Atherosclerosis and Lipoproteins

Association Between Complement Factor H and Proteoglycans in Early Human Coronary Atherosclerotic Lesions
Implications for Local Regulation of Complement Activation

Riina Oksjoki, Hanna Jarva, Petri T. Kovanen, Petri Laine, Seppo Meri, Markku O. Pentikäinen

Objective—Complement activation has been suggested to play a role in atherogenesis. To study the regulation of complement activation in human coronary atherosclerotic lesions, we examined the spatial relationships between the major complement inhibitor, factor H, and the complement activation products C3d and C5b-9.

Methods and Results—In early lesions (American Heart Association types II and III), factor H was immunohistochemically found in the superficial proteoglycan-rich layer in association with numerous macrophages and C3d, whereas C5b-9 was found deeper in the intima, where factor H was virtually absent. In vitro experiments involving surface plasmon resonance and affinity chromatography analyses demonstrated that isolated human arterial proteoglycans bind factor H, and functional complement assays showed that glycosaminoglycans inhibit the complement activation induced by modified low density lipoprotein or by a foreign surface.

Conclusions—The present observations raise the possibility that proteoglycans, because of their ability to bind the major complement inhibitor factor H, may inhibit complement activation in the superficial layer of the arterial intima. In contrast, deeper in the intima, where factor H and proteoglycans are absent, complement may be activated and proceed to C5b-9. Thus, the superficial and the deep layers of the human coronary artery appear to differ in their ability to regulate complement activation. (Arterioscler Thromb Vasc Biol. 2003;23:630-636.)

Key Words: atherosclerosis complement factor H proteoglycans

Increasing evidence implies that one of the key factors generating and maintaining inflammation in the arterial intima is the complement system.1 Immuno-staining experiments have shown the presence of large amounts of the terminal complement complex C5b-9 in association with smooth muscle cell α-actin and modified LDL.2,3 Lipids isolated from human atherosclerotic lesions and enzymatically modified LDL have been shown to activate complement via the alternative pathway in vitro.4 Most interestingly, in rabbits deficient in complement component C6 of the terminal complement cascade, the development of experimental atherosclerosis has been shown to be retarded.5 Thus, although the present evidence suggests a proatherogenic role for the terminal complement pathway, the role in atherosclerosis of the early complement cascade is not known. Interestingly, in C3-deficient apoE-null mice, the formation of fatty streaks was increased, but the fatty streaks failed to mature into fibrotic lesions.6

See cover

Complement activation depends critically on the balance between complement activators and complement-regulatory proteins. Human atherosclerotic lesions have previously been shown to contain the fluid-phase inhibitors (C1 inhibitor, factor H, factor B, and C4 binding protein), clusterin (apoJ), vitronectin/S-protein, and the cell membrane glycoproteins (complement receptors 1 and 3,7 decay-accelerating factor,11 and CD59).12 Consistent with the presence of complement activation products in atherosclerotic lesions, it has recently been shown that mRNAs for complement components, but not for complement inhibitors, are upregulated in atherosclerotic plaques.13

In the present study, our aim was to find out how complement activation is regulated in the arterial intima. For this purpose, we collected human coronary arteries and studied the spatial relationships between the complement activation markers (C3d and C5b-9) and the major complement inhibitor (factor H) and C-reactive protein (CRP), a protein that can activate the early classical complement pathway and inhibit complement amplification by binding factor H.14 Moreover, in vitro binding studies and complement consumption assays were carried out to study the complement-regulating properties of human arterial proteoglycans.

Methods

An extended Methods section can be found in the online supplement (available at http://atvb.ahajournals.org).
Coronary artery specimens (n=36) were obtained from 29 patients; their characteristics are shown in online Table I (see http://atvb.ahajournals.org). Frozen and paraffin sections were stained by the indirect immunoperoxidase method by using 3-aminopropyltriethylcarbazole or Vector NovaRED (Vector Laboratories) as substrate and were counterstained with hematoxylin. The primary antibodies used in the present study are listed in online Table II (see http://atvb.ahajournals.org).

Proteoglycans were isolated from the intima-media of human aortas. The proteoglycans were dissociated into monomers by incubation in buffer containing 4 mol/L guanidine HCl, and the proteoglycan monomers so formed were isolated by size-exclusion chromatography.

For surface plasmon resonance measurements, human factor H and CRP were immobilized on carboxylated dextran CM5 chips (Biacore) by an amine-coupling procedure. Analyses of their binding with human aortic proteoglycans were performed in 1:3 veronal-buffered saline (50 mmol/L NaCl, 0.6 mmol/L sodium barbital, and 1.1 mmol/L barbituric acid; pH 7.5).

