humid chamber with 25 μL/sec prehybridization solution containing 10 ng/μL RNA probe. After the hybridization, the sections were washed at 42°C twice in 2× SSC, once each in 0.2× SSC and 0.1× SSC, for 15 minutes per wash. Sections were then blocked for 30 minutes with PBS containing BSA, incubated with alkaline phosphatase-conjugated anti-DIG antibody for 30 minutes, and detected with color solution containing nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl-phosphate for 90 minutes according to the manufacturer’s instructions. After completion of the color reaction, each section was counterstained with carmine.

**Immunocytochemistry**

Immunostaining was carried out using the following antibodies: rat anti-mouse Mac-1 monoclonal antibody (Boehringer Mannheim Biochimica); rabbit anti-human CD3 polyclonal antibody (Dako); rat anti-mouse CD80 (B7–1) monoclonal antibody (PharMingen); rat anti-mouse GL1(B7–2) monoclonal antibody (PharMingen); goat anti-mouse IL-12 polyclonal antibody (R&D Systems). The rabbit anti-human CD3 polyclonal antibody exhibits cross-reactivity to mouse antigen. Tissue sections were pretreated with 3% H2O2 for 10 minutes at room temperature to exhaust endogenous peroxidase activities. Unless specified, sections were blocked in PBS containing 1% BSA and 1% goat serum at 37°C for 30 minutes, followed by treatment with the primary antibody for another 30 minutes. Sections were then incubated with horseradish peroxidase–conjugated secondary antibody for 30 minutes or biotin-conjugated secondary antibody followed by peroxidase-conjugated streptavidin for 30 minutes each at 37°C. After 3 washes in PBS, color was developed with 0.1% 3,3'-diaminobenzidine (DAB) and 0.01% H2O2 in Tris-HCl, pH 7.0. In some experiments, color was developed with 0.1% DAB in 0.1 mol/L Na-acetate, pH 6.0, containing 0.2% β-d-glucose, 130 U of glucose oxidase, and 2.5% NiCl2 to enhance the signals. A negative control was performed by incubating the sections with secondary antibody only (omission of primary antibody).

**Measurement of Oxidized LDL Antibody**

Human LDL was isolated as described previously. Oxidized LDL was prepared by incubating LDL (1 mg/mL) with 5 μmol/L CuCl2 in PBS overnight at 37°C. An enzyme-linked immunosorbent assay technique was used to determine antibody titers. Ninety-six–well polyvinylchloride microtitration plates were coated with native LDL (5 μg/mL) or oxidized LDL (5 μg/mL) in PBS overnight at 4°C followed by blocking with 1% BSA for 2 hours at room temperature. The plates were freshly prepared before all binding assays. Serial dilutions of sera from apoE-deficient mice were added into duplicate wells and incubated for 2 hours at room temperature. The amount of immunoglobulin (Ig) bound was quantified by incubating with a goat anti-mouse IgM conjugated with alkaline phosphatase (Sigma) or a rabbit anti-mouse IgG conjugated with alkaline phosphate (Sigma) at room temperature for 2 hours. After 3 washes, the alkaline phosphatase activity was determined using p-nitrophenyl phosphate as substrate. Color development was measured at 405 nm. Specific antibody to oxidized LDL was defined as the difference between 405 nm readings obtained from the binding to oxidized LDL and to native LDL. Serum IgG isotypes of oxidized LDL antibodies were determined using a MonoAb-ID EIA KIT (Zymed Laboratories). In brief, 50 μL of sera (1:40) from mice was added into wells of the antigen-coated plate and incubated for 2 hours at room temperature. After washing, subclass-specific rabbit anti-mouse Iggs were added and incubated for another 2 hours. After washing, incubation continued with a goat anti-rabbit IgG conjugated with alkaline phosphatase. Color was developed and data were calculated as described above. The standard curves for Ig isotypes were determined using plates coated with goat anti-mouse Iggs (light chains) antibody and known concentrations of mouse Ig isotypes which were then assayed as described above for serum samples. The optical density readings of mouse sera were converted to Ig concentrations by extrapolation from the standard curves for each isotype. To analyze the total Ig isotypes, plates coated with goat anti-mouse Iggs were incubated with serum samples (1:50 dilution), followed by incubation with subclass-specific rabbit anti-mouse Iggs antibodies as described above.

