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Inflammation and Complement Activation in Intracranial Artery Aneurysms

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Academic Dissertation

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by their Roman numerals:

I. Tulamo R, Frösen J, Junnikkala S, Paetau A, Pitkäniemi J, Kangasniemi M, Niemelä M, Jääskeläinen J, Jokitalo E, Karatas A, Hernesniemi J, Meri S. Complement activation associates with saccular cerebral artery aneurysm wall degeneration and rupture. Neurosurgery 59:1069-76, 2006.

II. Tulamo R, Frösen J, Junnikkala S, Paetau A, Kangasniemi M, Peláez J, Hernesniemi J, Niemelä M, Meri S. Complement system becomes activated by the classical pathway in intracranial aneurysm walls. Lab Invest 90:168-79, 2010.

III. Tulamo R, Frösen J, Paetau A, Seitsonen S, Hernesniemi J, Niemelä M, Järvelä I, Meri S. Lack of complement inhibitors from the outer intracranial artery aneurysm wall predisposes it to complement terminal pathway activation. Submitted.

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ABBREVIATIONS

AAA	Abdominal aortic aneurysm
AcomA	Anterior communicating artery
AMD	Age-related macular degeneration
APC	Antigen-presenting cell
Apo	Apolipoprotein
aSAH	Aneurysmal subarachnoid hemorrhage
bFGF	Basic fibroblast growth factor
C1-INH	C1-inhibitor
C4bp	C4b binding protein
cfh	Complement factor H gene
CR	Complement receptor
CRP	C-reactive protein
CT	Computer tomography
DAF	Decay-accelerating factor
ECM	Extracellular matrix
EM	Electron microscopy
eNOS	Endothelial nitric oxide synthase
EVT	Endovascular therapy
fH	Factor H
FHL-1	Factor H-like protein 1
GPI	Glycophosphatidylinositol
IA	Intracranial aneurysm
ICH	Intracerebral hematoma
IEL	Internal elastic lamina
IEM	Immuno-electron microscopy
IFN	Interferon
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
iNOS	Inducible nitric oxide synthase
I/R	Ischemia-reperfusion
ISAT	International Study of Aneurysm Treatment
ISUIA	International Study of Unruptured Intracranial Aneurysms
LDL	Low-density lipoprotein
LMM	Linear mixed model
MAC	Membrane attack complex
MAPK	Mitogen-activated protein kinase

MBL	Mannan binding lectin
MCA	Middle cerebral artery
MCP	Membrane cofactor protein
MCP-1	Monocyte chemotactic protein 1
MHC	Major histocompability complex
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
NAb	Natural antibody
NFκB	Nuclear factor kappa B
NK	Natural killer
NO	Nitric oxide
oxLDL	Oxidized low-density lipoprotein
PAMP	Pathogen-associated molecular pattern
PcomA	Posterior communicating artery
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PMN	Polymorphonuclear leukocyte
PRR	Pattern recognition receptor
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SAH	Subarachnoid hemorrhage
SCR	Scavenger receptor
SMC	Smooth muscle cell
SNP	Single-nucleotide polymorphism
TCC	Terminal complement complex
TF	Tissue factor
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinases
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TUNEL	Terminal deoxynucleotidyl transferase (dUTP) nick end labeling
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor

ABSTRACT

Background and purpose

Saccular intracranial artery aneurysm (IA) rupture causes subarachnoid hemorrhage (SAH), with up to 50% mortality. Of the population, 2.3% is estimated to harbor unruptured aneurysms. To date, rupture risk of an individual IA cannot be precisely predicted. Especially young patients with unruptured IAs with risk factors, such as smoking, hypertension, and family history, need active treatment to prevent the often fatal IA rupture. Current treatment options (endovascular or microsurgical therapy) are invasive and associated with some morbidity and mortality. Rupture-prone IAs should be identified and this requires a better understanding of the IA wall pathobiology. Inflammatory cell infiltration and IA wall degeneration are known to precede IA rupture. The complement system is a key mediator of inflammation and house-hold processing of injured tissue. The role of complement activation in IA wall pathobiology is unknown. This study aimed at identifying the role of complement activation in IA wall degeneration and the complement activators involved and determining how the complement system is regulated in the IA wall.

Materials and methods

The presence of complement activation, complement activators, and complement regulators was evaluated by immunostainings of samples of unruptured and ruptured IA fundi, resected perioperatively after microsurgical clipping. The relative localization of complement activation within the IA wall and in relation to complement activators and regulators was analyzed in IAs by light and fluorescence microscopy from single and double stainings. The extent of complement activation was correlated with clinical and histological parameters. The localization of the terminal complement complex at the ultrastructural level and its relation to cell death were analyzed by electron and immunoelectron microscopy. As complement activation may induce cellular death, a special emphasis was placed on assessing the potential role of complement activation in cell loss in the IA wall. The disease-associated Y402H polymorphism in complement inhibitor factor H was also investigated.

Results

The end-product of complement activation, the terminal complement complex (TCC), was located mainly in the outer part of the IA wall, in areas that had also sustained loss of cells. Complement activation correlated with IA wall degeneration and rupture, de-endothelialization, and T-cell and CD163-positive macrophage infiltrations. The area of

maximum TCC accumulation contained cellular debris and evidence of both apoptotic and necrotic cell death. The complement system was found to become activated by the classical pathway, with recruitment of alternative pathway amplification. This suggests the presence of chronic inflammation and complement activation in response to specific activators. Complement activation elicited by more than one specific activator was also suggested by the probable complement activators being scattered within IA walls, similarly to the early components of complement activation. Lipid accumulation was observed to clearly colocalize with C5b-9 and C-reactive protein. Lipid deposits thus likely have a role in complement activation and/or indicate impaired clearance mechanisms. Terminal pathway complement activation was limited to the outer part of the IA wall. In other areas, complement activation was limited by cellular expression of protectin (CD59) and extracellular matrix-bound inhibitors, C4b binding protein and factor H. The outer part of the wall was exposed to terminal complement activation due to a lack of cells expressing protectin as well as matrix-bound factor H. Unlike in age-related macular degeneration, factor H Y402H polymorphism was not associated with the presence of IAs or their rupture.

Conclusions

The data suggest that complement activation and TCC formation are involved in IA wall degeneration and rupture. The association of complement with de-endothelialization and expression of complement activators indicate a possible role of endothelial dysfunction in the pathobiological process. Furthermore, impaired clearance mechanisms may be involved in chronic inflammation. Whether complement activation is primary or secondary to cell death could not be determined. Impaired complement regulation seems to be associated with increased complement activation in IA walls. These results stress the role of chronic inflammation in IA wall pathobiology and the regulatory role of complement within this process. The IA wall is exposed to mechanical hemodynamic stresses, humoral inflammation, and innate immune attack. Complement deposits might thus serve as a marker of long-term stress in the IA wall, an attempt to remove debris, or disturbed complement regulation in the matrix. Whether inherent disturbances in function or regulation of the complement system exist should be examined in future studies. Imaging inflammation would enhance the diagnostics of rupture-prone IAs, and targeting IA treatment to prevent chronic inflammation might improve IA treatment.

I INTRODUCTION

Subarachnoid hemorrhage (SAH, Figure 1A), caused by a rupture of an intracranial artery aneurysm (IA), has been recognized as a disease entity already for thousands of years (Clarke, 1963). Today, SAH of aneurysmal origin accounts for 1-7% of all strokes in Western countries (Feigin *et al.*, 2003).

Intracranial artery aneurysm is a protrusion of a cerebral artery in the vicinity of the circle of Willis (Figure 1B), which is an arterial circle providing blood supply to the brain at the skull base. IAs are classified as saccular (or berry) or fusiform according to shape and relation to the conducting artery (Figure 1C). This study deals with saccular IAs only. IAs are usually located at the branching points of intracranial arteries, the most typical bifurcations being at the anterior communicating artery (AComA), posterior communicating artery (PComA), and middle cerebral artery (MCA).

IAs are usually symptomless until they rupture, with the exception of giant IAs, which may cause neurological symptoms by mass effect. When an IA ruptures, arterial blood bleeds into the subarachnoidal space, causing sudden headache, nausea, vomiting, and possibly neurological deficit. Sometimes IAs also bleed into the brain parenchyma, leading to intracerebral hemorrhage (ICH, Figure 1A).

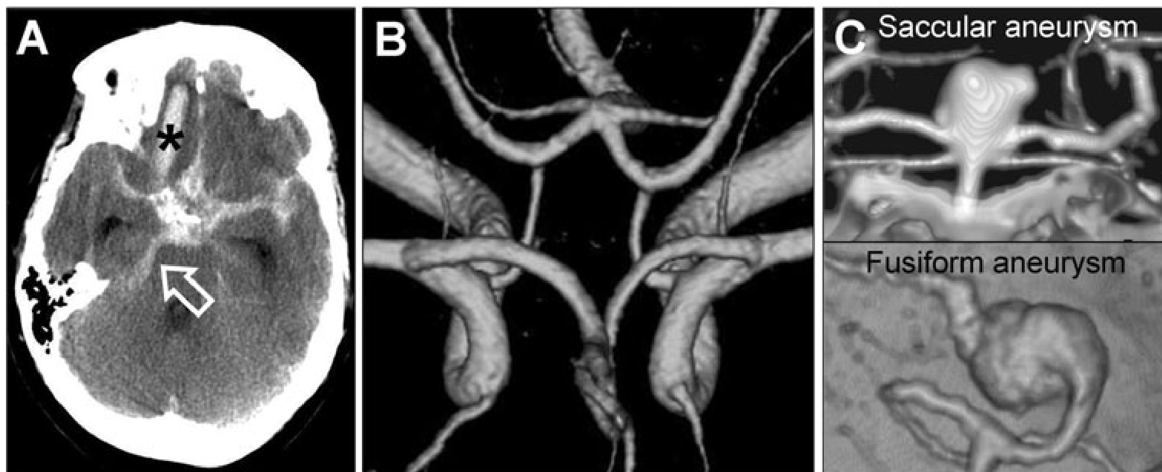

Figure 1. Subarachnoid hemorrhage (A), circle of Willis (B), and intracranial artery aneurysms (C). In subarachnoid hemorrhage, blood is seen in the subarachnoid space (arrow) around the brain in a computer tomography image, in this case with an associated intracerebral hematoma (asterisk, A). A magnetic resonance angiography image of the circle of Willis, an arterial ring located at the skull base (B). Computer tomography angiography images of a typical saccular aneurysm pouching at the arterial bifurcation and a fusiform aneurysm appearing as a dilatation of a cerebral artery (C). The images are from different patients.

There are several risk factors for IA growth and rupture, but the primary etiology remains unknown. IAs are considered acquired and not congenital lesions (Stehbens,

1989), and arise *de novo* (Graf and Hamby, 1964). Rarely, IAs may be associated with vascular anatomic variations, infections, traumas, neoplasms, systemic disorders, or drug abuse (reviewed by Biondi, 2006). Recent studies have strengthened the concept that a chronic inflammation predetermines IA wall degeneration towards a rupture-prone phenotype.

Inflammation is a normal tissue's reaction to physical, chemical, or biological stress or injury. It manifests as an immunological response and accumulation of inflammatory cells. Inflammation was first suggested to occur in IAs by Virchow (Virchow, 1847; Chyatte *et al.*, 1999). Further evidence comes from the 1930s, when Maass (Maass, 1937; Hassler, 1961) described a round-cell infiltration, most likely lymphocytic, especially in the IA neck. Later, after the introduction of modern molecular biological and immunostaining methods, several studies have shown the presence of inflammatory cell infiltration and inflammatory mediators in the IA wall, and their association with critical weakening and rupture of the IA wall. A well-known, but extremely rare cause of IAs and their rupture is a local bacterial infection associated with an inflammatory response triggered by the pathogen. This underlines the capability of local inflammation to destroy tissue.

The complement system can launch inflammatory reactions by approximately 35 components. It is a major part of innate immunity, which also regulates the adaptive immune system. Growing evidence points to an important role of the complement system in response to injury, involving the clearance of tissues from accumulating debris together with phagocytes. In general, components of the complement system can recognize foreign particles and alterations in host cell surfaces and structures. Many noninfectious factors are capable of activating complement, including antibodies, C-reactive protein (CRP), oxidatively modified lipids and proteins, and membrane phospholipids of apoptotic or necrotic cells. If complement activation is not inhibited by membrane-bound or soluble complement inhibitors, it ultimately leads to the formation of the membrane attack complex (MAC), which can cause death of a target cell (Sim and Tsiftsoglou, 2004; Biró *et al.*, 2007; Sjöberg *et al.*, 2009). The small fragments, anaphylatoxins, that are generated during complement activation are chemotactic to inflammatory cells and have many effects on the functions of inflammatory cells and the vascular wall (Klos *et al.*, 2009).

Although the presence of two complement components in the IA wall has been shown previously (Chyatte *et al.*, 1999), the characteristics and consequences of complement activation in the IA wall are still unknown. As molecular imaging and targeted pharmaceutical therapy continue to develop at an accelerating pace, defining the mechanisms of IA wall degeneration and rupture is increasingly important. This study adds to the knowledge of complement activation in IAs, based mostly on immunohistochemical studies. It describes the regulatory mechanisms of complement, as

well as the relation of complement activators to actual complement activation in IA walls. The association and potential mechanisms of complement activation and IA wall degeneration and rupture are also discussed.

II REVIEW OF THE LITERATURE

1 Intracranial artery aneurysm and subarachnoid hemorrhage

1.1 Epidemology

Of the population, 2.3% (0.4-6.0%) has been estimated to carry IAs, based on a series of radiological imaging and pathological examinations (Rinkel *et al.*, 1998). Eighty-five percent of SAHs are of aneurysmal origin (aneurysmal SAH; aSAH) (van Gijn *et al.*, 2007). In a meta-analysis, the incidence of SAH was 9.1 per 100 000 person-years in Europe and North America, halved in South and Central America (4.2 per 100 000) (de Rooij *et al.*, 2007), and twice as high in Finland (19.7 per 100 000) and Japan (22.7 per 100 000) (Linn *et al.*, 1996; Morita *et al.*, 2005; de Rooij *et al.*, 2007). The reason for a higher prevalence of SAH in Finland and Japan is unknown.

The reported annual rupture rate of IAs varies between 0.05% and 2.3%, depending on the inclusion criteria. It is higher in patients with previous SAH and larger IAs (Yasui *et al.*, 1997; ISUIA, 1998; Juvela *et al.*, 2000; Suga *et al.*, 2002; Wiebers *et al.*, 2003; Ishibashi *et al.*, 2009), as confirmed by the meta-analysis of Rinkel *et al.* (1998). Patients with familial background (Broderick *et al.*, 2009) or prior SAH and multiple IAs (Heiskanen, 1981; Winn *et al.*, 1983) have an annual rupture rate of 1-1.2%.

1.2 Risk factors for intracranial aneurysm rupture

The general risk factors for IA rupture are hypertension, smoking, female gender, alcohol and drug abuse, and family history. The size and location of the aneurysm as well as the genetic background of the patient and age affect the risk of rupture. As most SAHs are aneurysmal in origin (van Gijn *et al.*, 2007), it is also relevant to review here the risk factors for SAH in general.

1.2.1 Aneurysm-related risk factors

Location

IAs in posterior circulation have a higher rupture risk than those in anterior or medial circulation (Rinkel *et al.*, 1998; Wiebers *et al.*, 2003; Clarke *et al.*, 2005; Morita *et al.*, 2005; Ishibashi *et al.*, 2009). However, relative to other locations, anterior circulation IAs

tend to rupture when they are smaller (Orz *et al.*, 1997; Ohashi *et al.*, 2004; Beck *et al.*, 2006). In patients with multiple IAs, the proximal one has ruptured in 60-70% of cases (Jain, 1963; Crompton, 1966).

Size

Several studies and meta-analyses have reported a correlation between IA size and rupture rate (Rinkel *et al.*, 1998; Wiebers *et al.*, 2003; Morita *et al.*, 2005; San Millan Ruiz *et al.*, 2006; Ishibashi *et al.*, 2009). Size has been evaluated as an independent predictor for an IA rupture (relative risk 1.11 per mm) (Juvela *et al.*, 2000). However, small IAs are also at risk for rupture (Crompton, 1966; Orz *et al.*, 1997; Ohashi *et al.*, 2004; Beck *et al.*, 2006). In patients with multiple IAs, the largest IA has ruptured first in most cases (74-88%) (Wood, 1964; Crompton, 1966; Orz *et al.*, 1997).

Growth, morphology, and multiplicity

The growth of ruptured IAs is more marked than that of unruptured IAs (Björkesten and Troupp, 1962; Matsubara *et al.*, 2004). Secondary pouches and nonspherical shape, related to IA growth, are associated with a higher tendency for IA rupture (Crompton, 1966; San Millan Ruiz *et al.*, 2006; de Rooij *et al.*, 2009). Sometimes IAs grow to a size of several centimeters; these are referred to as giant aneurysms (Barth and de Tribolet, 1994). Multiple IAs are present in up to 34% of patients with diagnosed IAs (Ostergaard and Hog, 1985; Rinne *et al.*, 1994; Inagawa, 2009). Multiple IAs may rupture more easily, as the history of SAH (majority of multiple IAs) is a significant risk factor for IA rupture (Ishibashi *et al.*, 2009).

1.2.2 Patient-related risk factors

Age

The average age of an aSAH patient is 57 years (ACROSS, 2000). The prevalence of IAs is very low in the first two decades of life, but increases steadily after the third decade (Rinkel *et al.*, 1998). As the patient gets older, the IA rupture risk increases as the annual rupture risk accumulates (Juvela *et al.*, 2000). Patients with a family history have IA ruptures at a younger age (Ohashi *et al.*, 2004).

