Immunopathogenesis of Atherosclerosis

Endotoxin Accelerates Atherosclerosis in Rabbits on Hypercholesterolemic Diet

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Background—On the basis of our concept that atherosclerosis has an immunopathological background, we tested whether activation of the innate immune system influences its progression.

Methods and Results—Hypercholesterolemic (0.5% wt/wt diet) rabbits received either repeated intravenous injections of endotoxin (Escherichia coli lipopolysaccharide 1.25 to 2.5 μg, once per week) or a self-limiting cutaneous Staphylococcus aureus infection with or without a quinolone antibiotic. Measured laboratory parameters, including LDL and HDL cholesterols, were similar in the different groups of hypercholesterolemic animals. All endotoxin-treated animals developed transient episodes of fever after endotoxin administration. The extent of atherosclerosis was evaluated by computer-assisted morphometry in the aortas en face (Sudan IV) and by histology at 8 weeks after start of the experiments. Endotoxin-treated animals exhibited significantly accelerated atherosclerosis compared with control animals (141±38 versus 45±16 mm³ total lesion volume, n=7 to 9 rabbits each, P<0.001).

Conclusions—Nonspecific stimulation of the innate immune system accelerates cholesterol-induced atherosclerosis. These data support the concept that atherosclerosis has an immunopathological component and render it improbable that a single infectious agent should assume particular importance in its initiation or progression. (Circulation. 2001;104:914-920.)

Key Words: imaging ■ immunology ■ atherosclerosis ■ endotoxin

Atherosclerosis shares many features with inflammatory reactions (reviewed in Reference 1). Fatty streaks, the earliest lesions of atherosclerosis, are composed largely of lipid-laden macrophages derived from circulating blood monocytes that adhere to and emigrate across the endothelium of middle-sized and large arteries.

One of the authors (S.B.) hypothesized that enzymatic degradation of LDL is centrally important in atherogenesis. Proteolytic degradation of apolipoprotein B in conjunction with cleavage of cholesteryl esters generates lipoprotein droplets that are similar to lipoprotein derivatives that were earlier isolated from atherosclerotic lesions. Enzymatically modified LDL (E-LDL) induces foam cell formation, binds C-reactive protein (CRP) to activate complement, and induces upregulation of adhesion molecules on endothelial cells to promote selective transmigration of monocytes and lymphocytes across cell monolayers. In contrast, oxidized LDL does not activate complement. Immunohistochemical evidence has been obtained that E-LDL is extensively distributed in early atherosclerotic lesions and in colocalization with CRP and complement C5b-9 complexes.

Enzymatic transformation of LDL into a complement-activating molecule is thought to be physiologically meaningful because it initiates removal of stranded cholesterol from the vessel wall. Atherosclerosis is proposed to ensue when the physiological transport system suffers overload, because detrimental effects are evoked by the unhalted activation of complement and macrophages. This concept implies that atherosclerosis has an immunopathological component with central involvement of innate immune mechanisms. Inhibition of complement and/or macrophages might therefore counteract atherogenesis, whereas excessive coactivation of these components could accelerate disease progression. Evidence supporting the first expectation is available: complement C6 deficiency protects against diet-induced atherosclerosis in rabbits, and functional impairment of macrophages through abolishment or blockade of macrophage colony-stimulating factor is protective in mice. In
this study, we demonstrate that, conversely, nonspecific activation of the innate immune system markedly accelerates atherosclerosis in hypercholesterolemic rabbits.

Methods

Animal Model

Forty-nine female New Zealand White rabbits (12 weeks old at the beginning of the experiments) were maintained under standardized conditions (21°C, 41% to 62% humidity) with regular day/night cycle and free access to water and laboratory diets. The animals were randomly assigned to 1 of 8 groups. Groups 1 through 3 received standard maintenance diets (K-H4 pellets, Ssniff); groups 4 through 8 received the same diet but supplemented with 0.5% (wt/wt) cholesterol (Table). In animals of groups 2, 5, and 6, 4 through 8 received intravenous injections of 1.25 mg endotoxin until the end of the experiments.

