Lack of Complement Inhibitors in the Outer Intracranial Artery Aneurysm Wall Associates with Complement Terminal Pathway Activation

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Inflammation and activation of the complement system predispose to intracranial artery aneurysm (IA) rupture. Because disturbances in complement regulation may lead to increased susceptibility to complement activation and inflammation, we looked for evidence for dysregulation of the complement system in 26 unruptured and 26 ruptured IAs resected intraoperatively. Immunohistochemical and immunofluorescence results of parallel IA sections showed that deposition of the complement activation end-product C5b-9 was lacking from the luminal part of the IA wall that contained complement inhibitors factor H, C4b binding protein, and protectin as well as glycosaminoglycans. In contrast, the outer, less cellular part of the IA wall lacked protectin and had enabled full complement activation and C5b-9 formation. Decay accelerating factor and membrane cofactor protein had less evident roles in complement regulation. The Factor H Y402H variant, studied in 97 IA patients, was seen as often in aneurysm patients with or without aneurysm rupture as in the control population. The regulatory capacity of the complement system thus appears disturbed in the outer part of the IA wall, allowing full proinflammatory complement activation to occur before aneurysm rupture. Insufficient complement control might be due to matrix remodeling and cell loss by mechanical hemodynamics and/or inflammatory stress. Apparently, disturbed complement regulation leads to an increased susceptibility to complement activation, inflammation, and tissue damage in the IA wall. (Am J Pathol 2010, 177:3224–3232; DOI: 10.2353/ajpath.2010.091172)

Intracranial artery aneurysms (IA) have been estimated to be present in 2.3% of the population.1 IA rupture causes subarachnoid hemorrhage (SAH) with up to 50% mortality.1–2 The ultimate mechanisms behind IA structural weakening and rupture are unknown.

Chronic inflammation precedes IA rupture. This is indicated by inflammatory cell infiltration and complement activation in both unruptured and ruptured IAs.3–7 Two recent microarray analyses showed a differential expression of complement related genes in IAs compared to the control arterial tissue.8–9 In our recent study, we found that early pathway complement components are widely present in the IA wall,10 whereas the presence of an end-product of the terminal complement pathway [ie, the membrane attack complex (MAC)] is restricted to a band-like area in the outer part of the IA wall.7 We found evidence for activation of the classical pathway along with recruitment of the alternative pathway.10 This is comparable to other chronic inflammatory diseases like rheumatoid arthritis, atherosclerosis, and membranous nephropathy.11–13

Complement has an important role as a proinflammatory mediator when fully activated. Inflammatory cell infiltrations clearly associate with aneurysm wall degeneration and rupture.5–6 In animal models of hypertension-induced IAs, the inflammatory cells infiltrate to the IA wall already at an early phase and associate with IA growth.14 Smooth muscle cell proliferation typically occurs in the IA

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Complement may have a key role in the regulation of IA wall remodeling, as suggested by a recent experimental study, where the complement C1-inhibitor restricted neointima formation and reduced the number of accompanying macrophages and T-cells in a balloon injury model. Complement is strictly regulated in viable tissues by soluble and membrane bound complement inhibitors. This is to prevent potentially harmful effects of complement activation. The cells are protected from complement membrane attack by protectin (or CD59) that inhibits C5b-8 catalyzed polymerization of the terminal complement component C9 on all membranes. MAC-inhibition is also restricted by soluble factors clusterin and vitronectin (or S-protein) that keep the terminal complex (SC5b-9) in solution. An important inhibitor of the classical pathway is C4b binding protein (C4bp), which is usually in complex with the anticoagulant protein S. The alternative pathway is inhibited mainly by factor H.

Complement activation in the IA wall has been hypothesized to be due to accumulation of cell debris, possibly due to disturbances in the IA wall homeostasis. Recently, a single nucleotide polymorphism of factor H (variant Y402H, T1277C, rs 1061170) in the complement factor H gene (CFH) has been associated with age-related macular degeneration (AMD), a degenerative retinal disease with impaired clearance of eye pigment and/or cellular debris. Its potential role in aneurysmal diseases is unknown.

Based on previous studies, components of the complement system accumulate in the IA wall. However, whether complement activation is due to insufficient regulation in the IA wall or other reasons is unknown. In this work we studied complement regulation in the IA wall by immunohistochemical analysis. In addition, we screened a series of IA patients for the factor H Y402H polymorphism. Our results indicate a Y402H independent insufficiency of complement regulation in the IA wall. Insufficiency occurs at multiple steps (C3 and MAC stage) and predisposes to complement activation.

