Interleukin-1 Receptor Signaling Mediates Atherosclerosis Associated With Bacterial Exposure and/or a High-Fat Diet in a Murine Apolipoprotein E Heterozygote Model

Pharmacotherapeutic Implications

Hunghui Chi, DDS; Emmanuel Messas, MD, MSc; Robert A. Levine, MD; Dana T. Graves, DDS, DSc; Salomon Amar, DDS, PhD

Background—Current data demonstrate that progressive atherosclerosis is associated with activation of the inflammatory process, as evidenced by systemic elevations of molecules such as tumor necrosis factor, interleukin (IL)-6, and IL-1. It has been postulated that inflammatory events within an atherogenic lesion are induced by oxidized LDL. Recent evidence suggests that infectious agents, including those that cause periodontal disease, may also play an important role. Studies presented here tested the hypothesis that IL-1 receptor (IL-1R1) signaling plays a crucial role in bacteria- and/or high-fat diet (HFD)—enhanced atherosclerosis.

Methods and Results—Ten-week-old ApoE<sup>−/−</sup> mice lacking either 1 IL-1R1 allele (ApoE<sup>−/−</sup>/IL-1R1<sup>−/−</sup>) or 2 IL-1R1 alleles (ApoE<sup>−/−</sup>/IL-1R1<sup>−/−</sup>) fed either an HFD or regular chow were inoculated intravenously with live Porphyromonas gingivalis (P gingivalis) (10<sup>7</sup> CFU), an important periodontal pathogen, or vehicle once per week for 14 or 24 consecutive weeks. Histomorphometry of plaque cross-sectional area in the proximal aortas, en face measurement of plaque area over the aortic trees, and ELISA for systemic proinflammatory mediators were performed. Atherosclerotic lesions of proximal aortas and aortic tree were substantially reduced in ApoE<sup>−/−</sup>/IL-1R1<sup>−/−</sup> mice than in ApoE<sup>−/−</sup>/IL-1R1<sup>−/−</sup> mice challenged with P gingivalis. At 24 weeks after P gingivalis inoculation, proximal aortic lesion size quantified by histomorphometry was 5-fold–reduced in chow-fed ApoE<sup>−/−</sup>/IL-1R1<sup>−/−</sup> mice than in ApoE<sup>−/−</sup>/IL-1R1<sup>−/−</sup> mice (P<0.05). In the HFD group, ApoE<sup>−/−</sup>/IL-1R1<sup>−/−</sup> mice exhibited marked attenuation of the progression of atherosclerotic lesions (78% to 97%), with and without P gingivalis inoculation (P<0.05).

Conclusion—Ablation of IL-1R1 under P gingivalis challenge and/or an HFD reduced the progression of atherosclerotic plaques. These results indicate that IL-1 plays a crucial role in bacteria- and/or HFD-enhanced atherosclerosis. (Circulation. 2004;110:1678-1685.)

Key Words: atherosclerosis ■ infection ■ interleukins ■ cardiovascular diseases ■ Porphyromonas gingivalis

Atherosclerotic cardiovascular disease (ASCVD), contributing to an estimated one third of all deaths, is not clearly associated with its classic risk factors in more than one third of patients dying from it, which suggests other contributory mechanisms. There is increasing evidence for a systemic inflammatory process in patients with ASCVD and corresponding animal models, with the progression of atherosclerosis mirroring that of a chronic inflammation. The case for an inflammatory mechanism is strengthened by evidence for triggering or potentiation of atherosclerosis by exposure to infectious agents. The latter may involve the systemic consequences of infection or the effect of direct vascular invasion by microorganisms. The potential therapeutic implications of both inflammatory and infectious activations warrant investigation.