For affinity chromatography, human aortic proteoglycans were coupled to a N-hydroxysuccinimide-activated HiTrap column (Amersham Pharmacia Biotech). Human factor H (20 μg) was injected into the column that had been equilibrated with a buffer containing 10 mmol/L HEPES, pH 7.4, and either 2 mmol/L CaCl₂ or 100 μmol/L EDTA. The material bound to the column was then eluted with a gradient of NaCl (0–250 mmol/L) in the buffer.

For assays of complement consumption, LDL was modified enzymatically, as described previously, to yield E-LDL. Complement activation in normal human serum (NHS) was studied by incubating NHS (50 μL) in the absence or presence of Sepharose, heparin-Sepharose, proteoglycan-Sepharose, or CRP (50 μg/mL) in a total volume of 100 μL PBS. In other experiments, NHS (50 μL in a total volume of 100 μL PBS) was incubated with E-LDL (50 μg/mL) in the absence or presence of heparin (100 μg/mL). The samples were incubated for 20 minutes at 37°C in a shaker, and generation of the complement activation product C₃a-desarg was quantified by ELISA (Quidel Co.).

For analysis of factor H–like proteins in human atherosclerotic lesions, 2 samples from grossly normal human coronaries and samples from normal and atherosclerotic aortas were homogenized and analyzed by Western blotting. The blots were probed with goat α-human factor H (Calbiochem, diluted 1:5000), rabbit α-human short consensus repeat (SCR) 19-20 (diluted 1:1000, kindly provided by Dr Jens Hellwage, Hans Knoll Institute, Jena, Germany), or mouse α-factor H antibody 196X at 5 μg/mL.

### Results

The distribution patterns of complement activation products, the complement inhibitor factor H, and CRP were compared with those of proteoglycans and apoB by immunohistochemistry. As a control, online Figure I (data supplement; please see http://atvb.ahajournals.org) shows a grossly normal human coronary artery (American Heart Association type I lesion) with the adaptive intimal thickening typical of coronary arteries. Neither the superficial nor the deep layer of the intima showed staining for C3d or C5b-9, indicating the absence of immunohistochemically detectable complement activation. The narrow proteoglycan-rich layer stained positively for factor H and partially for CRP.

Figure I and online Figure II (data supplement; please see http://atvb.ahajournals.org) show early to intermediate atherosclerotic lesions with intimal thickening and fatty streak formation. Typically, the macrophages and apoB were located in the superficial thick proteoglycan-rich layer, which was visualized by immunostaining for versican. Interestingly, these areas were also positive for C3d, whereas C5b-9 was found as distinct deposits deeper in the intima (in the musculoelastic layer). Thus, in the superficial intima, complement activation had been limited to the C3 level, whereas in the deep intima, it had proceeded to the C5b-9 level. Importantly, factor H, the major complement inhibitor, was present only in the superficial intima, in which it showed strict colocalization with versican. The observed colocalization of proteoglycans and factor H was obvious in all the early atherosclerotic lesions studied in which a distinct superficial proteoglycan-rich layer and deep musculoelastic layer could be clearly distinguished (n=10). In addition, a reciprocal staining pattern for factor H and C5b-9 was found in most of the samples studied. Three examples of such staining, with the use of immunofluorescence double staining, are shown in Figure 2. When the overlap between C5b-9 and factor H was estimated in samples in which distinct staining for factor H and C5b-9 was found (n=24), it was found that a reciprocal staining pattern (≤20% overlap) was present in 62.5% of the lesions, partial overlap (21% to 80% overlap) in 25% of the lesions, and colocalization (>80% overlap) in

![Figure 1. Immunohistochemical analysis of an intermediate atherosclerotic lesion in a human coronary artery. Paraffin sections of an American Heart Association type III atherosclerotic lesion in a human coronary artery were stained with hematoxylin and eosin (H&E) and immunostained with antibodies against versican (LF99), apoB-100 (MB-47), macrophages (HAM-56), factor H, CRP, C3d, and C5b-9 by use of an indirect immunoperoxidase technique (red-brown) and counterstained with hematoxylin. Note the presence of factor H, CRP, and C3d in the proteoglycan (PG)-rich layer and the reciprocal distribution of factor H and C5b-9.](http://atvb.ahajournals.org)
12.5% of the lesions. Finally, CRP, which has the ability to initiate the classical complement pathway but also to terminate complement activation at the C3 level by binding factor H,14 was mainly found in the superficial intima colocalized with proteoglycans and factor H.

