Vascular Medicine

Impact of Interleukin-6 on Plaque Development and Morphology in Experimental Atherosclerosis

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Background—Vascular lipid accumulation and inflammation are hallmarks of atherosclerosis and perpetuate atherosclerotic plaque development. Mediators of inflammation, ie, interleukin (IL)-6, are elevated in patients with acute coronary syndromes and may contribute to the exacerbation of atherosclerosis.

Methods and Results—To assess the role of IL-6 in atherosclerosis, ApoE−/−/IL-6−/− double-knockout mice were generated, fed a normal chow diet, and housed for 53±4 weeks. Mortality and blood pressure were unaltered. However, serum cholesterol levels and subsequent atherosclerotic lesion formation (oil red O stain) were significantly increased in ApoE−/−/IL-6−/− mice compared with ApoE−/−, wild-type (WT), and IL-6−/− mice. Plaques of ApoE−/−/IL-6−/− mice showed significantly reduced transcript and protein levels of matrix metalloproteinase-9, tissue inhibitor of metalloproteinase-1, collagen I and V, and lysyl oxidase (by reverse transcriptase–polymerase chain reaction and immunohistochemistry). Recruitment of macrophages and leukocytes (Mac3- and CD45-positive staining) into the atherosclerotic lesion was significantly reduced in ApoE−/−/IL-6−/− mice. The transcript and serum protein (ELISA) levels of IL-10 were significantly reduced.

Conclusions—Thus, a lifetime IL-6 deficiency enhances atherosclerotic plaque formation in ApoE−/−/IL-6−/− mice and leads to maladaptive vascular developmental processes. These observations are consistent with the notion that baseline levels of IL-6 are required to modulate lipid homeostasis, vascular remodeling, and plaque inflammation in atherosclerosis. (Circulation. 2004;110:3493-3500.)

Key Words: interleukins ■ cholesterol ■ atherosclerosis ■ inflammation ■ metalloproteinases

Atherosclerosis is a chronic inflammatory process perpetuated by a variety of proinflammatory mediators, ie, cytokines and chemokines, during all stages of the disease, with myocardial infarction, stroke, or sudden cardiac death as fatal end points. Various lipid fractions, inflammatory cells, and fibrous elements accumulate in the vessel wall and determine progression of the disease.1 In particular, LDL retained in the intima of a vessel undergoes oxidative modification and subsequently may enhance the expression of chemokines and proinflammatory cytokines, ie, that of interleukin (IL)-6.2

Administration of supraphysiological concentrations of exogenous IL-6 in the murine apolipoprotein E–deficient (ApoE−/−) model of atherosclerosis may enhance atherosclerotic lesion formation dramatically, suggesting a pivotal role for IL-6 in plaque progression.3 However, the impact and role of physiological levels of endogenous IL-6 on atherosclerotic lesion formation remain unknown. In this regard, plasma levels of IL-6 are not only enhanced in patients with unstable angina but also predict the outcome of patients with acute coronary syndromes.4 In contrast, the antiatherosclerotic cytokine IL-10 is reduced in patients with acute myocardial infarction and downregulates IL-6 in vivo and in vitro,5,6 suggesting that the balance of proinflammatory (IL-6) and antiinflammatory (IL-10) mediators may determine the perpetuation of atherogenesis.

Because IL-6 may also be a pivotal regulator of extracellular matrix deposition and reorganization7,8 (which may be important for atherosclerotic plaque progression and stability9), we investigated whether a lifetime IL-6 deficiency could alter atherosclerotic lesion formation in the murine ApoE−/− model of atherosclerosis. Moreover, IL-6 is a cytokine with

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multiple and complex endocrine effects on lipid metabolism,10–12 and therefore, ApoE/−–IL-6/− double-knockout male mice were housed for 1 year while being maintained on a regular chow diet (instead of a cholesterol-enriched diet). ApoE/− single-knockout mice (C57BL/6 background), IL-6/− single-knockout (C57BL/6 background), and C57BL/6 wild-type (WT) mice served as controls.

Here we report that a lifetime deficiency of IL-6 in the ApoE/− model elicits opposing functions by enhancing atherosclerotic lesion formation and increasing serum cholesterol levels but by decreasing the accumulation of inflammatory cells in atherosclerotic plaque.

Methods

Animals

ApoE/− and C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, Me). IL-6/− mice13 were kindly provided by Prof. Wienand and Dr. Mossmann (Max-Planck-Institute for Immunobiology, Freiburg, Germany). Only male mice 53 ± 4 weeks of age, fed a normal rodent diet (Altromin) and water ad libitum, were used for analyses.