Materials and Methods

Sample and Clinical Data Collection

To study complement regulators in the IA wall, we used a series of human saccular IA domes (total n = 46; 26 unruptured, 26 ruptured) resected intraoperatively after clipping at the Department of Neurosurgery, Helsinki University Central Hospital. The specimens were immediately snap-frozen in liquid nitrogen and stored at −80°C. To study complement factor H polymorphism Y402H we collected a series (n = 97) of blood samples from IA patients. As controls we used a series (n = 100) of healthy blood donors. The clinical data of IA patients (Table 1) were collected from medical records, and IA dimensions were obtained from preoperative computer tomography angiography images. The study was approved by the local ethics committee.

Immunohistochemistry

For immunohistochemistry, IA-samples were cryosectioned at 4–10 μm. The sections were fixed with 4% paraformaldehyde, and blocked with 3 to 5% normal goat or horse serum (Vector Laboratories Inc., Burlingame, CA) in phosphate buffered saline (PBS), pH 7.4. Some
stainings were additionally blocked with 0.5% cold fish skin gelatin (Sigma-Aldrich Inc., St. Louis, MO) in 0.5% bovine serum albumin (Sigma) in PBS (BSA/PBS). The primary antibodies (Table 2) were diluted in 1% BSA/PBS and incubated for 30 minutes at room temperature or overnight at 4°C. The endogenous peroxidase was blocked with 0.7% hydrogen peroxide in PBS. The sections were incubated with biotinylated secondary antibodies (Vector) for 30 minutes at room temperature. The signal was enhanced by horseradish peroxidase-conjugated avidin-biotin complex and visualized with DAB (Sigma). The background was stained with Mayer’s hematoxylin. Human tonsil served as a positive control. For negative controls, the primary antibody was omitted or replaced with an irrelevant antibody of the same immunoglobulin subtype as the primary antibody.

**Immunofluorescence Stainings**

For immunofluorescence staining of IA-samples, fixation, blocking, and incubation with the primary antibody (Table 2) were performed as described above. The primary antibody was detected with fluorochrome-conjugated Alexa Fluor 488 (green), 546 (red), or 555 (red) secondary antibody (Molecular Probes Inc., Eugene, OR) diluted 1:100 to 1:200 in PBS. For double-stainings, the sections were thereafter incubated with another primary antibody originating from a different species (Table 2), that was detected with another non cross-reactive fluorochrome-conjugated secondary antibody. The slides were mounted in Vectashield with DAPI (Vector) to detect the nuclei.

**Alcian Blue Staining**

For staining of sulfated glycosaminoglycans, the cryosections were fixed in ice-cold acetone for 2 minutes, rinsed in running tap water, incubated in 1% Alcian Blue in 3% acetic acid at pH 2.5 for 3 minutes, rinsed in running tap water, and mounted in aqueous mounting media (VWR International Ltd., Poole, England).

**Imaging and Histological Analysis**

The immunohistochemical stainings were photomicrographed with an Axioplan 2 imaging microscope and AxioCam MRc digital camera (Carl Zeiss Vision GmbH, Aalen, Germany). The immunofluorescence stainings were viewed and photographed with Axioplan 2 and AxioCam (HR/MR) (Zeiss) or with Olympus BX51 and DP70 (Olympus corporation, Tokyo, Japan), with appropriate filters to create pseudocolored images.

The presence or absence of complement components and regulator proteins was screened in the areas of interest within parallel sections. Locations of positive staining within cells, and matrix were recorded. For comparison of terminal pathway complement activation and its regulation, the aneurysm wall was divided in three subareas according to positive staining for MAC (strong staining, moderate staining, or no staining). Similar semi-quantitative analysis for complement components and regulatory proteins was performed.
DNA Extraction and Factor H Y402H Single Nucleotide Polymorphism

DNA was extracted from blood mononuclear cells by the ethanol precipitation method (Gentra Puregene Genomic DNA Purification Kit, Gentra Systems, Minneapolis, MN). The region corresponding to the Y402H polymorphic site of the CFH gene was amplified by PCR and sequenced using the forward primer 5'-ctttgttagtaactttagttcg-3' and the reverse primer 5'-tttaagacattaagcttggg-3'. PCR amplifications were conducted in 50-μl volumes containing 80 ng of genomic DNA, 30 μM each primer, polymerase buffer, 10 mmol/L each nucleotide (dNTP), and 0.8 U of Dynazyme polymerase-enzyme (Finnzymes). Sequencing was performed using cycle sequencing with the Big Dye Terminator kit (version 3.1; Applied Biosystems Inc., Foster City, CA), and reactions were run on an ABI 3730 capillary sequencer according to the manufacturer’s instructions.

