Influence of C3 Deficiency on Atherosclerosis

Chiara Buono, MD*; Carolyn E. Come, BA*; Joseph L. Witztum, MD; Graham F. Maguire, BSc; Philip W. Connelly, PhD; Michael Carroll, PhD; Andrew H. Lichtman, MD, PhD

Background—The influence of complement activation on atherosclerosis is not well understood. The purpose of this study was to examine the effects of C3 deficiency on the extent and phenotype of atherosclerosis.

Methods and Results—Aortic atherosclerosis was analyzed in low-density lipoprotein receptor (ldlr)/C3-deficient mice (ldlr<sup>−/−</sup>C3<sup>−/−</sup>) and ldlr<sup>−/−</sup>C3<sup>+/−</sup> littermate control mice after 15 weeks on a 1.25% (wt/wt) cholesterol diet. Serum lipoprotein profiles and immunoglobulin levels were not significantly different between the 2 experimental groups. The lipid-positive en face lesion area in thoracic and abdominal aorta was greater in C3-deficient mice than in control mice (3.9% versus 2.1%, median, \( P=0.0076 \)). Similarly, the lipid-positive area in aortic arch sections was greater in C3-deficient mice than in controls (0.04 mm<sup>2</sup> versus 0.02 mm<sup>2</sup>, median, \( P=0.0089 \)). Analysis of aortic arch sections showed greater lesional macrophage content in C3-deficient versus control mice (8.24±1.36% versus 5.9±1.63% intimal area, mean±SEM, \( P=0.003 \)), less smooth muscle cell content in C3-deficient versus control mice (0.06±0.05% versus 0.92±0.32% intimal area, mean±SEM, \( P<0.0001 \)), and less collagen content in C3-deficient versus control mice (0.52±1.26% versus 11±10.43% intimal area, mean±SEM, \( P=0.008 \)).

Conclusions—The maturation of atherosclerotic lesions beyond the foam cell stage is strongly dependent on an intact complement system. (Circulation. 2002;105:3025-3031.)

Key Words: atherosclerosis • complement • inflammation

Atherosclerotic plaques are chronic inflammatory lesions of the vessel wall in which many components of the innate and adaptive immune system are active.1 Soluble immune effector molecules frequently found in arterial lesions include antibodies and complement proteins. The complement system plays a central role in protective immune responses to microbial pathogens and in the pathogenesis of immune-mediated disease processes. Complement activation, either by the antibody-dependent classical pathway or the alternative or lectin pathways, generates proinflammatory mediators such as C3a and C5a, which can activate endothelium and enhance leukocyte recruitment to inflammatory sites. C3 fragments bind covalently to antibodies or cell surfaces, thereby acting as opsonins that could enhance phagocytosis by macrophages. Generation of the terminal membrane attack complex (MAC) on the surface of lesional cells may kill these cells. Furthermore, complement is involved in the stimulation and regulation of antibody responses. In particular, the CD19/CD21 receptor complex on B cells links the complement system to the activation of B cells.2 In addition, the CD21 complement receptor expressed on follicular dendritic cells contributes to the maintenance of memory B cells and B-cell tolerance to self-antigens.3 Mice deficient in C3 have diminished protective inflammatory responses to infections, a profound defect in their antibody response to T-dependent antigens, diminished germinal center formation, and a failure in isotype switching.4,5

Individuals with atherosclerotic disease produce antibodies specific to atheroma antigens, such as oxidized low-density lipoproteins (LDLs)6 and heat shock protein 60/65.7 Antibody responses to oxidation-specific epitopes of oxidized LDL may modulate atherosclerotic disease.8,9 However, the mechanisms by which these antibodies influence atherogenesis are not understood. Because humoral immune responses to certain antigens are associated with and may modulate atherogenesis and because the complement system is involved in both the inductive and effector phases of humoral immune responses, it is plausible to hypothesize that complement is involved in modulating atherogenesis. Furthermore, antibody-independent activation of complement in arterial lesions may contribute to the inflammatory process of atherosclerosis.