We and others have described a model in which systemic exposure to an oral pathogen, Porphyromonas gingivalis (P gingivalis), potentiates the development of atherosclerosis in mice with genetic and dietary susceptibility. In both models, progressive atherosclerosis was associated with activation of the inflammatory process, as evidenced by increases in molecules such as serum amyloid–associated protein (SAA) and interleukin-1 (IL-1). The question remains, however, whether the inflammatory changes play a causal role in the atherosclerotic process or are only secondarily involved. This question can be addressed in a therapeutically relevant manner by genetically or pharmacologically modifying key components of the inflammatory process.
One such component is IL-1, a multifunctional cytokine secreted by monocytes/macrophages, polymorphonuclear neutrophils, and vascular endothelial and smooth muscle cells. IL-1 binding to the IL-1 receptor 1 (IL-1R1) and its accessory protein triggers intracellular signal transduction to activate the synthesis of vasoactive substances, growth factors, and cytokines, as well as increases the expression of vascular adhesion molecules and chemokines that attract monocyte-derived cells, enhance diapedesis, and stimulate their proliferation and differentiation. IL-1 is therefore a suitable target for modification in testing the role of inflammation in atherosclerosis. To date, several approaches have partially modified IL-1 or its effects. IL-1 has been neutralized by anti-IL-1 antibodies and soluble IL-1Rs. Its level is now successfully lowered by blocking its transcription and synthesis with corticosteroids, nonsteroidal antiinflammatory drugs, cyclooxygenase/looxygenase inhibitors, transforming growth factor-β, IL-4, IL-10, and IL-1β converting enzyme inhibitor. Short-term perfusion with an IL-1R antagonist has been shown to modify fatty-streak formation in apolipoprotein E (ApoE)-deficient mice, and genetic overexpression of this antagonist promoted atherosclerosis in LDL receptor–knockout mice fed a high-fat diet (HFD); absence of this antagonist conversely promoted neointimal formation after injury. Recently, genetic deletion of IL-1β was shown to decrease atherosclerotic severity in ApoE–/– animals, but only by ~30%, perhaps because of persistent activation of the IL-1R by IL-1α. In contrast, IL-1R2 is a nontransducing decoy receptor. The purpose of the present investigation was to test the hypothesis that blocking IL-1R signaling by genetic deletion of IL-1R1 reduces bacteria and/or diet-enhanced atherosclerosis. Specifically, in the model used, atherosclerosis is potentiated by exposure to an infectious agent, P. gingivalis, a common periodontal pathogen, in the presence of predisposing genetic and environmental factors (ApoE deficiency and HFD).

Methods

Bacterial Strain

P. gingivalis strain AT7436 was cultured on anaerobic blood agar plates (Becton Dickinson Co) in an aerobic chamber (Coy Laboratory Products Inc) with 85% N₂, 5% H₂, and 10% CO₂ for 3 to 5 days and then inoculated into Schaedler broth (Difco Laboratories) containing hemin and menadione for 24 hours until the culture reached an optical density of 0.8 at 660 nm, corresponding to ~10⁷ colony-forming units (CFU)/mL. Cultured P. gingivalis and culture medium were diluted with saline in the same dilution before inoculation.

Mice and Diet

The Institutional Animal Care and Use Committee of Boston University approved all animal protocols. Male ApoE homozygotes (ApoE−/−; strain B6.129P2-Apoetm1Unc, backcrossed for at least 10 generations to the C57BL/6J background) and female IL-1R1-knockout mice (IL-1R1−/−, strain B6.129S1−/−Il1r1tm15Hai/Hai) were obtained at 8 weeks of age from Jackson Laboratories (Bar Harbor, Me) and fed regular mouse chow. ApoE−/−/IL-1R1−/− mice were generated by crossing male ApoE−/− with female IL-1R1−/− mice. After an 8-week growing period, male ApoE−/−/IL-1R1−/− mice were backcrossed with female IL-1R1−/− mice, followed by genetic screening of the offspring through a polymerase chain reaction method (genotyping protocols from Jackson Laboratory). The experimental protocols are shown in Figure 1. ApoE−/−/IL-1R1−/− and ApoE−/−/IL-1R1−/− mice weaned at 4 weeks of age were randomly assigned to either an HFD containing 1.25% cholesterol, 15% fat, and 0.5% cholate (No. 21539, TestDiet) or regular mouse chow. ApoE−/−/IL-1R1−/− mice were backcrossed with female IL-1R1−/− mice, followed by genetic screening of the offspring through a polymerase chain reaction method (genotyping protocols from Jackson Laboratory).

Tissue Harvesting and Preparation

After an overnight fast, mice were heavily sedated with inhaled isoflurane (SOLVAY) and exsanguinated from the femoral arteries. The heart and aorta were perfused for 10 minutes with ice-cold phosphate-buffered saline containing 20 µmol/L butylated hydroxytoluene (Sigma) and 2 mmol/L EDTA, pH 7.4, through a left ventricular cannula. Perfusion was continued for another 10 minutes with cold formal-sucrose solution (10% neutral formalin, 5% sucrose, 20 µmol/L butylated hydroxytoluene, and 2 mmol/L EDTA, pH 7.4). The aortic tree and proximal aorta were separated close to the heart. The aortic tree, including ascending aorta, arch, descending thoracic aorta, and abdominal aorta, was processed for en face analysis as described by Palinski. The proximal aorta (ascending thoracic aorta or aortic annulus alone) was embedded together with the heart in Histo Prep (Fisher Scientific) for cryosectioning. Liver, kidney, and spleen were collected for routine paraffin sections and hematoxylin-eosin staining.

