Interleukin-18/Interleukin-18 Binding Protein Signaling Modulates Atherosclerotic Lesion Development and Stability

Ziad Mallat,* Anne Corbaz,* Alexandra Scoazec, Pierre Graber, Sami Alouani, Bruno Esposito, Yves Humbert, Yolande Chvatchko, Alain Tedgui

Abstract—Interleukin (IL)-18 is the interferon-γ–inducing factor and has other proinflammatory properties. The precise role of IL-18 in immunoinflammatory diseases remains poorly understood. In this study, we show that in vivo electrotransfer of an expression-plasmid DNA encoding for murine IL-18 binding protein (BP) (the endogenous inhibitor of IL-18) prevents fatty streak development in the thoracic aorta of apoE knockout mice and slows progression of advanced atherosclerotic plaques in the aortic sinus. More importantly, transfection with the IL-18BP plasmid induces profound changes in plaque composition (decrease in macrophage, T cell, cell death, and lipid content and increase in smooth muscle cell and collagen content) leading to a stable plaque phenotype. These results identify for the first time a critical role for IL-18/IL-18BP regulation in atherosclerosis and suggest a potential role for IL-18 inhibitors in reduction of plaque development/progression and promotion of plaque stability. The full text of this article is available at http://www.circresaha.org. (Circ Res. 2001;89:e41-e45.)

Key Words: atherosclerosis ■ inflammation ■ interleukin ■ cytokines

Atherosclerosis is the leading cause of mortality in industrialized countries and carries an important socioeconomic burden. Unabated inflammatory mechanisms are responsible for changes in atherosclerotic plaque composition leading to plaque disruption and to the occurrence of acute ischemic syndromes, namely myocardial infarction and stroke.1 Interleukin (IL)-18 is an inducer of interferon (IFN)-γ with potent activities on inflammatory and vascular cells2 and is thought to contribute to the pathogenesis of chronic immunoinflammatory processes.3,4 Interestingly, we have recently detected increased production of IL-18 by macrophages and smooth muscle cells in unstable human atherosclerotic plaques that were responsible for strokes compared with stable plaques from asymptomatic patients.5 An endogenous IL-18 binding protein (IL-18BP) that neutralizes IL-18 has been identified.6 However, the role of IL-18BP in the modulation of atherogenesis and other chronic immunoinflammatory diseases in vivo is currently unknown. In this study, we examined the role of IL-18/IL-18BP in a well-validated model of atherosclerosis.

Materials and Methods

In Vivo Intramuscular Electrotransfer of Murine IL-18BP Expression Plasmid

Fourteen male C57Bl/6 apoE knockout mice, 14 weeks old, received at 3-week intervals, 3 injections with the murine IL-18BP expression plasmid, pcDNA3-mIL18BP. The control mice (n=19) were injected with the control empty plasmid. Murine IL-18BP isoform d cDNA (accessory number #AF110803), isolated as described,6 was subcloned into the EcoR1/Not1 sites of mammalian cell expression vector pcDNA3 under the control of the cytomegalovirus promoter (Invitrogen). Control vector was a similar construct devoid of therapeutic cDNA. The construct with mL18BP isoform d in pCDNA3 plasmid was tested for expression and activity. This was performed using culture supernatants obtained from HEK 293/Ebna cells transfected with mL18BPd in pCDNA3 vector. We verified that the protein product was able to bind and neutralize mL18 in a bioassay performed with murine spleen cells, as previously described.7,8 The IL-18BP or control expression vector (60 μg) was injected in both tibial cranial muscles of the anesthetized mouse, as previously described. Briefly, transiently electric pulses (8 square wave electric pulses of 200 V/cm, 20 ms duration at 2 Hz) were delivered by a PS-15 electropulsator (Genetronics) using two stainless steel plate electrodes, placed 4.2 to 5.3 mm apart, at each side of the leg. To measure plasma levels of murine IL-18BP, microtiter 96-well ELISA plates (Maxisorb; Nunc) were coated overnight at 4°C with 5 μg/mL of rabbit polyclonal antibody raised against recombinant mL18BPd prepared in E. coli and then affinity purified on rmIL-18BPd produced in HEK 293/ebna cells. Soluble mL18BPd was detected using a biotinylated rabbit polyclonal anti–mIL-18BP (Peprotech) followed by incubation with peroxidase conjugated extravidin (diluted at 1:10,000) (Sigma). The plates were then developed by the addition of OPD peroxidase substrate (o-phenylenediamine dihydrochloride tablets, Sigma), and the absorbance was measured at 492 nm. Recombinant mL18BPd prepared in HEK 293/ebna cells was used as standard. The sensitivity of the
ELISA was 5 ng/mL. In addition, the serum IL-18BP activity was assessed in a bioassay using murine cells.7,8