**Quantification of Aortic Atherosclerotic Lesions**

For the quantitation of atherosclerotic lesions of apoE-deficient mice, 45 serial sections from aortic sinus or arch of each mouse were collected. Total 10 to 12 sections sampled from every 4 consecutive sections were H&E stained and the photomicrographs were taken. The cross-sectional area of a given photomicrograph was analyzed using a computer imaging graphic software (NIH Image 1.5). The lesion size of a particular location was then calculated from the average of the area quantitated from the 10 to 12 sections. Statistical analysis of the results was performed using Student’s t test for grouped data.

**Lipoprotein Analysis**

Plasma samples of 6 mice were pooled and subjected to density gradient ultracentrifugation for the fractionation of lipoproteins. SDS-PAGE analysis was performed on a 4% to 15% gradient gel. Protein was visualized by Coomassie Blue R-250 staining.

**Results**

**Immunocompetent Cells in Atherosclerotic Lesions of ApoE-Deficient Mice**

It has been shown that the apoE-deficient mice develop atherosclerotic lesions in aortic roots as early as 3 months. These lesions gradually extend throughout whole aortas and coronary arteries in older animals. With the use of antibodies specific against Mac-1 of macrophages and the CD3 antigen of T lymphocytes, immunostaining of the aorta sections of apoE-deficient mice revealed that macrophages are the predominant cell types present in early lesions as shown in Figure 1C. The presence of extracellular lipid and intracellular lipid accumulation in macrophages was also demonstrated by Sudan III staining (Figure 1A and 1B).
Likewise, the infiltration of T lymphocytes in atherosclerotic lesions was also evident (Figure 1E and 1F).

Cytokine Expression in Different Tissues From ApoE-Deficient Mice

The cytokine gene expression as analyzed by RT-PCR in different tissues of apoE-deficient mice of young (3 months) and older (6 months) ages was performed. As shown in Figure 2, mRNA encoding for IL-12p40, which is one of the subunits of IL-12, and its expression have been shown to be correlated with the secretion of the active cytokine,19 was evident in aortas of apoE-deficient mice at the age of 3 months. The expression of mRNA for IFN-γ, but not IL-10 and IL-4, was also observed in aortas of these mice. Because these cytokines were not detectable in aortas of control C57BL/6 mice of the same age, they were presumably products derived from the atherosclerotic lesions in aortas of apoE-deficient mice. When the cytokine pattern was examined in older apoE-deficient mice, it was very interesting to find that the expression of IL-10 mRNA was evident in aortas. The IL-4 mRNA, however, remained undetectable in aortas of the older mice. These results clearly demonstrate the preferential expression of IL-12 and IFN-γ in aortas of apoE-deficient mice in the earlier stage of the disease.

Localization of IL-12 in Macrophages of Atherosclerotic Lesions

To further confirm the cellular source of the IL-12p40 mRNA, in situ hybridization was performed. As illustrated in Figure 3A and 3B, the IL-12p40 mRNA was detected in macrophage-derived, foam-cell–like regions but not in the spindle-shape cells of the thickened intima. Hybridization using sense IL-12p40 RNA probe was virtually negative (Figure 3C). The presence of IL-12 protein in lesions was further examined by immunostaining of the serial sections with antibodies to murine IL-12 and macrophage surface antigen. As demonstrated in Figure 4, the immunoreactivity with IL-12 antibody was localized to the macrophage-rich area.