In the advanced atherosclerotic lesions studied (n=11005),14 staining for both proteoglycans and factor H was more diffuse and showed only partial colocalization, regardless of the presence of a distinct proteoglycan-rich layer (found in 4 samples). In the advanced lesions, in accord with the previous results, extensive deposition of C5b-9 was also occasionally detected in areas positive for factor H19 and CRP.20 Because the immunohistochemical findings demonstrated that factor H is present predominantly in the proteoglycan-rich layer of the intima, we experimentally tested for the ability of human arterial proteoglycans to bind factor H.

Using the surface plasmon resonance technique, we were able to observe binding of human arterial proteoglycans to immobilized factor H (Figure 3A). Moreover, because CRP colocalized with the proteoglycans in the arterial intima, we also tested for its ability to bind to the proteoglycans isolated from human aortas. However, the proteoglycans failed to bind to immobilized CRP in vitro (online Figure III; please see http://atvb.ahajournals.org).

Binding of factor H to surface-immobilized proteoglycans was analyzed further by affinity chromatography (Figure 3B). In this system, almost all of the factor H injected into the proteoglycan affinity column was eluted as a single peak at 50 mmol/L NaCl. This binding was found to be independent of the presence of Ca++. Because the results obtained by immunohistochemistry suggested a role for proteoglycans in complement inhibition, we studied whether glycosaminoglycans would inhibit complement activation in vitro. For this purpose, complement activation in NHS was studied by using enzymatically modified LDL and a foreign surface (Sepharose) as complement activator. We found that heparin glycosaminoglycan, when added to the solution with enzymatically modified LDL, strongly inhibited the production of C3a (Figure 4A). Moreover, heparin, when coupled to Sepharose, was able to fully inhibit the Sepharose-induced complement activation (Figure 4B). CRP, when added to the incubation mixture, caused a nonsignificant increase in Sepharose-induced complement activation and had no effect in the presence of heparin-Sepharose. Most important, proteoglycans isolated from human aortas were able to inhibit the complement activation induced by Sepharose beads (Figure 4C). Taken together, it appears that glycosaminoglycans can effectively inhibit complement activation in vitro.

To ascertain which proteins of the factor H family are deposited in human arterial tissue, the factor H–like proteins in homogenates of human coronary arteries and of the aorta were detected by immunoblotting (Figure 5).21 With a polyclonal antibody against factor H (Figure 5A), which recognizes all the proteins in the factor H family, we detected a band at 150 kDa corresponding to factor H, a band at 42/43 kDa corresponding to
both factor H–like protein-1 (FHL-1, ~42 kDa) and the α-form of factor H–related protein-1 (FHR-1, 43 kDa), and a band at 37 kDa corresponding to the β-form of FHR-1. We next probed the blot with an antibody against the SCR 1 of factor H and FHL-1 that does not bind to the FHRs. As shown in Figure 5B, relative to human serum, all the arterial preparations were enriched in FHL-1. Finally, we probed the blot with an antibody against

Figure 3. Surface plasmon resonance analysis and affinity chromatography of factor H binding to human arterial PGs. A, Large versican-like PG aggregates were isolated from human aortic intima-media. PGs (0.3 μg) were injected into a control flow cell (where no coating was performed) or into a flow cell coated with factor H in a BIACORE 2000 instrument, and the resonances (in arbitrary units) were monitored as a function of time. B, Human aortic PGs were coupled to a HiTrap NHS-activated column, as described in Methods. Factor H (20 μg) was injected into the column in a SMART chromatography apparatus, and the column was eluted with a gradient of 0–250 mmol/L NaCl. Elution of factor H and formation of a NaCl gradient were monitored by UV absorbance at 280 nm (A280) and conductometry (NaCl, mmol/L), respectively.

Figure 4. Effects of heparin and PGs on complement activation. Complement activation in NHS was studied by using E-LDL and Sepharose as complement activators. Generation of the complement activation product C3a-desarg was quantified by ELISA. A, E-LDL (50 μg/mL) significantly increased the generation of C3a-desarg (P<0.05), and heparin (100 μg/mL), when added to the mixture, was able to inhibit E-LDL–induced complement activation (P<0.05). B, Sepharose induced a significant (P<0.05) increase in the complement activation. Heparin, when coupled to Sepharose, was able to totally inhibit the generation of C3a-desarg (P<0.05). CRP caused a nonsignificant increase in the complement activation induced by Sepharose. Values are mean±SEM. C, Human aortic PG, when coupled to Sepharose, significantly inhibited the complement activation induced by Sepharose. Results are from 3 independent experiments. P<0.05 by paired t test.
SCR 19-20, which recognizes factor H and the different FHRs but not FHL-1. As shown in Figure 5C, the amount of FHR-1 in the vascular samples appeared not to be increased but reduced. Moreover, the relative intensities of the FHR-1 bands corresponded to those of albumin, suggesting that at least a fraction of the FHR-1 in the intima was plasma-derived.