Female gender

IAs become more common in females with age (Kongable *et al.*, 1996; de Rooij *et al.*, 2007), and women have a higher risk for IA rupture in general (Rinkel *et al.*, 1998; Morita *et al.*, 2005). Female gender is also an independent risk factor for *de novo* IA formation, IA growth (Juvela *et al.*, 2001), and multiple aneurysms (Ostergaard and Hog, 1985;

Kongable *et al.*, 1996; Inagawa, 2009). The risk for SAH increases after menopause (Stober *et al.*, 1985; Longstreth *et al.*, 1994), but can be reduced by hormone replacement therapy (Longstreth *et al.*, 1994; Mhurchu *et al.*, 2001). This might be mediated by estrogen effects on the vascular wall (Harrod *et al.*, 2006).

1.2.3 Environmental factors

Smoking and alcohol abuse

Smoking has been considered the strongest risk factor for SAH (Feigin *et al.*, 2005a), with a relative risk of 2.2-3.1 (Feigin *et al.*, 2005b), and is related to IA size (Qureshi *et al.*, 2000) and rupture (Juvela *et al.*, 2000). Excessive alcohol use correlates with IA rupture (de la Monte *et al.*, 1985), with a relative risk of 2.2 (Feigin *et al.*, 2005b). Reports also indicate increased risk of SAH with recent use of cocaine (Broderick *et al.*, 2003) or caffeine (Isaksen *et al.*, 2002; Broderick *et al.*, 2003).

Hypertension

Patients with an aSAH have been found to be hypertensive almost twice as often as nonaneurysmal SAH patients (Stober *et al.*, 1985; Inci and Spetzler, 2000), and hypertension increases the risk for SAH (Feigin *et al.*, 2005a) and small-sized IA ruptures (Ohashi *et al.*, 2004). Hypertension is also the most important risk factor for multiple IAs (Ostergaard and Hog, 1985).

1.2.4 Family history

Approximately 10% of SAH patients have a family history of IAs (Ruigrok *et al.*, 2001). The prevalence of carrying IA, as a member of a family with two or more affected members, increases to 8.7% (Ronkainen *et al.*, 1997), and in the presence of acquired risk factors, the risk increases further to 32% (Nakagawa *et al.*, 1999). However, first-degree relatives have only a slightly increased incidence (4%) (Raaymakers, 1999; Wang *et al.*, 2002). IA patients with a family history are more often women (80%) (Leblanc, 1996), the patients are slightly younger (by 2-4 years), and the aneurysms are smaller and more often multiple than in patients with sporadic IAs (Ronkainen *et al.*, 1995).

1.3 Diagnosis, prevention of rupture, and treatment

IA rupture causes sudden onset of headache with vomiting, neck stiffness, often short loss of consciousness, and/or focal neurological symptoms, especially in the case of an intracerebral hemorrhage. SAH is diagnosed by computer tomography (CT), magnetic resonance imaging (MRI), or lumbal puncture. IA is finally identified in CT, MR, or

catheter angiography (van Gijn *et al.*, 2007). Nowadays, a growing number of IAs in clinical practice are unruptured, incidental findings in patients imaged primarily for reasons other than IAs or to screen families with IAs. The risks of active treatment must then be weighed against the natural course of the disease in individual patients and the probable risk of lifetime rupture estimated.

IAs are treated to prevent rupture or re-rupture. An IA that has ruptured once has an increased risk of up to 39% to re-bleed without an intervention (Winn *et al.*, 1983; Hijdra *et al.*, 1987; Ohkuma *et al.*, 2001; Brilstra *et al.*, 2002). The current options to prevent rupture or re-rupture of an IA are clipping and endovascular embolization (endovascular therapy, EVT), both invasive and somewhat risky (King *et al.*, 1994; Raaymakers *et al.*, 1998; Molyneux *et al.*, 2002; Lanterna *et al.*, 2004; Fraser *et al.*, 2006).

1.4 Outcome

Approximately half of the patients with SAH die and half of those surviving have hindered daily living (van Gijn *et al.*, 2007). The fatality rate of SAH in the 1960s to the 1990s was as high as 67-32%, showing a decreasing profile with an annual minimum decrease of 0.5% in the case fatality rate (Hop *et al.*, 1997; Nieuwkamp *et al.*, 2009). Patients surviving from SAH have been reported to suffer from altered personality, memory problems, daytime sleepiness, problems sleeping at night, reduced ability to work, and headaches (Ogden *et al.*, 1997; van Gijn *et al.*, 2007). Epilepsy develops in 5-7% of patients (Claassen *et al.*, 2003; Buczacki *et al.*, 2004). A factor affecting the outcome is delayed vasospasm, which develops in up to half of the patients, leaving 30% of these patients with permanent neurological deficit (Dorsch and King, 1994; Pluta *et al.*, 2009).

1.5 Histopathology of intracranial aneurysms

The primary reasons for IA formation and rupture remain obscure. As the rates of IA rupture and incidental unruptured IAs increase with age, and IAs have been found to arise *de novo* (Graf and Hamby, 1964), IAs are thought to be acquired rather than congenital lesions (Stehbens, 1989). IA wall histology reveals a vascular wall that varies in histology. At gene-expression level, the most impacted functional pathways in IAs, in comparison with extracranial arteries, are pathways involved in focal adhesion, extracellular matrix receptor interaction, cell communication, inflammatory response, and apoptosis (Shi *et al.*, 2009).

1.5.1 Cerebral artery wall

Cerebral arteries, like extracranial arteries, normally comprise three histologically different layers: the intima consisting of endothelial cells, the media rich in smooth muscle cells (SMCs), and the adventitia with fibroblasts as the main cellular component. The intima and media are separated by a well-preserved, homogeneous, but fenestrated internal elastic lamina (IEL) (Nyström, 1963). As an age-related change, IEL may reduplicate (Hassler, 1961). The media consists of regular layers of SMCs surrounded by collagenous bundles and sparse elastic fibers (Nyström, 1963). Distinct from extracranial arteries, cerebral arteries lack the fully developed external elastic lamina that otherwise separates the media and the adventitia. The adventitia of cerebral arteries is only weakly developed (Nyström, 1963) (Figure 2A).

Cerebral arteries rarely have vasa vasori. Vasa vasorum-like vessels have been found only in the proximal segments of the internal carotid and of the vertebral arteries with a thickness of more than 250 µm (Aydin, 1998). Small vascular channels have also been described in the outer media and adventitia of more proximal segments of anterior and medial cerebral arteries (A1 and M1) (Connolly *et al.*, 1996). They are seen more often in cerebral arteries with atherosclerosis (Atkinson *et al.*, 1991; Aydin, 1998) or thrombosis (Aydin, 1998). Vasoconstriction and vasodilatation of intracranial arteries are partly controlled by the perivascular fiber system of the autonomous nervous system, seen as longitudinal bundles in the periadventitia-adventitia and as a network-like reticular structure at the adventitia-media border (Buki *et al.*, 1999).

1.5.2 Intracranial aneurysm formation

Typical saccular IAs form at the bifurcation of cerebral arteries. Contrary to the normal cerebral artery structures (i.e. intima, IEL, media, and adventitia), the apical part of the cerebral artery bifurcation lacks the medial layer of SMCs (Hassler, 1961) (Figure 2B). The gap in the medial layer is called a ‘medial raphe’ (Stehbens, 1999a), and the media is replaced by tendon-like, linearly organized collagen (Finlay *et al.*, 1998). A role for the medial raphe in IA formation is unlikely, but still being explored (Stehbens, 1999a) (Figure 2C).

IEL disruption and an associated vascular wall remodeling have been thought to precede IA formation. Tears in IEL have been detected at cerebral artery bifurcations of normal cerebral arteries, and at even a higher incidence in IA patients (Hassler, 1961). The site of the tear in IEL is often associated with a pad of myointimal hyperplasia in the intima (Stehbens, 1960; Hassler, 1961). Half of the degenerative changes of IEL and media are located at some distance from the apex (Kayembe *et al.*, 1987). In experimental models of aneurysms, the hemodynamics only causes transversely oriented tears and

fragmentation of IEL (Stehbens, 1999b). Experimental studies have shown that the primary aneurysmal changes did not develop at the apical tip, but a small distance away, next to the intimal pad (Jamous *et al.*, 2007; Wang *et al.*, 2009) (Figure 2C). However, during IA growth, also the bifurcation becomes involved, manifesting typical macroscopic feature of an IA.

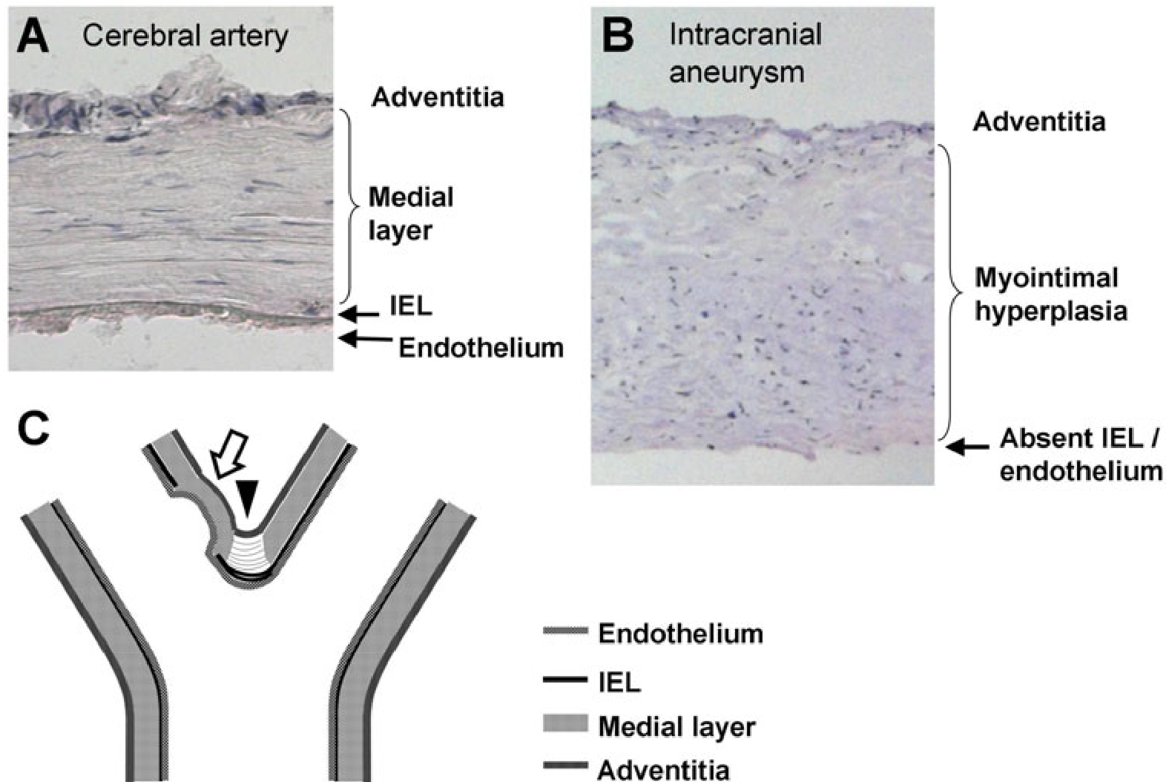

Figure 2. Histological structures of a normal cerebral artery wall (A) and an intracranial aneurysm wall (B), and a schematic presentation of aneurysm formation (C). The normal cerebral artery wall has endothelium, internal elastic lamina (IEL), a medial layer of linearly organized smooth muscle cells, and adventitia, whereas the wall of an intracranial aneurysm lacks IEL and often also endothelium, and shows varying amounts of myointimal hyperplasia. The aneurysmal nidus, typically located a small distance from the arterial bifurcation (open arrow), reaches the bifurcation by growth so that the ‘medial raphe’ (arrowhead) soon becomes a part of the IA.

IA patients may have either a hereditary or acquired predisposition for structural disturbances in their cerebral arteries. IA patients, contrary to healthy individuals, have a significantly higher incidence of small evaginations, thinnings of the media, and dilatations at the apical area in their cerebral artery bifurcations (Kayembe *et al.*, 1987). In the cerebral arteries of IA patients, the amount of reticular fibers is reduced by up to 35% (Ostergaard *et al.*, 1987b), predominantly in the outer part of the media (Hegedus, 1984). In normal cerebral arteries, fine reticular fibers form a dense network that surrounds the SMCs of the media and is present independently of intimal proliferation with age.

Moreover, the content of hyaluronan, an extracellular matrix (ECM) component that probably contributes to the viscoelastic resistance of cerebral arteries, is increased in cerebral arteries of IA patients compared with normal controls (Klekner *et al.*, 2005).

1.5.3 Intracranial aneurysm wall

The IA wall often shows a gradual change from almost intact to highly degenerated wall in the neck to fundus direction (Frösen *et al.*, 2004; Rajesh *et al.*, 2004). Degenerative changes in the IA wall include a decrease in the number of mural cells, mainly SMCs, an excess in the amount of fibrous tissue, and, ultimately, thinning of the wall. However, the IA wall is also under a constant remodeling process, indicated by disorganization of the wall structures, myointimal hyperplasia, and SMC proliferation.

IA wall is characterized by loss of IEL, which normally gives mechanical strength to the cerebral artery wall. The IEL typically ends at the level of the IA neck, where the IEL divides into branches ending in connective tissue (Hassler, 1961; Scanarini *et al.*, 1978a), leaving only remnants, if any, in the aneurysmal wall (Nyström, 1963; Scanarini *et al.*, 1978a; Frösen *et al.*, 2004). The endothelium in IAs often shows various degrees of damage (Kataoka *et al.*, 1999; Frösen *et al.*, 2004). In electron microscopy, the normally smooth and linearly organized endothelium has an irregular surface and intracytoplasmic, probably lipidic, vacuoles (Scanarini *et al.*, 1978a). The medial layer is disorganized (Frösen *et al.*, 2004; Rajesh *et al.*, 2004), often with scarce SMCs within the fibrohyaline tissue rich in collagen (Scanarini *et al.*, 1978a; Sakaki *et al.*, 1997). In histology, the fibrohyaline tissue appears eosinophilic and might occur together with inflammatory cells (Hassler, 1961). SMCs have undergone at least a partial phenotypic change from contractile towards synthetic type (Nakajima *et al.*, 2000). In electron microscopy, the SMCs are stretched, elongated, irregularly arranged, and separated by thick connective tissue processes (Nyström, 1963). The adventitia of the IA resembles that of a healthy cerebral artery (Hassler, 1972; Scanarini *et al.*, 1978b) (Figure 2C).

Vasa vasori (Scanarini *et al.*, 1978a; Atkinson *et al.*, 1991), periadventitial vessels (Holling *et al.*, 2009), and capillaries (Nyström, 1963) can also be found in the wall of an IA. As a sign of intramural bleedings, the IA walls sometimes contain erythrocytes (Nyström, 1963; Scanarini *et al.*, 1978a; Kataoka *et al.*, 1999) and hemosiderin (Schlote and Gaus, 1994; Rajesh *et al.*, 2004; Holling *et al.*, 2009). These indicate earlier, local, clinically silent bleeding. In IAs, the distribution of the perivascular fiber system, responsible for vasoconstriction and vasodilatation, does not follow any organized pattern. Instead, it shows wave and whirl-like patterns and cannot be detected in the circumscribed areas at all (Buki *et al.*, 1999). Sometimes the IAs have a luminal thrombus, fresh or organized with thin-walled capillaries, fibroblasts, and minimal amounts of extracellular mucoid material (Frösen *et al.*, 2004; Rajesh *et al.*, 2004). The IA wall also has signs of

atheromatous and atherosclerotic changes, cellular death, inflammatory cell infiltrations (of polymorphonuclear leukocytes (PMNs), macrophages, T-cells, B-cells, and natural killer cells (NK-cells)), and ECM remodeling, described in detail in the following sections and summarized in Section 1.5.4.8.

Rupture site

IAs show an uneven distribution of rupture sites; 83-84% rupture at the apex, 13-14% at the body, and 2% at the neck (Crawford, 1959; Crompton, 1966). The margin of the rupture site contains fibrin (Crompton, 1966), seen with, but never without, an (inflammatory) cell infiltration. The inflammatory cells at the site of the IA rupture have been identified as activated macrophages, T-cells, and NK-cells (Kosierkiewicz *et al.*, 1994). There is also endothelial damage and partial degeneration at and near the rupture site (Scanarini *et al.*, 1978a; Kataoka *et al.*, 1999). Apoptotic cell death is more common near the rupture site than in other parts of the wall (Hara *et al.*, 1998). A fresh, unorganized, extraluminal thrombus may also be seen at the rupture site (Nomura *et al.*, 2000).

Differences between unruptured and ruptured aneurysms

Ruptured IAs often show a decreased SMC content in their walls (Sakaki *et al.*, 1997; Kataoka *et al.*, 1999; Frösen *et al.*, 2004; Kilic *et al.*, 2005). Interestingly, in the study of Kataoka *et al.* (1999), the symptomatic but unruptured IAs revealed similar histological changes as the ruptured IAs. Thus far, only two major studies comparing the histological parameters of unruptured and ruptured IAs have been performed (Kataoka *et al.*, 1999; Frösen *et al.*, 2004).

