Complement Components, but Not Complement Inhibitors, Are Upregulated in Atherosclerotic Plaques


Abstract—Complement activation occurs in atherosclerotic plaques. The capacity of arterial tissue to inhibit this activation through generation of the complement regulators C1 inhibitor, decay accelerating factor, membrane cofactor protein (CD46), C4 binding protein (C4BP), and protectin (CD59) was evaluated in pairs of aortic atherosclerotic plaques and nearby normal artery from 11 human postmortem specimens. All 22 samples produced mRNAs for each of these proteins. The ratios of plaque versus normal artery pairs was not significantly different from unity for any of these inhibitors. However, in plaques, the mRNAs for C1r and C1s, the substrates for the C1 inhibitor, were increased 2.35- and 4.96-fold, respectively, compared with normal artery; mRNA for C4, the target for C4BP, was elevated 1.34-fold; and mRNAs for C7 and C8, the targets for CD59, were elevated 2.61- and 3.25-fold, respectively. By Western blotting and immunohistochemistry, fraction Bb of factor B, a marker of alternative pathway activation, was barely detectable in plaque and normal arterial tissue. These data indicate that it is primarily the classical, not the alternative pathway, that is activated in plaques and that key inhibitors are not upregulated to defend against this activation. (Arterioscler Thromb Vasc Biol. 2001;21:1214-1219.)

Key Words: CD59 ■ CD46 ■ C4 binding protein ■ classical pathway ■ inflammation

It has long been known that there is full activation of the complement cascade in atherosclerotic plaques. The membrane attack complex (MAC) is generated, which could result from activation of either the classical or the alternative pathway, or both.1-4 Association of the MAC with damaged plaque tissue has pointed to the autodestructive nature of this phenomenon. Several theories based on antigen-antibody interactions have been advanced to account for the complement activation. Infectious agents, such as herpesvirus, cytomegalovirus, or Chlamydia pneumoniae, have been proposed as antigenic stimulants,5-8 but it is not clear how this would result in selective opsonization of fibrous plaque tissue. It has also been proposed that antibody-independent mechanisms activate complement, including cholesterol and oxysterols,9 which activate the alternative pathway, and C-reactive protein (CRP), which activates the classical pathway.10 CRP is an attractive candidate because it colocalizes with the MAC in atherosclerotic lesions10,11 and is known to activate complement in vitro12 as well as in vivo.13 The reason for CRP localization in plaques has been unclear because it was generally believed to be an acute-phase reactant made exclusively in the liver.14 However, we have reported that human arterial tissue is capable of generating CRP along with proteins of the classical complement pathway.11 There is upregulation of all these components in atherosclerotic plaques. Colocalization of CRP and C4 mRNAs and the C5b-9 protein complex occurs on transformed cells of the thickened intima, indicating that transformed cells within the plaques may be responsible for the autodestructive damage.

There are many regulators that are designed to control unwanted complement activation. These include C1 inhibitor (C1-INH), C4 binding protein (C4BP), decay accelerating factor (DAF), membrane cofactor protein (MCP, CD46), and protectin (CD59).15 C1-INH arrests the complement cascade at the stage at which C1q binds to a target, causing dissociation of the C1 complex. It is a suicide inhibitor that attaches covalently to the exposed esterase sites of C1r and C1s. C4BP inhibits C3 convertase by binding to the active fragment C4b. DAF is a glycosphosphatidylinositol-linked surface protein that accelerates the decay of C4b2a. MCP accelerates cleavage of C3b, primarily in the alternative pathway,16 but it may also have inhibitory activity in the classical pathway.17 CD59 is another glycoprotein 1-linked cell surface protein that binds to the C7 and C8 components of the MAC as it assembles on the surface of host cells. Thus, it prevents C9 attachment and full insertion of the MAC into the membrane. It is also known as protectin because it protects host cells against bystander lysis.18 In the present study, we describe the production of all of these inhibitors by normal artery and plaque tissue. We compare their upregulation in plaques with that of their main complement protein targets to assess their ability to defend against the self-damaging effects of complement activation.
Single-strand cDNA synthesis was performed on 5- 

by the acid guanidinium thiocyanate–phenol–chloroform method. 

we19,20 and others21,22 have reported, mRNAs are remarkably stable 

7 to 96 hours, but the variation was not a factor in the results. As 

University Human Ethics Committee. Postmortem delay varied from 

types of malignancy, but none was the result of an acute thrombosis. 