Morphometric Analysis

En Face Morphometric Analysis of the Aortic Tree

The extent of atherosclerosis in the aortic tree was determined by en face quantification. The aortic tree was briefly rinsed in 70% ethanol, followed by staining with a solution containing 0.5% Sudan IV (Sigma), 35% ethanol, and 50% acetone for 6 minutes, and destaining in 80% ethanol for 5 minutes. Images of the aortic tree were
captured with a Sony color video camera mounted on a stereomicroscope. Sudan red lesions were quantified with a computer-assisted image analysis system (Image Pro Plus 4.0; Media Cybernetics). The extent of atherosclerosis was expressed as a percentage of the aortic surface area covered by lesions.

**Histomorphometric and Histopathologic Analysis of Atheroma Lesions in the Proximal Aorta**

Proximal aortic cross sections for quantitative and histopathologic evaluation of atherosclerotic lesions were prepared as previously described. Four sections per animal, each separated by 80 μm, were stained (Sudan IV), counterstained (hematoxylin), and then evaluated quantitatively with a computer-assisted image analysis system. The luminal edge of the lesions was traced with the help of the edge detection function, and the intimal-medial border was traced manually. Lesion cross-sectional areas from 3 images were added to obtain the total lesion area per cross section slide. The percentage of total aortic lumen occupied by lesions per section was then calculated. The total lesion area and the percentage of total aortic lumen occupied by lesions were averaged over 4 sections per animal and expressed as mean lesion area and percentage of total lumen of the proximal aorta occupied by lesions per section per animal.

**Serum Amyloid A**

The acute-phase protein SAA was detected by ELISA (Tri-Delta Labs). Venous blood was collected at euthanasia. After clotting at room temperature, blood samples were centrifuged for 30 minutes at 3500 rpm, and the supernatant was collected and stored at −20°C. Dilutions and determination of standards were performed according to the instructions of the manufacturer. Optical density was determined within 30 minutes of colorimetric reaction at 450 nm; corrections were made at 550 nm.

**Statistical Analysis**

All of the en face specimens and histomorphometric measurements were coded so that the measurements were done blindly. All quantitative measurements were confirmed by random reanalysis of approximately one fourth of the specimens by the same examiner (R > 0.92) and by another independent examiner (pathologist) to ensure consistency. The intraexaminer and interexaminer variation was < 10%. A level of P < 0.05 was considered significant. Extent of atherosclerosis was analyzed by ANOVA (2-way) among groups and subsequently by Student’s paired 2-tailed t test.

**Results**

**Clinical Assessment**

No clinical signs of infection or mortality were noted in any of the animals at any time. There were no significant differences in body weight between P gingivalis–inoculated and noninoculated mice. The heart, kidney, spleen, gall bladder, and small intestine showed normal histological structure.

**Morphometric Analysis**

**En Face Morphometric Analysis of the Aortic Tree**

In chow-fed groups, the sensitivity of this gross pathological technique did not allow detection of any lesion at any time point in any of the groups. In HFD groups, red-stained, lipid-rich lesions were present in both P gingivalis–inoculated and noninoculated mice, and the percentage of affected area increased in subsequent weeks. At 14 weeks, there was no statistically significant difference between the small proportions of affected surface area (≤ 0.1%) in the ApoE+/+/IL-1R1+/- and ApoE+/+/IL-1R1−/− groups with or without P gingivalis inoculation. At 24 weeks, with HFD, there were notable increases in lesion surface area in ApoE+/+/IL-1R1+/- mice, greater with P gingivalis inoculation than without inoculation; in contrast, with complete deletion of IL-1R1, there was no significant progression in lesion area from 14 to 24 weeks, and area was considerably lower than in the ApoE+/+/IL-1R1−/− mice (P < 0.05). This significant reduction of atherosclerosis development was observed in both P gingivalis–inoculated and noninoculated groups (93% reduction with P gingivalis HFD and 79% with only HFD; Figure 2). These lesions were flat or slightly protruding into the vascular lumen and were scattered along the inner curvature of the arch and near the orifices of the intercostal and large abdominal arteries, with the gross appearance of early-stage lesions (Figure 3A through 3D).