Kataoka investigated a series of 27 unruptured and 44 ruptured IAs, observing that ruptured IAs had marked endothelial damage. The endothelial cells represented a disrupted arrangement and shape, with intra-endothelial gaps filled with blood cells. In some cases, even total destruction of the endothelium was seen. The wall of the ruptured IAs was also more degenerated with disorganization of type IV collagen, scattered, diminished, or totally lost SMCs, and even hyalinization. Macrophage and leukocyte infiltration and expression of the proteolytic enzymes cathepsin D and G were greater in ruptured IAs. Unruptured IAs, in turn, often showed an almost intact, longitudinally organized endothelium of the IA wall, dense SMCs, regular layers of type IV collagen, and only a few macrophages and cathepsin D-positive SMCs in the outer layer of the wall (Kataoka *et al.*, 1999).

Frösen classified 24 unruptured and 42 ruptured IA walls into types A, B, C, and D according to their proliferative and degenerative changes. The lowest risk of rupture was found within normal-looking IA walls (A-type). The rupture risk increased gradually for

thicker walls with myointimal hyperplasia and an occasional thrombus (B-type), thicker thrombosed walls with a reduced number of mural cells (C-type), and thin hypocellular walls with an organized thrombus (D-type). The ruptured IAs showed an increased proportion of apoptosis, de-endothelialization, organizing luminal thrombosis, SMC proliferation, and infiltration of macrophages and T-cells (Frösen *et al.*, 2004). An increased incidence of apoptotic cells (Sakaki *et al.*, 1997; Pentimalli *et al.*, 2004), inflammatory cells (PMNs and lymphocytes), and partially organized thrombi (Schlote and Gaus, 1994) have been associated with IA rupture also in other studies.

The pattern of vascular growth factor expression differs between unruptured and ruptured IAs. Vascular endothelial growth factor (VEGF) is expressed more abundantly in ruptured than unruptured IAs, especially in the adventitia. In contrast, the basic fibroblast growth factor (bFGF) is slightly less expressed in ruptured than unruptured IA walls (Kilic *et al.*, 2005). In a study of 12 different growth factor receptors in 21 unruptured and 35 ruptured IAs, only VEGF receptor 1 and the transforming growth factor beta (TGF-β) receptor type II were found to be associated with IA rupture (Frösen *et al.*, 2006b).

There are also differences in ECM expression and turnover and IA wall remodeling between unruptured and ruptured IAs. Fibronectin and collagen III are more densely expressed and type IV collagen expression is decreased in ruptured IAs compared with unruptured IAs (Kilic *et al.*, 2005). Matrix degradation appears to be increased in ruptured IAs, as judged by increased expression of matrix-degrading enzymes matrix metalloproteinase (MMP) -2 and MMP-9 relative to their endogenous inhibitors (tissue inhibitor of metalloproteinase; TIMP) (Jin *et al.*, 2007). The vasa vasori of ruptured IAs show sclerotic changes, disturbances of the internal elastic lamina, and partial occlusion of the lumen by probable thrombotic material (Scanarini *et al.*, 1978a).

1.5.4 Mechanisms altering intracranial aneurysm wall structure

Many of the histological changes seen in the IA wall relative to the normal cerebral artery wall reflect degenerative and compensatory remodulatory processes. The following sections discuss the possible roles of these stress-response mechanisms in IA wall pathobiology.

1.5.4.1 Endothelial dysfunction

De-endothelialization is associated with IA rupture (Kataoka *et al.*, 1999; Frösen *et al.*, 2004). Endothelial damage has been shown to be one of the first changes in hypertension-induced experimental IAs in rodents (Jamous *et al.*, 2007). Pressure and shear stress from circulating blood are necessary for the normal functioning of endothelial cells (Chien, 2008). As the flow alters during IA progression, the hemodynamic impact on the endothelium changes. This probably sustains molecular signaling of pro-inflammatory and

proliferative pathways (Chien, 2008). As a probable sign of dysfunction, the endothelial cells of human IAs express monocyte chemoattractant protein-1 (MCP-1) (Cao *et al.*, 2002; Krischek *et al.*, 2008; Aoki *et al.*, 2009b) and vascular cell adhesion molecule-1 (VCAM-1) (Chyatte *et al.*, 1999; Shi *et al.*, 2009). MCP-1 is chemotactic to inflammatory cells, macrophages, T-cells, NK-cells, and basophils, and VCAM-1 aids leukocyte recruitment. The induction of MCP-1 and VCAM-1 likely occurs through an activation of transcription factor nuclear factor kappa B (NFκB), as seen in the early phase of experimentally induced IAs in rodents (Aoki *et al.*, 2007c). NFκB plays a key role in regulating immune responses. It is involved in cellular responses to stress, cytokines, free radicals, and oxidatively modified low density lipoproteins (oxLDL). Strong activation of NFκB has been observed in human IAs, mainly in intimal cells (Aoki *et al.*, 2007c).

1.5.4.2 Inflammatory cell infiltration

Inflammatory cell infiltrations have been detected in unruptured IAs at locations where the IA wall typically breaks upon an IA rupture (Crawford, 1959; Crompton, 1966). Crompton (1966) identified the inflammatory cells seen beneath the endothelium and deep in the IA wall as polymorphonuclear leukocytes (PMN), plasma cells, and small round cells. Later, immunohistochemical analyses have verified the inflammatory cells in IA walls to also be $CD68^+$ macrophages, $CD163^+$ macrophages and T-lymphocytes (T-cells), some natural killer cells (NK-cells), and a few B-lymphocytes (B-cells) (Kosierkiewicz *et al.*, 1994; Chyatte *et al.*, 1999; Kataoka *et al.*, 1999; Frösen *et al.*, 2004). The magnitude of inflammatory cell infiltrations has been associated with IA rupture (Kataoka *et al.*, 1999; Frösen *et al.*, 2004), stressing the role of inflammation in the degenerative processes of the IA wall. Interestingly, macrophage infiltration has also been associated with the proliferation of mural cells in IA walls (Frösen *et al.*, 2004). In experimental IA studies, macrophages have been the first inflammatory cells invading the progressing IA wall following endothelial dysfunction (Jamous *et al.*, 2007). Macrophages produce numerous effectors, i.e. TGF-β, reactive oxygen species (ROS), tumor necrosis factor alpha (TNF-α), and interleukin-1 (IL-1), to induce apoptotic cell death, cell proliferation and migration, ECM production and degradation, and modulation of IA wall structures, as reviewed in Boyle (2005). The numerous potential functions of macrophages in IA walls are presented in Figure 3. Inflammatory cells in general likely also have more functions in IA wall pathobiology.

The initial trigger for inflammatory cell infiltration into IAs is unknown. In experimental IAs in rodents, macrophage infiltration follows IA formation and endothelial dysfunction (Aoki *et al.*, 2007a; Jamous *et al.*, 2007). This is probably a response to chemotaxis mediated by MCP-1 (see Section 1.5.4.1). Components of the complement system along with immunoglobulins G and M (IgG and IgM) have also been seen in IAs

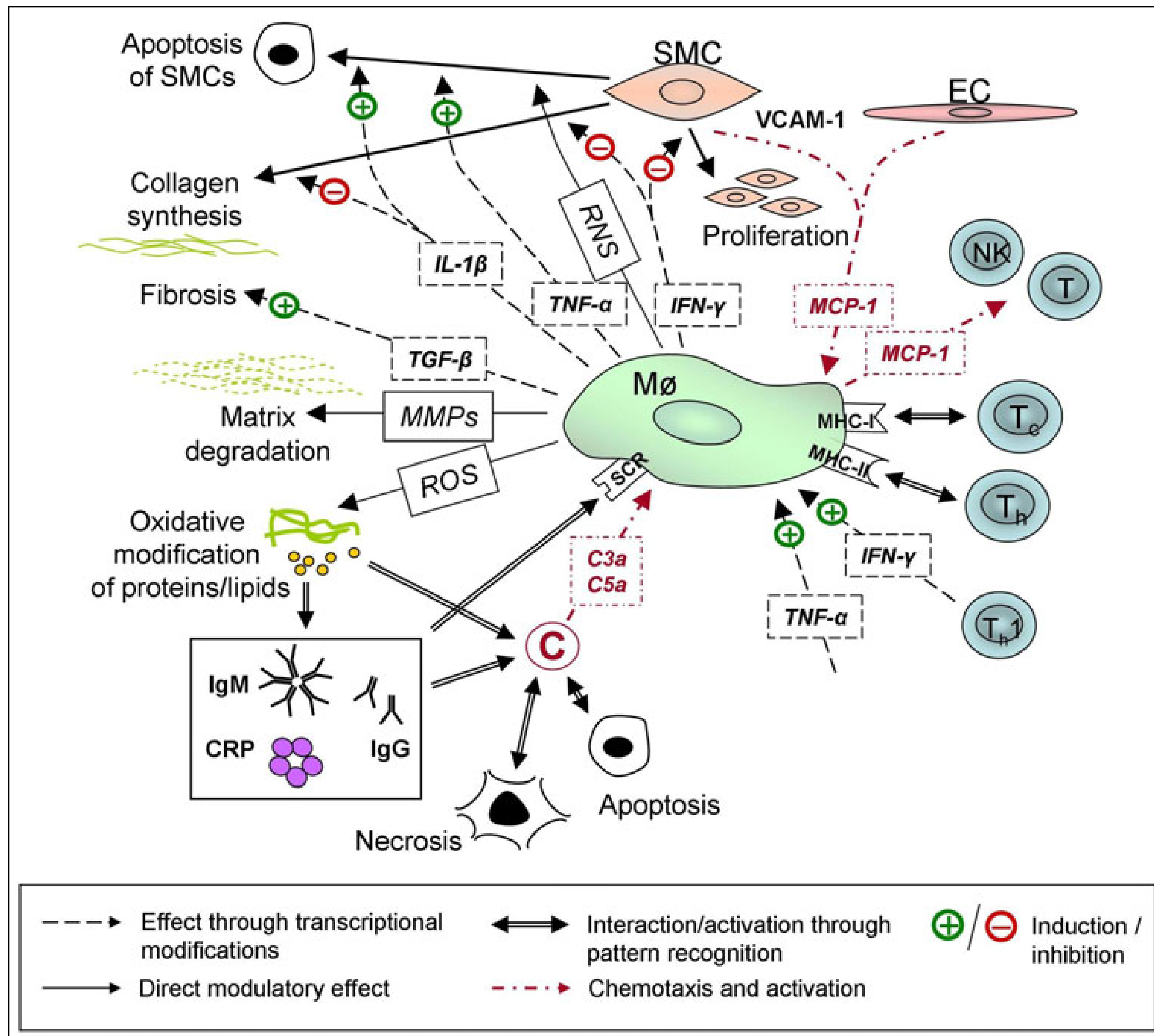

Figure 3. Probable activators and main functions of macrophages in intracranial aneurysms. MCP-1, monocyte chemotactic protein; SMC, smooth muscle cell; EC, endothelial cell; Mø, macrophage; VCAM-1, vascular cell adhesion molecule; IL-1β, interleukin 1 beta; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon gamma; MMP, matrix metalloproteinases; TGF-β, tissue growth factor beta; ROS, reactive oxygen species; RNS, reactive nitrogen species; SCR, scavenger receptor; IgG, immunoglobulin G; IgM, immunoglobulin M; CRP, C-reactive protein; C, complement system; C3a and C5a, anaphylatoxins; MHC-I and MHC-II, major histocompability complexes I and II; T, T-cell; NK, natural killer cell.

harboring inflammatory cells (Chyatte *et al.*, 1999). Target-bound immunoglobulins activate the complement system, and the activated complement system is chemotactic to inflammatory cells. The possible involvement of PMNs in recruiting macrophages and destroying tissue enzymatically has not been verified.

The role of lymphocytes in IA wall pathobiology remains obscure. The timing of their invasion into the IA wall during IA progression has not been verified in experimental IA studies in animals. However, lymphocytes have a central role in acquired immunity, and polarization of the T-cell response to cytotoxic, effector, or regulatory T-cells may orientate the vascular wall response to inflammation in either a proliferative or

degenerative direction. The potential functions of lymphocytes in IA walls are reviewed more thoroughly in Section 2.2.1.

In an autopsy series of 79 unruptured IAs, (inflammatory) cell infiltrations occurred in the fundus and were always accompanied by fibrosis. More importantly, fibrosis was never seen without an (inflammatory) cell infiltration, suggesting it to be secondary to inflammatory cell invasion (Crompton, 1966). Fibrosis is considered to be an end-phase of chronic inflammatory reactions (Wynn, 2008). Fibrosis develops as a response of SMCs and myofibroblasts to cytokines and growth factors secreted by inflammatory and other activated cells. Macrophages are known to induce fibrosis through secretion of TGF-β (Boyle, 2005).

1.5.4.3 Remodeling of extracellular matrix

The tensile strength of the IA wall originates from many very thin, relatively coherently organized sublayers of collagen that collectively provide a balanced distribution for bearing the biaxial tensile stress of the IA wall (Canham *et al.*, 1996). However, the distribution of the structural proteins in IAs often differs from their organization in cerebral arteries, and their expression in immunohistochemistry is often less intense. Moreover, in IAs, the total collagen content and the number of strength-providing deoxypyridinoline cross-links are decreased (Gaetani *et al.*, 1997).

Whereas normal cerebral arteries often show linear organization of different collagens, type I, III, IV, and V collagens, and fibronectin are diffusely expressed in the IA wall. Type IV collagen, which is normally expressed in the adventitia, is missing from the abluminal part of the IA wall (Austin *et al.*, 1993; Skirgaudas *et al.*, 1996; Mimata *et al.*, 1997; Kilic *et al.*, 2005). The IA wall also harbors less laminin than the normal cerebral artery wall (Kilic *et al.*, 2005). It also nearly lacks desmin, which is normally expressed around medial SMCs (Mimata *et al.*, 1997). The content of hyaluronan, a highly hydrated anionic macromolecule and an essential component of the ECM responsible for the viscoelastic resistance of a thinned vessel wall, is increased in IAs (Klekner *et al.*, 2005). Although immunostaining shows slight diminishment of type III collagen relative to the normal control artery, *in situ* hybridization of the IA wall reveals its synthesis being increased, indicating a faster turnover of collagen III in the IA wall (Mimata *et al.*, 1997).

A definite feature related to IA wall degeneration is the disruption of its matrix. MMPs are proteolytic enzymes expressed by SMCs and macrophages in the IA wall (Caird *et al.*, 2006; Aoki *et al.*, 2007a). MMP activity can be upregulated in response to hemodynamic factors, mechanical strain, vascular injuries, inflammatory cytokines, and ROS (Raffetto *et al.*, 2008). In IA patients, MMP-2 (Todor *et al.*, 1998) has been found to be responsible for increased serum gelatinase activity and decreased levels of serum procollagens I and III (Chyatte and Lewis, 1997). The expression levels of membrane-type

1 MMP (MT1-MMP) and gelatinases MMP-2 and -9 are increased (Bruno *et al.*, 1998; Caird *et al.*, 2006; Jin *et al.*, 2007) in IA walls. Also cathepsins D, G, S, B, and K, proteolytic enzymes expressed differentially by SMCs, macrophages, leukocytes, and endothelial cells, have been found in IAs (Kataoka *et al.*, 1999; Aoki *et al.*, 2008a). The activities of proteinases are in part regulated by their endogenous inhibitors (Raffetto *et al.*, 2008). In IAs, levels of both TIMP (Jin *et al.*, 2007; Jayaraman *et al.*, 2008) and cystatin C (Aoki *et al.*, 2008a) are decreased, thus potentiating the effects of proteinases. Similar changes have been seen in experimental IAs (Aoki *et al.*, 2007b; Aoki *et al.*, 2008a).

IA patients have significantly higher plasma elastase levels than normal nonaneurysmal control patients. Furthermore, elastin in hyperplastic extracranial arteries is more deranged in IA patients than in controls (Connolly *et al.*, 1997). However, the disturbance in the balance of proteinases and their inhibitors is more likely local than systemic, as IA walls have markedly increased levels of both elastase MMP-9 and its major inhibitor TIMP (Kim *et al.*, 1997).

In addition to proteolytic activities, MMPs may have other functions in the IAs. MMPs are needed for vascular remodeling, cellular migration, and processing of ECM proteins in order to provide vascular homeostasis. MMPs also have a role in the formation of neointima, MMP-2 and -9 inhibit rat SMC contraction, MT1-MMP and MMP-2 enhance angiogenesis, and degradation of ECM by MMPs releases several angiogenic growth factors (Raffetto *et al.*, 2008). Thus, the balance between activation of proteolytic enzymes and their inhibition might be critical for IA wall degeneration.

1.5.4.4 Proliferation and myointimal hyperplasia

IA wall thickness is independent of patient age or gender or aneurysm size or location (Inagawa and Hirano, 1990), indicating that it depends on local factors. In a series of 91 ruptured IAs, SMC hyperplasia was seen in 40% (Holling *et al.*, 2009), and the proliferation ratio increased in ruptured IAs (Frösen *et al.*, 2004). Moreover, stress-induced mitogen-activated protein kinases (MAPKs) are expressed in IAs (Laaksamo *et al.*, 2008). They are involved in vascular remodeling, apoptosis, cell growth, and survival. Furthermore, strong expression of the proinflammatory cytokine TNF-α in ruptured IAs and its activity are linked to IA growth (Jayaraman *et al.*, 2005). TNF-α can be induced by all of the common IA risk factors (hypertension, aging, female gender, smoking, alcohol abuse, and genetics) for IA rupture (Jayaraman *et al.*, 2008).