Discussion

In the present study, we found that in early atherosclerotic lesions, the superficial layer of the arterial intima containing the versican proteoglycan also contained the major complement inhibitor, factor H. In most lesions, this area was also positive for CRP and C3d. However, in the deeper parts of the intima and also to some extent in the media, there was a relative absence of factor H and prominent deposition of the terminal complement complex C5b-9. This mutually exclusive staining pattern of factor H and C5b-9 suggests that factor H tends to prevent the assembly of the terminal complement complex in the arterial intima. In addition, the immunoblotting experiments revealed that various arterial preparations were relatively enriched not only in factor H but also in FHL-1, a protein that is encoded by an alternatively spliced mRNA derived from the factor H gene and that shares the complement-regulatory properties of factor H. Macrophages have previously been shown to synthesize factor H and FHL-1.22 Thus, these proteins, in addition to infiltration from the plasma, may also have originated from local synthesis in the macrophage-rich areas of the coronary intima.

Because proteoglycans colocalized with factor H in the arterial intima, we hypothesized that they might be involved in controlling excessive complement activation. Two independent types of analysis showed that factor H binds to isolated arterial proteoglycans in vitro. This binding is likely to involve ≥1 of the heparin-binding sites present in SCRs 7, 13, and 20 of factor H.23–26 Importantly, we could also show that human arterial proteoglycans, like heparin, can inhibit complement activation in vitro. The inhibitory effect of heparin appears to depend on its ability to potentiate the effect of factor H in C3b inactivation.27,28 Taken together, our observations made in vivo and in vitro suggest that factor H binds to the intimal proteoglycans and may restrict complement activation locally. In the glomerular basement membranes of pigs genetically deficient in factor H29 and in humans either genetically deficient in factor H30 or with dysfunction of factor H due to autoantibodies,31 the in vivo findings of extensive complement deposition in the glomerular membrane of the kidney demonstrate the importance of factor H in protecting the extracellular matrix components against attack by complement. The present analogous findings in the human coronary artery extend the repertoire of protected tissues to the proteoglycan-rich layer of the arterial intima. Indeed, this intimal layer can be considered functionally analogous to the glomerular membrane in terms of its high content of glycosaminoglycans and in its capacity to retain molecules with an affinity for heparin.

The present study did not elucidate the nature of the agents that activate the complement system in the arterial intima. However, the findings of (1) C3d in the absence of C5b-9 in the proteoglycan-rich layer of the arterial intima and (2) the deposition of C4 also in this layer (not shown) led us to infer that the classical pathway of complement is activated in this layer, although activation via mannose-binding lectin cannot be excluded.32 One of the mediators of the classical complement pathway activation is CRP. We, among others, have found CRP in the arterial intima,20,31–37 and interestingly, intimal cells in addition to hepatocytes have been shown to encode a high level of mRNA for CRP.37,38 Previous studies have shown that human atherosclerotic lesions also contain deposits of IgM and IgG,39,40 both of which have been shown to activate the classical pathway of complement in vitro.41 CRP and immunoglobulins have been shown to bind to modified lipoproteins,36,42 and CRP also binds to apoptotic cells,43 chromatin,44 and fibronectin,45 all of which are present in atherosclerotic lesions. The presence, in the deep musculoelastic layer of the arterial intima, of C5b-9 and extracellular lipids, including cholesterol crystals, is compatible with the view that the extracellular lipids have activated...
the alternative pathway of complement. This view is supported by previous reports demonstrating that intimal extracellular liposomes, enzymatically modified LDL, and cholesterol crystals can all activate the alternative pathway of complement in vitro.

As demonstrated by a study involving experimental atherosclerosis in C6-deficient rabbits, excessive activation of the terminal complement pathway, like excessive activation of other pathways of the inflammatory system, is proatherogenic. Although activation of the early complement cascade may lead to full activation of complement with ensuing inflammation, its role in opsonization, the evolutionarily old function of complement, may be protective. Indeed, this is supported by the recent findings showing that the formation of fatty streak lesions is increased in apoE-null mice deficient in C3. According to this view, CRP and factor H may work together in clearing debris from the intima via opsonization of other pathways of the inflammatory system, is proatherosclerosis in C6-deficient rabbits, excessive activation of complement in vitro.