Expression of B7–1 and B7–2 Molecules on Macrophages of Atherosclerotic Lesions

To assess whether macrophages in atherosclerotic lesions may function as the antigen-presenting cells to activate the adjacent infiltrated T cells after the interaction of surface MHC molecule and TCR, the expression of B7 molecules, which are required for generating costimulatory signal for T-cell activation on binding to CD28 receptor of T cells, was examined. As shown in Figure 5, immunostaining of the serial sections of an atherosclerotic lesion with specific antibodies to Mac-1, B7–1, and B7–2 revealed that both B7–1 and B7–2 immunoreactivities were detectable in regions that were enriched in macrophages. This observation suggests that macrophages may regulate the T-cell activation and subsequent immune response via surface antigens in atherosclerotic lesions.

Figure 2. Expression of various cytokine genes in different tissues of apoE-deficient mice. The expression of mRNAs for IL-12p40, IFN-γ, IL-4, IL-10, and β-actin in liver (L), spleen (S), heart (H), and aorta (A) in C57BL/6 control mice (3 months old) and apoE-deficient mice, 3 and 6 months old, was examined by RT-PCR. The total RNA was isolated from the indicated tissues of 5 mice in each group. The amplified cDNA fragments had lengths of 394 bp for IL-12p40, 324 bp for IL-10, 245 bp for IFN-γ, 180 bp for IL-4, and 348 bp for β-actin.

Figure 3. In situ hybridization of IL12p40 mRNA in atherosclerotic lesions of apoE-deficient mice. Sections from a 5-month-old apoE-deficient mouse were blocked in PBS containing 1% BSA followed by staining without (A) or with antIL12(B), or antimacrophage (C) antibody. Sections were then incubated with peroxidase-conjugated secondary antibody followed by color development with DAB and were counterstained with hematoxylin. Magnification: A to C, ×200.

Figure 4. Immunostaining of IL-12 in atherosclerotic lesions of apoE-deficient mice. Serial paraffin-embedded sections from a 6-month-old apoE-deficient mouse were blocked in PBS containing 1% BSA followed by staining without (A) or with antIL12(B), or antimacrophage (C) antibody. Sections were then incubated with peroxidase-conjugated secondary antibody followed by color development with DAB and were counterstained with hematoxylin. Magnification: A to C, ×200.

Figure 5. Immunostaining of B7 molecules in lesions of apoE-deficient mice. Serial sections were stained with anti-Mac-1 (A), anti-B7–1 (B), and anti-B7–2 (C) antibodies, followed by incubation with biotin-conjugated secondary antibody and peroxidase-conjugated streptavidin, sequentially. Color was then developed by the glucose oxidase-DAB-nickel method as described in Methods. D, negative control. Magnification: A to D, ×200.
Immunoglobulin Isotype Distribution of Antioxidized LDL Antibodies in Sera of ApoE-Deficient Mice

The presence of antioxidized LDL antibodies in sera and lesions was previously demonstrated in humans with atherosclerosis. A similar observation was later reported in apoE-deficient mice. As shown in Figure 6A, both IgM and IgG to oxidized LDL were detected in sera of these mice. Determination of the subclasses of oxidized LDL IgG revealed the predominance of IgG2a, which represented ~50% of the total antioxidized LDL IgG in sera of young mice. The subclasses IgG2b and IgG1 accounted for another 30% and 15%, respectively, whereas IgG3 was only ~5%. However, when the sera of the older mice (6 months) were examined, the amount of IgG2a had declined to 35%, which was accompanied by an increase in IgG3 13%, with no significant changes in both IgG2b (33%) and IgG1 (18%) (Figure 6B). Because the level of total IgG2a in these mice was not significantly changed with age (Figure 6C), the decrease in antioxidized LDL IgG2a in older mice was not due to an alteration in the abundance of this subclass.