Table: Lipid Profile and Hematological Parameters at the End of the Experiments

<table>
<thead>
<tr>
<th>Group</th>
<th>Normal Diet</th>
<th>Cholesterol-Supplemented Diet</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>S aureus</td>
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<td>S aureus + Antibiotic</td>
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<td>Control</td>
<td>S aureus</td>
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<tr>
<td></td>
<td></td>
<td>S aureus + Antibiotic</td>
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<tr>
<td>Cholesterol, mmol/L</td>
<td>1.5 ± 0.52</td>
<td>1.46 ± 0.2</td>
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<tr>
<td>LDL cholesterol, mmol/L</td>
<td>0.48 ± 0.44</td>
<td>0.51 ± 0.15</td>
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<tr>
<td>HDL cholesterol, mmol/L</td>
<td>0.76 ± 0.26</td>
<td>0.65 ± 0.18</td>
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<tr>
<td>Triglycerides, mmol/L</td>
<td>0.64 ± 0.21</td>
<td>0.66 ± 0.34</td>
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<tr>
<td>Fibrinogen, g/L</td>
<td>3.36 ± 0.72</td>
<td>3.3 ± 0.67</td>
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<tr>
<td>Leukocytes, 10⁹/L</td>
<td>8.7 ± 1.76</td>
<td>8.0 ± 1.49</td>
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<tr>
<td>Monocytes, %</td>
<td>5.9 ± 4.5</td>
<td>5.4 ± 1.3</td>
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<tr>
<td>Neutrophils, %</td>
<td>32.5 ± 21.7</td>
<td>34.6 ± 17.4</td>
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<tr>
<td>Lymphocytes, %</td>
<td>59.1 ± 26.1</td>
<td>58.4 ± 19.6</td>
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<tr>
<td>Erythrocytes, 10¹²/L</td>
<td>3.67 ± 0.3</td>
<td>5.98 ± 0.27</td>
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<tr>
<td>Hematocrit, %</td>
<td>35.6 ± 2.0</td>
<td>34.1 ± 1.3</td>
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*P < 0.05 vs group 4 (Wilcoxon) (n = 5 rabbits per group).

Image Analysis

Images of Sudan IV–stained aortas were taken with a standard digital camera (Camedia C-1400XL, Olympus Optical Co) with a macro conversion lens (f = 40 cm), imported into the S-VHS port of a personal computer (G3 Macintosh computer with built-in graphic capture board) using the Import command of Photoshop (Adobe). Images were imported into Photoshop with a Kodachrome slide scanner (LS-1000, Nikon, with SilverFast software). Images were stored in Photoshop or PICT format on the hard drive or on an external storage device (ZIP drive, Iomega). Image analysis was performed as previously described. Figure 1 describes how atherosclerotic lesion area was assessed. The area is given in number of pixels, which is then converted into mm², depending on the resolution of the image. To facilitate the conversion of pixels into mm², a 50-mm tape measure was placed next to each aorta during photography. This calculation was performed for the aorta in its entirety as well as separately for 4 quadrants of the aorta, namely aortic arch (1/4), thoracic aorta (1/4), suprarenal abdominal aorta (1/4), and infrarenal abdominal aorta (1/4). For analysis of the respective histological sections, the fatty streak lesions were selected by tracing their contours with the Lasso tool of the toolbox (Figure 2). Areas were quantified by use of the Histogram command and calculated into mm² on the basis of the resolution of the image and a reference slide (a 1-cm² square printed on a glass slide, imported and quantified similarly with Photoshop). The base of the lesion was quantified by tracing the interface between foam cell lesion and media and the average thickness of the foam cell lesion calculated as a fraction of cross-sectional area and length of the lesion base (Figure 2). The mean thickness of the lesion area was thus assessed in 4 to 6 representative sections per aortic quadrant, and the statistical mean was calculated. The product of lesion thickness (in mm, assessed from the histological slides) and lesion surface area (in mm², assessed from the Sudan-stained en face preparation) was calculated and the sum of all 4 aortic quadrants used as a 3D measure of total atherosclerotic lesion load (Figure 5).
Chromogen density and distribution in immunohistochemical stains were quantified by Photoshop-based image analysis as described. Pixels with similar chromogen characteristics were selected with the Magic Wand tool and the Select Similar command, and gray levels/luminosities of the areas were calculated with the Histogram command in Photoshop. For MIF slides, which exhibited an even distribution of MIF immunoreactivity over the entire neointimal space (Figure 4, top), the immunostaining intensity was evaluated separately in the media and the neointima, and a mean luminosity value was calculated for the red channel of the color spectrum. For E-LDL slides, in which the chromogen distribution was patchy and localized in the vicinity of the lamina elastica interna (Figure 4, bottom), the chromogen distribution was assessed and calculated as percentage of neointimal space.

Statistical Analyses
Data were analyzed by the Wilcoxon test and by ANOVA analysis.

Results
All animals survived through the course of the experiment. All animals on hypercholesterolemic diet had comparable serum levels of total, LDL, and HDL cholesterol, ranging between 18.4 and 27.8 mmol/L, 17.3 and 26.5 mmol/L, and 0.47 and 0.73 mmol/L, respectively (Table). Endotoxin-treated animals developed fever on day 1 after each injection, body temperature rising from baseline temperatures between 38.4°C and 39.2°C (mean 38.6±0.1°C) to values between 40.3°C and 41.1°C (mean 40.8±0.02°C; P<0.01 versus baseline values) at 6 hours after endotoxin injection. There was no difference between the animals of groups 3 and 8 and no difference in the development of fever over the course of the study. All animals reacted similarly, and no animal failed to develop fever after any of the endotoxin injections. Animals with the local S aureus infection did not develop fever. The cutaneous infections were self-limiting, resolving within 3 to 4 weeks in animals with no antibiotic treatment and within 1 week in quinolone-treated animals.