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Methods

Patients and coronary artery specimens
Coronary artery specimens (n=36) from 29 patients were obtained at autopsy 27 to 216 hours after death of the patients. Patient characteristics are described in Table I. Segments with and without evident atherosclerotic plaques were taken from the left anterior descending arteries. The samples were either embedded in OCT and snap-frozen in liquid nitrogen or fixed in 10% neutral-buffered formalin and embedded in paraffin according to standard procedures. Frozen (8 µm) or paraffin (4 µm) sections were cut, stained with hematoxylin-eosin, and evaluated microscopically to classify the atherosclerotic lesions according to the guidelines of the American Heart Association (AHA). Two of the coronary specimens were classified as normal, 20 as early atherosclerotic lesions (AHA types II-III), and 14 as advanced coronary plaques (AHA types IV-V).

Histology
Frozen and paraffin sections were stained by the indirect immunoperoxidase method with ABC Elite kits (Vector laboratories). Briefly, the paraffin sections were deparaffinized in xylene and rehydrated through a series of graded alcohols. The frozen section were fixed in methanol for 10 minutes and washed three times in PBS (pH 7.4). All slides were treated with 2% H2O2 for 30 minutes to block endogenous peroxidase activity, followed by 2 x 5min PBS washings. Then, slides were incubated with 3% normal serum (rabbit, horse, or goat) diluted in 0.1% Tween-3%BSA in PBS for 30 min at room temperature (RT) to reduce non-specific binding of antibodies. Primary antibodies (listed in Table II) were incubated overnight +4°C in the above buffer. As negative controls, the primary antibodies were replaced with non-specific rabbit IgG or isotype-matched mouse IgG. The primary antibodies were detected using the avidin-biotin complex system (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA) with 3-amino-9-ethylcarbazole (AEC; Sigma) or Vector NovaRED peroxidase substrate (Vector laboratories, Burlingame, CA, USA) as a chromogen. Thereafter, the sections were counterstained with hematoxylin and mounted using Aquamount or Permount. For the simultaneous localization of factor H and C5b-9, we used immunofluorescence double-staining method. Slides were
incubated with primary antibodies against both factor H and C5b-9 overnight at +4°C. After washing 2 x 5 min in PBS, biotinylated secondary antibody (Goat Elite Kit) and TRITC-conjugated rabbit α-mouse (1:50, Dakopatts) were applied and the slides were incubated for 45 min at RT followed by washing 2 x 5 min in PBS. To detect the biotinylated secondary antibody, the slides were incubated with streptavidin-FITC (10 µg/ml, DAKO) for 1 hour at RT. The slides were washed 3 x 3 min in PBS and the nuclei were counterstained with DAPI (Molecular probes). The slides were mounted using fluorescent mounting medium (DAKO).

The overlap between areas positive for factor H and C5b-9 was quantitatively analyzed in samples in which distinct staining for factor H and C5b-9 was found, independent of the staining method (double-immunofluorescence or indirect immunoperoxidase-method). The area positive for C5b-9 was estimated and the overlap between C5b-9 and factor H was expressed as a percentage of C5b-9 in an area positive for factor H. The staining patterns were categorized as reciprocal (≤ 20% overlap), partial overlap (21-80% overlap) or, colocalization (>80% overlap).

**Isolation and purification of human proteoglycans**

Proteoglycans from the intima-media of human aortas were obtained at autopsy within 24 h of accidental death and were prepared essentially by the method of Hurt-Camejo et al., as described previously. Briefly, proteoglycans were extracted from intima-media at 4°C for 24 h with 15 volumes of 6 M urea, 1 M NaCl in the presence of 10 mM EDTA, 10 mM ε-aminocaproic acid, 0.2 mM phenylmethyl sulfonyl fluoride, and 0.02% (w/v) NaN3. After extraction, the mixture was centrifuged at 100,000 g for 60 min. The supernatant was diluted with 6 M urea to give a final concentration of 0.25 M NaCl and loaded on a HiTrap Q column (5 ml) equilibrated with 6 M urea, 0.25 M NaCl, 10 mM CaCl2, and 50 mM acetate, pH 6.2, and the protease inhibitors. The column was washed with the above buffer, and the proteoglycans were eluted with a linear gradient of 0.25 to 1.0 M NaCl in the buffer (120 ml) at a flow rate of 3 ml/min. The peaks at 280 nm were collected, dialyzed against water and lyophilized. Glycosaminoglycans were quantified by the method of Bartold and Page. The amounts of the proteoglycans are expressed in terms of their glycosaminoglycan contents. The proteoglycans were dissociated into monomers by
incubation in buffer containing 4 M guanidine HCl and the proteoglycan monomers formed were isolated by chromatography in 50x1 cm column packed with Sephacryl 400 HR.\textsuperscript{6}

**Surface plasmon resonance analysis**
Surface plasmon resonance measurements were performed using the BIACORE 2000® instrument and BIAevaluation software V 3.0 (Biacore AB, Uppsala, Sweden) as described previously.\textsuperscript{7} Human factor H (Calbiochem Corp., La Jolla, CA, USA) and human CRP (Sigma) were immobilized on carboxylated dextran CM5 sensor chips (Biacore), using the amine coupling procedure according to the protocol of the manufacturer. Analyses of binding with human aortic proteoglycans were performed, using 1/3 veronal-buffered saline buffer (50 mM NaCl, 0.6 mM sodium barbital, 1.1 mM barbituric acid, pH 7.5) at a flow rate of 5 µl/min at 22 °C. In control experiments, the sensor chips were treated identically, except that no protein was coupled to the chip.