Mural cells of the IA wall, mainly the SMCs, may proliferate as a response to several growth factors detected in the IA wall. Especially VEGF, functioning in angiogenesis, vasodilatation, and chemotaxis for inflammatory cells, is strongly expressed in IA walls (Skirgaudas *et al.*, 1996; Kilic *et al.*, 2005). Expression of VEGF receptors is

associated with IA wall rupture, vascular remodeling, T-cell and macrophage infiltration (VEGF receptor-1), and cell proliferation and myointimal hyperplasia (VEGF receptor-2) (Frösen *et al.*, 2006b). The differential expression of VEGF receptors indicates multiple alternative effects of VEGF in the IA wall. Platelet-derived growth factor (PDGF) and bFGF seem to be expressed less in IAs than in control arteries (Ohkuma *et al.*, 2003; Kilic *et al.*, 2005). In addition, the receptors for PDGF, bFGF, and insulin-like growth factor (IGF), which promote cell proliferation, are expressed in IAs (Frösen *et al.*, 2006b). Based on the detected expression of different growth factors, growth factor receptors, and MAPKs, the IA wall seems to undergo a constant remodeling process, probably in response to inflammation and local stress.

1.5.4.5 Decellularization

The loss of medial SMCs is evident upon degeneration of the IA wall. In apoptosis, the cells die in a programmed way as a response to death-signaling with a minimum proinflammatory effect. Apoptotic cells have been seen especially in the necks and domes of IAs, whereas only a few apoptotic cells have been described in control arteries (Sakaki *et al.*, 1997; Hara *et al.*, 1998; Pentimalli *et al.*, 2004; Guo *et al.*, 2007). Apoptosis has been associated with IA formation also in experimental settings (Kondo *et al.*, 1998). Moreover, necrotic cell death is seen in half of IAs (Holling *et al.*, 2009). In contrast to apoptosis, necrotic cell death that occurs in response to an acute injury leads to a burst of highly proinflammatory cellular contents. The exact reasons for cellular death in the IA wall are unknown, although many of the proinflammatory mediators present in the IA wall, e.g. the proinflammatory cytokine TNF-α (Jayaraman *et al.*, 2005), are known to induce apoptotic or necrotic cell death. Proteolytic enzymes may detach the SMCs from the surrounding ECM, leading to anoikis, another type of apoptotic cell death (Chiarugi and Giannoni, 2008). In addition, local hypoxia has been proposed as a possible mechanism for cell death in the IA wall (Inci and Spetzler, 2000). Notably, hypoxia would also induce collagen synthesis (Fahling *et al.*, 2004).

1.5.4.6 Lipid accumulation and atherosclerotic changes

Atherosclerosis has been thought to be related to IA formation (Carmichael, 1950; Walker and Allegre, 1954). The deposition of lipids into the vascular wall, an early phenomenon of atherosclerosis, is most frequent in the fundus of the IA. In normal cerebral arteries, neutral lipids accumulate in the subintimal space and only rarely reach the media (Zugibe and Brown, 1961). In IAs, lipid droplets have been seen in both the media and the adventitia together with macrophages and intracellularly in endothelial cells, distended SMCs, other intramural cells (Nyström, 1963), and macrophages (foam cells) (Crompton, 1966). The foam cells have been detected together with fibrin deep in the IA wall (Crompton, 1966). Other histological changes considered to be atherosclerotic, such as

proliferation of SMCs (Kataoka *et al.*, 1999; Frösen *et al.*, 2004), hyalinization of the connective tissue (Hassler, 1961), infiltration of the media and adventitia by macrophages (Kosierkiewicz *et al.*, 1994; Kataoka *et al.*, 1999; Frösen *et al.*, 2004), vacuolization of endothelial cells (Nyström, 1963), and intimal neovascularization (Aydin, 1998), have also been seen in IAs.

The atheromatous changes are often focal (Rajesh *et al.*, 2004) and seem to be strictly localized in the aneurysm sac. They are only infrequently present in the IA neck, while parent vessels are usually spared (Scanarini *et al.*, 1978a). In some IAs, the atherosclerotic changes colocalize with endothelial damage (Kataoka *et al.*, 1999). Advanced atherosclerotic lesions with cellular infiltrates of mostly macrophages, SMCs, and lymphocytes are detected in 32-48% of IAs in general (Kosierkiewicz *et al.*, 1994; Caird *et al.*, 2003). They are even more often seen in large IAs (Kosierkiewicz *et al.*, 1994). Notably, if the definition of atherosclerotic changes is strictly followed, nearly all IAs could be considered ‘atherosclerotic’.

Apolipoprotein(a) (Apo(a)), an active moiety of lipoprotein(a), has been observed in layers between the intimal pad and the deep aneurysmal wall and in most of the feeding vessels (Caird *et al.*, 2003), suggesting a more generalized phenomenon. Normal intracranial arteries may show atherosclerotic plaques and Apo(a) (Jamieson *et al.*, 1995). Serum lipoprotein(a) levels are also higher in IA patients, especially in females, than in clinically nonatherosclerotic healthy controls (Bolger *et al.*, 1995).

Oxidative modification of lipids (and proteins) by reactive oxygen and nitrogen species induces the formation of epitopes that can be recognized by the so-called pattern recognition receptors (PRRs). These include CRP, complement C1q, and macrophage scavenger receptors (Chou *et al.*, 2008). Oxidized epitopes are also the main targets for naturally occurring antibodies (NAbs), which mainly belongs to the IgM class (Chou *et al.*, 2009). The ultimate oxidation level of the tissue depends on the balance of ROS production and destruction. ROS are produced in the arterial wall by electron leakage from the mitochondrial respiratory chain, phagocytes’ oxidative burst (NAPDH oxidase), as a by-product of normal cellular metabolism, and by the action of nitric oxide synthases (i.e. iNOS and eNOS) (Szasz *et al.*, 2007). Of the known arterial ROS producers, inducible nitric oxide synthase (iNOS) has been found in human IA walls (Fukuda *et al.*, 2000). In experimental IAs, the expression of iNOS has been found to increase and that of endothelial nitric oxide synthase (eNOS) to decrease during IA progression (Fukuda *et al.*, 2000). The knockout of iNOS diminishes the size of experimental IAs and the number of apoptotic SMCs (Sadamasa *et al.*, 2003). Recently, Aoki *et al.* (2009c) showed that ROS inhibition by edavarone diminished the size of IA, IEL disruption, and macrophage infiltration, and enhanced the medial thickness of rat experimental IAs. These effects

occurred, probably through inhibition of NFκB and the consequent decrease in expression of MCP-1, VCAM-1, and MMP-2.

1.5.4.7 Thrombus

The presence of an organizing thrombus is frequently seen in ruptured IAs (Schlote and Gaus, 1994; Frösen *et al.*, 2004). The organizing thrombus often carries thin-walled capillaries, fibroblasts, and minimal amounts of extracellular mucoid material (Rajesh *et al.*, 2004). Normally, the intact and healthy endothelium protects the luminal surface of the vascular wall from thrombosis and platelet aggregation by the expression of CD39 and secretion of nitric oxide (NO) and prostacyclin (Watson, 2009). The coagulation cascade may be initiated by a local endothelial injury or by a release of inflammatory mediators from damaged or stressed endothelial cells (Wynn, 2008). In total endothelial injury, also the subendothelial prothrombotic structures that come into contact with platelets facilitate activation of the coagulation cascade and thrombus formation. Altered hemodynamics and decreased blood flow promote endothelial dysfunction and thrombus formation (Wohner, 2008). Both erythrocytes and platelets become trapped in the fibrin meshwork of a fresh thrombus. Chemotactic signals and expression of adhesion molecules aid neutrophil and macrophage infiltration into the site of injury (Wohner, 2008; Wynn, 2008).

To promote fibrinolysis and thrombus deformation, platelets and neutrophils secrete MMPs, cathepsin G, and elastase. The proteolytic enzymes released by neutrophils and macrophages are probably involved in further degradation of the IA wall as well as in the induction of anoikis (Fontaine *et al.*, 2002; Fontaine *et al.*, 2004). Later, the inflammatory cells may migrate into an IA wall as PMNs, and other inflammatory cells have been found in the IA wall (Hassler, 1961; Crompton, 1966). A thrombus begins to organize by invading cells, SMCs, or myofibroblasts that originate at least in part from the vascular wall (Frösen *et al.*, 2006a; Lee *et al.*, 2007; Wynn, 2008). The thrombus subsequently becomes an indistinguishable part of the IA wall.

1.5.4.8 Summary of mechanisms altering intracranial aneurysm wall structure

Both degenerative and reparative mechanisms seem to operate in the IA wall. The likely mechanisms leading to IA progression and critical thinning of the IA wall are summarized in Figure 4. They are based on combined data from studies on human and experimental IAs.

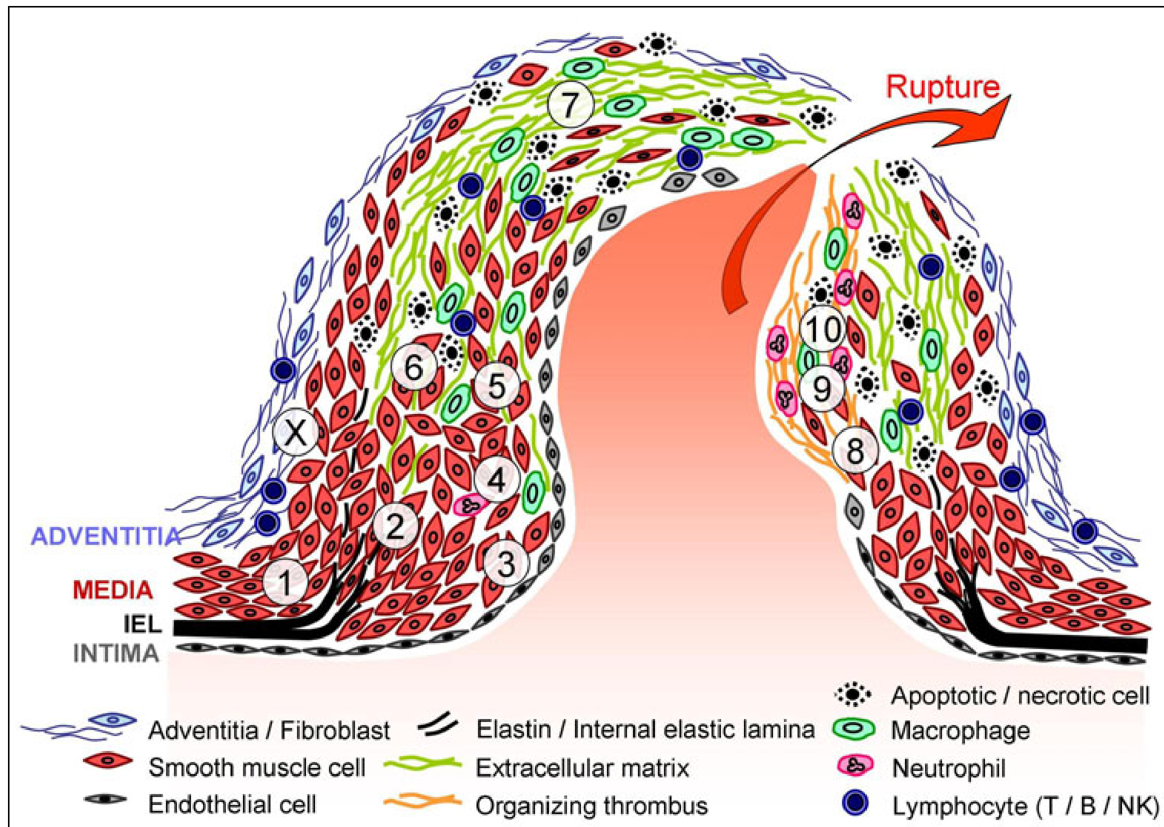

Figure 4. Mechanisms likely leading to IA progression and critical thinning of the IA wall. An aneurysm forms at the site of disrupted internal elastic lamina (IEL) (1) associated with myointimal hyperplasia (2). Hemodynamic stress, primary or secondary to the structural modifications of the vascular wall, leads to endothelial dysfunction (3) and chemotaxis for invasion of macrophages and/or other leukocytes (4). At the same time, matrix synthesis is increased to support the tensile strength of the aneurysm wall due to function of growth factors secreted by, for instance, macrophages (5). However, because of the cytotoxic milieu caused by inflammatory cells, increased matrix, and possibly decreased oxygen supply arising from an increased wall thickness, the mural cells start to die (6). Matrix turnover slows down due to decreased production secondary to decellularization and increased lysis by the action of matrix metalloproteinases (MMPs) (7). Over time, as the aneurysm wall becomes mechanically more fragile, it finally ruptures when the intraluminal pressure overcomes the tensile strength. Lymphocytes also have an important role in aneurysm wall degeneration by guiding the immune response and the surveillance of mural cells. However, their timing in aneurysm progression is unclear (X). Depending on the hemodynamics and shear stress, the endothelium may die or severely dysfunction, leading to thrombus formation (8). In addition to other inflammatory cells, the thrombus attracts neutrophils (9), which are potent producers of proteolytic enzymes. Over time, the thrombus may become organized by migrating mural cells (10). However, due to cell death and decreased matrix turnover, the aneurysm wall becomes fragile enough to rupture. This figure is a simplification of the pathobiological processes within the aneurysm wall. Thus, it does not represent all factors with potentially crucial roles in aneurysm wall degeneration, e.g. complement activation, lipid accumulation, and neovascularization.

1.5.5 Role of extra-aneurysmal factors

During IA formation the hemodynamics in the IA changes. Blood flow inside the IA has two components: pressure and shear stress to the IA luminal surface. In a follow-up study of 7 IA patients by MR-angiography, aneurysm growth and development of

atherosclerotic plaques were shown to be related to low local wall shear stress (Boussel *et al.*, 2008; Tateshima *et al.*, 2008). Laminar flow is required for endothelial survival (Chien, 2008). Excessively high or low shear stress affects the endothelium and induces endothelial dysfunction. Smooth muscle cells are also sensitive to shear stress and stretch (Zou *et al.*, 1998). Thus, flow conditions in IAs affect the biology of the IA wall.

IAs grow into the subarachnoidal space, composed of arachnoideal strains and spaces filled by cerebrospinal fluid. In general, the extra-arterial pressure most likely affects the cellular organization of the cerebral artery wall, and thus, IA wall modification. In experimental settings, decreased intracranial pressure allows the cerebral artery wall to become thicker and lose the tight packing of linearly organized SMCs (Hassler, 1961). The perianeurysmal environment, serving to constraint contact between the IAs and anatomical structures, mechanically influences the IA shape and rupture. When optimally positioned, contact constraints can have a protective effect, as they may decrease the stresses near the IA fundus (Seshaiyer and Humphrey, 2001). The contact constraints of ruptured IAs and the perianeurysmal environment are more unbalanced than those of unruptured IAs, allowing ruptured IAs to grow larger and irregular in shape (San Millan Ruiz *et al.*, 2006).

1.5.6 Role of intrinsic and acquired risk factors

Hypertension

Vascular hypertension has been proposed to affect the IA wall by three different mechanisms: by inducing endothelial injury, by promoting occlusion of the vasa vasori, and by disturbing the synthesis of elastin and collagen (Inci and Spetzler, 2000). Hypertension is also known to induce TNF-α expression, which affects IA growth and may initiate degenerative changes at the cellular level (Jayaraman *et al.*, 2008). Experimental IAs can only be induced by disturbed hemodynamics (Hashimoto *et al.*, 1984; Stehbens, 1989).

Smoking

Smoking (also passive) is proinflammatory, as it induces inflammatory injury by increasing VCAM-1, MCP-1, and IL-8 expression in the subendothelial and medial layers of the arterial wall (Zou *et al.*, 2009). Cigarette smoke also downregulates lysyl oxidase, an enzyme initiating cross-linkage between elastin and collagen, in fibroblasts (Chen *et al.*, 2005). It also increases the metalloproteinase activity of rat plasma and human fibroblasts (Machida *et al.*, 2004; Yin *et al.*, 2000) and decreases collagen I and III synthesis (Yin *et al.*, 2000). At least part of the effects of cigarette smoke on cellular function might be mediated through induction of a nonfunctional form of TGF-β1 and

downregulation of the TGF-β1 receptor (Yin *et al.*, 2003). The modifications on type IV collagen by cigarette smoke and lipid peroxidation products trigger macrophages by their type A scavenger receptor and promote MCP-1 release and oxidative burst from macrophages (Kirkham *et al.*, 2003).

Female gender

Decreasing circulating estrogen levels and a probable decrease in expression of estrogen receptors have been suggested as mechanisms for IA progression in women (Harrod *et al.*, 2006). Estrogen receptors are important modulators of the vascular wall. In vascular injury, estrogen acts as an anti-inflammatory, as it inhibits the local induction of proinflammatory mediators and adhesion molecules, leukocyte activation, infiltration, and accumulation, adventitial activation and local release of cytokines, and remodeling of matrix proteins (reviewed by Xing *et al.*, 2009). In an experimental setting, estrogen deficiency caused an increased incidence and size of experimental IAs, probably by endothelial dysfunction and associated ROS generation, whereas estrogen replacement therapy counteracted the effect (Jamous *et al.*, 2005a; Jamous *et al.*, 2005b; Tamura *et al.*, 2009).

Aging

Aging causes many changes in tissue structures and cellular functions in general. Cerebral arteries also undergo changes with age. The relative proportion of different fibers in elastic lamina is altered gradually, with collagen becoming more abundant, and with the appearance of type 3 pseudoelastic fibers the arterial wall losing its elasticity (Hegedus and Molnar, 1989). Accumulation of neutral lipids within IEL and intima along with the expression of acid mucopolysaccharides also becomes more abundant with age (Zugibe and Brown, 1961). The total lipid concentration increases and the relative amounts of different glycosaminoglycan change (Murata, 1985). In the elderly, the effect of acquired risk factors accumulates.