Augmentation of Atherosclerosis in Mice Administered Recombinant IL-12

To further elucidate the role of IL-12 in the progression of atherosclerosis, young mice were injected with recombinant murine IL-12 daily for 30 days, and the effects on the severity of the atherosclerotic lesions and titers of antioxidized LDL antibodies were examined. C57BL/6 mice treated with IL-12 did not develop atherosclerotic lesions (data not shown), indicating that IL-12 alone is not sufficient to induce atherosclerosis in normal animals under these experimental conditions. When apoE-deficient mice were examined, the body weight of mice treated with IL-12 was not significantly different from that of control littermates (26.8±1.8 g versus 27.2±1.7 g, n=9). Likewise, the serum cholesterol level of IL-12-treated animals was comparable with that of control mice (614.2±86.6 mg/dL versus 684.0±125.1 mg/dL, n=9). SDS-PAGE analysis of the lipoproteins isolated from plasma of these animals did not reveal significant changes in levels of apoB, apoAIV, and apoAI after IL-12 treatment (Figure 7). However, when the area of atherosclerotic lesions was assessed, the IL-12-treated group had lesion areas that were >100% larger than that of the control group in the aortic sinus and arch, respectively (P<0.005) (Figure 8). Furthermore, the lesions of the IL-12-treated group of animals were more advanced and contained more CD3-positive cells, as shown in Figure 9A. Examination of cytokine gene expression by RT-PCR revealed that the expression of IFN-γ mRNA in atherosclerotic aorta from IL-12-treated apoE-deficient mice was substantially higher (Figure 9B), indicating that the CD3-positive cells detected are primarily Th1 cells. The effect of IL-12 on the humoral immune response was also...
assessed. As shown in Figure 10, the antioxidized LDL IgM and IgG2a, but not other isotypes, detected in sera of the IL-12-treated mice, were also significantly higher than those of the control animals ($P < 0.025$). Because the level of antioxidized LDL antibodies in C57BL/6 mice are significantly lower than that of the apoE-deficient mice, the effect of IL-12 on antioxidized LDL antibodies in C57BL/6 mice was not considered significant.

**Discussion**

Similar to observations in humans and rabbits, immunocytochemistry revealed that macrophages and T lymphocytes were present within the atherosclerotic lesions of apoE-deficient mice. These lipid-laden foam cells originated from the Mac-1 positive macrophages and appear to be the predominant cell types in early lesions. Infiltrated lymphocytes, as detected by immunostaining with specific antibody for CD3 surface antigen, were also present in lesions from these mice, which was consistent with recent reports by others. The coexistence of macrophages and T lymphocytes in lesions of apoE-deficient mice makes these animals a superb murine model for studying the role of the immune system in the development of atherosclerosis. Because the cytokines derived from these cells are important for immune function, we examined the cytokine profile in aortas of apoE-deficient mice using a highly sensitive RT-PCR method. Our data showed that the mRNAs encoding IL-12p40 and IFN-γ were readily detectable in aorta of 3-month-old apoE-deficient mice. The mRNA for IL-10 was not evident in younger mice but was detectable in older mice. Furthermore, the level of message encoding IL-4 was undetectable in aorta from either young or old mice. These results are consistent with a recent report by Uyemura et al who demonstrated the expression of IL-12 and IFN-γ in human atherosclerotic lesions. In their study, the expression of IL-10 mRNA was also detected in some of the human plaques. Furthermore, an in vitro cell culture study showed that IL-10 inhibits the LDL-induced IL-12 synthesis in monocytes, suggesting that the balance between IL-12 and IL-10 produced in lesions may influence the immune response in atherosclerosis. Together with previous observations that T lymphocytes in human atherosclerotic lesions are activated and produce IFN-γ and IL-2 (which are the characteristics of Th1 cells), the coexpression of IL-12 and IFN-γ in atherosclerotic lesions implies that the Th1-mediated immune response is involved in the pathogenesis of this vascular disease.