Several studies provide evidence that complement activation is involved in atherogenesis. Components of the terminal complement pathway are frequently found in human atheromas.10–12 C3 and C4 deposition in arterial lesions has also

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been demonstrated. Furthermore, RNA analysis indicates that complement genes are expressed locally within the plaques. Although there is evidence for complement activation in atherosclerotic lesions, little is known about how complement is activated in lesions or the influence of complement activation on disease progression or phenotype. Several components of the arterial wall may trigger complement activation and C5b-9 assembly. The classical pathway of complement may be activated by C-reactive protein (CRP) bound to enzymatically degraded LDL. Consistent with this view is the finding that the terminal complement proteins colocalize with CRP in the intima of early human lesions. Likewise, immunoglobulin (Ig) M or IgG antibodies specific for oxidized LDL within lesions may activate the classical pathway. Chlamydia pneumoniae, a microbe frequently associated with atherosclerotic lesions, may also activate the alternative pathway of complement.

A limited number of studies on the influence of complement protein deficiencies on atherogenesis have been reported. In rabbits, there is an apparent protective effect of C6 complement protein deficiencies on atherogenesis have been reported. The serum C3 levels in other studies. The serum C3 levels in heterozygous littermates were used as controls to ensure that the C3 genotype was identified by polymerase chain reaction with the use of LDLR gene–specific primers, whereas the C3 genotype was followed by ELISA determination of serum C3 protein levels (sensitive to ~5 ng/mL), as described. C3 heterozygous littermates were used as controls to ensure that enough age-matched, genetically comparable mice were available for the studies. The serum C3 levels in C3 heterozygous mice were not significantly different from wild-type C3 mice, and the C3 heterozygotes do not have any detectable phenotypic differences from wild-type animals relevant to these studies. Mice were maintained in the Longwood Medical Research Center facility in accordance with guidelines of the Committee on Animals of the Harvard Medical School (Boston, Mass).

**Methods**

**Mice**

Male ldlr/−/− mice, backcrossed on the C57Bl/6J background 6 times, purchased from Jackson Laboratories (Bar Harbor, Maine), were crossbred with female C3−/− mice on a mixed 129/B6 background to generate ldlr−/−C3−/− double-knockout mice and ldlr−/−C3+/- littermate controls. The C3−/− gene was identified by polymerase chain reaction with the use of LDLR gene–specific primers, whereas the C3+/- genotype was followed by ELISA determination of serum C3 protein levels (sensitive to ~5 ng/mL), as described. C3+/- heterozygous littermates were used as controls to ensure that enough age-matched, genetically comparable mice were available for the studies. The serum C3 levels in C3+/- heterozygotes were not significantly different from wild-type C3+/- mice, and the C3+/- heterozygotes do not have any detectable phenotypic differences from wild-type animals relevant to these studies. Mice were maintained in the Longwood Medical Research Center facility in accordance with guidelines of the Committee on Animals of the Harvard Medical School (Boston, Mass).

**Diet Protocols**

Two separate experiments were performed with identical diet protocols but different methods of lesion analysis. In both experiments, ldlr−/−C3−/− and 10 age- and sex-matched ldlr−/−C3+/- littermate control mice were recruited at 5 to 8 weeks of age. At day 0 (initiation of study, before diet) serum samples were collected from overnight-fasted individual mice by tail-vein bleeding. The mice were then fed ad libitum a semipurified D12108-based, 40% kcal lipid, 1.25% (wt/wt) cholesterol diet synthesized by Research Diets, Inc. After 15 weeks of diet, mice were fasted overnight and killed by ether inhalation, blood was collected for serum analyses by vena cava nicking, and the arterial tree was perfused with PBS (25 mL). Perfused aortas from exanguinated mice were dissected from the aortic valve to the iliac bifurcation.