Alcohol and drug abuse

Heavy alcohol use is a common cardio- and cerebrovascular risk factor for hemorrhagic (SAH) and ischemic strokes as well as cardiomyopathy. Although the exact cellular mechanisms underlying alcohol abuse-altering arterial homeostasis are widely unknown, increased blood pressure related to alcohol intoxication and alterations in cerebral artery tone are thought to promote the degeneration of the cerebral artery wall (Hillbom, 1998). Cocaine, in turn, may cause a severe elevation in blood pressure, vasospasm, and vasculitis. Naturally, some of the IAs in cocaine users may also be mycotic in origin due to intravenous administration of the drugs (Neiman *et al.*, 2000).

1.6 Molecular pathology of intracranial aneurysms

Genetic background plays a role in IA pathobiology, as different nationalities have different rates of aSAH. Even rat species differ in their susceptibility to IAs. The genetic variation is evident, as of the several susceptible gene loci only four (1p34.3-p36.13, 7q11, 19q13.3, and Xp22) have been replicated in different populations (reviewed in Ruigrok and Rinkel, 2008). Recently, the susceptibility locus 19q13.3 for Finnish and Japanese populations (van der Voet *et al.*, 2004; Mineharu *et al.*, 2007) remained unconfirmed in another study of Finnish, Japanese, and Dutch cohorts (Bilguvar *et al.*, 2008).

Several candidate genes are included in the IA susceptibility loci. However, the linkage studies of their associations have yielded varying results depending on the study population (Krischek and Inoue, 2006). Of these, the genes for IL-1β, collagen type I and III, elastin, eNOS, endoglin, angiotensin-converting enzyme, MMP-9, and heme-oxygenase 1 have revealed positive associations. Several candidate genes for IAs have also been investigated at the level of single-nucleotide polymorphisms (SNPs), with varying results depending on the study population.

2 Inflammation in the vascular wall

Inflammation is a normal tissue's reaction to physical, chemical, or biological stress or injury. It is manifested as activation of innate immune response, release of inflammatory mediators, and inflammatory cell accumulation. The classical clinical symptoms caused by inflammatory reaction – rubor (redness), calor (heat), tumor (swelling), and dolor (pain) – are also seen during microbial *infections*, where inflammation has an important role as a defense mechanism against invading *microbes*. However, in most acute and chronic inflammatory diseases, the inflammatory reaction occurs aseptically, i.e. in the absence of microbes. In aneurysms, similarly to atherosclerosis, the inflammatory reaction has become persistent and chronic. As there is no evidence of a persistent infection in IAs (excluding the rare cases of mycotic aneurysms), the next sections focus on the inflammatory mechanisms responding to apathogenic irritants.

Inflammatory reactions are mediated by the immune system, which can be broken down into two responses: innate and adaptive immunity. The division is based on the ability to respond to certain surface structures either inherently (innate immunity) or by modification of the response by genetic rearrangements (adaptive immunity). In practice, both arms of immunity are usually involved in the inflammatory reaction, and they act in concert with each other. The complement system, an ancient immunological defense mechanism, is a core element of the innate immune response, also having an important role in directing adaptive immune responses.

2.1 Inflammation and immunity

Inflammation is a normal response to tissue trauma or irritants, whether microscopic or macroscopic, and necessary for removing the irritant and for healing to occur. The innate immune system recognizes the irritant and/or the trauma and recruits inflammatory cells (polymorphonuclear leukocytes and monocytes) to the site of inflammation. The recognition is based on the inherent ability of certain molecules (pattern recognition receptors, e.g. complement components, acute phase proteins, toll-like receptors (TLRs), and macrophage scavenger receptors (SCRs)) to bind certain molecular patterns, leading to further activation of innate immunity. Antibodies may as well be involved in the recognition of harmful stimuli (Chou *et al.*, 2008). At an early stage, the trauma activates also the coagulation cascade, promoting the inflammatory reaction by, for example, platelet degranulation (Wynn, 2008).

Cytokines, chemokines, and growth factors secreted mainly by inflammatory cells create a milieu that favors healing of the injury. Cytokines produced by the innate inflammatory cells are also chemotactic to the cells of the adaptive immunity (lymphocytes; T-cells, B-cells). If adaptive immunity cells become activated, they can further affect the nature and outcome of the inflammatory reaction by secreting cytokines and/or producing antibodies. Fibrosis and neovascularization are important late-stage processes in the route to complete healing. Fibrosis, promoted by myofibroblasts, takes place to restore mechanical strength at the site of injury and to build an ECM framework for migrating cells. Neovascularization, in turn, provides the newly formed tissue with a sufficient supply of oxygen and nutrients (Beutler, 2004; Stavitsky, 2007; Wynn, 2008).

As inflammatory cells and chemical mediators also have the potential to harm uninvolved, healthy tissue beyond the trauma, the inflammatory reaction is tightly controlled and healthy tissues are protected by several inhibitors. If control of the inflammatory reaction fails, it may lead to various diseases depending on the site and failed mechanisms, e.g. glomerulonephritis, acute pancreatitis, vasculitis, angioedema, hemolysis, disseminated intravascular coagulation, and shock.

2.1.1 Acute and chronic inflammation

Acute inflammation is the initial response to harmful stimuli, where characteristically the innate immunity, with an inherent ability to recognize the stimuli and react fast, is recruited. After removal of the irritant, aided by the acquired immunity, the injured tissue heals and the inflammation ceases in the shortage of proinflammatory mediators. However, if the immune system cannot overcome the irritant, the acute inflammatory reaction becomes chronic, as seen in atherosclerosis, rheumatoid arthritis, and psoriasis. In chronic inflammation, tissue healing and destruction occur simultaneously, leading to

permanent changes in the tissue causing the disease phenotype. Remarkably, in the case of autoimmune diseases and possibly also in other chronic inflammatory diseases, the inflammatory mechanisms themselves may maintain the problem and cause the tissue destruction.

2.2 Innate and adaptive immunity

The innate immune system has an inherent ability to recognize and become activated by a wide range of molecular patterns, to respond quickly to activation, and to act in a targeted fashion against the activating stimulus, sparing otherwise healthy tissues. The activating molecular patterns or individual molecules often resemble surface antigens found on pathogens, and are thus called pathogen-associated molecular patterns (PAMPs). The majority of PAMPs arise from oxidative modification of proteins and lipids or from cell death. PAMPs are recognized by TLRs, complement components, naturally occurring immunoglobulins, and SCRs. Antibodies and complement factors (C1q, C3b, iC3b, MBC, ficolins) are, in turn, recognized by Fc receptors and complement receptors (Chou *et al.*, 2008). The cellular components of the innate immunity are polymorphonuclear phagocytes (neutrophils, basophils, and eosinophils), and mononuclear phagocytes (macrophages and dendritic cells) that are recruited to the site of inflammation by chemokines originating from the activated complement system, other inflammatory cells, or parenchymal or endothelial cells. Of the polymorphonuclear phagocytes, neutrophils are specialized in target destruction and opsonophagocytosis, whereas eosinophils and basophils are mainly involved in the production of inflammatory mediators in response to cytokines of the adaptive immune system (Beutler, 2004).

Innate immunity can be regarded as primitive and inherent in nature. Adaptive immunity, in turn, is acquired, requiring prior activation and modulation by the innate immunity system. Adaptive immunity cells comprise B-lymphocytes (B-cells) and T-lymphocytes (T-cells), to which the antigen is presented by macrophages, dendritic cells, and B-cells. B-cells develop into plasma cells that can produce specific antibodies. The function of T-cells depends on their phenotype: cytotoxic CD8-positive T-cells secrete mainly proinflammatory cytokines. The actions of CD4-positive T-cells at the site of inflammation depend on their differentiation into either proinflammatory effector T-cells (T_h1 or T_h17), humoral immunity-promoting T_h2 cells, or immunosuppressive regulatory T-cells (T_h3 or T_r1). This differentiation is largely determined by the local cytokine milieu. In addition to the cytokines present, the natural killer cells (NK cells), and the regulatory T-cells (T_{reg}), the adaptive immune response is facilitated by the complement system (Carroll, 2004b; Kemper and Atkinson, 2007). The covalent binding of complement opsonins to antigens reacting with B-cells leads to a more efficient antibody production. In addition, complement activation affects cytokine production by antigen-

presenting cells (APCs), further directing the T-cell response. As most IgGs are products of the adaptive immune system, complement activation through the classical pathway in response to the antigen-bound IgG can be considered an adaptive immune response. The interactions between innate and adaptive immune systems are outlined in Figure 5.

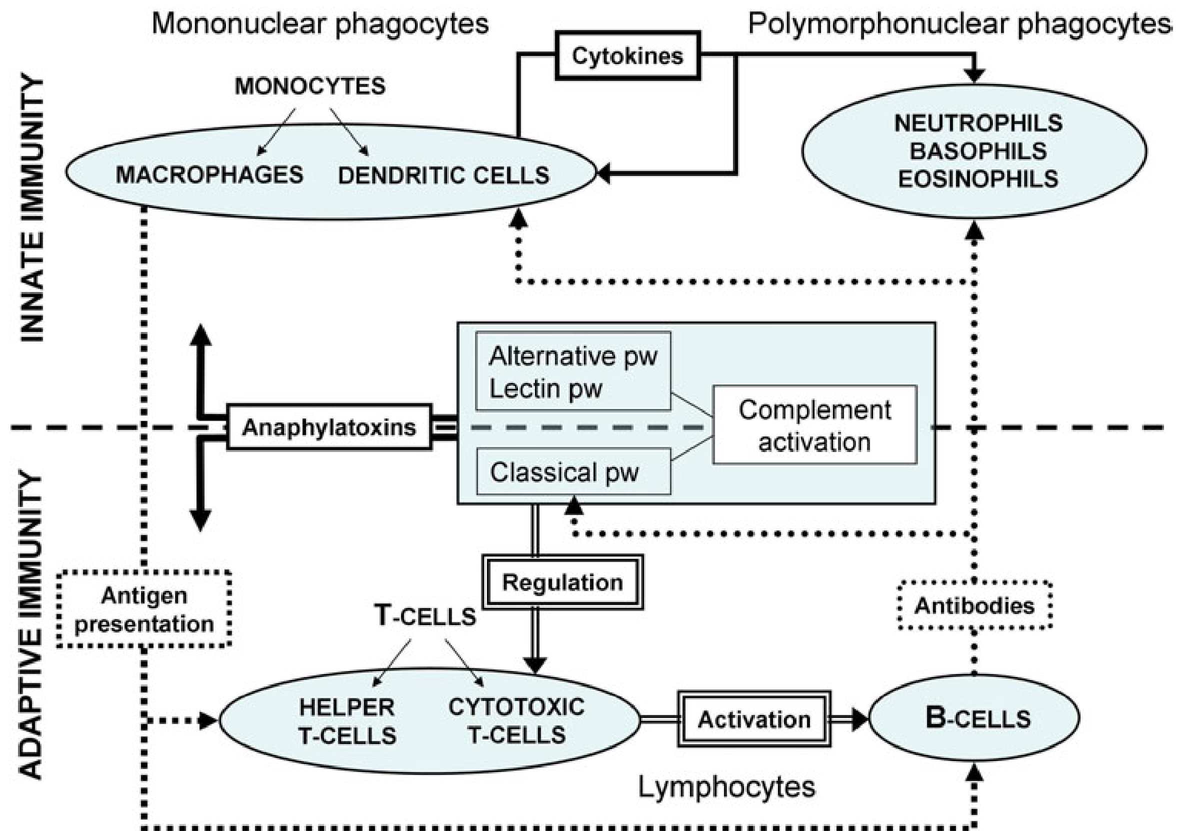

Figure 5. Interactions between innate and adaptive immunity. Pw stands for pathway.

2.2.1 Inflammatory cells

Many types of inflammatory cells are present in the IA wall, and some are associated with IA rupture. These include macrophages, T-cells, B-cells, plasma cells, NK cells, and PMNs. The nature of these cells is briefly described in the following sections.

Macrophages

Macrophages are inflammatory cells that differentiate from blood monocytes at the site of inflammation. They are a morphologically diverse group of cells expressing a varying repertoire of surface antigens and receptors. To recognize the PAMPs, macrophages express SCRs and TLRs (Chou *et al.*, 2008). The antigen-bound immunoglobulins are recognized through Fc receptors, and the complement activation products (opsonins and anaphylatoxins) through C1qR, CR1, CR2, CR3, C3aR, and C5aR (Morgan and Gasque, 1997). Activation of macrophage receptors can lead to the production of cytokines (e.g.

IFN-γ, TNF-α, IL-1β, IL-6, and IL-8) and growth factors (e.g. TGF-β) to recruit other inflammatory cells to the site of inflammation and to modulate the tissue's response to injury for healing purposes (Figure 3) (Osterud and Bjorklid, 2003). Thus, although macrophages are also capable of killing microbes in infections, their most important function is supervisory. Interestingly, the reactive oxygen and nitrogen species produced for killing purposes are also cytotoxic to viable cells and may modulate proteins and lipids to become proinflammatory (Chou *et al.*, 2008). Moreover, macrophages express major histocompability complex (MHC)-I and II molecules, through which they introduce phagocytosed and processed antigens to T-cells (Beutler, 2004). MHC-I- and II-expressing macrophages have been detected in IAs, some of them in close contact with T-cells (Kosierkiewicz *et al.*, 1994). In general, macrophage infiltration is associated with IA rupture (Kataoka *et al.*, 1999; Frösen *et al.*, 2004).

Dendritic cells

Dendritic cells are phagocytic cells that present antigens on MHC-II to activate T-cells (Beutler, 2004). They also express CD1d, needed for activation of NK cells (Bobryshev and Lord, 2002). Dendritic cells have been shown to be present in the periadventitial tissue of extracranial arteries (Millonig *et al.*, 2002). However, as dendritic cells share common receptors used for detecting macrophages in immunostainings, the presence of dendritic cells in IAs has remained uncertain.

T-lymphocytes

T-lymphocytes are classified into cytotoxic CD8-positive T-cells (T_c-cells) and CD4-positive helper T-cells that can be further classified as proinflammatory effector T-cells and immunosuppressive regulatory T-cells. The functions of T-cells depend on their phenotype. T_c-cells can secrete proinflammatory cytokines interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), and may also induce apoptosis by a Fas ligand-dependent interaction. The actions of effector and regulatory T-cells depend on their differentiation into the direction of T_h1, T_h17, T_h2, T_h3, T_r1, or T_{reg}. IL-12, IL-23, or IL-1β differentiates effector T-cells into either the T_h1 or T_h17 direction, whereas the regulatory T_h2-phenotype occurs in the presence of IL-4. Regulatory T_r1 differentiation requires IL-10 or IFN-α. The differentiation of regulatory T_h3 cells requires the presence of several cytokines (van Roon *et al.*, 2006; Romagnani *et al.*, 2009). The subtypes of effector and regulatory T-cells differ in their functions depending on the cytokines produced upon activation. The activation and polarization of an adaptive immune response and the main functions of different T-cell subtypes are summarized in Figure 6. T-cells are also responsive to complement activation, as they express several receptors (CR1, CR2, CR4, C3aR, CD46, CD55, and CD59) for complement components and anaphylatoxins that regulate T-cell activation, proliferation, and cytokine production (Morgan and Gasque,

1997; Kemper and Atkinson, 2007). Human IAs harbor T-cells, and T-cell infiltration in general is associated with IA rupture.

