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−/−C3−/−) and ldlr−/−C3+/+ 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 lesional 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 mm² versus 0.02 mm²; 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
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 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 deficiency. In rabbits, there is an apparent protective effect of C6 in the intima of 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.

**Methods**

**Mice**

Male ldrl−/− 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 ldrl−/− C3−/− double-knockout mice and ldrl−/− C3−/− littermate controls. The ldrl 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 levels in age-matched, genetically comparable mice were available 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, 10 ldrl−/− C3−/− and 10 age- and sex-matched ldrl−/− 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% (w/vt) 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 veno

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Size distribution of serum lipoprotein particles in complement-deficient and control ldrl−/− mice after 15 weeks on a cholesterol-enriched diet. Typical profiles from 2 individual mice of 12 analyzed are shown.

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**TABLE 1. Serum Lipoprotein/Cholesterol Profiles in ldrl−/−C3−/− and ldrl−/−C3−/− Mice**

<table>
<thead>
<tr>
<th>Mouse Genotype</th>
<th>Total Cholesterol, mg/dL</th>
<th>VLDL</th>
<th>LDL</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>ldrl−/− 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>ldrl−/− 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

cava nicking, and the arterial tree was perfused with PBS (25 mL). Perfused aortas from exsanguinated 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. Five 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.
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′)2 (ICN), and HRP-conjugated goat IgG (Jackson ImmunoResearch Laboratories) as isotype control. Staining was visualized by adding 3-amino-9-ethylcarbazole (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.

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 collagen-rich regions in C3-deficient and control animals, respectively.

### Results

Serum Lipid and Immunoglobulin Analyses

Analyses of serum lipids from the mice in these studies indicate that complement deficiency does not alter serum lipid content. After 15 weeks of high-fat/high-cholesterol diet, serum triglycerides were 4.4-fold higher in C3-deficient compared with control animals (P<0.01). Serum triglycerides were elevated in both diet groups, but the increase was significantly greater in C3-deficient mice (P<0.01).

### Table 2. Serum Immunoglobulin Levels in ldlr−/−C3−/− and ldlr−/−C3+/+ Mice

<table>
<thead>
<tr>
<th>Mouse Genotype</th>
<th>Total Ig Before Diet, mg/dL</th>
<th>Total Ig After Diet, mg/dL</th>
<th>Anti-MDA-LDL IgM After Diet, RLU</th>
<th>Anti-MDA-LDL IgG After Diet, RLU</th>
<th>Anti-POVPC-LDL IgM After Diet, RLU</th>
<th>Anti-POVPC-LDL IgG After Diet, RLU</th>
</tr>
</thead>
<tbody>
<tr>
<td>ldlr−/−C3−/−</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>ldlr−/−C3+/+</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-phosphatidylcholine; and RLU, relative light units.

*P<0.41; †P<0.31; ‡P<0.42; §P<0.25; ¶P<0.39; ¶¶P<0.44.

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 collagen-rich regions in C3-deficient and control animals, respectively.

Statistical Analysis

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 collagen-rich regions in C3-deficient and control animals, respectively.

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Figure 2. Oil-red O staining of aortic atherosclerotic lesions in complement-deficient and control mice. Aortas from cholesterol-fed ldlr−/−C3−/− mice (top) and ldlr−/−C3+/+ (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.
diet, the mean total, very-low-density lipoprotein (VLDL), LDL, and HDL cholesterol levels in sera of \textit{ldlr} \textsuperscript{-/-} mice were not statistically different from \textit{ldlr} \textsuperscript{+/-} 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 \textit{ldlr} \textsuperscript{-/-} and \textit{ldlr} \textsuperscript{+/-} 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 \textit{ldlr} \textsuperscript{-/-} \textit{C3} \textsuperscript{-/-} and \textit{ldlr} \textsuperscript{-/-} \textit{C3} \textsuperscript{+/-} mice (data not shown).