**Affinity chromatography**
Human aortic proteoglycans (1 mg) were coupled to a 1-ml NHS-activated HiTrap column (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer’s instructions. Human factor H (20 µg) was injected into the column that had been equilibrated with a buffer containing 10 mM HEPES, pH 7.4, and either 2 mM CaCl\textsubscript{2} or 100 µM EDTA. The material bound to the column was then eluted with a gradient of NaCl (0 → 250 mM) in the buffer at a flow rate of 0.5 ml/min in a high-performance FPLC system (SMART®; Amersham-Pharmacia Biotech). The elution was monitored by UV absorbance at 280 nm and the NaCl gradient by conductometry.

**Effects of heparin and proteoglycans on complement activation**
LDL was enzymatically modified by trypsin and cholesterol esterase to yield E-LDL, as described previously.\textsuperscript{9} Briefly, LDL (2 mg/ml protein) was first treated with trypsin from bovine pancreas (Sigma) in PBS at +37°C for 2 h after which trypsin was inhibited with 64 µg/ml Soybean trypsin inhibitor (Sigma) in PBS. Finally the mixture was incubated with 80 µg/ml CEase from Candida Cylindracea (Boehringer
Mannheim, Germany) in PBS at +37°C for 2 h. E-LDL was purified by gel filtration on a Bio-Gel A-0.5m column (1x50 cm, Bio-Rad, Hercules, CA, USA) and its concentration was determined from its cholesterol content. Normal human serum (NHS) was obtained from healthy volunteers and stored at –70°C until use. Human aortic proteoglycans (2mg) were coupled to NHS-activated HiTrap Sepharose columns, as described above. Control Sepharose was prepared by blocking a NHS activated Sepharose column according to the manufacturer’s instructions. Complement activation in NHS was studied by incubating NHS (50 µl) in the absence or presence of Sepharose, heparin-Sepharose (22.5 µl; Amersham Pharmacia Biotech), proteoglycan-Sepharose (22.5 µl) or CRP (50 µg/ml; Calbiochem, San Diego, CA, USA) in a total volume of 100 µl PBS. In other experiments, NHS (50 µl in a total volume of 100 µl PBS) was incubated with E-LDL (50 µg/ml) in the absence or presence of heparin (100 µg/ml; Sigma). The samples were incubated for 20 minutes at 37°C in a shaker, after which complement activation was terminated by addition of 10 mM EDTA and by cooling the samples in an ice bath. Generation of the complement activation product C3a-desarg was quantified by enzyme-linked immunoassay (ELISA) (Quidel Co, San Diego, CA) according to manufacturer’s instructions.

**Analysis of factor H-like proteins in human atherosclerotic lesions**

Samples of two grossly normal human coronary arteries and samples from grossly normal and atherosclerotic areas of a human aorta were obtained at autopsy. The samples were minced and homogenized in buffer containing 5 mM Tris-HCl, 0.5% Triton X-100, 10 mM NaOH, 1 mM PMSF, 2 µg/ml leupeptin, 2 µg/ml aprotinin and 15 mM EDTA. Aliquots of the homogenates (10 µg/lane) were run into a 10% SDS-page gel under nonreducing conditions and electrotransferred onto a nitrocellulose membrane; 2% normal human serum was run in parallel as a control. Nonspecific binding sites were blocked by incubating the membrane for 1 hour at 22°C in 5% fat-free milk in PBS. The membranes were then incubated overnight at +4°C with the following antibodies: goat α-factor H (Calbiochem Corp., diluted 1:5000), rabbit α-SCR19-20 (diluted 1:1000, kindly provided by Dr. Jens Hellwage, Hans Knoell Institute, Jena, Germany) or mouse α-factor H antibody 196X8 (5 µg/ml). After incubation, the membranes were washed and incubated for 1 h at room temperature
with HRP-conjugated donkey α-goat, goat α-rabbit, or rabbit α-mouse-IgG (all
diluted 1:5 000; all from Jackson Immunoresearch Laboratories, West Grove, PA).
Finally, the membranes were washed and binding was visualized on an x-ray film by
enhanced electrochemiluminescence (Super RX, Fuji Photo Film Co, Tokyo, Japan).

