Interleukin-6 Exacerbates Early Atherosclerosis in Mice

S.A. Huber, P. Sakkinen, D. Conze, N. Hardin, R. Tracy

Abstract—Acute-phase proteins, which respond to systemic proinflammatory cytokines such as interleukin-6, are elevated in cardiovascular disease and are predictive markers of future ischemic events, even over decades. This suggests a role for proinflammatory cytokines and/or acute phase proteins in early lesion development. To explore this issue, we fed C57Bl/6 and nonobese diabetic male mice high-fat (20% total fat, 1.5% cholesterol) diets and ApoE-deficient male mice both high-fat and normal chow diets for 6 to 21 weeks, injecting them weekly with either 5000 U recombinant interleukin-6 (rIL-6) or saline buffer. Blood was collected when animals were euthanized and assayed for cytokines, acute-phase proteins, and cholesterol. Across all mice, IL-6 injection resulted in significant increases in proinflammatory cytokines (IL-6, 4.6-fold; IL-1β, 1.6-fold; and tissue necrosis factor-α, 1.7-fold) and fibrinogen (1.2-fold) and with decreased concentrations of albumin (0.9-fold) in plasma. Total cholesterol levels were unchanged between rIL-6–treated and nontreated groups. Serial sections through the aortic sinus were stained with oil red O to detect fatty streaks, and area of the lesions was determined by image analysis. Although no fatty streaks were detected in the nonobese diabetic mice with or without rIL-6 treatment, rIL-6 treatment increased lesion size in C57Bl/6 and ApoE-deficient mice 1.9- to 5.1-fold over lesions in saline-treated animals. These results suggest that under the appropriate circumstances changes in circulating proinflammatory cytokines and acute-phase proteins may be more than just markers of atherosclerosis but actual participants in early lesion development. (Arterioscler Thromb Vasc Biol. 1999;19:2364-2367.)

Key Words: inflammation ■ atherosclerosis ■ interleukin-6

Interleukin-6 (IL-6) belongs to a family of 20kDa polypeptide cytokines having a four-long-chain α-helix-bundle structure.1–3 This family includes IL-11, leukemia inhibitory factor, oncostatin M, ciliary neurotrophic factor, and cardiotropin-1. The IL-6–type cytokines use the same receptor gp130 subunit for signal transduction resulting in activation of the Janus kinase–signal transducer and activator of transcription pathway. Thus these cytokines may induce similar physiological responses and participate in various aspects of cell proliferation and differentiation. Macrophage/monocytes, T cells, endothelial cells, fibroblasts, smooth muscle cells, osteoblasts, and chondrocytes are primarily identified with IL-6 production, usually as a result of interleukin-1β (IL-1β), tissue necrosis factor-α (TNFα), transforming growth factor-β, or lipopolysaccharide stimulation. The major biological effects of IL-6 are proliferation and differentiation of B and T lymphocytes4 and regulation of the acute-phase response.5

Cardiovascular disease (CVD) likely represents, at least in part, a chronic low-level inflammatory process characterized by increased circulating levels of proinflammatory cytokines (IL-6, TNFα, and IL-1β), soluble adhesion molecules (intracellular adhesion molecule-1 and P-selectin), and cytokine-responsive acute phase proteins including C-reactive protein (CRP), plasminogen-activator inhibitor-1 (PAI-1), and fibrinogen.6 Increased levels of CRP7–8 and IL-69 in patients with unstable angina are associated with poor prognostic outcome. More recently, several studies indicate that CRP can be an independent predictive risk factor for CVD in apparently healthy middle-aged men over long-time periods, even decades,10,11 suggesting a possible association with early lesion development.

A major question is whether increased levels of risk markers such as proinflammatory cytokines, CRP, and/or fibrinogen12 simply result from the developing underlying disease or whether these factors also participate directly in the disease process. To investigate whether IL-6 directly promotes fatty lesion development, we placed atherosclerosis-prone (C57Bl/6, ApoE-deficient [ApoE−]) and atherosclerosis-resistant (nonobese diabetic [NOD]) mice on high-fat, high-cholesterol diets and gave the animals weekly injections of recombinant mouse IL-6. The results show that exogenously administered IL-6 significantly enhanced fatty lesion development in the atherosclerosis-prone, but not in the atherosclerosis-resistant, animals, suggesting that inflammatory factors likely play an active role in the disease process.

Methods

Mice

Male C57Bl/6, NOD, and ApoE− mice were purchased from Jackson Laboratories (Bar Harbor, ME) at 3 weeks of age and fed ad libum...
Increased Atherosclerosis in Mice Treated with Recombinant Mouse IL-6

<table>
<thead>
<tr>
<th>Experimental Pair</th>
<th>Mice Age (weeks) Diet</th>
<th>IL-6 n</th>
<th>Weight, gm</th>
<th>Blood Glucose, mg/dl</th>
<th>Lesion Size (μm²)</th>
<th>Plasma Concentration</th>
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<td>Cholesterol, mg/dl</td>
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<td>24</td>
<td>Hi-Fat</td>
<td>7</td>
<td>28±3</td>
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<td>ApoE</td>
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* P<0.05 compared to non-IL-6-treated animals.

either a low-fat diet (Teklad # 7012: 5.67% fat, 0% cholesterol) or a high-fat diet (Teklad # 96354: 20% fat, 1.5% cholesterol, 0.5% sodium cholate) for either 6 or 21 weeks.