None of the animals on regular diet developed any macroscopic or histological evidence of atherosclerosis (Figure 3, groups 1 to 3). In contrast, all animals on the cholesterol-supplemented diet developed atherosclerosis, which was comparable in groups 4 through 7: similar values were observed for plaque surface area (Figure 3, top), average plaque thickness (Figure 3, middle), and overall plaque load (Figure 3, bottom). The distribution of atherosclerotic lesion area and total lesion load over the entire aorta was similar in all groups of hypercholesterolemic animals, with the majority of lesions (67% to 84%) present in the aortic arch. The infrarenal abdominal aorta contributed 3% and the thoracic and suprarenal abdominal aortas contributed between 6% and 17%, respectively. Of particular interest was the observation that repeated local infection with S aureus (group 5) failed to elicit increased lesion formation (Figure 3). The fact that antibiotic treatment did not attenuate atherogenesis compared with the hypercholesterolemic control animals of group 4 underscores the specific pathogen–free environment in which the animals were kept during the experiment: previous studies
have suggested that rabbits maintained under non-specific pathogen-free conditions can show increased atherogenesis because of infections with *Pasteurella multocida* and that this is reversed by antibiotic treatment.\(^1\)

In contrast, weekly injections of the animals with endotoxin significantly accelerated atherosclerosis, as evidenced by increased aortic lesion area and lesion volume, but not lesion thickness (Figure 3). For lesion area and lesion volume, the difference reached statistical significance at a level of \(P<0.01\) and \(P<0.001\), respectively, versus the 9 animals of the hypercholesterolemic control group. Indeed, lesion volume was higher in each individual animal of the endotoxin group (group 8) than in any of the animals in the control group (group 4). This was also confirmed by ANOVA, \(P<0.025\). Histological examination of hearts, kidneys, and livers showed no abnormalities other than striking fatty change in the livers of all cholesterol-fed rabbits. There was no difference between animals from the different groups, however, as assessed in a 3-tier score of fatty change severity (data not shown). Of all the hematological and clinical chemistry data assessed in a subset of 5 animals per group, only 1 parameter showed a significant difference: total monocyte counts were significantly increased in the animals of group 8 (Table). All animals showed comparable, steady weight gain over the course of the study (not shown). Also, no effect of endotoxin was observed on the level of serum triglycerides or on the level of serum and LDL cholesterol.

The image analysis of the immunohistochemical studies using E-LDL and MIF antibodies showed no significant differences between the treatment groups. For each individual tissue section, MIF immunoreactivity was stronger in the neointima than in the media (Figure 4, top), but the immunostaining intensity for neointima and media was comparable in groups 4 through 8 (not shown). Likewise, we saw no significant differences in E-LDL–associated chromogen distribution in the neointimas of animals from groups 4 through 8 (data not shown).

**Discussion**

The present experiments were conducted to test a hypothesis that followed from a new concept about the pathogenesis of atherosclerosis.\(^3\) We propose that each LDL molecule con-
involving repetitive weekly application of bacterial lipopoly-
the animals never developed fever. The second protocol,
no systemic reactions: lesions resolved spontaneously, and
endeavor met with only partial success. The infections caused
S aureus
implemented with the intent of establishing a chronic, dermal
infection in hypercholesterolemic animals. The
macrophage foam cell formation in vitro. 29 Studies in rats
and smooth muscle cells.28 Endotoxin can reportedly induce
implantation of an endotoxin-soaked thread induces intimal infiltration of monocytes, neutrophils,
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of MIF,\textsuperscript{30,31} an effect that has also been demonstrated in vitro.\textsuperscript{32,33} Rabbit studies have linked MIF expression to upregulation of adhesion molecules on the surface of endothelial cells over cholesterol-induced atherosclerotic lesions\textsuperscript{34} and also to the appearance of foam cells. We therefore quantified the presence of MIF but found no difference between lesional MIF expression in endotoxin-treated and control rabbits.

Irrespective of underlying mechanisms, this study renders it clear that nonspecific activation of innate immune effectors accelerates atherosclerosis even in the absence of a genuine infection. This agrees with the data of Huber et al.,\textsuperscript{35} who observed enhancement of atherosclerosis in hypercholesterolemic mice on application of IL-6. It is also in accord with the observation by Richardson et al.\textsuperscript{17} that atherosclerosis was accelerated in hypercholesterolemic rabbits simultaneously suffering from respiratory tract (but not vascular) infections with Pasteurella multocida. Because myriad other situations will probably induce similar effects, a search for a single infectious cause of atherosclerosis becomes essentially meaningless. Any chronic infection (eg, periodontitis and chronic obstructive lung disease) should be detrimental, and noninfectious causes of immune stimulation, such as smoking and renal dialysis, are likely to be equally important. Immuno-pathogenesis of atherosclerosis should best be avoided through reduction of the E-LDL load in tissues. The success of all clinical lipid-lowering trials as opposed to the failure of antioxidant trials is completely in line with this concept.

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