In addition to cytokines, activation of T lymphocytes by macrophages can be mediated through the interactions between surface molecules. It is theorized that the degradation of oxidized LDL by macrophages may result in the generation of some antigenic epitopes on small peptides carrying oxidation-modified amino acid residues, such as malondialdehyde-lysine, which then activate T lympho-

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**Figure 8.** Effect of IL-12 on the extent of atherosclerosis in apoE-deficient mice. Male apoE-deficient mice, 4 months old, were treated with or without IL-12 for 30 days. The atherosclerotic lesions that developed in the aortic sinus and aortic arch were quantitated as described in the Methods. The number of animals in each group is shown in parentheses. *$P < 0.005$.**

**Figure 9.** (A) Infiltration of T cells in lesions of apoE-deficient mice treated without (a) or with (b) IL-12. High-power views of the framed area in (a) and (b) are shown in (c) and (d), respectively. The CD3 immunoreactive cells are indicated with arrows. Magnification: a and b, ×20; c and d, ×100. (B) The expression of IFN-γ, IL-4, and β-actin in aortic tissues of control or IL-12-treated mice was examined by RT-PCR. RNA was isolated from 6 mice in each group.

**Figure 10.** Effect of IL-12 treatment on antioxidized LDL antibodies in sera of C57BL/6 and apoE-deficient mice. The antioxidized LDL IgM and IgG subclasses of apoE-deficient mice treated with or without IL-12 were assessed (serum dilution, 1:40) as described in the Methods. The number of animals in each group is shown in parentheses. *$P < 0.025$.**
cytes via MHC-TCR engagement. Although direct evidence is lacking, the recent report that T-cell clones isolated from human atherosclerotic lesions are activated by oxidized LDL29 may be the first evidence to support this notion. Because full activation of T cells requires signals from the activation of both TCR and coreceptor CD28, we were interested in examining the expression of the ligand for CD28, B7 molecules, on macrophages of these lesions. The immunostaining results clearly demonstrated that both B7-1 and B7-2 molecules were present on macrophages, further supporting the potential regulatory role of macrophages on the immune function of T lymphocytes in atherosclerotic lesions. Recently, evidence has accumulated which demonstrates that IL-12 synergizing with CD28/B7 interaction is important for the activation of quiescent T cells and induction of Th1 cells.40,41 It is conceivable that in coordination with B7/CD28 interaction and the presence of IL-12, T cells present in lesions preferentially undergo differentiation toward Th1 type cells, which may then assist with the activation of B cells and the production of antioxidized LDL antibodies.

Experiments were further conducted to investigate the potential role of IL-12 in humoral immune response in atherosclerosis. When the distribution of the isotypes of antioxidized LDL IgG in the sera of apoE-deficient mice was examined, we found that all 4 types of IgG subclasses were present. Furthermore, a higher titer of IgG2a compared with other subclasses was observed in younger mice. This result was consistent with a recent study by Zhou et al42 who reported the preferential production of IgG2a isotype to MDA-LDL in the early phase of the disease in apoE-deficient mice fed with chow diet. These observations suggest that the humoral response was primarily regulated by the cytokines favoring Th1 cell development in the early phase of atherosclerosis. However, the predominance of IgG2a becomes less evident in older mice. Zhou et al42 were able to show that, in severe hypercholesterolemia induced by feeding a high-cholesterol diet to apoE-deficient mice, the expression of IL-4 as well as a switch to Th2-dependent IgG1 isotype was evident, indicating that the cytokine expression and immune response in these animals are subjected to modulation by hypercholesterolemia. This was also revealed in a study53 showing that immunodeficiency by Rag-1 gene knockout in apoE-deficient mice reduced the foam cell lesion formation in these animals when they were placed on a chow diet, supporting the theory that immune response plays a role at least in the early stage of lesion development. However, this effect was eliminated in mice that were fed a high-cholesterol diet to accelerate lesion development. Similar results were reported in immunodeficient Rag-2/ apoE double-gene knockout mice who were placed on a cholesterol-enriched diet for 3 months; they developed fibrous plaques to the same extent as apoE-deficient mice.44 It will be of great importance to discover whether the switch to Th2 response after the high-cholesterol feeding in these mice was associated with the reduced impact of the immune system on lesion development. Because the level of serum cholesterol did not fluctuate significantly between 3- and 6-month-old apoE-deficient mice,24,25 the underlying mechanism responsible for the alteration in antioxidized LDL IgG isotypes with age is unclear. Whether IL-10 and/or other cytokines produced in older animals has an impact on the immunoglobulin isotype switch in the later stage of the disease awaits further investigation. A recent study on the isotypes of oxidized LDL antibodies in humans demonstrated that both IgG1 and IgG3 predominate.45 Because the effect of Th1/Th2 cytokines on immunoglobulin isotypes observed in the murine system is not yet established in humans, the results from humans and mice are not comparable. Nevertheless, it is of great interest to note that the IgG1/IgG3 and IgG2a/IgG2b are the most potent immunoglobulin subclasses to activate the classical complement pathway in humans46 and mice,47 respectively.