**Preparation and Analysis of Aortic Tissue**

In experiment No. 1, the proximal 1 cm of aorta starting from the aortic valve was cut with a scalpel and rapidly frozen in optimal cutting temperature embedding medium (OCT medium, Tissue-Tek). Serial 5-μm-thick cryostat sections were prepared, and every tenth section of the proximal 1000 μm of aorta was stained with oil-red O and counterstained with Wrights/Geimsa solution. To quantify cross-sectional area of lesions, digital photomicrographs of these sections were subject to image analysis with IMAGEPRO PLUS software (Media Cybernetics). The fractional area of the lesions, expressed as percentage of total sectional area of aortic wall, was calculated, as described. Adjacent cryostat sections were analyzed by immunohistochemistry, as described below. The remaining thoracic and abdominal aorta from each mouse in experiment No. 1 was fixed in 10% buffered formalin, stained with oil-red O, opened longitudinally, pinned out, and photographed with a digital camera to obtain images of lesions en face, as previously described. The percent surface area occupied by oil-red O–stained lesions viewed en face was determined with IMAGEPRO PLUS software.

In experiment No. 2, the aortic arches from mice were dissected from the aortic valve, separated from the remaining aorta, and frozen in OCT medium. Four micron-thick longitudinal-cryostat sections were prepared, which included greater and lesser curvatures of the aortic arch from the aortic root to beyond the takeoff of the left subclavian artery. These sections were stained with oil-red O and photographed with a digital camera attached to a microscope. A defined portion of the aortic arch was quantitatively analyzed in each section, consisting of a 3-mm segment of the lesser curvature, starting at the point of origin of the left subclavian artery. Lesions seen en face were analyzed by a microscopic eyepiece micrometer and quantitated by measuring the area of lesions and dividing by the area of the aortic wall. This value was used to estimate lesion area as percentage of the cross-sectional area of the aorta in that segment. The percent surface area of each aortic arch was determined by measuring the area of lesions viewed en face and dividing by the area of the aortic wall in that segment.

**TABLE 1. Serum Lipoprotein/Cholesterol Profiles in ldlr−/−C3−/− and ldlr−/−C3+/- Mice**

<table>
<thead>
<tr>
<th>Mouse Genotype</th>
<th>Total Cholesterol, mg/dL</th>
<th>VLDL Cholesterol, mg/dL</th>
<th>LDL Cholesterol, mg/dL</th>
<th>HDL Cholesterol, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>ldlr−/−C3−/−</td>
<td>1215±271*</td>
<td>628±137†</td>
<td>445±125‡</td>
<td>74±24§</td>
</tr>
<tr>
<td>(n=6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ldlr−/−C3+/-</td>
<td>1265±221*</td>
<td>776±213†</td>
<td>359±115‡</td>
<td>62±21§</td>
</tr>
<tr>
<td>(n=6)</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Values are expressed as mean±SEM. Overnight fasting serum samples for cholesterol analysis were obtained after 15 weeks of cholesterol diet feeding.

*P=0.84; †P=0.35; ‡P=0.29; §P=0.62

**Figure 1.** Size distribution of serum lipoprotein particles in complement-deficient and control ldlr−/− mice after 15 weeks on a cholesterol-enriched diet. Typical profiles from 2 individual mice of 12 analyzed are shown.
defined proximally by the aortic root and distally by a perpendicular axis dropped from the distal side of the left common carotid artery origin.\textsuperscript{24} Medial, intimal, and lipid-positive areas within this 3-mm stretch of aorta were calculated for each mouse by computerized image analysis with IMAGEPRO PLUS software.