**Histomorphometric Analysis of Atheroma Lesions in the Proximal Aorta**

As shown in Figure 4, percentage area occupied by lesions increased over time in all 4 different conditions. At 14 weeks, chow-fed animals showed only mild foam cell lesions, both with or without P gingivalis inoculation, with no statistically significant differences between the ApoE+/+/IL-1R1+/- and ApoE+/+/IL-1R1−/− mice. At 24 weeks in chow-fed animals, this percentage parameter was 5-fold higher in ApoE+/+/IL-1R1+/- mice compared with ApoE+/+/IL-1R1−/− mice (P < 0.05) in P gingivalis–challenged groups. These differences were more pronounced in HFD animals, which exhibited larger atherosclerotic lesions. At 14 weeks, the differences between ApoE+/+/IL-1R1+/- and ApoE+/+/IL-1R1−/− mice were not statistically significant. However, at 24 weeks, ablation of IL-1R1 markedly attenuated the otherwise progressive atherosclerotic lesions, both with and without P gingivalis inoculation (P < 0.05; Figure 4). Comparison of the lesion area progression at the 2 time points in ApoE+/+/IL-1R1+/- mice challenged with HFD and P gingivalis (Figure 5) disclosed a 35-fold increase of lesion area from 14 weeks to 24 weeks. However, in the ApoE+/+/IL-1R1−/− mice, the
lesion area was only 2.5 times greater at 24 weeks than at 14 weeks, demonstrating significant reduction of atherosclerosis development (14-fold) in ApoE \( \text{H11001}/\text{H11002}/\text{H11002}/\text{IL-1R1}/\text{H11002} \) mice compared with ApoE \( \text{H11001}/\text{H11002}/\text{IL-1R1}/\text{H11001}/\text{H11002} \) mice. 

Histopathologic Aspect of Atherosclerotic Lesions at the Proximal Aorta

Histopathologic observation of sudanophilic lesions was performed on each proximal aorta cross section (Figure 6). Lesions progressed more slowly in vehicle-challenged mice than in \( P \) \( \text{gingivalis} \)-challenged mice. Also, chow-fed mice had smaller lesions than did HFD mice. ApoE \( ^{+/+} \)/IL-1R1 \( ^{+/+} \)-mice exhibited milder atheromatous lesions than did ApoE \( ^{+/+} \)/IL-1R1 \( ^{+-} \)-mice. Two stages were observed in the progression of the lesions. The first stage was characterized by sudanophilic lesions resembling foam cell lesions (Figure 6). Lesions related to the aortic valve attachments and Valsalva sinus were more frequent and developed earlier than the lesions in the free aortic wall. The second stage consisted of a mixture of sudanophilic cells and spindle-shaped cells (Figure 6). Stained cells were observed adhering to the surface of the endothelial lining within the lesions. In general, lesions occurred preferentially in 2 locations: the aortic valve attachments (commissures) and the free aortic wall.

Systemic Proinflammatory Factor: SAA

ELISA was used to assess the serum acute-phase reactant SAA, the mouse counterpart of human C-reactive protein (CRP), at 14 weeks and 24 weeks after bacterial inoculation with and without HFD. As shown in Figure 7A and 7B, animals that had the highest risk of developing atherosclerosis (ApoE \( ^{+/+} \)/IL-1R1 \( ^{+-} \)) fed the HFD and challenged with \( P \) \( \text{gingivalis} \) had by far the highest level of SAA. SAA levels were 4 to 8 times higher when they were heterozygous for IL-1R1 than when they had complete ablation of IL-1R1, regardless of the treatment group, demonstrating the critical
that exposure to microbial pathogens can potentiate atherosclerosis and its associated inflammatory changes. Studies have attempted to reduce the level or action of cytokines such as IL-1, which has been shown to recruit and stimulate cells into the inflammatory process. Recently, Kirii et al recently showed that, in ApoE<sup>−/−</sup> mice, genetic ablation of IL-1β reduced atherosclerosis by only 30%, perhaps because of persistent IL-1α stimulation of the IL-1R or because of alternative pathways. To establish a causative role for bacteria-enhanced atherosclerosis and dissect the inflammatory pathway, we have used our murine model (ApoE<sup>−/−</sup>) genetically deficient in IL-1R1 signaling.