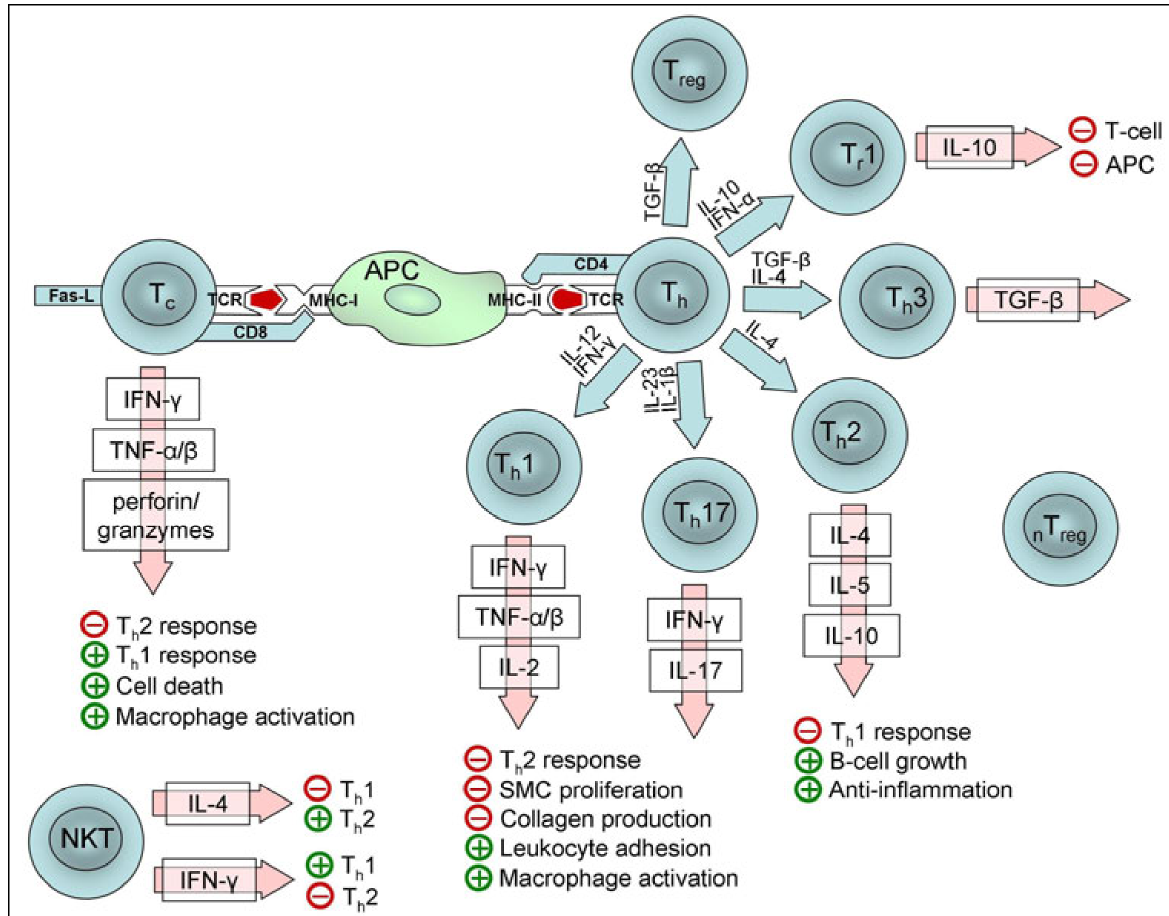

Figure 6. Potential T-cell differentiation and activation mechanisms and functions in intracranial aneurysms. An antigen-presenting cell (APC) presents antigens in a specific groove of its major histocompability complex I or II (MHC-I or MCH-II) molecules depending on the nature and origin of the antigen. T-cell recognizes antigen and MHC through T-cell receptor (TCR) and CD4 (helper T-cells, T_h) or CD8 (cytotoxic T-cells, T_c). Activated T-cells secrete many cytokines and inflammatory mediators (e.g. interferon gamma, IFN-γ; tumor necrosis factors alpha and beta, TNF-α and TNF-β; transforming growth factor beta, TGF-β; and interleukins, IL) that further guide the inflammation in the aneurysm wall. The polarization of T_h-cells into the effector (T_h1 or T_h17) or regulatory (T_h2, T_h3, T_r1, or T_{reg}) direction depends on the presence of distinct sets of interleukins. Natural killer T-lymphocytes (NKT) play an important role in this process, as they can guide the polarization by secreting either IFN-γ or IL-4. The regulatory T-cells (T_{reg}) and the naturally occurring regulatory T-cells ($_nT_{reg}$) function by a direct cell-cell interaction. Note that T-cell regulation is a complex process, and only the most important mediators are presented here. T-cell regulation is also a flexible system and this figure represents a simplification of the current view.

B-lymphocytes

B-cells are lymphocytes that mediate humoral immune response by differentiating into antibody-producing plasma cells or memory B-cells. They can also act as APCs. B-cells become activated through an antigen-receptor interaction independently or by

costimulation of T_h2 cells. In addition to the B-cell receptor (BCR), B-cells express receptors for complement components C3d and C3b (CD21 and CD35), which aid in antigen recognition and stimulate antibody production (Carroll, 2004b). After activation, the B-cell undergoes clonal expansion and eventually develops into a memory B-cell. The cytokines from T_h2 cells promote B-cell progression into plasma cells that secrete soluble antibodies (Manz *et al.*, 2006). Both B-cells and plasma cells have been detected in IA walls (Crompton, 1966; Chyatte *et al.*, 1999).

Natural killer cells

Natural killer cells differ from other lymphocytes in that they produce cytokines in response to antigens presented in the CD1 molecule. NK cells kill virus-infected cells and tumor cells, which lack or have low levels of MHC-I expression. The main cytokines produced by NK cells are IL-4 and IFN-γ, and thereby, NK cell can direct the T-cell response into either a T_h1 or T_h2 direction. NK cells have been detected in human IAs (Kosierkiewicz *et al.*, 1994).

Polymorphonuclear leukocytes

Polymorphonuclear leukocytes (PMNs) are also called granulocytes due to their cytoplasmic contents of granules containing degrading enzymes and substances that shape the inflammatory milieu. PMNs are divided into basophils, eosinophils, and neutrophils according to the morphology of the nucleus and the contents of the granules (Beutler, 2004; Kennedy and DeLeo, 2009). As eosinophils and basophils are mainly involved in parasitic infections, they will not be discussed in more detail here. Basophils may also function in directing the T_h2 immune response by IL-4, but this and other possible basophil-mediated mechanisms remain poorly understood (Beutler, 2004; Min, 2008).

Neutrophils are the most abundant polymorphonuclear leukocytes and especially important in first-line immune defense. They are primed and recruited to the site of inflammation and/or tissue injury by released inflammatory mediators and by, for example, activated complement. The extravasation (trans-endothelial migration) is also mechanically aided by endothelial expression of selectins. Neutrophils contain and secrete many cytotoxic molecules that can cause significant damage to host tissues. They also produce ROS and phagocytose the opsonized or PAMP-expressing particles through PRR-mediated recognition (Kennedy and DeLeo, 2009). In tissues and circulation, neutrophils are short-lived, undergo spontaneous death, and become phagocytosed by macrophages in order to maintain homeostasis of the immune system (Savill *et al.*, 1989; Beutler, 2004). Even in the presence of proinflammatory cytokines (e.g. IL-1β, TNF-α, IFN-γ), which prolong neutrophil survival, they remain viable for only up to two days (Kennedy and DeLeo, 2009). As neutrophils die, they may release matrix-degrading enzymes (i.e. cathepsin G, elastase, protease-3, MMP-9) and other granule proteins to the surroundings,

thus inadvertently promoting the inflammation. The granule proteins together with MCP-1 are chemoattractants to monocytes, T-cells, and dendritic cells (Soehnlein *et al.*, 2009).

2.3 Complement system

The complement system is a first-line defense mechanism with a central role in the innate immune response. Complement also regulates adaptive immunity by activation and stimulation of inflammatory cells. Complement participates in the removal of foreign substances and cell debris via opsonization and strongly enhances adaptive immune responses by, for instance, antigen delivery. Activation of the complement system leads to the production of anaphylatoxins C3a and C5a, which are chemoattractants for leukocytes and generate an inflammatory reaction.

Complement becomes activated in a cascade-like manner by three optional activation pathways – classical, alternative, and lectin – depending on the activating factor. A particular role of the ‘alternative pathway’ is to amplify complement activation in areas lacking sufficient regulatory activity (Meri and Pangburn, 1990a). Complement may become activated via the ‘classical pathway’ in response to endogenous ligands such as immunoglobulins (e.g. antigen-bound IgG or IgM), pentraxins (e.g. CRP), dying cells (apoptotic, ischemic, or necrotic), and ECM proteins. The ‘lectin pathway’ is activated by surfaces rich in acetylated oligosaccharides or mannan. The ‘alternative pathway’ becomes activated by a surface that lacks complement inhibitors, e.g. by bacteria and lipopolysaccharides. In addition, cholesterol-containing lipids and enzymatically or oxidatively modified low-density lipoproteins, which may accumulate in the walls of stressed blood vessels, e.g. in atherosclerosis, are capable of activating complement.

If not inhibited, complement activation leads to the formation of a C3-convertase, activation of the ‘terminal pathway’, and formation of terminal complement complexes (TCCs) or membrane attack complexes (MACs) (Figure 7). TCCs can be noncytolytic complexes where the binding of soluble inhibitors, S-protein (vitronectin), or clusterin has prevented the formation of a polymeric C9 pore (SC5b-9) or cytolytic MAC. MAC consists of the C5b-8 protein complex and a varying number of C9 proteins that polymerize and form lytic pores on cell membranes. Formation of MAC may induce cell death or activate cells by proinflammatory mechanisms, e.g. by increasing Ca^{2+} influx into cells. Because of the tendency to cause cell damage, complement activation is strongly regulated by soluble and membrane-bound proteins (Cole and Morgan, 2003; Bohana-Kashtan *et al.*, 2004; Sim and Tsiftsoglou, 2004; Sjöberg *et al.*, 2009). Complement activation has been shown to occur in various vascular inflammatory diseases, e.g. atherosclerosis and abdominal aortic aneurysms, and is associated with tissue degeneration and inflammatory cell infiltration (Capella *et al.*, 1996; Niculescu and Rus, 1999; Oksjoki *et al.*, 2003b; Kostner, 2004; Szeplaki *et al.*, 2009). Apparently, the complement system is

involved in the pathogenesis of these vascular diseases, but the reasons for complement activation and its consequences are poorly understood.

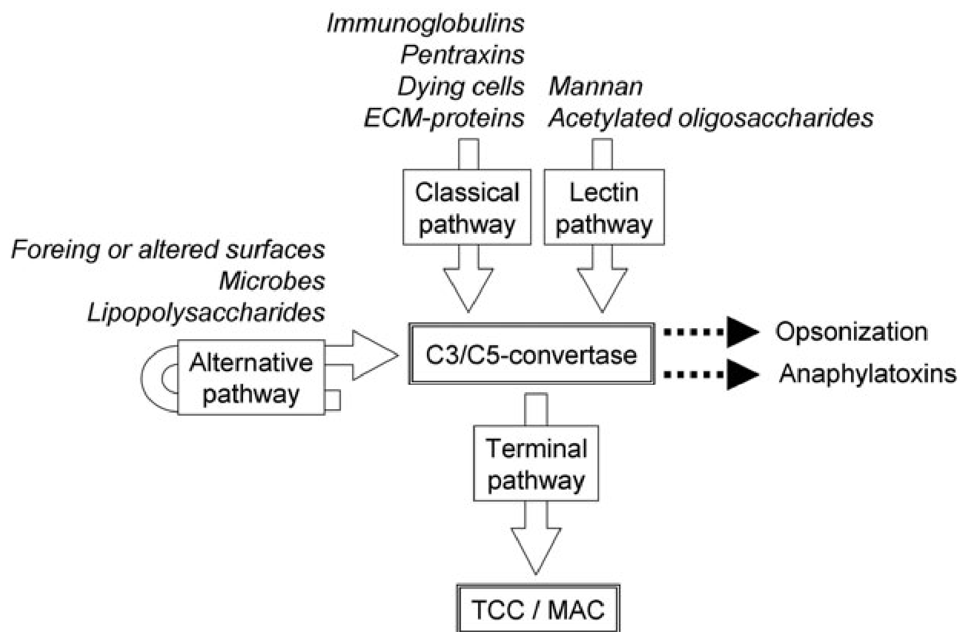

Figure 7. Simplified presentation of complement activation. The complement activation by either classical or lectin pathway, and activation or amplification by alternative pathway leads to formation of C3/C5 convertases, which activate the terminal pathway leading to the formation of the terminal complement complex (TCC) or the membrane attack complex (MAC). Opsonins and anaphylatoxins form as split products of the activated complement components C3, C4, and C5.

2.3.1 Complement activation

The complement system consists of approximately 35 proteins, which become activated in a cascade-like manner through sequential proteolytic cleavages and formation of protein complexes. The classical pathway is activated by C1q-mediated recognition of the target, which may be a tissue structure or another pattern recognition receptor, e.g. immunoglobulin or CRP. After binding to a target, C1q changes its conformation, triggering self-activation of C1r, which in turn activates C1s, responsible for the cleavage of C2 and C4 (Arlaud *et al.*, 2002). The cleavage of C4 causes C4b and its cleavage product iC4b to accumulate in tissues. C4b binds to C2 to generate C4b2a, the C3 convertase enzyme of the classical pathway. The same enzyme acts as a C5 convertase. The C2a component of the convertase is responsible for the serine protease activity in cleavage of C3 and C5. Activation of the lectin pathway is initiated analogously by activation of mannan binding lectin (MBL) or ficolins, followed by activation of the MBL-associated serine protease MASP-2 (and possibly MASP-1), leading to cleavage of C2 and C4.

The alternative pathway undergoes constant turnover in the fluid phase. The spontaneous hydrolysis of C3 generates C3(H_2O), which then forms the C3(H_2O)Bb complex with the cleavage fragment of factor B. The C3(H_2O)Bb complex has C3 convertase activity on C3, and new C3bBb enzymes cleave further C3 molecules. This spontaneous "tickover" enables fast alternative pathway amplification by (bacterial) lipopolysaccharides or by a self-surface lacking sufficient regulatory activity (Meri and Pangburn, 1990a). Moreover, the C3/C5 convertases formed by activation of the classical or lectin pathways are responsible for initiating amplification of the alternative pathway by cleaving C3. After cleavage of C3, the additional binding of C3b to the C3bBb complex generates C5 convertase activity. Bb in the C3Bb complex originates from a cleavage of factor B by factor D and is responsible for the serine protease activity of the complex. The alternative pathway activation or spontaneous amplification via C3/C5 convertases results in the deposition of surface-bound cleavage products C3b/iC3b and C3d. By being the end-product of C3b cleavage, C3d remains covalently bound to tissues, indicating prolonged complement activation (Davis *et al.*, 1984; Nakagawa *et al.*, 2000).

The C3/C5 convertases activate the terminal pathway by cleaving C5 to C5a and C5b. C5b forms a complex with C6, C7, C8, and up to 12 C9 components. Upon formation of the C5b-9 complex, the binding of the latter component is enabled by a conformational change in the former. The forming C5b-9 complex may remain soluble by binding to vitronectin (S-protein) or clusterin (ApoJ). Otherwise, the C5b-9 complex is inserted into the cell membrane as a membrane attack complex (MAC), a lytic transmembranous pore. When MAC formation on a target membrane is massive and uncontrolled, the target cells lyse and die. However, sublytic concentrations of MAC may still affect the cell fate by inducing apoptosis or proliferation (Sim and Tsiftsoglou, 2004; Sjöberg *et al.*, 2009). Complement activation and actions of the regulators are described in detail in Figure 8.

Opsonins

Binding of complement components to the surfaces of self-particles, immune complexes, or pathogens opsonizes the target for phagocytic cells. The opsonins of complement origin are recognized by several membrane-bound complement receptors (CRs) (Sim and Tsiftsoglou, 2004; He *et al.*, 2008; Sjöberg *et al.*, 2009). In addition to complement split products, also immunoglobulins act as opsonins through recognition of macrophage Fc receptors. The traditional complement opsonins are covalently bound C3b and C4b as well as their inactivated forms iC3b and iC4b, cleaved from C3b and C4b by factor I. Some of the complement receptors, such as CR1 (CD35), also recognize the surface-bound C1q or MBL. C3b and C4b are recognized by CR1, which has the main function of capturing immune complexes to erythrocytes for transport to the spleen (Cornacoff *et al.*, 1983). CR2 (CD21), which recognizes the C3dg fragment, lowers the threshold of B-cell

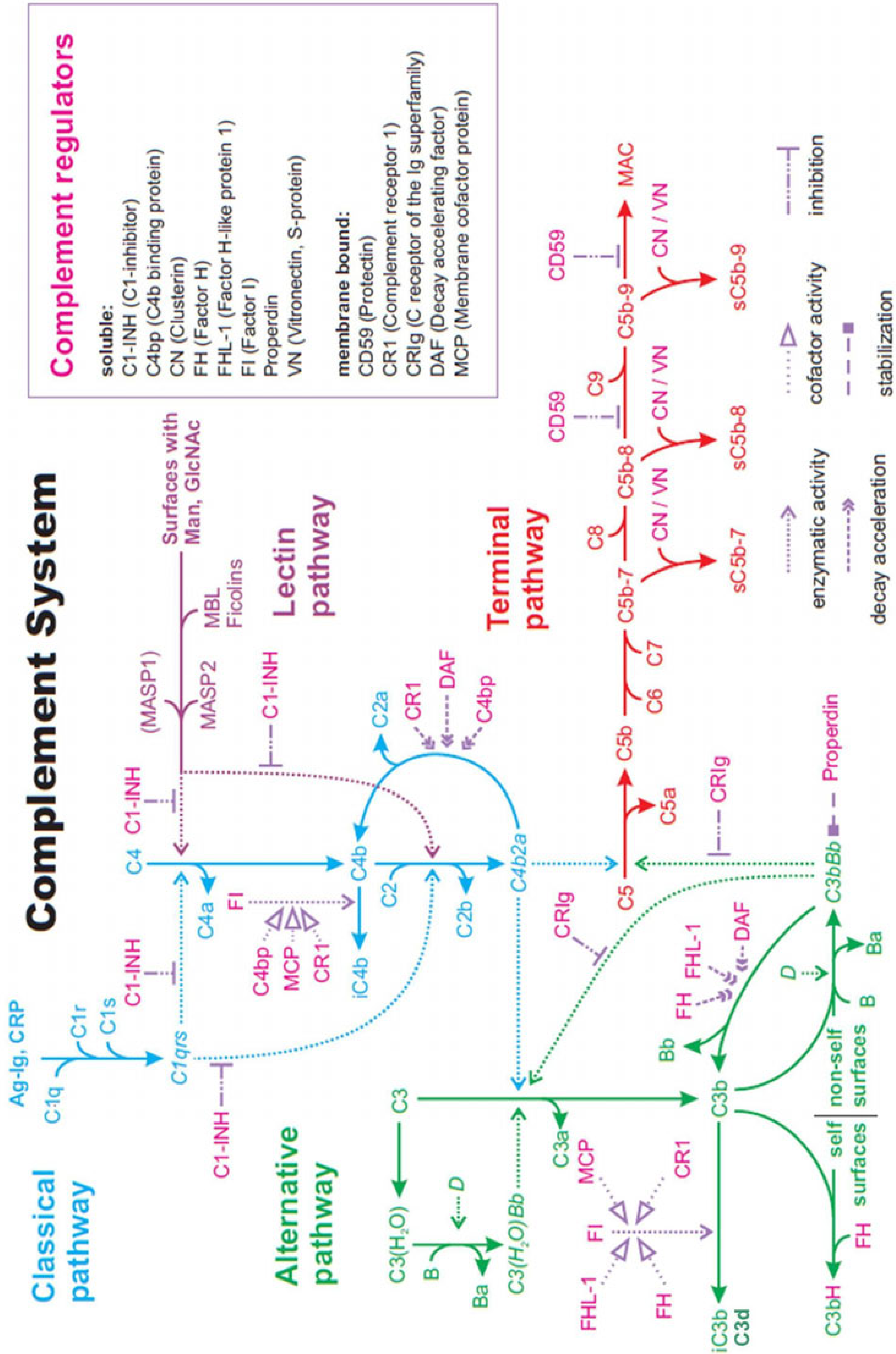

Figure 8. Activation pathways of the complement system. The figure was created by Matti Laine.

activation, thereby enhancing B-cell immunity and survival (Matsumoto *et al.*, 1991; Carter and Fearon, 1992; Carroll, 2004a). CR3 and CR4 are transmembranous heterodimers composed of an alpha subunit (CD11b or CD11c, respectively) and a common beta chain (CD18). In addition to orchestrating phagocytosis of iC3b-opsonized particles, they also have functions in leukocyte trafficking and migration (reviewed by He *et al.*, 2008). CRIg on tissue macrophages binds C3b, iC3b, and C3c (Wiesmann *et al.*, 2006) for the ingestion of complement-opsonized particles (Helmy *et al.*, 2006). As complement receptors may activate intracellular signaling pathways during phagocytosis (Rutherford and Schenkein, 1983; Couturier *et al.*, 1990; Thieblemont *et al.*, 1995), the complement opsonins may have a more versatile role in inflammation than earlier recognized.