Plasma Analysis
Blood was collected by cardiac puncture when animals were euthanized (one week after the final injection), and ethylenediamine-tetraacetic acid plasma was prepared. Cholesterol, glucose, and albumin were determined using the Vitros CHOL and Vitros ALB slide methods (Johnson & Johnson Clinical Diagnostics, Inc.). Fibrinogen was determined by a clot-rate assay, modified from the original Clauss assay13 for use with ethylenediamine-tetraacetic acid plasma.14

Cytokine and ELISA
Recombinant mouse IL-6 was purchased from Genzyme (Cambridge, Mass). Plasma concentrations of TNFα, IL-1, and IL-6 were determined by capture ELISA using commercial kits from Pharmingen and performed according to manufacturer’s directions.

Histology
The heart and ascending aorta, including the aortic arch, were removed and evaluated for atherosclerotic lesions according to the method of Plump et al12 using oil Red O stained serial sections. Hearts were fixed in 10% buffered formalin, embedded sequentially in 5%, 10%, and 25% gelatin, grossly cut through the ventricles parallel to the atria, and frozen in O.C.T (Miles Laboratories). Every other 10-micron section was placed on gelatin coated slides, stained with 0.24% oil Red O, and counterstained with 2.4% hematoxylin and 0.25% light green. Stained sections were evaluated by image analysis in transmitted light mode with an Olympus BX50 compound light microscope (4x objective lens; numerical aperture, 0.13). True-color digital images (640 by 480 pixels) were captured with a Sony DXC-960 MD/LLP video camera connected via an RS170 cable to a video frame grabber on a Sun SPARCstation 5. Image processing and analysis were accomplished with IMIX software (Princeton Gamma Tech, Inc). Final lesion size was calculated as the mean lesion area in 4 sections, 80 μm apart.

Experimental Design and Statistics
As shown in the Table, we defined 4 experimental pairs in this study: (I) C57Bl/6, high-fat diet, ±IL-6; (II) NOD, high-fat diet, ±IL-6; (III) ApoE, high-fat diet, ±IL-6; and (IV) ApoE, low-fat diet, ±IL-6. Starting group sizes for mice ranged from 5 to 7. The smallest group size examined at the end of an experiment was 3, due to the death of 2 mice. Levels of blood components were analyzed 2 ways. First, we compared raw values between IL-6–treated and nontreated mice for each experimental pair. Second, we combined data from all animals across the 4 experiments by normalizing each animal’s value for a given assay to the mean for the non-IL-6–treated animals in their experimental pair. Data were evaluated by Student’s t test.

Results
Male atherosclerosis-prone (C57Bl/6, ApoE+) and atherosclerosis-resistant (NOD) mice were maintained on high-fat or normal diet from 3 weeks of age until euthanization. ApoE+ mice on high-fat diet (Experimental pair III) were euthanized at 9 weeks of age because fatty streak development in these mice is known to progress rapidly.16 In contrast, lesion progression is slower in ApoE− mice on low-fat diet and in C57Bl/6 wild-type mice. Thus the animals in Experimental pairs I, II, and IV were euthanized at 24 weeks of age to allow adequate lesion development. Plasma from individual animals was evaluated for cholesterol, glucose, fibrinogen, albumin, IL-6, IL-1, and TNFα concentrations, and hearts were sectioned through the aortic sinus and lesion size by the method of Plump et al.15 Results are shown in the Table by experimental pair. The most significant observation from this study is that rIL-6 treatment of atherosclerosis-prone mice (C57Bl/6 and ApoE+) significantly increased lesion size over noncytokine-treated animals. NOD mice failed to develop fatty lesions with or without rIL-6. Lesions in all mice appeared similar by microscopy, although different in extent.

No significant differences were observed between IL-6–treated and nontreated mice for total body weight or plasma cholesterol levels. Two of seven NOD mice developed hyperglycemia in both cytokine-treated and nontreated groups; however, no significant difference in glucose levels was observed between groups. Mean fibrinogen concentrations tended to be elevated and albumin concentrations tended to be lower in C57Bl/6 and ApoE+ mice given rIL-6 compared with identical mice without cytokine, whereas cholesterol levels were similar between IL-6–treated and nontreated mice.

Proinflammatory cytokine concentrations were elevated in the plasma of IL-6–treated mice. This was most dramatic for IL-6 levels, especially in ApoE+ mice on high-fat diets where plasma IL-6 concentrations increased from 0.14 ng/mL in noncytokine-treated animals to 1.10 ng/mL in IL-6–treated
mice. The other 2 proinflammatory cytokines, TNFα and IL-1β, were also increased in the plasma of IL-6–treated animals.