When the young apoE-deficient mice were injected with recombinant IL-12 daily for a month, the progression of atherosclerosis was accelerated. The infiltration of CD3-positive T cells and the expression of IFN-γ mRNA were markedly increased in lesions of IL-12-treated animals. Apparently, this observation is quite different from the report by Roselaar et al,57 who demonstrated that T-cell density tends to decrease with disease progression in apoE-deficient mice. Because the mice in their study were placed on a high-cholesterol diet which would promote the T-cell response toward the Th2 type,42 it is conceivable that the type of immune activation as well as the effect on lesion progression in those animals is different from that of the IL-12-treated animals. Whether the density of T cells in atherosclerotic lesions is influenced by the Th1/Th2 ratio remains to be clarified. In conjunction with the severity of the disease, the relative abundance of the antioxidized LDL IgG2a subclass in IL-12-treated mice was significantly higher than that in control mice. These results clearly demonstrate that IL-12 augments the immune response of the T cells and modulates the subsequent humoral antibody production in atherosclerosis in experimental animals. It is generally believed that this effect of IL-12 is mediated by the production of IFN-γ by Th1 cells. In addition to its role in regulating the humoral immune response, IFN-γ has also been shown to up-regulate the expression of VCAM-1, MHC II, and scavenger receptor in vascular cells,48–50 which conceivably would exacerbate the lesion progression. This may explain the observation that the T cell–independent IgM titer to oxidized LDL was also significantly increased in IL-12-treated mice. Apparently, the present observation was consistent with a recent study by Gupta et al,51 who showed that apoE/IFN-γ receptor double-knockout mice exhibited substantial reduction in lesion formation. The detrimental role of IFN-γ in the pathogenesis of arterial disease was also demonstrated in a study showing that IFN-γ deficiency prevents coronary arteriosclerosis in transplanted mouse hearts.52 In apoE/IFN-γ receptor double-knockout mice, the expression of atheroprotective apoA-IV was found to be up-regulated, suggesting that IFN-γ may promote atherosclerosis through effects on vascular walls and plasma lipoproteins.51 However, in the present study we did not find a significant alteration in the level of apoA-IV in serum lipoproteins of apoE-deficient mice after IL-12 treatment. It is possible that the amount of IFN-γ produced in control apoE-deficient mice already caused maximal suppression of apoA-IV gene expression. In contrast to its ability to protect against infectious diseases and tumors, IL-12 apparently has a deleterious effect on vascular atherosclerotic disease. This negative role of IL-12 has also been reported recently in other murine disease models. Administration of IL-12 accelerates autoimmune diabetes in NOD mice53 and glomerulonephritis in MRL/lpr mice,54 and induces severe arthritis in DBA/1 mice.55
In summary, this study clearly demonstrates that IL-12 is produced in the early stage of atherosclerosis in apoE-deficient mice. The observation of coexpression of IL-12 and B7 molecules in macrophages suggests that macrophages can play an active role in activating T lymphocytes to undergo preferential differentiation to the Th1 phenotype in atherosclerotic lesions. On the other hand, IL-12 together with IFN-γ produced by Th1 cells may also exert an effect on the isotype switch of oxidized LDL antibodies in these mice. Although atherosclerosis is a disease with a complicated etiology, the immune system obviously has an important impact on the development of the disease. We suggest that the blockade of IL-12 function or alteration in the Th1/Th2 balance may serve as a potential therapeutic intervention for the progression of atherosclerosis.

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References


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