Anaphylatoxins

During complement activation C3/C5 convertase cuts off small fragments from C3, C4, and C5, producing C3b, C4b, and C5b. The small fragments, C3a, C4a, and C5a, are collectively called anaphylatoxins. In addition to the action of the C3/C5 convertases, anaphylatoxins may be generated also by the serine protease activity of coagulation factors FXa and FXIa or thrombin as well as by plasmin, a serine protease of the fibrinolytic system (Huber-Lang *et al.*, 2006; Amara *et al.*, 2008). Interestingly, also phagocytic cells are capable of generating biologically active C5a (Huber-Lang *et al.*, 2002). Anaphylatoxins are chemotactic to phagocytes, cause vasodilatation probably by an endothelial NOS-dependent mechanism (Park *et al.*, 1999; Kurizaki *et al.*, 2004), increase the permeability of small blood vessels, and induce contraction of smooth muscle cells (reviewed by Klos *et al.*, 2009). The effects of C3a and C5a are mediated by binding to specific C3a, C5a, and C5L2 (C5a receptor-like 2) receptors. A functional receptor for C4a has not been recognized. On inflammatory cells, the anaphylatoxins C3a and C5a trigger an oxidative burst, histamine release from basophils and mast cells, and adhesion and migration of eosinophils, and also modulate synthesis of IL-6 and TNF-α from B-cells and monocytes (Klos *et al.*, 2009). Anaphylatoxins are able to induce the endothelial expression of cell adhesion molecules VCAM-1, intercellular adhesion molecule (ICAM-1), and selectins (Foreman *et al.*, 1996; Albrecht *et al.*, 2004; DiScipio and Schraufstatter, 2007). C5a may activate the exogenous coagulation pathway by inducing tissue factor (TF) activity in endothelial cells (Ikeda *et al.*, 1997; Amara *et al.*, 2008). Anaphylatoxins also regulate tissue degeneration and fibrosis (Klos *et al.*, 2009). To some extent, anaphylatoxins may also act in an autocrine manner, as the APCs within allograft-induced inflammation as well as T-cells synthesize and cleave C3 to C3a (reviewed by Klos *et al.*, 2009). Both C3a and C5a receptors have been detected in smooth muscle cells, T-cells, macrophages, and endothelial cells within atherosclerotic lesions (Oksjoki *et al.*, 2007).

2.3.2 Regulation of complement activation

Complement C3 convertase enzymes have catalytic activity, but are intrinsically unstable ($T_{½}$ 1-3 min). Due to the potentially harmful effects of complement activation on host tissues, the activation is regulated by several soluble (C1-INH, C4bp, fH, FHL-1, clusterin, and vitronectin) and membrane-bound (CR1, MCP, DAF, and protectin) regulators.

C1 inhibitor

C1 esterase inhibitor (C1-inhibitor; C1-INH) is a heavily glycosylated single-chain polypeptide of 105 kDa, with a plasma concentration of 240 μg/ml (Schapira *et al.*, 1985; Nuijens *et al.*, 1989). Cells including fibroblasts, monocytes, macrophages, and endothelial cells as well as hepatocytes are responsible for synthesis of C1 inhibitor (reviewed by Caliezi *et al.*, 2000). Being an acute phase protein, its synthesis is induced by several proinflammatory cytokines such as IFN-γ, TNF-α, IFN-α, monocyte colony-stimulating factor, and IL-6. However, C1 inhibitor can be inactivated by elastase and proteinase 3 as well as by thrombin and plasmin, e.g. in an inflammatory process. C1 inhibitor is a naturally occurring serine protease inhibitor of C1r and C1s and also controls MASP-1 and MASP-2 within the lectin pathway. C1 inhibitor inhibits C1r and C1s either by binding them and inhibiting their autoactivation or by dissociating them from C1q in the C1 complex (Sim *et al.*, 1979; Sim *et al.*, 1980; Liszewski *et al.*, 1996). In addition to the complement system, C1 inhibitor also inhibits the contact system (FXIIa and kallikrein), intrinsic activation of the coagulation system (FXIa), and fibrinolysis (plasmin). The presence of glycosaminoglycans enhances complement inhibition by C1 inhibitor (reviewed by Caliezi *et al.*, 2000).

C4b binding protein and protein S

C4b binding protein (C4bp) is a large glycoprotein (570 kDa) synthesized mainly by the liver, although synthesis by, for instance, monocytes can occur under certain conditions (Dahlback and Muller-Eberhard, 1984; Lappin and Whaley, 1990). Together with factor H, C4bp is the main soluble complement inhibitor, found in plasma at a concentration of 200 μg/ml (Dahlback, 1983). However, C4bp is an acute phase protein, whose levels in blood may increase up to 400% during an inflammatory response (Rezende *et al.*, 2004).

The main function of C4bp is to inhibit classical pathway activation by preventing formation of the C3 convertase C4bC2a (Scharfstein *et al.*, 1978). To some extent, C4bp also inhibits the alternative pathway, acting as a factor I cofactor in C3b cleavage (Seya *et al.*, 1995). The majority of C4bp is composed of seven α-chains and a single β-chain that is responsible for binding of the anticoagulant protein S (Hillarp and Dahlback, 1988). In the α-chains, C4bp has a binding site for heparin (Hessing *et al.*, 1990). Protein S is a 75-

kDa glycoprotein synthesized mainly by hepatocytes, but also by endothelium and vascular smooth muscle cells (DiScipio and Davie, 1979; Fair and Marlar, 1986; Dahlback, 1991). The C4bp-unbound fraction of protein S (40%) acts as a cofactor for the activated anticoagulant protein C (Rezende *et al.*, 2004).

In human tissue homeostasis, C4bp has an important role in limiting complement activation on apoptotic cells to prevent the formation of anaphylatoxins and MAC (Blom *et al.*, 2004; Rezende *et al.*, 2004). Whereas apoptotic cells are capable of binding several complement components to potentially induce complement activation (Nauta *et al.*, 2002), C4bp binds via protein S to negatively charged phosphatidylserine to inhibit further complement activation. C4bp is also able to bind CD40 and induce cell proliferation and upregulation of ICAM-1 in B-cells (Brodeur *et al.*, 2003). However, in addition to B-cells, CD40 is also expressed on monocytes, dendritic cells, and endothelial cells (Blom *et al.*, 2004). In atherosclerotic plaques, C4bp is present in the proteoglycan-rich superficial intima, where it probably binds to arterial proteoglycans (Oksjoki *et al.*, 2007). In some specimens, C4bp is only seen deeper in the intima and in the necrotic cores of advanced lesions. A fraction of C4bp is associated with protein S and caspase-3 -positive apoptotic cells.

Factor H and FHL-1

Together with C4bp, factor H (fH) is the most important soluble complement inhibitor. Its main function is to inhibit the alternative pathway. Factor H is a single polypeptide chain glycoprotein (155 kDa) found in plasma in varying concentrations (110-615 μg/ml) (Rodriguez de Cordoba *et al.*, 2004). In addition to factor H, there are also at least six structurally related proteins such as factor H-like protein 1 (FHL-1), an alternative transcript of the factor H-coding gene *cfh* (Estaller *et al.*, 1991; Rodriguez de Cordoba *et al.*, 2004).

Factor H inhibits the alternative pathway by accelerating the decay of the alternative pathway C3 convertase (C3bBb) and acting as a cofactor for factor I to inactivate C3b. Factor H is able to act both in solution and on surface-bound C3b. In addition to several binding sites for C3b and iC3b, factor H also binds to sialic acids, glycosaminoglycans, and sulfated polysaccharides such as heparin (Rodriguez de Cordoba *et al.*, 2004). By binding to CRP, factor H may limit alternative pathway amplification on otherwise proinflammatory surfaces (Jarva *et al.*, 1999). Factor H and FHL-1 are mainly synthesized in the liver, although extrahepatic synthesis also occurs by, for example, lymphocytes, fibroblasts, and endothelial cells (Friese *et al.*, 1999). The proinflammatory cytokine IFN-γ seems to upregulate the synthesis of factor H and FHL-1 (Ripoche *et al.*, 1988; Rodriguez de Cordoba *et al.*, 2004). In atherosclerotic arteries, factor H is present in

the superficial layer of the intima, colocalizing with CRP and arterial proteoglycans, and restricting terminal pathway complement activation in that area (Oksjoki *et al.*, 2003a).

Several known mutations exist in the factor H gene. These are associated with, for instance, type II membranoproliferative glomerulonephritis or aHUS (atypical hemolytic uremic syndrome), where a deficient protection of host cells exposes them to complement activation (Rodriguez de Cordoba *et al.*, 2004). Factor H single-nucleotide polymorphism Y402H was found in 2005 to be associated with age-related macular degeneration (AMD) (Edwards *et al.*, 2005; Haines *et al.*, 2005; Klein *et al.*, 2005). AMD is featured by impaired clearance of debris and accumulation of drusen in the subretinal space.

Properdin

Properdin is synthesized by monocytes/macrophages, T-cells, neutrophils, and mast cells (Schwaeble *et al.*, 1993; Schwaeble *et al.*, 1994; Wirthmueller *et al.*, 1997; Stover *et al.*, 2008) at the site of inflammation upon stimulation (Wirthmueller *et al.*, 1997). It is found only in small concentrations (4-6 μg/ml) in plasma (Nolan and Reid, 1993), where it enhances activation of the alternative complement pathway. Properdin is a highly positively charged (pI>9.5) protein (Fearon and Austen, 1975) and it binds heparin, a major glycosaminoglycan (Yu *et al.*, 2005). Properdin is secreted by leukocytes and endothelial cells and is capable of leukocyte activation (Bongrazio *et al.*, 2003; Ruef *et al.*, 2008). Properdin has traditionally been thought to stabilize only the C3bBb complex attached to nonself surfaces (e.g. bacterial capsules) for more efficient alternative pathway activation (Fearon and Austen, 1975). However, recent studies of early apoptotic T-cells and late apoptotic Jurkat cells suggest that properdin itself is capable of danger target recognition and launching subsequent alternative pathway activation (Kemper *et al.*, 2008; Xu *et al.*, 2008). In the case of apoptotic T-cells, the sulfated glycosaminoglycans are a prerequisite for properdin interaction. In atherosclerotic coronary arteries, properdin is associated with the C5b-9 complex, and thus, indicates complement activation through the alternative pathway (Oksjoki *et al.*, 2007).

S-protein/Vitronectin

Vitronectin or S-protein is a glycoprotein (75 kDa) synthesized mainly by the liver and present in plasma and serum at concentrations of 200-400 μg/ml (Podack and Muller-Eberhard, 1979; Barnes and Silnutzer, 1983; Ekmekci and Ekmekci, 2006). It is also present in several tissues as a component of loose ECM and on the cell surface of, for instance, fibroblasts (Hayman *et al.*, 1983; Dahlback *et al.*, 1986). It binds to the C5b-7 complex, preventing its insertion into the cell membrane. The SC5b-7 complex remains hydrophilic and is thus soluble (Bhakdi and Roth, 1981). Vitronectin also prevents the polymerization of C9, inhibiting the formation of the tubular structure of MAC (Podack *et al.*, 1984). In addition, vitronectin binds to plasminogen activator inhibitor-1 (PAI-1),

thrombin-antithrombin III complexes, heparin, and probably also collagen type I. It has been suggested to have functions in cell adhesion, migration, vascular remodeling, thrombosis, hemostasis, fibrinolysis, and immune defense (reviewed by Ekmekci and Ekmekci, 2006). In human atherosclerotic arteries, vitronectin has been found to partially colocalize with C5b-9, especially in intimal fatty streaks, intimal thickenings, and fibrous plaques as well as in the ECM and cell debris (Niculescu *et al.*, 1987). Vitronectin was associated with C5b-9, particularly in cell debris embedded in elastin, whereas cell debris embedded in collagen was positive only for C5b-9 (Niculescu *et al.*, 1989). Recently, vitronectin was observed to be one of the seven differently expressed proteins in unruptured and ruptured abdominal aortic aneurysms (AAAs) (Urbonavicius *et al.*, 2009). Its downregulation was associated with AAA rupture.

Protectin

Protectin or CD59 is a small (~20 kDa) glycophosphatidylinositol (GPI)-anchored glycoprotein that is widely expressed on cell membranes of almost all circulating cells and tissues in the body (Meri *et al.*, 1991). It belongs to the leukocyte antigen 6 (Ly-6) protein superfamily (Davies *et al.*, 1989). Protectin inhibits MAC formation by preventing the binding of C9 to the C5b-8 complex (Rollins and Sims, 1990; Meri *et al.*, 1991; Farkas *et al.*, 2002). In addition to complement inhibition, protectin contributes to T-cell signaling and autoregulation (Longhi *et al.*, 2006; Kimberley *et al.*, 2007) as well as to B-cell signaling (Kimberley *et al.*, 2007). In human keratinocytes, protectin acts as an LPS receptor, instead of CD14, leading to NFκB–mediated production of granulocyte-macrophage colony-stimulating factor, IL-6, and TNF-α (Yamamoto *et al.*, 2003).

Protectin is essential for viable cells to survive complement lysis, as patients with paroxysmal nocturnal hemoglobinuria (PNM) suffer from hemolytic anemia due to an acquired lack of GPI-anchored proteins or inherited deficiency of protectin on erythrocyte cell membranes (Yamashina *et al.*, 1990; Parker, 1991; Motoyama *et al.*, 1992). Patients with PNM have a small tendency to develop vascular thrombosis. In atherosclerotic arteries, protectin has been seen on macrophages, T-cells, endothelial cells, and SMCs, partially overlapping with the C5b-9 complex (Seifert *et al.*, 1992; Yasojima *et al.*, 2001a). Extensive glycosylation may inactivate protectin and is a mechanism postulated to be responsible for diabetic vascular complications (Acosta *et al.*, 2000; Qin *et al.*, 2004).

CR1

Complement receptor 1 (CR1), or CD35, is a membranous receptor for C1q (Klickstein *et al.*, 1997), MBL (Ghiran *et al.*, 2000), C3b, and C4b (Klickstein *et al.*, 1988). It has cofactor activity for factor I in cleaving C3b and C4b (Ross *et al.*, 1982). By binding to C3b/C4b, CR1 destabilizes and enhances the decay of C3/C5 convertases, thereby inhibiting complement activation (Khera and Das, 2009). CR1 is expressed on the plasma

membrane of several cell types, including erythrocytes, monocytes, macrophages, B-lymphocytes, a subpopulation of CD4+ T-cells, and dendritic cells (Khera and Das, 2009). CR1 also circulates in plasma in a soluble form (sCR1) generated by proteolytic cleavage of the leukocyte surface-bound CR1 (Hamer *et al.*, 1998). Being expressed on all blood cells, CR1 aids in the transport of C3b/C4b-coated immune complexes to the liver and spleen.

Decay-accelerating factor

Decay-accelerating factor (DAF), or CD55, is a 75-kDa GPI-anchored protein expressed on the cell membranes of most plasma-exposed cells (Nicholson-Weller *et al.*, 1985). Many bacteria and viruses use DAF as a receptor for adhesion and often also for invasion (reviewed by Lea, 2002). DAF inhibits complement activation by dissociating the catalytic subunits C2a and Bb of the classical pathway and alternative pathway target enzymes C4b2a and C3bBb, respectively (Brodbeck *et al.*, 2000). In malignancies, DAF increases the invasiveness by protecting the tumor from complement and apoptosis as well as by inducing neoangiogenesis (Mikesch *et al.*, 2006). DAF is also expressed in atherosclerotic lesions. The expression is mainly extracellular, although a variable proportion of cells (20-60%) also express DAF (Seifert and Hansson, 1989a). DAF is the most inducible of the complement membrane regulators on endothelial cells (Meri *et al.*, 1993).