Statistical Analysis

The clinical and radiological data on patients and aneurysms and CFH genotype were compared using the SPSS 16.0 software (SPSS Inc., Chicago, IL). Medians and ranges were calculated, and for comparisons the χ² and Mann-Whitney U tests were used.

Results

Patients and Aneurysms

The clinical and radiological data of patients and aneurysms studied for histology and complement factor H Y402H polymorphism are presented in Table 1. In the two series studied, patients with unruptured or ruptured IAs did not differ in age, gender, or multiplicity of aneurysms. The patients differed neither in the known family history, nor in known hypertension (data not shown). The ruptured IAs were slightly bigger than the unruptured ones.

C4b Binding Protein and Factor H Colocalize with Glycosaminoglycans in the IA Wall

The important complement inhibitors, C4bp and factor H, stained positive in the extracellular matrix in all studied IA samples. The positive staining for C4bp was seen in the luminal as well as in the outer part of the IA wall (Figure 1, A, C–E, H, and K). The positive staining for factor H was especially prominent in the luminal part of the IA wall (Figure 2, A–C and E–G). In parallel sections, the same area stained positive with Alcian Blue, indicating the presence of sulfated glycosaminoglycans (Figure 2D). The outer part of the IA wall showed a remarkably fainter staining for Alcian Blue (Figure 2D).

Soluble Complement Regulators and C5b-9 Deposition

The staining for C4bp and factor H was seen in the luminal part of the IA wall that lacked positive staining for C5b-9. However, in the outer part of the IA wall, at the area of intensive staining for C5b-9, also C4bp and some factor H were seen (Figure 1; Figure 2, E–I). Both C4bp and factor H were also seen deposited on a few separate cells that were negative for C5b-9. C4bp usually occurs in complex with the anticoagulant protein S. As expected, protein S was seen in identical areas with C4bp in parallel sections in 18/18 IAs studied for both C4bp and protein S (9 unruptured and 9 ruptured) (Figure 1), although C4bp sometimes was seen in larger areas than protein S. Of the soluble MAC-inhibitors both clusterin and vitronectin were seen in the matrix in similar areas as C5b-9. However, in 4 of 7 samples the positive staining area for...
vitronectin was not as large as for C5b-9 and for clusterin the staining was faint (Figure 3, A–F).

Protectin (CD59) Is the Main Membrane Attack Complex-Limiting Protein in the IA Wall

The MAC inhibitor, protectin, stained positive in all 44 studied IAs (24 unruptured and 20 ruptured IAs). In general, the staining was seen reciprocally to the C5b-9 complex: the areas of intensive staining for protectin were virtually negative for C5b-9 (Figure 4, A–F, and I). Only faint or no staining for protectin, was seen in the matrix in areas of strong positive staining for C5b-9 at the outer band-like part of the IA wall (Figure 4, A–F). In areas of fainter positive staining for protectin, both C5b-9–positive and –negative cells and C5b-9 on the matrix surrounding the cells were seen depending on the sample (Figure 4, C–F). The staining was located mostly to cell membranes, but also some protectin was seen bound diffusely in the extracellular matrix (Figure 4, G and H). In double stainings, few cell were found double-positive. However, in a large scale, the staining was reciprocal. The other membrane bound complement inhibitors, DAF and MCP, were expressed only by separate mural cells. DAF was mainly detected in the adventitial area and MCP in endothelial cells (Figure 3G-H).

Factor H Y402H Polymorphism

The factor H genotype 1227C (402H) predisposes to age-related macular degeneration (AMD), while 1227T (402Y) seems to protect from it. Of the IA patients 36/97 (37%) represented the TT genotype. Heterozygous CT genotype was seen in 53/97 (55%) IA patients, and the most strongly AMD-associated CC genotype in 8/97 (8%) IA patients. Of the aneurysm patients with TT, CT, and CC genotypes 26/36 (72%), 35/53 (66%), and 3/8 (38%) had had a ruptured aneurysm (aneurysmal SAH). The distribution did not differ significantly from the IA patients with only unruptured IAs. The CFH genotype did not correlate to recorded hypertension. Neither did the IA patients’ genotypes differ statistically significantly from the control-group’s TT, CT, and CC genotypes: 41/100 (41%), 42/100 (42%), 17/100 (17%), respectively.