Eleven pairs of atherosclerotic plaque tissue and nearby normal 

transcription (RT)–polymerase chain reaction (PCR) products, as well 

CD59, and fraction Bb of factor B were also carried out. 

Western blotting 

analysis. Levels of the mRNAs for the inhibitors and their substrate 

CD59 (between 24 and 30 cycles), DAF (between 27 and 33 cycles), 

C1-INH, 56°C for DAF, 60°C for C4BP and MCP, and 57°C for 

94°C for 1 minute, an annealing step of 30 seconds at 61°C for 

primers used, along with GenBank accession numbers and the 

TABLE 1. Primer Sequences Used, Restriction Enzymes, and Product Fragment Lengths 

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<tr>
<th>Gene and Sequence of Primers (5’ to 3’)</th>
<th>Product Length, bp</th>
<th>Restriction Enzyme</th>
<th>Digestion Fragments</th>
<th>GenBank Accession No.</th>
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<td>Sau3A</td>
<td>246, 86</td>
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<td>XM002009</td>
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<td>Reverse TCTTACCTTCTTGTGCTGTA</td>
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<td>C4BP</td>
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<td>Rsal</td>
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<td>AluI</td>
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<td>Reverse TCCACACCTTTTCAGCTGTTGTT</td>
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Methods 

Eleven pairs of atherosclerotic plaque tissue and nearby normal arterial tissue were obtained from the aorta of 11 separate postmortem cases. There were multiple causes of death, including various types of malignancy, but none was the result of an acute thrombosis. Tissues were obtained through the Pathology Service of the University of British Columbia Hospital under conditions approved by the University Human Ethics Committee. Postmortem delay varied from 7 to 96 hours, but the variation was not a factor in the results. As we19,20 and others21,22 have reported, mRNAs are remarkably stable in cooled tissue after death. Total RNA and proteins were extracted from the samples, and portions were taken for immunohistochemical analysis. Levels of the mRNAs for the inhibitors and their substrate complement proteins were subsequently measured. Western blotting of the proteins and immunohistochemical analysis for C1-INH, CD59, and fraction Bb of factor B were also carried out. 

The techniques used for RNA extraction and preparation of reverse transcription (RT)–polymerase chain reaction (PCR) products, as well as for protein extraction, Western blotting, and immunohistochemistry, were as previously reported in detail.11,18,19,23 

Briefly, total RNA was extracted from ~500 mg of tissue samples by the acid guanidinium thiocyanate–phenol–chloroform method. Single-strand cDNA synthesis was performed on 5-μg samples of total RNA extract. The resultant cDNAs (1-μl aliquots) were amplified by using appropriate primers and standard buffer. The primers used, along with GenBank accession numbers and the endonucleases used for identifying the RT-PCR products, are shown in Table 1. The thermal profile consisted of a denaturation step of 94°C for 1 minute, an annealing step of 30 seconds at 61°C for C1-INH, 56°C for DAF, 60°C for C4BP and MCP, and 57°C for CD59, and an extension step of 72°C for 1 minute, except for 3 minutes in the first cycle. All samples were initially denatured for a total of 9 minutes (94°C). Preliminary experiments were carried out with each set of primers to determine the range of cDNA concentration and PCR cycle amplification number that would give reliable comparative values for each cDNA product. Cyclophilin mRNA was chosen as the internal standard because of the consistency of its level from tissue to tissue and its postmortem stability. A linear relationship was found between the amount of cDNA product obtained and the original cDNA added within the range corresponding to 0.01 to 0.5 μg total RNA. Accordingly, standard conditions were followed in which cDNA (1 μL) corresponding to 0.1 μg total RNA was added, and the cyclophilin product was amplified for 27 cycles. In parallel experiments, the C1-INH and CD59 products were amplified for 29 cycles; the DAF, C4BP, and MCP products, for 30 cycles; and the complement products, for 35 cycles. 