## Discussion

This study shows that IL-6, a proinflammatory cytokine, enhanced fatty lesion development in atherosclerosis-prone mice. The effect was most pronounced in strains of animals that normally develop modest lesions. IL-6 treatment of C57Bl/6 mice resulted in a 5.1-fold increase in fatty streak size (from 410 to 2090 μm²), whereas treatment of ApoE−/− mice on low- or high-fat diets resulted in 2.4- and 1.9-fold increases. ApoE−/− mice on either diet developed substantially greater lesions than C57Bl/6 parental mice, most likely because total cholesterol levels were considerably higher. Because the ApoE−/− mice develop such rapid and extensive lesions, the effects of exogenously administered IL-6 may be less obvious than in C57Bl/6 animals, which have modest cholesterol levels and develop smaller lesions at a much lower rate.

Fatty streaks result from the accumulation of fat-laden macrophages (foam cells) in the subendothelial spaces. Monocytes, the progenitors of early foam cells, are involved in lesion development and must adhere to and migrate through the endothelium at the site of fatty streak/atheroma formation.17–19 Cell adhesion molecules on endothelial cells, such as ICAM-1, VCAM-1, E-selectin, and P-selectin, promote leukocyte migration.18 These cell-adhesion molecules interact with specific ligands on leukocytes: ICAM-1 with LFA-1, Mac-1; VCAM-1 with VLA-4.19 Expression of these adhesion molecules can be greatly enhanced by specific cytokines (IL-1β, TNFα, and IFNγ). These cytokines also activate macrophage-monocytes, which increases their stickiness, motility, phagocytosis, and enzyme levels. All of these effects would be expected to increase lipid uptake, LDL oxidation, and cell migration into the intima. Furthermore, these proinflammatory cytokines promote proliferation of various cell types, including smooth muscle cells, and increase extracellular matrix production.20,21

Although endothelial cells are likely targets of proinflammatory mediators, other cells are targets, and also producers, of these cytokines. Substantial numbers of cells expressing T-cell markers are found in fatty streaks and advanced lesions in humans17,22,23 and in fatty streaks in mice.24 Although controversial, several investigators have found that depleting mice of CD4+ T cells significantly reduces the size of fatty lesions compared with immunocompetent control animals.24,25 IL-6 is a potent lymphocyte growth factor, which could extend clonal expansion of pathogenic CD4+ lymphocytes in the fatty lesion. IL-6 also regulates T cell differentiation and cytokine production, which may select for immune responses conducive to lesion formation.

In addition, the effects of IL-6 on hepatic acute phase reactants can also impact atherogenesis. IL-6–treated animals showed significantly increased fibrinogen and decreased albumin when normalized to nontreated controls. Increasing fibrinogen concentrations can augment blood clotting,12 whereas decreasing albumin may increase platelet reactivity,26,27 both of which could contribute to disease progression in humans. Other potentially important inflammation mediators, which we did not examine, include the fibrinolytic regulators tissue plasminogen activator, PAI-1, and CRP, which has been shown to increase tissue factor expression on monocytes.28

One of the intriguing observations in the present study is that IL-6 did not cause fatty streak induction in atherosclerosis-resistant NOD mice. Plasma cholesterol levels and proinflammatory cytokine concentrations were as high or higher in these animals than in the susceptible C57Bl/6 animals. The results indicate that although a high-cholesterol diet or circulating proinflammatory cytokines such as IL-6 can promote fatty streak formation, these risk factors cannot overcome inherent genetic traits causing disease resistance. The identity of these genetic resistance trait(s) is unknown.

The relationship between fatty streaks in mice and atheromatous plaques in humans is controversial. To date, the complex advanced atherosclerotic lesions observed in humans has not been duplicated in the mouse, raising the issue of relevance of the mouse model system. Fatty streaks may occur in areas of the aorta not normally associated with atheromatous plaques and in children living in geographical regions in which atheromatous plaques are infrequent. Undoubtedly, not all fatty streaks progress to atheromas.29–32 In contrast, fatty streaks in coronary arteries occur in anatomical sites prone to atheromatous plaque development and appear to correlate to and precede mature lesions in humans.33,34 Evidence from experimental models further links fatty streaks to atherosclerotic plaques. Faggiotto and Ross35 studied nonhuman primates and demonstrated that advanced lesions developed in anatomical sites first associated with fatty streaks. Thus, although undoubtedly all fatty streaks do not progress to atheromas, some do. T lymphocytes and foam cells are found in fatty streaks of both humans and mice, and extensive studies over the last 40 years indicate that most immunological processes are either identical or quite similar between these 2 species. Thus, although the mouse may not be applicable to all studies in atherogenesis, it may provide important insights into the role of immune and inflammatory mediators in fatty streak development.

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References

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