Discussion

The rupture of an intracranial artery aneurysm is a life-threatening event and represents an important cause of death. The reasons underlying the development and structural weakening of the IA wall are unknown. We have previously shown that the magnitude of terminal pathway complement activation associates with IA wall degeneration and rupture. We have now carefully analyzed complement regulators by immunohistochemical and immunofluorescence stainings to see possible evidence for dysregulation of complement activation in unruptured or ruptured IA walls. Also, the frequency of the AMD-associated factor H single nucleotide polymorphism Y402H in IA patients was studied. Here, we show that complement activation in the IA walls is controlled in the luminal part.
L indicates lumen. Scale bar corresponds to 100 μm. The adventitia (arrowheads; cells (arrowheads; protein stained mainly endothelial cells (arrowheads., C5b-9, whereas only some factor H staining was weaker and only partially overlapped with C5b-9. In atherosclerotic lesions, C4bp is widely deposited but luminal to C5b-9 accumulation.21 This is similar to our observations, but without an overlap with C5b-9. In the IA wall, the area positive for C5b-9 has lipid-containing particles,19 a decreased number of viable cells, apoptotic and necrotic cells, and apparent cell debris.7 C4bp has an inherent capacity to bind apoptotic cells through the interaction between protein S and phosphatidylserine on apoptotic blebs.22 As the area of heavy C4bp and C5b-9 accumulation in the outer part of the IA wall has only few nuclei, the local synthesis of such amounts of C4bp only by cells in the arterial wall seems unlikely.23 Rather, we suggest, that C4bp has become accumulated to that area because of a passive diffusion or physiological mass transport through the IA wall.24 Why factor H did not show similar heavy accumulation as C4bp could be explained by a difference in the affinity of factor H and C4bp for extracellular matrix components. We found vitronectin and clusterin in the subareas that stain positively for C5b-9. This suggests that part of the terminal complexes could be in the soluble form SC5b-9. Clusterin and vitronectin, however, also have potential functions (eg, in vascular remodeling)25–26 in addition to functions (eg, in vascular remodeling)25–26 in addition to inhibition of MAC assembly on the cell membrane. Therefore clusterin and vitronectin could have additional roles in the IAs.

The factor H polymorphic variant Y402H has been strongly associated with age-related macular degeneration, a disease characterized by eg, impaired eye pigment and debris clearance.17 The role of the Y402H polymorphism in cardiovascular diseases has remained controversial. One study has supported an association of Y402H with myocardial infarction,27 but further studies have failed to confirm the finding.28–30 The factor H variant with 402 histidine has a reduced affinity for C-reactive protein,31–32 an acute phase reactant associated with tissue damage and inflammation found also in IA walls.10 Thus, it was in our interest to study this factor H polymorphism in patients with IAs. However, we saw no significant difference in Y402H distribution between 97 IA patients and 100 individuals representing healthy normal Finnish population. Neither did we see a statistically significant difference in Y402H distribution between patients with only unruptured IAs and those with a history of subarachnoid hemorrhage from ruptured IA. Although the latter comparison was based on a relatively small sample material, it seems likely that the Y402H polymorphism does not have a major role in predisposing to IA or its rupture.

We found that the MAC-inhibitor protectin was located widely in the mural cells, mainly reciprocally to the stron-
gest band-like accumulation of the C5b-9 complex in the outer IA wall. In contrast, the other membrane-bound complement inhibitors, MCP and DAF, were expressed only by few mural cells and in the adventitia. In general, smooth muscle cells are protected against complement attack by expressing the glycophosphatidylinositol-anchored complement regulator protectin, but loss of protectin expression may occur, e.g., in myocardial infarction. In our series, the strongest C5b-9 accumulation was mostly seen in areas lacking protectin. Often these areas had only few cells. Our finding underlines the importance of cell-mediated complement inhibition in IAs. Many cells in the IA walls have gone through either apoptotic or necrotic death. While ischemic cells lose protectin, the possible role of hypoxia in the IA wall pathobiology remains still hypothetical. However, whether the lack of protectin is a cause or consequence of the cell loss in the same area cannot be determined in this type of a study. In either case, however, it could lead to increased local C5b-9 formation. The assembly of MAC has multiple consequences like an increased Ca2+ influx, and a detergent-like