Blood Pressure

Systolic blood pressure was measured by a noninvasive tail-cuff method with the BP-2000 blood pressure analysis system (Visitech Systems). All animals underwent repeated measurements on 3 different days 2 weeks before preparation.

Tissue Preparation for Collagen Measurement

Animals were humanely killed after the indicated observation period by intraperitoneal injection of ketamine/xylazine, and aortas were isolated. The adventitia was removed by enzymatic digestion (30 U/L collagenase, (Biochrom) and 12 μg/mL Elastase, Serva) in phosphate-buffered saline, pH 7.4, for 15 minutes at 37°C. The vessel was opened longitudinally and stained with oil red O (Merek). Sections were mounted on slides, and lesion size was quantified while being viewed under a stereomicroscope (Zeiss) coupled to a computerized morphometry system (Quantimet 500, Leica).

Immunohistochemical Analysis

Serial cryostat cross sections were incubated with the following primary antibodies: matrix metalloproteinase (MMP)-9, collagen I, collagen IV, Mac3 for macrophages, and CD45 for leukocytes. The biotinylated secondary antibody was detected by a peroxidase–avidin-biotin-complex system (Vectastain, Vector Laboratories) and diaminobenzidine (Boehringer Mannheim).

Plasma Lipid and Lipoprotein Analyses

Plasma samples were obtained by retrobulbar puncture with heparinized capillary tubes after a 12-hour overnight fast, and lipoproteins were isolated from mouse plasma and determined as described previously.17

Reverse Transcriptase–Polymerase Chain Reaction

Aortic tissue was immediately shock-frozen in LN2.

Gelatin Zymography

Protein samples from aortic tissue were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, supplemented with 1 mg/mL gelatin, as described by Grote et al.5 Please refer to the online-only Data Supplement for details.

Results

WT, IL-6/−, ApoE/−, and ApoE/−–IL-6/− double-knockout male mice were housed for 53 ± 4 weeks while being maintained on a regular rodent chow diet. No differences with regard to survival, morbidity, or behavior were observed. Systolic blood pressure was not different between the strains (101 ± 2 mm Hg in ApoE/−, 106 ± 1 mm Hg in ApoE/−–IL-6/−, 105 ± 3 mm Hg in IL-6/−, and 102 ± 2 mm Hg in WT mice). Aortic plaque extension was predominantly detected in the aortic arch; in branching of the subclavian, renal, and femoral arteries; and in the abdominal aorta, as described before.18 Figure 1A shows representative aortic en face preparations from the 4 mouse strains; plaques were visualized in red (oil red O stain). ApoE/−–IL-6/− animals developed a greater number of and more extensive lesions, and random lipid deposits appeared throughout the entire aorta. Quantitative analysis of relative lesion area revealed significantly enhanced lesion formation in ApoE/−–IL-6/− double-knockout mice compared with ApoE/− mice (Figure 1B; 28.1% versus 14.9%, P < 0.008). IL-6/− and WT control mice did not develop significant lipid deposits (0.2% in IL-6/− and 0.1% in WT mice). Morphometric analysis of defined sections showed that lesion size was significantly increased in ApoE/−–IL-6/− mice (Figure 1C, P < 0.05). Serum lipids were determined in plasma samples after a 12-hour overnight fast (Figure 2). ApoE/−–IL-6/− developed significantly higher levels of total cholesterol, LDL, and VLDL compared with ApoE/− mice, whereas triglycerides were unaffected. Levels of total cholesterol, LDL, and VLDL were significantly higher in ApoE/−–IL-6/− and ApoE/− mice compared with IL-6/− and WT mice because of the apoE knockout; the controls themselves did not differ. Because IL-6 levels may be regulated by IL-10, its synthesis and release were determined (Figure 3). Both aortic IL-10 mRNA expression (Figure 3A, P < 0.01) and IL-10 serum levels were significantly reduced in ApoE/−–IL-6/− mice compared with ApoE/− mice (Figure 3B, P < 0.01). In IL-6/− mice and WT controls, IL-10 transcripts were not detectable, and IL-10 plasma levels were significantly lower compared with those in ApoE/− mice. Because IL-6 and IL-10 may modulate inflammatory cell maturation, differentiation, and recruitment,19,20 we determined the accumulation of inflammatory cells in atherosclerotic lesions of the aortic arch of the apoE-deficient strains with atherosclerosis. We observed a significant reduction of Mac3- and CD45-positive cells (P < 0.01), representing macrophages and leukocytes within sections of ApoE/−–IL-6/− mice (Figure 4), although the plaque load was higher. In IL-6/− mice and WT controls, Mac3- and CD45-positive cells were only occasionally detectable and did not differ between the groups.