Each PCR reaction product was electrophoresed through a 6% polyacrylamide gel, and the product was visualized by incubation for 10 minutes in a solution containing 10 ng/mL ethidium bromide. Resulting gel bands were imaged by using a GDS 7600 image analyzer (Ultra Violet Product). The relative intensities of the bands, expressed as optical density units, were quantitatively analyzed by using NIH image software 1.62. Each mRNA analysis was made in parallel with a cyclophilin mRNA analysis to provide an internal standard. Values normalized to cyclophilin, as well as uncorrected data, were analyzed. Cyclophilin values were almost constant from sample to sample. Most were within 1% of each other, with the range <4%, so that the normalization corrections were very small. Polaroid photographs of the gels were taken. The PCR products were of the expected size, and endonuclease digestion in each case gave fragments of the predicted size as previously reported.11,18,19 In all experiments, the presence of possible contaminants was checked by control reactions in which amplification was carried out for up to 40 cycles on samples in which we omitted from the RT-PCR reaction mixture either (1) the reverse transcriptase or (2) a template cDNA. No product was obtained under these conditions. 

Western blots were performed on extracts of the soluble fraction of homogenates of normal or plaque tissue as previously described.11 Briefly, tissue samples were homogenized in 5 times (vol/wt) extraction buffer (0.02 mol/L Tris-HCl, pH 7.5, and 0.1% Triton X) containing the protease inhibitors phenylmethylsulfonyl fluoride (10 μg/mL) and aprotinin (10 μg/mL) and 1 mmol/L EDTA. Homogenates were centrifuged at 18 000g at 4°C for 30 minutes. The protein content of the supernatants was determined by Lowry’s method. The samples were diluted in SDS sample buffer (60 mmol/L Tris, pH 6.8, 2.5% SDS, and 5% 2-mercaptoethanol) to a final protein content of 5 mg/mL and were boiled for 3 minutes. Samples containing 70 μg of protein were loaded onto 7.5% polyacrylamide minigels. Life Technologies high-range prestained standards were used as mole-
Figure 1. Polaroid photographs of ethidium bromide–stained gels of electrophoresed RT-PCR products for C1-INH, C1r, C1s, DAF, C4BP, CD46, CD59, C7, and C8. Lanes 1 to 11 are paired samples from 11 postmortem cases with normal artery (N) and plaque (P) representing the pairs. Size markers are in lane M, with the size of the RT-PCR products indicated by the arrows on the right. Notice the constant intensity bands for M, with the size of the RT-PCR products indicated by the arrows on the right. Notice the constant intensity bands for M, with the size of the RT-PCR products indicated by the arrows on the right.

Table 2 gives quantitative values for the mRNAs. The housekeeping gene cyclophilin was almost constant for all tissues, indicating the stability of the mRNAs. Moreover, the values for normal tissue from case to case and for plaques from case to case varied little for any of the other mRNAs. This indicates that such confounding factors, such as cause of death and postmortem delay, are not significant contributors to the values under study.

Figure 2 also gives the average ratio of plaque to normal artery mRNA levels for the 11 matched pairs. For the regulators, they were as follows: C1-INH 1.04, C4BP 0.97, MCP 0.94, and CD59 1.06. None of these ratios was significantly different from 1. However, C1r, C1s, C4, C7, and C8 all showed significant increases ($P<0.001$), with ratios of 2.35, 4.96, 1.34, 2.61, and 3.25, respectively.

Figure 2 shows typical Western blots comparing protein extracts from a normal artery and a paired nearby plaque. This is illustrated for C1-INH compared with its 2 substrates C1r and C1s, C4BP compared with C4 and C4d, and CD59 compared with C7 and C8. Figure 2 also compares Western blots of fraction Bb of factor B in normal human serum compared with the same serum activated by IgG, a normal artery, and a nearby plaque. The figure illustrates that no discernible differences between normal arterial and plaque tissue can be seen for the regulators C1-INH, C4BP, CD59, and fraction Bb, with the last being a specific marker of alternative pathway activation. In contrast, compared with its companion normal artery, plaque tissue shows strong upregulation for C1r and C1s, for C4 and C4d, and for C7 and C8. C4d is a specific marker of classical pathway activation. These results are consistent with the mRNA data of Table 2. Additionally, serum activated by IgG, but not normal serum,
TABLE 2. Levels of mRNAs in Paired Normal and Atheromatous Aortic Tissue for C1-INH, DAF, C4BP, MCP, CD59, and the Complement Proteins With Which They Interact