The present study shows that the complete absence of IL-1R1 markedly reduces the progression of atherosclerosis in ApoE<sup>−/−</sup> mice at 24 weeks of exposure to HFD and P gingivalis injections, experimental conditions known to aggravate vascular lesions. This effect of IL-1R1 absence persists whether the inciting factors are genetic, dietary, or infectious, alone or in combination; thus, ApoE<sup>−/−</sup>/IL-1R1<sup>−/−</sup> mice have reduced severity of atherosclerosis at 24 weeks compared with ApoE<sup>−/−</sup>/IL-1R1<sup>−/+</sup> mice, whether fed chow or an HFD, and whether inoculated with P gingivalis or not. These results confirm the crucial role that IL-1 plays in the inflammatory cascade involved in the progression of atherosclerosis and suggests that both bacteria and diet mediate the response through an IL-1 signaling pathway, yet the nodal point of conversion for these 2 pathways remains unknown.

IL-1 gene polymorphisms have been associated with a number of inflammatory and infectious diseases, including periodontitis, leading to the suggestion that the IL-1 locus may be responsible for a link between periodontal disease and ASCVD. Other studies suggest a crucial role for IL-1 in the atherosclerotic process, even at its earliest foam cell stage. On the basis of incomplete effects of IL-1 on atherosclerosis in prior studies, we planned to neutralize the effect of IL-1 as completely as possible with a knockout IL-1R1 receptor mouse model. In the present study, histopathologic analysis of the ApoE<sup>−/−</sup>/IL-1R1<sup>−/−</sup> mice exhibited reduced atherosclerotic lesions, suggesting that IL-1R knockout diminishes the hemoattractant effect on circulating monocytes. IL-1R absence also attenuates the systemic rise in SAA protein seen in P gingivalis–injected ApoE<sup>−/−</sup> mice fed an HFD. Studies show that CRP, the human counterpart of SAA, can have a protective role in limiting the inflammatory response. However, substantial data suggest that CRP exhibits direct proinflammatory effects on endothelial cells. Elevated serum CRP is being taken as a powerful predictive marker of adverse outcome in ASVCD. Thus, IL-1R deletion may attenuate atherosclerosis both at the local cellular and systemic inflammatory levels.

If this effect of blocking IL-1 on atherosclerosis is confirmed, this cytokine could become a target of antiatherosclerosis therapy. Care should be taken, however, not to block IL-1 synthesis in the postinfarction period, as a recent animal study showed that anti–IL-1β treatment suppressed procollagen gene expression and delayed wound healing mechanisms, which may promote left ventricular remodeling. The hope is that blocking this cytokine will help limit the progression of atherosclerotic plaque and so decrease acute coronary syn-
Figure 6. Cross sections of aortic lesions in chow-fed or HFD mice 24 weeks after *P. gingivalis* or vehicle inoculation. In HFD group, larger and more advanced lesions are observed in *P. gingivalis*-challenged ApoE^{+/−}/IL-1R1^{+/−} mice (A) than in ApoE^{+/−}/IL-1R1^{−/−} mice (B). Significant number of sudanophilic stained cells can be seen at ×200 (A). Smaller but well-established lesions are present in vehicle-challenged ApoE^{+/−}/IL-1R1^{−/−} mice (C) and ApoE^{+/−}/IL-1R1^{−/−} mice (D) (ApoE^{+/−}/IL-1R1^{−/−} greater than ApoE^{+/−}/IL-1R1^{−/−}). In chow-fed group, lesions are observed in ApoE^{+/−}/IL-1R1^{−/−} mice with *P. gingivalis*-challenge (E) but greatly reduced in ApoE^{+/−}/IL-1R1^{−/−} (F). Smaller lesions are found in ApoE^{+/−}/IL-1R1^{−/−} mice (G) and ApoE^{+/−}/IL-1R1^{−/−} mice that were vehicle challenged (H). L indicates aortic lumen; I, intima; M, media; A, adventitia; and arrow, sudanophilic cells. Abbreviations are as defined in text.
dromes or stroke. Moreover, some anti-IL-1 drugs such as a recombinant version of the human IL-1R antagonist (Kineret) are already in clinical use for arthritis without notable side effects.49

The present study applied only one infectious agent of the type associated with atherosclerosis.50 However, P gingivalis shares some pathogenic characteristics with other reported infectious agents, including endothelial and smooth cell invasion and presence in human atherosclerotic plaques.17 Longer follow-up will be needed to fully explore the effects of IL-1R deletion in atherosclerosis.

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**References**

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