**Immunohistochemical and Histochemical Analyses of Aortic Lesions**

In experiment No. 1, cross sections of proximal aorta were stained for C3 and IgG. Cryostat microtome cross sections (5 µm thick) were fixed in cold acetone (4°C, 7 minutes), rehydrated in PBS, and blocked with PBS, 1% hydroperoxide in PBS, and 10% normal goat serum. The sections were stained with horseradish peroxidase (HRP)--conjugated goat IgG specific for mouse C3 (ICN Pharmaceuticals), HRP-conjugated goat anti-mouse IgG F(ab')\textsuperscript{2} (ICN), and HRP-conjugated goat IgG (Jackson ImmunoResearch Laboratories) as isotype control. Staining was visualized by adding 3-aminobenzidine (AEC) substrate kit for peroxidase (Vector Laboratories), and sections were counterstained with hematoxylin.

In experiment No. 2, immunohistochemistry and histochemistry of longitudinal sections of aortic arch were also performed to analyze and quantify the cellular and collagen content of atherosclerotic lesions. Serial longitudinal 5-µm cryostat sections of aortic arch were fixed in cold acetone, rehydrated in PBS, and blocked with 10% normal rat serum/PBS (room temperature, 20 minutes), avidin, and biotin solutions (Vector Laboratories). Subsequently, sections were stained with the respective cell-specific and isotype control antibodies. To stain macrophages we used anti-mouse Mac3 Clone M3/84 (BD Pharmingen) as primary antibody and biotinylated goat anti-rat IgG, 1:200 (Jackson ImmunoResearch) as secondary antibody. The sections were blocked with 0.3% hydroperoxide/PBS (room temperature, 15 minutes) and incubated with avidin-biotin complex solution (ABC Elite Peroxidase kit, Vector Laboratories). The staining was visualized by adding 3-amino-9-ethylcarbazole (AEC) substrate kit for peroxidase, (Vector Laboratories) and sections were counterstained with hematoxylin.

**Results**

**Serum Lipid and Immunoglobulin Analyses**

Serum lipid and Ig data, as well as data from quantitative image analysis of immunohistochemically stained sections, were determined to be normally distributed (Prism software). For these data sets, the Student’s t-test was used to analyze differences between the groups, and the data are expressed as mean±SEM. Data on lesional intimal area and lipid positive area, shown in Figure 3, were not uniformly normally distributed; therefore, the differences between ildlr\textsuperscript{−/−}C3\textsuperscript{−/−} and ildlr\textsuperscript{−/−}C3\textsuperscript{+/−} groups were analyzed by the Mann-Whitney U test and expressed as the median. A value of $P \leq 0.05$ was considered significant for both types of analysis.

**Figure 2.** Oil-red O staining of aortic atherosclerotic lesions in complement-deficient and control mice. Aortas from cholesterol-fed ildlr\textsuperscript{−/−}C3\textsuperscript{−/−} mice (top) and ildlr\textsuperscript{−/−}C3\textsuperscript{+/−} (bottom) are shown. In experiment No. 1 (left), cross sections of the proximal ascending aorta were stained, and in experiment No. 2 (right), longitudinal sections of the aortic arch were stained. Arrows in left panels indicate corresponding lipid-rich and collagen-rich regions in C3-deficient and control animals, respectively.

**TABLE 2. Serum Immunoglobulin Levels in ildlr\textsuperscript{−/−}C3\textsuperscript{−/−} and ildlr\textsuperscript{−/−}C3\textsuperscript{+/−} Mice**

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Total Ig Before Diet, mg/dL</th>
<th>Total Ig After Diet, mg/dL</th>
<th>Anti-MDA-LDL IgG After Diet, RLU</th>
<th>Anti-MDA-LDL IgG After Diet, RLU</th>
<th>Anti-POVPC-LDL IgG After Diet, RLU</th>
<th>Anti-POVPC-LDL IgG After Diet, RLU</th>
</tr>
</thead>
<tbody>
<tr>
<td>ildlr\textsuperscript{−/−}C3\textsuperscript{−/−}</td>
<td>936±224*</td>
<td>974±76†</td>
<td>19 318±6238‡</td>
<td>4196±2683§</td>
<td>1791±1006‖</td>
<td>1118±952§</td>
</tr>
<tr>
<td>ildlr\textsuperscript{−/−}C3\textsuperscript{+/−}</td>
<td>956±168</td>
<td>908±244</td>
<td>19 739±2185‡</td>
<td>2789±1418§</td>
<td>1931±936‖</td>
<td>1024±431§</td>
</tr>
</tbody>
</table>

MDA indicates malondialdehyde; POVPC, 1-palmitoyl-2-5-oxovaleroyl-phosphatidyl-choline; and RLU, relative light units.