Analysis of Mice
Cryostat sections (8 μm) were obtained from the aortic sinus and were used for detection of lipid deposition using Oil red, for detection of collagen using Sirius red, and for immunohistochemical analysis, as previously described.10 The sections were stained with specific primary antibodies: antimouse macrophage, clone MOMA-2 (BioSource), phosphatase alkaline-conjugated anti-α-actin for smooth muscle cells, and anti-CD3 for T lymphocytes (Dako), as previously described.9 Detection of cell death was performed using the TUNEL technique.11 CD3 positive cells were microscopically counted in a blinded manner. Atherosclerotic plaques in the aortic sinus and areas that stained positive for macrophages, smooth muscle cells, collagen, or TUNEL were measured blindly by one observer using computer-assisted-image semi-quantification (NS15000, Microvision), as previously described.10 For each stain, the threshold was predetermined10 and remained the same for all analyzed sections from the 2 groups of mice. Intraobserver variability was less than 10%. Specificity of the immunostaining was assessed by staining with nonimmune isotype-matched immunoglobulins. Specificity of TUNEL was assessed by omission of the enzyme terminal deoxynucleotidyl transferase. The thoracic aorta, spanning from the left subclavian artery to the renal arteries, were fixed with 10% buffered formalin and stained for lipid deposition with Oil red. They were then opened longitudinally, and the percentage of lipid deposition was calculated using computer-assisted image semiquantification (NS15000, Microvision).

Statistical Analysis
Values are expressed as mean±SEM. Comparisons between groups were made by use of ANOVA. A value of P<0.05 was considered statistically significant.

Results
We tested the hypothesis that endogenous IL-18 affects atherosclerosis development and stability by using apoE knockout (KO) mice that spontaneously develop human-like atherosclerotic lesions. Fourteen 14-week-old male mice received IL-18BP supplementation through in vivo intramuscular electrotransfer of an expression-plasmid DNA encoding for isoform d of murine IL-18BP, while 19 age-matched mice received the empty plasmid (controls). Plasmid electrotransfer was repeated every 3 weeks, and the mice were euthanized at 23 weeks of age after 9 weeks of treatment. Plasma levels of murine IL-18BP were below the detection limit of the assay in apoE KO mice injected with the empty plasmid. However, a single injection of the IL-18BP plasmid resulted in high levels of IL-18BP in the blood with a maximal rise 2 days after the injection (323.5±100.9 ng ml−1) and persistently high levels measured after 2 weeks (127.4±35.4 ng ml−1). In addition, a serum dilution of 1:90 from mice transfected with the IL-18BP plasmid completely abrogated IFN-γ production in the murine spleen cell bioassay, indicating an in vivo inhibitory activity against IL-18. Following 9 weeks of treatment with either IL-18BP or empty plasmid, total cholesterol (489.4±34.6 versus 480.8±36.3 mg/dL, respectively) and high-density lipoprotein serum levels (52.3±9.4 versus 48.8±5.1 mg/dL, respectively) were not different between the 2 groups. We observed a modest but significant increase in animal weight in the IL-18BP plasmid group compared with the control group (31.8±0.9 versus 28.6±0.8 g, respectively, P<0.05).

Figure 1. Reduction of atherogenesis by IL-18BP treatment. Representative photomicrographs (lipid staining) show thoracic aorta from control mice treated with the empty vector (a) and from mice treated with IL-18BP (b). c, Quantitative computer-assisted image analysis of lipid deposition. Data represent mean±SEM (n=19 for empty plasmid, n=14 for IL-18BP plasmid). ****P<0.0001.

IL-18BP cDNA Transfer Prevents Atherosclerosis Development and Progression
We examined the effects of IL-18BP supplementation on atherosclerosis in 2 different locations: the descending thoracic aorta and the aortic sinus. The thoracic aorta was chosen to determine the role of IL-18BP in fatty streak development (atherogenesis) because thoracic atherosclerotic lesions are almost absent at the age of 14 weeks (data not shown) when IL-18BP transfection was started. The aortic sinus, where atherosclerotic lesions are already present at 14 weeks of age (data not shown), was examined for advanced plaque progression and composition, an important determinant of plaque stability. Treatment of apoE KO mice with the IL-18BP plasmid significantly affected atherosclerotic lesion development and progression. Examination of the thoracic aorta showed a marked reduction in lipid deposition in mice treated with the IL-18BP plasmid compared with controls (Figure 1). Quantitative computer-assisted image analysis showed 69% reduction in the extent of atherosclerotic lesions (P<0.0001) (Figure 1), pointing to a critical permissive role for IL-18 in atherogenesis. In addition, treatment with IL-18BP plasmid for only 9 weeks significantly limited the progression of advanced atherosclerotic plaques in the aortic sinus (24% reduction in plaque size, P<0.01) compared with treatment with the empty plasmid (Figure 2).