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Normal</th>
<th>Plaque</th>
<th>P†</th>
<th>Pair Ratio‡</th>
<th>P for Ratio†</th>
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<tbody>
<tr>
<td>C1-INH</td>
<td>90.54±2.41</td>
<td>93.81±2.41</td>
<td>0.70</td>
<td>1.04±0.03</td>
<td>0.60</td>
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<td>C1r</td>
<td>9.23±0.54</td>
<td>20.97±1.09</td>
<td>&lt;0.001</td>
<td>2.35±0.19</td>
<td>&lt;0.001</td>
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<tr>
<td>C1s</td>
<td>4.89±0.61</td>
<td>20.21±0.91</td>
<td>&lt;0.001</td>
<td>4.96±0.76</td>
<td>&lt;0.001</td>
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<tr>
<td>DAF</td>
<td>15.61±1.21</td>
<td>17.53±0.91</td>
<td>0.22</td>
<td>1.12</td>
<td>0.21</td>
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<tr>
<td>C4BP</td>
<td>19.94±0.54</td>
<td>19.30±0.98</td>
<td>0.58</td>
<td>0.97</td>
<td>0.48</td>
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<td>MCP</td>
<td>18.71±2.44</td>
<td>17.69±1.41</td>
<td>0.72</td>
<td>0.94</td>
<td>0.35</td>
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<td>C4</td>
<td>34.90±0.94</td>
<td>46.69±1.98</td>
<td>&lt;0.001</td>
<td>1.34</td>
<td>&lt;0.001</td>
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<tr>
<td>CD59</td>
<td>112.7±3.6</td>
<td>117.2±2.7</td>
<td>0.96</td>
<td>1.06±0.05</td>
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<td>C7</td>
<td>9.50±0.85</td>
<td>22.66±1.09</td>
<td>&lt;0.001</td>
<td>2.61±0.33</td>
<td>&lt;0.001</td>
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<td>C8</td>
<td>6.80±0.55</td>
<td>20.81±1.04</td>
<td>&lt;0.001</td>
<td>3.25±0.27</td>
<td>&lt;0.001</td>
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<tr>
<td>Cyclophilin</td>
<td>80.84±0.52</td>
<td>80.60±0.37</td>
<td>0.71</td>
<td>1.00±0.003</td>
<td>0.38</td>
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</table>

Values are mean±SEM.

*Reported values are not normalized to cyclophilin. Cyclophilin values are almost constant; thus, P values for normalized data are unchanged.

†Corrected for multiple comparisons by the stepdown procedure of Holm.26
‡The ratio of plaque/normal was calculated for each of the 11 pairs, and ANOVA was used to calculate the mean±SEM and the significance of the difference from 1.00±0.00.

gave a strong band for fraction Bb, indicating that aggregated IgG, when added to serum, will activate the alternative pathway and that no apparent activation of the alternative pathway is occurring in plaque tissue.

Figure 3 illustrates immunohistochemical results for C1-INH, CD59, and fraction Bb staining. Overall, they show a similar intensity of staining in plaque compared with normal artery for both C1-INH and CD59, although the staining in plaque tissue is more widespread. The distribution is somewhat different. C1-INH staining is more prominent in the intimal and adventitial layers than in the medial layer (Figure 3A). In plaque tissue, C1-INH staining is widespread in the proliferating fibrous zone and appears to be intracellular and extracellular (Figure 3B). CD59 staining is intense in the endothelial layer, as previously described (Figures 3C and 3D), with weaker staining in the other layers. In fibrous plaque tissue, the antibody to CD59 weakly stains some elongated fibromyocytes and some round cells resembling leukocytes (Figure 3D). Overall, the immunohistochemical findings for C1-INH and CD59 are consistent with the mRNA and Western blot data, showing a failure to upregulate the inhibitors in inflamed plaque tissue. Fraction Bb staining was very faint (Figure 3E), with no structures being identifiable.