In aortic cross sections from ApoE/−–IL-6/− animals stained with Sirius red for total collagen content, we observed disintegration and alteration of extracellular matrix assembly within the medial and adventitial layers of the vessel wall (Figure 5A). Quantification of Sirius red-stained sections of the aortic arch revealed significantly reduced collagen content in plaques of ApoE/−–IL-6/− mice (Figure 5B, P < 0.05 versus ApoE/−). In IL-6/− and WT mice, plaques were too rare and too small to analyze. Similarly, gene expression
levels (summarized in Figure 6) and immunohistochemical staining for collagen I and V (Figure 5C) revealed reduced synthesis of these collagens. In addition, MMP-9 mRNA levels (Figure 6, $P < 0.05$ versus ApoE$^{-/-}$) and protein (Figure 5D), responsible for collagen I and V degradation, were significantly reduced in ApoE$^{-/-}$–IL-6$^{-/-}$ mice. In contrast, the gelatinolytic activity of MMP-9 was significantly increased in the double-knockout mice (Figure 5E, $P < 0.05$ versus ApoE$^{-/-}$). This may be explained by a reduced tissue inhibitor of metalloproteinase (TIMP)-1 mRNA levels (Figure 6, $P < 0.05$), which negatively controls MMP-9 activity. MMP-9 activity was significantly lower in both controls.

Vascular development is also modulated by maturation of extracellular collagens and elastin. Lysyl oxidase controls this process, oxidizes peptidyl lysine within the collagen and elastin molecule, and thereby initiates formation of the covalent cross-linkages between molecules that insolubilize the extracellular matrix proteins. In ApoE$^{-/-}$–IL-6$^{-/-}$ mice, aortic mRNA levels of lysyl oxidase were reduced and not detectable in controls (Figure 6).

**Discussion**

Here we report that a lifetime deficiency of IL-6 in the ApoE$^{-/-}$ model of atherosclerosis resulted in enhanced formation of atherosclerotic lesions, reduced collagen metabolism, and elevated levels of serum cholesterol. IL-6 deficiency blunted the synthesis and release of its counteracting

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Figure 1. A, Impact of IL-6 deficiency on atherosclerotic plaque development in ApoE$^{-/-}$ model. Aortas were prepared for en face analysis and stained with oil red O, whereafter lipid-laden areas appear red. ApoE$^{-/-}$–IL-6$^{-/-}$ mice developed fewer atherosclerotic lesions compared with ApoE$^{-/-}$ mice, and IL-6$^{-/-}$ and WT controls showed no visible lipid deposits. B, Quantitative analysis of whole aortas revealed more extensive lesion area of en face–prepared aortas of ApoE$^{-/-}$–IL-6$^{-/-}$ compared with ApoE$^{-/-}$ mice ($P < 0.008$ vs ApoE$^{-/-}$; $P < 0.001$ vs IL-6$^{-/-}$; and $P < 0.001$ vs WT). Mean ± SEM of n=10 animals per group. C, Larger total plaque area of all plaques measured in sections in defined region of aortic arch. $P < 0.05$. nd indicates not detectable; mean ± SEM of n=10 animals per group. All other abbreviations are as defined in text.
cytokine IL-10 and suppressed the recruitment of proinflammatory cells into the atherosclerotic plaque. Therefore, we suggest that IL-6 elicits a dual modulatory role in atherosclerosis by reducing the lipid accumulation in atherosclerotic lesions and enhancing the recruitment of inflammatory cells to the atherosclerotic plaque, thus modulating vessel extracellular matrix assembly.

IL-6 has long been associated with unstable angina as a potential indicator of plaque destabilization, but a direct link between IL-6 and plaque decomposition has never been shown. In vivo analysis revealed that IL-6 induces monocyte differentiation, MMP expression, and complement activation through acute-phase proteins that may affect the plaque’s fibrous cap composition. In vivo analysis further demonstrated that short-term administration of excessive amounts of exogenous IL-6 resulted in enhanced plaque development in young ApoE−/− mice, also owing to the upregulation of other proinflammatory modulators such as tumor necrosis factor-α and IL-1, especially with high-fat diet feeding. Together with the results that indicate that IL-6 is an important predictor of the recurrence of cardio-

Figure 2. Effect of IL-6 deficiency on serum lipid levels. Total cholesterol and LDL were significantly enhanced in ApoE−/−−IL-6−/− mice, whereas HDL was lowered significantly and triglycerides were not altered. *P<0.05 vs ApoE−/−; †P<0.05 vs IL-6−/−; and ‡P<0.05 vs WT. Mean±SEM of n=7 animals per group. Abbreviations are as defined in text.