\*P=0.41; †P=0.31; ‡P=0.42; §P=0.25; ‖P=0.39; ¶P=0.44.

stained with Picrosirius red, as described,\textsuperscript{25} and the sections were analyzed by polarization microscopy.
diet, the mean total, very-low-density lipoprotein (VLDL), LDL, and HDL cholesterol levels in sera of ldlr<sup>−/−</sup>C3<sup>−/−</sup> mice were not statistically different from ldlr<sup>−/−</sup>C3<sup>+/−</sup> mice, as determined by gel-filtration fast-performance liquid chromatography (Table 1, Figure 1). Enzymatic assays of total, LDL, and HDL cholesterol also did not reveal significant differences between ldlr<sup>−/−</sup>C3<sup>−/−</sup> and ldlr<sup>−/−</sup>C3<sup>+/−</sup> mice (data not shown).

C3 deficiency in mice is associated with reduced IgG production in response to T-dependent antigens, but serum IgM and IgG levels are not significantly different in C3-null versus wild-type mice. Total serum Ig levels of C3-deficient mice did not differ from control mice at time 0 (before diet) or after diet (Table 2). Furthermore, we did not see significant differences between ldlr<sup>−/−</sup>C3<sup>−/−</sup> and ldlr<sup>−/−</sup>C3<sup>+/−</sup> mice (data not shown).

Quantitative Analysis of Atherosclerotic Lesions
After 15 weeks of cholesterol-diet feeding, both ldlr<sup>−/−</sup>C3<sup>−/−</sup> and ldlr<sup>−/−</sup>C3<sup>+/−</sup> mice had aortic atherosclerotic lesions with intimal proliferation and foam cells, although the fat content appeared greater in the lesions from complement-deficient mice (Figure 2). In experiment No. 1, the median en face aortic lesion area in descending and abdominal aorta in ldlr<sup>−/−</sup>C3<sup>−/−</sup> was significantly greater than in ldlr<sup>−/−</sup>C3<sup>+/−</sup> mice (Figure 3). The median cross-sectional area of the proximal aortic lesions, as a percentage of total arterial wall sectional area, was not significantly different between ldlr<sup>−/−</sup>C3<sup>−/−</sup> and ldlr<sup>−/−</sup>C3<sup>+/−</sup> mice.

In experiment No. 2, analysis of longitudinal sections of aortic arch revealed significantly greater lipid positive areas in ldlr<sup>−/−</sup>C3<sup>−/−</sup> mice compared with ldlr<sup>−/−</sup>C3<sup>+/−</sup>, whereas total intimal areas were not different between the 2 groups (Figure 3, bottom). These combined data indicate that C3 enhances lesional lipid content but not total lesional size. Medial areas were also not statistically different between the 2 groups (data not shown).

Phenotypic Analysis of Atherosclerotic Lesions
Lesional C3 and IgG depositions were detectable in proximal aortic cross sections from ldlr<sup>−/−</sup>C3<sup>−/−</sup> mice, whereas IgG but not C3 was detectable in lesions from ldlr<sup>−/−</sup>C3<sup>+/−</sup> mice (Figure 4). Both C3 and IgG were deposited in the lesional neointima and the subjacent media.