**Discussion**

The data described in the present study show that normal arterial cells produce the key complement regulators C1-INH, DAF, C4BP, MCP, and CD59. This is consistent with a previous study, which reported that rat arterial smooth muscle cells produce DAF, MCP, and CD59 as well as the complement proteins C3, C4, and C5. The regulator levels are not significantly increased in plaque tissue, but the complement proteins that they regulate are significantly enhanced. Thus, these regulatory mechanisms are unable to defend the host against complement activation within the plaque. There is full activation of the classical complement pathway in plaque tissue, and localization of the autolytic MAC on damaged transformed myocytes emphasizes the harmful aspects of this activation. In contrast, the failure to identify more than trace amounts of fraction Bb of factor B, a specific marker for activation of the alternative pathway, in either normal artery or nearby plaque tissue would suggest that activation of the alternative pathway is not a significant factor.

We have previously shown that all components of the classical complement pathway are produced within arterial tissue and that they are all significantly upregulated in plaques. Moreover, we have shown that CRP, a known complement activator, is also produced by arterial tissue and is more upregulated than any of the complement proteins. Thus, the mechanism for a self-sustaining complement attack generated within plaque tissue itself is clearly present.

In several previous publications, it has been shown that CRP and the MAC colocalize on damaged cells in the proliferating zone of plaque tissue. The fact that the relevant mRNAs are also colocalized to these same cells indicates that the process is internal to plaque tissue. Although the plaque deposits build up over time and are, thus, the end result of a long-standing pathological process, the half-life of mRNAs is usually minutes to hours. Therefore, the mRNA levels give a dynamic picture of what is continuously occurring in plaque tissue.

The reason for the proliferation of cells in the deep intimal layers of evolving plaque tissue is unclear. It is proposed that oxidized LDL is a stimulant for such proliferation. This may be an initial stimulus, but it is possible that continuing complement attack is also acting as a stimulus, thus enhancing the plaque enlargement process. Macrophage attack occurs on opsonized targets, and such an attack is easily discerned within the proliferating plaque shoulder area. The process leads to 2 unfortunate outcomes. The first is dissolution of the fibrous coat by macrophage collagenase activity, releasing material that causes platelet aggregation and throm-
barely detectable bands of equal intensity were obtained from fraction Bb, was obtained in activated human serum, but only for C1-INH appear to be of equal strength in plaque compared with normal artery, but considerably stronger bands for C1r and C1s can be seen in the plaque samples. The comparative strength in plaque vs normal artery is noticeably greater for C1s than C1r. Notice also a faint minor band for C1s. Second row, C4BP compared with C4 and C4d. Bands of ~70 kDa, corresponding to the reported molecular weight of C4BP, were detected in normal artery and plaque tissue. They were relatively equal in intensity. In contrast, a much stronger band for C4 of ~75 kDa was obtained in plaque compared with normal artery. A clearly detectable band for C4d, a specific marker of classical pathway activation, of ~45 kDa was obtained from plaque tissue, but no detectable band was obtained from normal artery. Third row, CD59 compared with C7 and C8. Bands of ~18 kDa for CD59, 115 kDa for C7, and 85 kDa for C8 can be seen, which is consistent with previously reported values. The bands for CD59 appear to be of comparable strength in normal and plaque tissue. Only faint bands (indicated by arrowheads) can be visualized in normal artery for C7 and C8, whereas strong bands can be seen in plaque tissue. C8 shows a stronger main band than does C7, and a faint secondary band is also visible. Bottom row, Comparison of fraction Bb expression in normal serum (NS) diluted 1:20, the same serum activated (AS) for 1 hour at 37°C with heat-aggregated IgG (see detailed methodology), normal artery, and plaque tissue. A prominent band of ~63 kDa, corresponding to the reported molecular weight of fraction Bb, was obtained in activated human serum, but only barely detectable bands of equal intensity were obtained from normal artery and plaque tissue.