IL-18BP cDNA Transfer Induces a Stable Plaque Phenotype
Interestingly, the composition of advanced lesions, a major determinant of plaque instability, was profoundly affected by IL-18BP treatment. Atherosclerotic lesions of mice treated with the IL-18BP plasmid exhibited a very significant 50%
reduction in macrophage infiltration \( (P<0.0001) \) (Figures 3 and 4), contained 67\% fewer T lymphocytes \( (P<0.005) \) (Figure 4), and showed a 2-fold increase in smooth muscle cell accumulation \( (P<0.05) \) (Figures 3 and 5). In addition, these important changes in lesion cellular composition were associated with a significant 85\% increase in collagen content \( (P<0.0005) \), as determined by staining with Sirius red (Figures 3 and 5), and a decrease in total lipid content (Figure 3). Therefore, IL-18BP treatment significantly attenuated the inflammatory process within the atherosclerotic lesions and induced a healing process characteristic of stable atherosclerotic plaques. Furthermore, the marked reduction in the inflammatory component of the lesions in IL-18BP–treated mice was associated with a substantial reduction in the occurrence of cell death within the plaques \( (2.9 \pm 0.9\% \text{ in IL-18BP–treated mice versus } 10.5 \pm 3.6\% \text{ in controls, } P<0.05) \) (Figure 3), therefore limiting the expansion and thrombogenicity of the acellular lipid core.\textsuperscript{12}

**Discussion**

Using a well-validated mouse model of human-like atherosclerosis, our results clearly establish an unsuspected and crucial role for IL-18 signaling pathway in atherosclerotic plaque development, progression, and stability. While preventing early lesion formation in the thoracic aorta, inhibition of IL-18 signaling by IL-18BP supplementation also profoundly affected advanced lesion composition in the aortic sinus, inducing a switch toward a stable plaque phenotype.

Severe clinical manifestations of atherosclerosis (infarctions of the heart and brain) are mainly due to vessel lumen occlusion by a thrombus formed at the contact of a disrupted atherosclerotic plaque.\textsuperscript{13} Pathological studies have shown that vulnerable or unstable plaques are rich in inflammatory cells and exhibit a substantial loss in smooth muscle cell and collagen content.\textsuperscript{14,15} Moreover, such plaques show significant increase in apoptotic cell death leading to the formation of a highly thrombogenic lipid core.\textsuperscript{5,12,16} It is noteworthy that all these signs of increased plaque instability were markedly attenuated in IL-18BP treated mice, indicating that IL-18 activity is a major determinant of plaque instability. The relevance of the results obtained in apoE KO mice to human
disease is strengthened by our finding of increased IL-18 production in unstable carotid atherosclerotic plaques responsible for stroke.5

Mainly produced by activated macrophages, IL-18 promotes the action of IL-12, which favors T-lymocyte differentiation along the Th1 lineage. IL-18 and IL-12 act synergistically to induce the production of IFN-γ in T cells, natural killer cells, and subsets of macrophages. There is a unique synergism between IL-18 and IL-12 in the induction of IFN-γ.17 This may explain the critical role of IL-18 in atherosclerotic lesion since IFN-γ signaling has been previously reported to promote atherosclerosis.18 In this context, it is noteworthy that IL-18BP has been shown to decrease endogenous IL-18 activity by reducing IFN-γ-mediated responses.19 This mechanism also leads to enhanced prostaglandin E2 production,19 which could be deleterious to the atherogenic process. However, it is unlikely that this latter mechanism is involved in our findings because Pratico et al20 recently showed that inhibition of thrombomodulin, but not prostaglandin E2 production, decreases atherosclerosis development in LDL-R knockout mice fed an atherogenic diet. Other mechanisms may be involved in the proatherogenic effects of IL-18. By inducing TNF-α, IL-1-β, Fas ligand, and the expression of chemokines (eg, IL-8, MIP-1α, MCP-1) and adhesion molecules3 on vascular cells, IL-18 may directly control both mononuclear cell accumulation and cell death within the plaque: two important determinants of plaque disruption and thrombosis. Moreover, IL-18 has been recently shown to contribute to cardiac dysfunction following ischemia-reperfusion in vitro.21 These findings, taken together, identify inhibitors of IL-18 signaling as new important therapeutic tools to prevent atherosclerotic plaque development and to limit plaque complications.

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Figure 4. Effect of IL-18BP treatment on lesion inflammatory cell content. Quantitative computer-assisted image analysis was used to determine the percentage of macrophage-positive areas (black bars) and the number of infiltrating T lymocytes per mm² (gray bars) in aortic sinus lesions of control (n=12 for macrophage staining, n=15 for T-lymocyte staining) or IL-18BP-treated mice (n=13 for macrophages, n=12 for T lymocytes). Immunostainings for macrophages and T lymocytes were performed as described in Materials and Methods. Data represent mean±SEM. ***P<0.005, ****P<0.0001.

Figure 5. Effect of IL-18BP treatment on lesion smooth muscle cell and collagen content. Quantitative computer-assisted image analysis was used to determine the percentage of smooth muscle cell–positive areas (black bars) and collagen accumulation (gray bars) in aortic sinus lesions of control (n=6 for smooth muscle cells, n=11 for collagen) and IL-18BP-treated mice (n=6 for smooth muscle cells, n=13 for collagen). Immunostainings for smooth muscle cells and collagen were performed as described in Materials and Methods. Data represent mean±SEM. *P<0.05, **P<0.01.
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