Quantitative Analysis of Atherosclerotic Lesions

After 15 weeks of cholesterol-diet feeding, both \textit{ldlr} \textsuperscript{-/-} \textit{C3} \textsuperscript{-/-} and \textit{ldlr} \textsuperscript{-/-} \textit{C3} \textsuperscript{+/-} 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 \textit{ldlr} \textsuperscript{-/-} \textit{C3} \textsuperscript{+/-} was significantly greater than in \textit{ldlr} \textsuperscript{-/-} \textit{C3} \textsuperscript{-/-} 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 \textit{ldlr} \textsuperscript{-/-} \textit{C3} \textsuperscript{-/-} and \textit{ldlr} \textsuperscript{-/-} \textit{C3} \textsuperscript{+/-} mice.

In experiment No. 2, analysis of longitudinal sections of aortic arch revealed significantly greater lipid positive areas in \textit{ldlr} \textsuperscript{-/-} \textit{C3} \textsuperscript{-/-} mice compared with \textit{ldlr} \textsuperscript{-/-} \textit{C3} \textsuperscript{+/-}, 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 \textit{ldlr} \textsuperscript{-/-} \textit{C3} \textsuperscript{-/-} mice, whereas IgG but not C3 was detectable in lesions from \textit{ldlr} \textsuperscript{-/-} \textit{C3} \textsuperscript{+/-} 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 \textit{ldlr} \textsuperscript{-/-} \textit{C3} \textsuperscript{-/-} versus \textit{ldlr} \textsuperscript{-/-} \textit{C3} \textsuperscript{+/-} (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 results in a complete block versus slower kinetics of lesion maturation, but both possibilities are of significance in the natural history of atherosclerotic disease.

The C3-derived peptide C3ades-Arg is reported to regulate triglyceride synthesis and apobetalipoprotein. However, the importance of C3ades-Arg in systemic lipid metabolism remains controversial. We did not find any differences in fasting lipoprotein profiles between C3-deficient and control mice, consistent with a previous study, indicating that the complement system does not have a major impact on circulating lipoprotein levels in mice. We also did not find significant differences in serum Ig levels between the experimental groups. The serum Ig and lipid data support the conclusion that the differences we did observe in the extent and phenotype of lesions in C3-deficient and control mice is most likely attributable to direct local effects of complement activation within the lesions. Consistent with this conclusion is the fact that there was IgG and C3 deposition within aortic lesions of the control animals in this study and IgG but not C3 deposition in the lesions of C3-deficient mice.

Byproducts of complement activation are known to have many biological properties that could influence the development of atherosclerotic lesions. Local complement activation may induce cell lysis and generate at least some of the cell debris found in the necrotic core of advanced lesions. This would be consistent with our finding of more macrophages in lesions from C3-deficient mice. Sublytic assembly of C5b-9 on smooth muscle cells are reported to induce cell activation and proliferation. In support of this, we found fewer smooth muscle cells in the lesion of C3-deficient mice compared with controls.

Previous studies of atherosclerosis in complement-deficient animals have focused on terminal lytic pathway components and are contradictory. C6-deficient rabbits seemed to be protected from lesion development, whereas aortic root lesion size in C5-deficient ApoE-null mice was not different from control ApoE mice. The latter mouse study did not examine more distal regions of the aorta, nor did it examine lesion phenotype. We also did not see statistically significant differences in lipid-positive area of proximal aortic cross sections in complement-deficient and control mice, but we did see differences when aortic arch or 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.

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References

### Table 3. Quantitative Analysis of Cell and Matrix Content of Aortic Arch Lesions in ldrlcr C3−/ and ldrlcr C3+/ 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>ldrlcr C3−/</td>
<td>8.24±1.36*</td>
<td>0.06±0.05†</td>
<td>0.52±1.26‡</td>
</tr>
<tr>
<td>(n=10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ldrlcr C3+/</td>
<td>5.9±1.63*</td>
<td>0.92±0.32†</td>
<td>11±10.43‡</td>
</tr>
<tr>
<td>(n=10)</td>
<td></td>
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</tbody>
</table>

*P<0.003; †P=0.0000003; ‡P=0.008.
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