Figure 3. Effect of IL-6 deficiency on IL-10 mRNA synthesis and protein release. A, Summary of transcript levels of IL-10 in murine aorta normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression evaluated by semiquantitative RT-PCR (n=9 animals per group). Significant difference between ApoE−/− and ApoE−/−−IL-6−/− mice was observed; in controls, transcription was not detectable. *P<0.01 vs ApoE−/−. nd indicates not detectable, mean±SEM of n=7 animals per group. B, IL-10 plasma levels determined by ELISA technique. IL-10 serum levels were significantly reduced in ApoE−/−−IL-6−/− mice compared with ApoE−/−. Plasma levels of controls (IL-6−/− and WT) were significantly lower than in apoE-deficient groups. *P<0.01 vs ApoE−/−; †P<0.05 vs IL-6−/−; and ‡P<0.05 vs WT, mean±SEM of n=7 animals per group. All other abbreviations are as defined in text.

Figure 4. Effect of IL-6 deficiency on recruitment of macrophages and leukocytes to atherosclerotic lesion, as demonstrated by immunohistochemistry. Quantitative analysis of longitudinal serial sections of aorta revealed significantly smaller relative plaque area stained positive for macrophages (Mac3) and leukocytes (CD45) in ApoE−/−−IL-6−/− animals. *P<0.01 vs ApoE−/−. nd indicates not detectable, mean±SEM of n=9 animals per group. All other abbreviations are as defined in text.
Figure 5. Effect of IL-6 deficiency on extracellular matrix assembly. In Sirius red-stained cross sections under polarized light (A, bar-150 μm), less organized extracellular matrix scaffolding (white arrows) was observed in aortic vessel wall of ApoE−/−IL-6−/− mice and weaker plaque cap (black arrows). B, Morphometric quantification of Sirius red-stained sections of aortic arch revealed that total collagen content was significantly reduced in plaques of ApoE−/−IL-6−/− mice. *P<0.05 vs ApoE−/−. nd indicates not detectable, mean±SEM of n=9 animals per group. In IL-6−/− and WT animals, plaques were not present. C, Immunohistological stainings for collagen I and collagen V demonstrate that collagen proteins were reduced in ApoE−/−IL-6−/− animals (magnification ×100). D, MMP-9 protein distribution in aortic cross sections (immunohistochemistry; magnification ×100). E, Increased MMP-9 activity in ApoE−/−IL-6−/− mice demonstrated by gelatin zymography compared with ApoE−/− and IL-6−/− and WT mice. *P<0.05 vs ApoE−/−; †P<0.05 vs IL-6−/−; and ‡P<0.05 vs WT, mean±SEM of n=7 animals per group. Typical gel zymography is shown. All other abbreviations are as defined in text.
vascular events and observations that have demonstrated an association between IL-6 and acute-phase proteins in patients with unstable angina,4 we hypothesized that a deficiency of IL-6 may reduce plaque formation in experimental atherosclerosis.

To test this hypothesis, we generated ApoE−/−IL-6−/− double-knockout mice. Unexpectedly, a lifetime deficiency of both IL-6 and apoE resulted in enhanced plaque formation, compared with the single apoE-knockout, raising the possibility that IL-6 is involved in basic (patho)physiological regulatory mechanisms of cholesterol homeostasis and vessel development (ie, opposing effects for atherosclerosis). One mechanism may involve the downregulation of IL-10 in ApoE−/−IL-6−/− mice, which is known to decrease serum cholesterol levels through an as-yet-unknown mechanism.23 Thus, the expression and mutual regulation of IL-6 and IL-10 seem to reflect a crucial step not only in the modulation of inflammation but also in the regulation of cholesterol homeostasis. Depletion of IL-6 and IL-10, as observed in the present study, may increase serum cholesterol levels and subsequently enhance plaque formation, presuming a basal inflammation status in both ApoE−/− models, because we did not observe an effect due to IL-6 deficiency alone (IL-6−/− compared with WT). IL-6 deficiency in ApoE−/− mice may also have a direct effect on cholesterol metabolism. In our study and in the absence of a cholesterol-rich diet, these mice had a proatherogenic lipoprotein profile with significantly enhanced plasma total cholesterol levels. IL-6 is a cytokine with multiple and complex endocrine effects on lipid metabolism: administration of IL-6 to nonhuman primates29 and cancer patients31 resulted in a decrease of total cholesterol levels, whereas in healthy volunteers, it caused a significant increase in total cholesterol.24 In patients with myocardial infarction,25 after major surgery,26,27 or on hemodialysis,28 circulating IL-6 levels are correlated negatively with total cholesterol levels. Thus, the molecular and cellular mechanisms that elicit IL-6 effects on lipoprotein metabolism need further investigation. On the basis of current observations, we speculate that in ApoE−/−IL-6−/− mice, a defect in VLDL and LDL catabolism may exist.