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<td>II</td>
<td>Intoxication</td>
<td>Breast cancer, operated</td>
<td>Aspirin</td>
</tr>
<tr>
<td>3</td>
<td>71/F</td>
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<td>IV</td>
<td>Ketonacidosis</td>
<td>Diabetes, asthma</td>
<td>Insulin, verapamil</td>
</tr>
<tr>
<td>4</td>
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<td>V</td>
<td>Cranial fracture, intracerebral hemorrhage</td>
<td>Data not available</td>
<td>No medication</td>
</tr>
<tr>
<td>5</td>
<td>83/F</td>
<td>frozen</td>
<td>IV</td>
<td>Aortic dissection</td>
<td>Healthy</td>
<td>No medication</td>
</tr>
<tr>
<td>6</td>
<td>85/F</td>
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<td>III</td>
<td>Pneumonia, hip fracture</td>
<td>Dementia, diabetes, asthma</td>
<td>Furosemide, verapamil, thyroxin, budesonide, insulin</td>
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<tr>
<td>7</td>
<td>86/F</td>
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<td>I</td>
<td>AMI</td>
<td>Diabetes, asthma</td>
<td>Metformin, beclomethasone, salbutamol</td>
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<tr>
<td>8</td>
<td>34/M</td>
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<td>III</td>
<td>Gunshot</td>
<td>Data not available</td>
<td>No medication</td>
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<tr>
<td>9</td>
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<td>II</td>
<td>Hypothermia</td>
<td>Alcohol abuse</td>
<td>Data not available</td>
</tr>
<tr>
<td>10</td>
<td>40/M</td>
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<td>II</td>
<td>Trauma</td>
<td>Schizophrenia</td>
<td>Lorazepam, clozapine</td>
</tr>
<tr>
<td>11</td>
<td>42/M</td>
<td>frozen</td>
<td>IV</td>
<td>Trauma</td>
<td>Healthy</td>
<td>No medication</td>
</tr>
<tr>
<td>12</td>
<td>44/M</td>
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<td>II</td>
<td>Dilating cardiomyopathy with cardiac failure</td>
<td>Alcohol abuse</td>
<td>No medication</td>
</tr>
<tr>
<td>13</td>
<td>51/M</td>
<td>paraffin</td>
<td>III</td>
<td>Crash injury</td>
<td>Healthy</td>
<td>Oxazepam</td>
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<tr>
<td>14</td>
<td>53/M</td>
<td>frozen</td>
<td>IV, V</td>
<td>Intoxication</td>
<td>Alcohol abuse</td>
<td>Chlorpromazine</td>
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<tr>
<td>15</td>
<td>55/M</td>
<td>frozen</td>
<td>II, III, V</td>
<td>Aortic dissection</td>
<td>Alcohol abuse</td>
<td>Nimesulide</td>
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<tr>
<td>16</td>
<td>59/M</td>
<td>paraffin</td>
<td>III</td>
<td>Violent death</td>
<td>Healthy</td>
<td>No medication</td>
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<tr>
<td>17</td>
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<td>frozen</td>
<td>III, III</td>
<td>Perioperative death</td>
<td>Aortic valve insufficiency, endocarditis</td>
<td>Warfarin, atenolol, furosemide, captopril, glimepiride, allopurinol</td>
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<tr>
<td>18</td>
<td>61/M</td>
<td>frozen</td>
<td>III</td>
<td>Mesothelioma</td>
<td>Data not available</td>
<td>Ibuprofen, lactulose, dexamethasone, lorazepam, haloperidol</td>
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<tr>
<td>19</td>
<td>63/M</td>
<td>paraffin</td>
<td>II, III, IV</td>
<td>Hanged</td>
<td>Healthy</td>
<td>No medication</td>
</tr>
<tr>
<td>20</td>
<td>64/M</td>
<td>paraffin</td>
<td>V</td>
<td>Arrhythmia</td>
<td>Ventricular ulcer</td>
<td>Lansoprazole</td>
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<tr>
<td>21</td>
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<td>paraffin</td>
<td>III</td>
<td>AMI with cardiac failure</td>
<td>Data not available</td>
<td>Zopiclone</td>
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<tr>
<td>22</td>
<td>69/M</td>
<td>frozen</td>
<td>III</td>
<td>Hypothermia</td>
<td>Data not available</td>
<td>No medication</td>
</tr>
<tr>
<td>23</td>
<td>69/M</td>
<td>frozen</td>
<td>V</td>
<td>Intoxication</td>
<td>Diabetes, asthma, Addison's disease</td>
<td>Insulin, hydrocortisone, fludrocortisone acetate, salbutamol</td>
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<tr>
<td>24</td>
<td>70/M</td>
<td>paraffin</td>
<td>II</td>
<td>Pulmonary embolism</td>
<td>Brain infarction</td>
<td>Diclofenac, tolterodine, isradipine, candesartan</td>
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<tr>
<td>25</td>
<td>70/M</td>
<td>frozen</td>
<td>I</td>
<td>AMI</td>
<td>Chronic atrial fibrillation</td>
<td>Digoxin, warfarin, bisoprolol</td>
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<tr>
<td>26</td>
<td>74/M</td>
<td>frozen</td>
<td>V</td>
<td>Cardiac failure</td>
<td>AMI and cardiac failure</td>
<td>Data not available</td>
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<tr>
<td>27</td>
<td>83/M</td>
<td>paraffin</td>
<td>III</td>
<td>Lobar pneumonia</td>
<td>Parkinson's disease, CHD</td>
<td>Morphine, furosemide</td>
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<tr>
<td>28</td>
<td>88/M</td>
<td>frozen</td>
<td>II</td>
<td>Pulmonary embolism</td>
<td>CLL, CHD</td>
<td>Digoxin, furosemide, ISMN, citalopram, haloperidol, cefuroxime</td>
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</table>