Cell and matrix content of aortic arch lesions appeared quantitatively different in ldlr<sup>−/−</sup>C3<sup>−/−</sup> versus ldlr<sup>−/−</sup>C3<sup>+/−</sup> (Figure 5), with more macrophages but less collagen and smooth muscle cells in the lesions of C3-deficient mice. Smooth muscle actin was evident in the region of early fibrous cap formation in control mice but not C3-deficient mice. Image analysis confirmed that macrophage content was significantly greater in lesions of C3-deficient mice compared with controls, and smooth muscle cells and collagen content was significantly greater in control mice (Table 3).

Discussion
A principal finding of the present study is that the extent of total aortic atherosclerosis, determined by lipid staining, was
greater in aortas from C3-deficient mice compared with controls. The differences were evident from analysis of en face preparations of descending thoracic and abdominal aorta and in longitudinal sections of aortic arch. In addition, the median lipid-positive area of proximal aortic cross sections was greater in C3-deficient versus control mice, but the differences between the experimental groups was not statistically significant.

Figure 4. C3 and immunoglobulin deposition in aortic atherosclerotic lesions. Cross sections of proximal ascending aorta from a C3-deficient mouse (top) or control mouse (bottom) in experiment No. 1 were stained with antibodies specific for C3 (left) or mouse Ig (right), as described in Methods. Sections shown are representative of all animals in the respective groups.

Figure 5. Macrophage, smooth muscle cell, and collagen content of aortic arch atherosclerotic lesions in complement-deficient and control mice. Aortic arch sections from 2 complement-deficient mice (Nos. 483 and 485) and 2 control mice (Nos. 806 and 803) in experiment No. 2 were stained for smooth muscle actin (top 4 panels), macrophages (Mac 3) (middle 4 panels), and collagen (bottom 4 panels), as described in Methods. Approximately the same areas of each lesion are shown for macrophage and smooth muscle cell stains, and these areas are typical of lesions quantitatively analyzed in Table 3. Smooth muscle cell staining in the position of an early fibrous cap in control mice is indicated by arrows.
A second important finding in this study was the difference in lesion phenotype seen in C3-deficient versus control mice. Macrophages were more abundant in lesions from C3-deficient mice, whereas collagen and smooth muscle cells were more abundant in the control mice. Taken together with the lesion lipid staining, these data support the conclusion that in the absence of C3, lesions remain predominantly lipid and foam cell rich, and lesion maturation, indicated by smooth muscle cell migration or proliferation and the deposition of fibrous matrix, is impaired. One implication of this finding is that complement activation promotes changes that are associated with plaque stability. Our data from the 15-week time point do not distinguish the possibilities that C3 deficiency is the fact that there was IgG and C3 deposition within aortic arch and descending aorta were analyzed. Although activation of the terminal lytic pathway requires C3, there are likely to be effects of C3 on atherosclerosis that are independent of the terminal pathway.

In conclusion, we have demonstrated that the progression of atheromas from foam cell–rich and lipid-rich lesions to lesions with prominent smooth muscle cells and collagen depends in part on the presence of an intact complement system. Our findings indicate that complement activation should be considered when evaluating the mechanisms and prognostic significance of other inflammatory parameters associated with atherosclerosis, including CRP, and humoral immune responses.

**Acknowledgments**

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**References**


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**TABLE 3. Quantitative Analysis of Cell and Matrix Content of Aortic Arch Lesions in *ldlr* and *ldlr* Mice**

<table>
<thead>
<tr>
<th>Mouse Genotype</th>
<th>Macrophages, % Intimal Area</th>
<th>Smooth Muscle Cells, % Intimal Area</th>
<th>Collagen, % Intimal Area</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ldlr</em> (n=10)</td>
<td>8.24±1.36*</td>
<td>0.06±0.05†</td>
<td>0.52±1.26‡</td>
</tr>
<tr>
<td><em>ldlr</em> (n=10)</td>
<td>5.9±1.63*</td>
<td>0.92±0.32†</td>
<td>11±10.43‡</td>
</tr>
</tbody>
</table>

*P=0.003; †P=0.0000003; ‡P=0.008.


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