Preliminary results indicate that the production rate of the apoB-containing lipoproteins VLDL and LDL is not altered in IL-6−/− deficient animals compared with controls (U.J.F. Tietge, unpublished observations). Therefore, differences only emerge when the turnover rate of lipoproteins is decreased in genetic models like the ApoE−/− mouse. Further studies are currently under way to provide more detailed characterization of the metabolic basis of increased plasma cholesterol levels in ApoE−/−IL-6−/− mice.

Although previous findings indicated that ovariectomized IL-6−/− mice may show enhanced plaque formation,19 we hardly observed any plaque formation in 1-year-old male IL-6−/− mice being fed normal rodent chow. However, in the ApoE−/− model, IL-6 deficiency resulted in enhanced atherosclerosis and also provided evidence that IL-6 may be a major regulator of normal vessel development. Suppression of IL-10 synthesis and release in ApoE−/−IL-6−/− double-knockout mice compared with ApoE−/− mice was associated with a decrease in recruited macrophages and leukocytes at the atherosclerotic lesion, suggesting a reduced inflammatory balance at the atherosclerotic plaque. A balanced interplay between proinflammatory and antiinflammatory mediators (IL-6 and IL-10) may be involved in the perpetuation of atherosclerosis within the vessel wall and may modulate the development of atherosclerotic plaques by recruiting inflammatory cells (Mac3- and CD45-positive) to the plaque.29 This hypothesis is supported by the observation that in IL-6−/− and WT animals without atherosclerotic plaques, IL-10 transcripts and protein levels were not different but were significantly lower than in both ApoE−/− knockout strains. Moreover, previous observations concerning IL-10 suggest that a baseline synthesis of IL-6 is required to maintain IL-10 homeostasis.30 However, IL-10 synthesis and release are not completely blunted despite a lack of IL-6. This observation might be explained by the enhanced serum LDL concentration in the ApoE−/−IL-6−/− mouse model, which also stimulates IL-10 synthesis.31 In addition, IL-6 could, at least in part, be functionally replaced by other cytokines of the IL-6 family, ie, oncostatin M or leukemia...
inhibitory factor, that signal through the same receptor component, gp130.19 Thus, knocking out gp130 would enable us to study not only the proatherogenic effects of the IL-6–cytokine family but also that of the IL-6–dependent acute-phase reaction.

In ApoE<sup>−/−</sup>–IL-6<sup>−/−</sup> mice, reduced concentrations of collagens I and V in aortic plaques were associated with a significantly higher gelatinolytic activity of MMP-9, although MMP-9 protein levels were also reduced. The latter may represent a vascular developmental defect that is induced matrix assembly, which was observed in the present study, 

basic mechanism for organ development.32 MMP activation, which has previously been described as a

assistance.

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may be crucial for lipid homeostasis, plaque formation, and

special vessel development or atherosclerosis. Moreover, hypercholesterolemia, as observed in ApoE<sup>−/−</sup>–IL-6<sup>−/−</sup> mice, downregulates lysyl oxidase44 and subsequently may contribute to lesion formation. Thus, alteration of extracellular matrix assembly, which was observed in the present study, may represent a vascular developmental defect that is induced by a lifetime deficiency of IL-6.

In summary, we have demonstrated that a deficiency of IL-6 in the ApoE<sup>−/−</sup> model elicits Janus-like opposing functions by enhancing atherosclerotic lesion formation and increasing serum cholesterol levels but decreasing the accumulation of inflammatory cells at the atherosclerotic plaque. Together with a reduced synthesis and release of IL-10 in ApoE<sup>−/−</sup>–IL-6<sup>−/−</sup> mice, these results are consistent with the notion that in the process of atherogenesis (in the presence of a hypercholesterolemia), the balance between IL-6 and IL-10 may be crucial for lipid homeostasis, plaque formation, and plaque morphology.

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References


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