AMI indicates acute myocardial infarction; CHD, coronary heart disease; CLL, chronic lymphatic leukemia; and ISMN, isosorbide mononitrate
Table II. List of antibodies used.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Host</th>
<th>Conc./dilution</th>
<th>Source/ref.</th>
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</thead>
<tbody>
<tr>
<td>SC5b-9 neoantigen</td>
<td>A239</td>
<td>mouse</td>
<td>10 µg/ml</td>
<td>Quidel</td>
</tr>
<tr>
<td>apoB</td>
<td>2-B4</td>
<td>mouse</td>
<td>10 µg/ml</td>
<td>ICN Pharmaceuticals</td>
</tr>
<tr>
<td>macrophage surface antigen</td>
<td>HAM56</td>
<td>mouse</td>
<td>0.7 µg/ml</td>
<td>Dako</td>
</tr>
<tr>
<td>CRP</td>
<td>CRP-8</td>
<td>mouse</td>
<td>29 µg/ml</td>
<td>Sigma</td>
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<tr>
<td>CRP</td>
<td></td>
<td>rabbit</td>
<td>1:500</td>
<td>Biomedica</td>
</tr>
<tr>
<td>C3d</td>
<td></td>
<td>rabbit</td>
<td>47 µg/ml</td>
<td>DAKO</td>
</tr>
<tr>
<td>versican LF99</td>
<td></td>
<td>rabbit</td>
<td>1:100</td>
<td>Bernstein et al.¹</td>
</tr>
<tr>
<td>factor H</td>
<td></td>
<td>goat</td>
<td>1:300</td>
<td>Incstar Corp.</td>
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</table>

Fig. I. Immunolocalization of complement activation products, factor H, and CRP in a grossly normal human coronary artery. Frozen sections of a grossly normal human coronary human coronary (AHA type I lesion) were immunostained with antibodies against C3d, C5b-9, factor H, C-reactive protein, apolipoprotein B-100 (MB-47), versican (LF99), and macrophages (HAM-56) using the indirect immunoperoxidase technique (red-brown) and counterstained with hematoxylin. Note the near absence of a proteoglycan-rich layer in this grossly normal artery and the near absence of LDL and of complement proteins.
Figure II.

Versican

ApoB-100

Macrophages

Factor H

CRP

C3d

C5b-9

Proteoglycan-rich layer

Musculoelastic layer

Intima

Media

Adventitia

250 µm
Fig. II. Immunohistochemical analysis of an early atherosclerotic lesion in a human coronary artery. Frozen sections of an AHA type II-III atherosclerotic lesion in a human coronary artery were immunostained with antibodies against C3d, C5b-9, factor H, C-reactive protein, apolipoprotein B-100 (MB-47), versican (LF99), and macrophages (HAM-56) using the indirect immunoperoxidase technique (red-brown) and counterstained with hematoxylin. Note the diffuse distribution of factor H, CRP, and C3d in the proteoglycan-rich layer, and the reciprocal distribution of factor H and C5b-9. Arrows indicate the border between the intimal and medial layers.
Fig. III. Surface plasmon resonance analysis of CRP binding to human arterial proteoglycans.

Large versican-like proteoglycan aggregates were isolated from human aortic intima-media. Proteoglycans (0.3 µg) were injected into a control flow cell (where no coating was performed) or into a flow cell coated with human C-reactive protein (CRP) in a BIACORE 2000® instrument, and the resonances were monitored as a function of time.