Table 3. Correlation of Complement Factor H Polymorphism Y402H and Variables Related to Intracranial Artery Aneurysms

<table>
<thead>
<tr>
<th>Complement factor H T1277C genotype</th>
<th>TT n = 36</th>
<th>CT n = 53</th>
<th>CC n = 8</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient age (years)*</td>
<td>50 (22–77)</td>
<td>56 (34–84)</td>
<td>51 (28–75)</td>
<td>0.048</td>
</tr>
<tr>
<td>Females†</td>
<td>72% (26/36)</td>
<td>66% (35/53)</td>
<td>63% (5/8)</td>
<td>0.777</td>
</tr>
<tr>
<td>Patients with SAH from ruptured IA†</td>
<td>72% (26/36)</td>
<td>66% (35/53)</td>
<td>38% (3/8)</td>
<td>0.188</td>
</tr>
<tr>
<td>Patients with multiple (≥2) IAs†</td>
<td>42% (15/36)</td>
<td>43% (23/53)</td>
<td>13% (1/8)</td>
<td>0.197</td>
</tr>
<tr>
<td>IA neck diameter (mm)*</td>
<td>4 (2–7)</td>
<td>4 (2–9)</td>
<td>4 (2–7)</td>
<td>0.645</td>
</tr>
<tr>
<td>IA fundus length (mm)*</td>
<td>6 (2–17)</td>
<td>5 (2–20)</td>
<td>7 (4–14)</td>
<td>0.173</td>
</tr>
<tr>
<td>IA fundus width (mm)*</td>
<td>5 (2–14)</td>
<td>4 (2–13)</td>
<td>6 (3–13)</td>
<td>0.443</td>
</tr>
</tbody>
</table>

*Mann–Whitney U test used, median and range are given for continuous variables.
†χ² test used, proportions are given.
‡ SAH, subarachnoidal hemorrhage; IA, intracranial artery aneurysm.
membrane destabilizing effect, which both could contribute to inflammation and cell loss.

In some IA wall areas, usually in areas between the strongest protectin expression and the strongest C5b-9 accumulation, the staining for protectin partially overlapped with that of C5b-9. A similar phenomenon has also been seen in atherosclerotic lesions. This may indicate an imbalance between complement inhibition and activation in the IA tissue. If the degeneration of IA wall is considered as a continuous degenerative process ultimately leading to critical thinning and rupture of IA wall, this area of partially decreased inhibitory capacity of complement activation might represent a transitional zone in this process. Protectin has a role in prevention of vascular inflammation as indicated by studies on atherosclerosis-prone ApoE−/− mice, in which the protectin-deficient mice showed signs of accelerated atherosclerosis. In our previous study, we have seen lipid-deficient mice showed signs of accelerated atherosclerosis-prone ApoE−/− mice, in which the protectin-deficient mice showed signs of accelerated atherosclerosis.35 In conclusion, complement activation and MAC formation in the IA wall seem to be controlled mainly by the glycosaminoglycan-binding inhibitors factor H and C4bp. In the cellular and luminal parts, MAC formation seems to be prevented by the expression of protectin. The outer part of the IA wall has a limited capacity to inhibit complement, as suggested by decreased expression of the complement inhibitor factor H, and complement inhibitor-binding glycosaminoglycans. Also cells expressing protectin are scarce in this region. This may facilitate the proinflammatory complement activation that associates with wall degeneration7 and might subsequently facilitate the infiltration of the IA wall by inflammatory cells, previously associated with rupture.5–6

The insufficient complement regulation we observed might be due to matrix remodeling and loss of mural cells. Such matrix remodeling can be in part caused by exposure to mechanical hemodynamic stress. By facilitating the activation of the complement cascade, wall remodeling may further enhance the inflammatory reaction that lead to destruction of the wall by eg, proteolytic enzymes released from leukocytes or their cytotoxic activity.

Our findings suggest that a disturbed complement regulation associates with an increased susceptibility to complement activation and inflammation, and maybe even cell loss, in the IA wall. Whether the role of complement in the IA wall could be altered eg, pharmacologically, should be studied.

Acknowledgments

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protein negatively regulates the classical complement pathway, and that C5b-9 is formed via the alternative complement pathway. Atherosclerosis 2007, 192:40–48