Figure 2. Typical Western blot results on protein extracts of normal (N) vs plaque (P) tissue and normal vs IgG-activated serum. Size markers are shown on the right, and arrows on the left indicate the location of the protein bands. Top row, C1-INH compared with C1r and C1s. It can be seen that C1-INH gave single bands of ~100 kDa and that C1r and C1s gave bands of ~85 kDa, consistent with previously reported values. The bands for C1-INH appear to be of equal strength in plaque compared with normal artery, but considerably stronger bands for C1r and C1s can be seen in the plaque samples. The comparative strength in plaque vs normal artery is noticeably greater for C1s than C1r. Notice also a faint minor band for C1s. Second row, C4BP compared with C4 and C4d. Bands of ~70 kDa, corresponding to the reported molecular weight of C4BP, were detected in normal artery and plaque tissue. They were relatively equal in intensity. In contrast, a much stronger band for C4 of ~75 kDa was obtained in plaque compared with normal artery. A clearly detectable band for C4d, a specific marker of classical pathway activation, of ~45 kDa was obtained from plaque tissue, but no detectable band was obtained from normal artery. Third row, CD59 compared with C7 and C8. Bands of ~18 kDa for CD59, 115 kDa for C7, and 85 kDa for C8 can be seen, which is consistent with previously reported values. The bands for CD59 appear to be of comparable strength in normal and plaque tissue. Only faint bands (indicated by arrowheads) can be visualized in normal artery for C7 and C8, whereas strong bands can be seen in plaque tissue. C8 shows a stronger main band than does C7, and a faint secondary band is also visible. Bottom row, Comparison of fraction Bb expression in normal serum (NS) diluted 1:20, the same serum activated (AS) for 1 hour at 37°C with heat-aggregated IgG (see detailed methodology), normal artery, and plaque tissue. A prominent band of ~63 kDa, corresponding to the reported molecular weight of fraction Bb, was obtained in activated human serum, but only barely detectable bands of equal intensity were obtained from normal artery and plaque tissue.

Figure 3. Immunostaining of a normal artery (A and C) and a nearby plaque (B and D) for C1-INH (A and B) and CD59 (C and D). Lumen is at the top. Panel E shows immunostaining for fraction Bb of a cross section with a normal area indicated by the arrow and a plaque indicated by an asterisk. See Methods for details. In the normal artery (A), C1-INH staining was most intense in the endothelial layer. It was prominent in the intima and adventitia but very weak in the media. Immunoreactivity seemed to occur both extracellularly and intracellularly. In the plaque (B), immunostaining for C1-INH covered a larger area but was of similar intensity to normal artery. The intima, which is considerably thickened and peeled away from the media, showed moderate staining, whereas immunoreactivity in the media was very weak. CD59 immunoreactivity followed a similar pattern but was less intense than that of C1-INH. In normal artery (C), CD59 immunoreactivity was very strong over the endothelial layer and was visible in the intima and adventitia, whereas the media was nearly unstained. In plaque tissue (D), a similar pattern was seen, although the intimal layer was greatly thickened. Staining was often weakly associated with elongated cells. Additionally and in the diseased tissue only, some cells of probable leukocytic origin were stained moderately by the CD59 antibody. Bars = 100 μm in panel C (for panels A and C) and 180 μm in panel D (for panels B and D). In panel E, only trace immunoreactivity was observed for fraction Bb, with no detectable difference between the region of normal arterial wall (arrow) and a prominent fibrous plaque (asterisk). Bar = 200 μm.
modified LDL, and even IgG. But these are all inhibited, including the lectin pathway, by the regulators described in the present study. Of course, there may be other less specific inhibitors, such as α2-macroglobulin and α1-antitrypsin, which play a role in inhibiting the complement cascade, but it cannot be a decisive role because robust activation is clearly taking place in plaque tissue.

In summary, the data reported in the present study suggest that complement activation is at the core of the inflammatory process that characterizes atherosclerosis. The inflammation is silent because arteries lack pain fibers. Therapeutic intervention to reduce the inflammatory process may be an effective addition to reducing the levels of cholesterol and other lipids. Once the inflammatory process in a plaque is initiated, arrest may not be achieved by simple lowering of circulating lipid levels. Controlling the autodestructive local process by inhibiting complement activation may be essential.

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References
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