Membrane cofactor protein

Membrane cofactor protein (MCP), or CD46, is a 60- to 65-kDa complement inhibitor expressed widely on cells throughout the body, except erythrocytes (Liszewski *et al.*, 1991). MCP is associated with the plasma membrane via its C-terminal transmembrane domains. In addition to the membrane-bound form, MCP can be released from cancer cells with an MMP as a soluble form and with an analogous complement inhibitory capacity as the membrane-bound form (Hakulinen *et al.*, 2004). MCP binds to C3b and C4b deposited on the cell surface and acts as a cofactor for plasma serine protease factor I to promote the generation of iC3b and iC4b (Liszewski *et al.*, 1991). MCP may transduce cellular signaling, as MCP can downregulate IL-12 secretion on monocytes and affect T-cell cytotoxicity, proliferation of helper T-cells, and production of IL-2 and IL-10 (Karp *et al.*, 1996; Marie *et al.*, 2002).

2.3.3 Role of complement in clearance of cellular debris

Growing evidence points to the role of complement in the clearance of cellular debris. Apoptotic cells are highly immunogenic and proinflammatory if not promptly cleared (Chang *et al.*, 2004). This is due to mitochondrial disruption, leading to oxidation of various epitopes expressed on apoptotic blebs (Chang *et al.*, 1999; Huber *et al.*, 2002).

During early apoptosis negatively charged phosphatidylserine is transferred to the outer cell membrane leaflet. It is thought to be responsible for binding of complement components and natural IgM antibodies. Apoptotic cells also downregulate complement inhibitors (Attali *et al.*, 2004), exposing the cells to complement activation and complement-mediated necrosis. Apoptotic cells bind several complement components, most importantly C1q (Nauta *et al.*, 2002). In addition, other complement activators (e.g. pentraxins CRP and PTX-3, IgM, and IgG), MBL, and ficolins bind to apoptotic cells and activate complement to enhance phagocytosis of apoptotic bodies. The alternative pathway also plays a role in priming apoptotic cells for phagocytes, as bound Bb is seen especially prominently on apoptotic/necrotic cells (reviewed by Trouw *et al.*, 2008). However, in addition to complement components, complement inhibitors also bind to apoptotic cells; C4bp binds to the blebs of apoptotic cells via protein S to prevent complement activation and formation of anaphylatoxins and MAC (Blom *et al.*, 2004). The binding of both complement activators and inhibitors by apoptotic cells is believed to orchestrate organized removal of apoptotic cells with a minimum inflammatory response (Trouw *et al.*, 2008). The binding of complement components to other tissue structures may serve a similar function.

2.3.4 Role of terminal complement activation in cell fate

If not inhibited, the terminal complement pathway leads to the assembly of MACs on target cell membranes. When assembled in massive amounts, the transmembrane pores of MACs result in the free passage of solutes and water through the membrane. This leads to lysis of the target and to a proinflammatory, necrotic cell death (Bhakdi and Tranum-Jensen, 1991). Necrotic cells also rapidly lose their membrane inhibitors DAF, MCP, and protectin, promoting further complement activation (Trouw *et al.*, 2008). However, if the amounts of MAC remain sublytic, the cells may escape lysis by internalization of the MACs by endocytosis and membrane shedding (Carney *et al.*, 1985; Scolding *et al.*, 1989). However, sublytic amounts of MAC have been noted to increase Ca2+ influx, activate phospholipases and protein kinase C, generate arachidonic acid-derived inflammatory mediators, induce proto-oncogenes, activate the cell cycle, and inhibit apoptosis (reviewed in Rus *et al.*, 2001). In human aortic SMCs, sublytic MAC promotes the cell cycle, resulting in cell division by the activation of ERK-1 (extracellular signal-regulated kinase 1) of the MAPK pathway (Niculescu *et al.*, 1999). This will enhance cell survival and induce resistance to a subsequent MAC attack. Importantly, in addition to cell protection, sublytic MAC is also capable of inducing apoptosis through a caspase-3-dependent pathway (Nauta *et al.*, 2002). As MAC has both proapoptotic and antiapoptotic effects, the final cell fate is probably affected by the extracellular environment, e.g. by cytokines.

2.3.5 Complement system and coagulation

In large tissue traumas, activation of both the complement and coagulation cascades is evident. Moreover, the complement C1 esterase inhibitor (C1-INH) has long been known to inhibit the coagulation cascade and kinin enzymes (FXIIa and kallikrein). However, recent studies have revealed more versatile interactions of the complement and coagulation cascades (Amara *et al.*, 2008). In addition to the cofactor activity of C4bp-free protein S on activated protein C in the anticoagulant pathway (Rezende *et al.*, 2004), C5a may activate the exogenous coagulation pathway by inducing tissue factor (TF) activity in endothelial cells (Ikeda *et al.*, 1997; Amara *et al.*, 2008). Coagulation factors may also be responsible for complement activation and especially for the generation of anaphylatoxins, particularly since thrombin is capable of acting as a potent C5 convertase (Huber-Lang *et al.*, 2006). The coagulation factors FXa and FXIa, generated during activation of the intrinsic coagulation cascade, are also capable of cleaving C3 and C5 (Amara *et al.*, 2008). Finally, plasmin, the strongest serine protease of the fibrinolytic system, is capable of cleaving both C3 and C5 (Amara *et al.*, 2008).

2.4 Complement in the vascular wall

Complement activation has been studied in only selected arterial diseases and in some disease states that also have an effect on arteries. As IAs are dilatations of middle-sized, musculoelastic arteries, a special emphasis is placed on reviewing complement activation in atherosclerotic arterial remodeling and aneurysms as well as on the role of complement in arterial injury caused by ischemia-reperfusion (I/R).

2.4.1 Complement in normal arteries

Normal arterial wall is protected from complement by membrane-bound inhibitors MCP, DAF, and protectin, which are ubiquitously expressed on most nucleated cells (Seifert and Hansson, 1989a; Liszewski *et al.*, 1991; Meri *et al.*, 1991; Seifert *et al.*, 1992). C4bp (Oksjoki *et al.*, 2007) and factor H have been detected diffusely in the proteoglycan-rich areas of the intima and media of arterial walls (Oksjoki *et al.*, 2003a). No studies of the presence of complement regulators in normal cerebral arteries are available. Of the complement components, C3 has been detected occasionally in normal basilar arteries. C9 has been absent or occasionally present as a diffuse staining pattern in normal basilar arteries (Chyatte *et al.*, 1999). In normal coronary arteries, no staining for C3d, or C5b-9 has been observed (Oksjoki *et al.*, 2003a). Of potential complement activators, IgG has not been detected in normal basilar arteries, and IgM has been detected only occasionally as a diffuse staining pattern in normal basilar arteries (Chyatte *et al.*, 1999). In coronary

arteries, CRP was seen only in the superficial intima together with factor H (Oksjoki *et al.*, 2003a).

Although the majority of the complement components are produced in the liver, there is also some extrahepatic, local production of complement components, especially during inflammation (Laufer *et al.*, 2001). The local production of complement components is regulated by several cytokines, e.g. IL-1, IL-2, IL-6, IL-13, IL-17, TNF-α, and IFN-γ, and growth factors PDGF, EGF, FGF, and TGF-β1 (reviewed in Laufer *et al.*, 2001). Endothelial cells are capable of synthesizing C3, C5-9, and factor H, and fibroblasts produce C2, C3, and C5-9 (Andrews *et al.*, 1995). Smooth muscle cells synthesize at least C3 and C4 (Ueda *et al.*, 1996; Yasojima *et al.*, 2001b). In addition, platelets and inflammatory cells, monocytes, macrophages, T-cells, and polymorphonuclear leukocytes are capable of producing complement components (C1q, r, s, C2, C4, C3), regulators (C1-INH, C4bp, fH, factor I, properdin, factor B, factor D), and several receptors for complement components (C1qR, CR1, CR2, CR3, CR4, C3aR, C5aR) (Andrews *et al.*, 1995; Morgan and Gasque, 1997).

2.4.2 Complement in vascular diseases

Aneurysms

Two recent microarray analyses have shown a differential expression of complement-related genes in IAs compared with control arterial tissue (Krischek *et al.*, 2008; Shi *et al.*, 2009). In immunostainings, Chyatte *et al.* (1999) found complement components C3 and C9 in mostly unruptured IAs and suggested that the complement system is activated in IAs. They also detected IA-associated IgG and IgM, which are known to activate complement. Earlier, Ryba *et al.* (1992) had reported accumulation of IgM and/or C3 on the luminal surface of IAs after aSAH. In AAAs, the amounts of IgG subtypes, measured from tissue extraction, are enormously increased: IgG1 (193-fold), IgG2 (160-fold), IgG3 (389-fold), and IgG4 (627-fold) compared with a normal aortic wall. The presence of several C3 degradation products has also been noted in AAAs (Capella *et al.*, 1996).

Vasospasm

Delayed vasospasm following aSAH is manifested by smooth muscle cell contraction, which is probably secondary to an inflammatory reaction (Pluta *et al.*, 2009). Plasma C3d and C4 levels of aSAH patients increase at the time of vasospasm, but remain normal in aSAH patients without vasospasm (Ostergaard *et al.*, 1987a; Kawano and Yonekawa, 1990). Complement activation may have a role in the induction of a delayed vasospasm following aSAH. In experimental models, artificial particles alone (latex and dextran) can activate complement (Carreno *et al.*, 1988; Luck *et al.*, 1999) and induce vasospasm

(Peterson *et al.*, 1990; Yanamoto *et al.*, 1994). The complement system has an inherent activity to recognize foreign surfaces and become activated. The complement activation products, anaphylatoxins (C3a, C4a, C5a), are chemotactic to inflammatory cells. They are also able to induce the endothelial expression of cell adhesion molecules VCAM-1, ICAM-1, and selectins (Foreman *et al.*, 1996; Albrecht *et al.*, 2004; DiScipio and Schraufstatter, 2007). The complement system has been found to become activated in the cerebrospinal fluid after SAH. Levels of the terminal complement complex are highest during the first two days after SAH, decreasing to the level of plasma concentrations in 7-10 days (Lindsberg *et al.*, 1996). Complement depletion inhibits vasospasm in experimental models of cerebral vasospasm (German *et al.*, 1996).

Atherosclerosis

In extracranial arteries, atherosclerotic lesions develop as progressive thickening of the intima and lipid accumulation within the plaque. The IEL remains intact and probably protects the media from the pathologic changes. Complement activation has been detected in atherosclerotic arteries, and it is widely accepted as a potential pathogenetic mechanism in the progression of atherosclerosis. Complement activation has even been suggested as an initiating event of the atherosclerotic changes because C5b-9 has been found to accumulate in the intima with lipids and to precede inflammatory cell infiltrations and other atherosclerotic changes in an experimental model in rabbits (Seifert *et al.*, 1989). Both early complement components C1q, iC3b, C3d, C4, and C4c and components of the terminal pathway (C9 and C5b-9) have been detected in atherosclerotic arteries (Vlaicu *et al.*, 1985b; Seifert *et al.*, 1989; Oksjoki *et al.*, 2003a; Oksjoki *et al.*, 2007).

The intensity of C5b-9 deposition has been correlated with the disease state (Vlaicu *et al.*, 1985a). The heaviest deposition of C5b-9 by immunohistochemistry has been seen in fibrous plaques, suggesting a pathogenetic involvement of complement in chronic progression of the lesions (Vlaicu *et al.*, 1985a). C5b-9 accumulation has occurred especially in the deeper musculoelastic layer of the intima, whereas the superficial, proteoglycan-rich layer is protected from terminal complement activation by C4bp and factor H, which have been shown to bind to the arterial proteoglycans (Oksjoki *et al.*, 2003a). In IEL, C5b-9 has been localized particularly on apoptotic cells and cellular debris (Rus *et al.*, 1986). Atherosclerotic arteries also harbor IgG, IgM, and IgA (Vlaicu *et al.*, 1985a; Oksjoki *et al.*, 2007).

IgM class NAbs (Chou *et al.*, 2009) have been suggested to have a role in the induction of complement classical pathway activation in myocardial infarction (Zhang *et al.*, 2006). NAbs may also have a protective role in atherosclerosis, as they recognize the proinflammatory oxidative epitopes that could reduce the scavenger-receptor-mediated uptake of oxLDL by macrophages and thereby decrease atherosclerosis (Hartvigsen *et al.*,

2009; Lewis *et al.*, 2009). In atherosclerosis, the complement system has been proposed to become activated through the classical pathway (Yasojima *et al.*, 2001a; Oksjoki *et al.*, 2003a), but evidence has also emerged in support of purely alternative pathway-mediated complement activation by the colocalization of properdin and C5b-9 (Oksjoki *et al.*, 2007).

Ischemia-reperfusion injury

Ischemia-reperfusion (I/R) injury occurs as a response to reperfusion in a tissue that has sustained ischemia because of a depletion in oxygen supply due to insufficient blood circulation. Reperfusion leads to the generation of immunogenic reactive oxygen species, release of cytokines, and expression of adhesion molecules. The restored circulation imports immune reactants (e.g. immunoglobulins, acute phase proteins, leukocytes) that recognize ischemia-injured tissue elements and activate innate immune responses. These may lead to an advanced inflammatory reaction and tissue damage within hours (reviewed by Fleming, 2006; Diepenhorst *et al.*, 2009). Both IgM and IgG class natural antibodies in association with neutrophils and complement play a role in inducing the injury (Hernandez *et al.*, 1987; Williams *et al.*, 1999; Fleming *et al.*, 2002). Complement activation has been accompanied by the loss of the complement inhibitor protectin, e.g. in acute myocardial infarction (Väkevä *et al.*, 1992). In experimental studies, the complement system has been found to play a key role in mediating the injury (Hill and Ward, 1971). In a rat model of brain infarction, an inhibition of all complement pathways by C1-INH protected the brain from I/R injury (Akita *et al.*, 2003; De Simoni *et al.*, 2004). In I/R injury, both the classical and the lectin complement pathways are involved initially via the recognition of exposed neo-epitopes in the ischemic tissue. Subsequently, complement activation is amplified by the alternative pathway (Fleming, 2006; Diepenhorst *et al.*, 2009).

III AIMS OF THE STUDY

Complement activation seems to play a key role in many chronic inflammatory diseases, including arterial diseases. However, practically very little is known about the possible role of complement activation in IA wall pathobiology and its relation to IA wall degeneration and rupture.

The objectives of this study were the following:

1. To investigate whether complement activation has occurred in unruptured or ruptured IAs and whether it is associated with IA wall degeneration and inflammatory cell infiltrations.

2. To identify complement activation pathways in unruptured and ruptured IAs and to determine whether complement activators are present in the IA wall.

3. To evaluate the complement regulatory mechanisms in the IA wall.

IV PATIENTS AND METHODS

1 Patients and samples

The samples of IA fundi distal to the aneurysm clip were resected during microsurgery at the Department of Neurosurgery, Helsinki University Central Hospital. The specimens were immediately snap-frozen in liquid nitrogen or fixed for electron microscopy. The snap-frozen samples were stored at -80˚C. Clinical data and demographics were collected from medical records (patient age, gender, multiplicity of IAs, previous SAHs, smoking, alcohol abuse, hypertension, and family history). IA dimensions (fundus lengths, fundus width, and neck width) were obtained from the preoperative vascular imaging studies (computed tomographic angiography, magnetic resonance angiography, or digital subtraction angiography). The venous whole blood samples were drawn postoperatively. The study protocol was approved by the local Ethics Committee (Departments of Neurology, Neurosurgery, Ophthalmology, and Otorhinolaryngology, Helsinki University Central Hospital). A total of 162 patients were included in the study, 43 of whom had previously been histologically characterized (Frösen *et al.*, 2004).

2 Histological studies (I-III)

For histological studies, the snap-frozen tissue specimens were embedded in OCT Tissue Tek compound (Sakura, Torrance, CA, USA) and cryosectioned at 4 µm.

2.1 Histological stainings (I-III)

For basic morphological analysis, the sections were stained with Mayer's hematoxylin and eosin G.

The neutral lipids were stained with Oil-Red-O in unfixed cryosections. The sections were incubated in absolute propylene glycol (Sigma-Aldrich, St. Louis, MO, USA) for 5 min, followed by incubation in 0.5% Oil-Red-O solution in propylene glycol for 7 min, and then washed twice in 85% propylene glycol, running tap water, and aqua. The nuclei were background-stained with Mayer's hematoxylin.

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

2.2 Immunohistochemistry and immunofluorescence stainings (I-III)

Antibodies

The names, sources, and dilutions of primary antibodies used in the immunohistochemical and immunofluorescence stainings are listed in Table 1. The functions of their target antigens in complement activation and regulation are briefly described in Table 2.

Table 1. Primary antibodies used in immunohistochemical and immunofluorescence stainings.

Antigen	Antibody (labeling)	Type	Dilution	Source or reference	Doublestained with	Studies
Complement components						
C1q	anti-C1q	Rabbit pAb	1:1000	DAKO	Lipoproteins	II
C3c	anti-C3c	Rabbit pAb	1:1000	DAKO	-	II
C3d	4C2	Mouse mAb	1:100	(Koistinen *et al.*, 1989)	-	II
C4c	anti-C4c	Rabbit pAb	1:400	DAKO	-	II
SC5b-9 (TCC)	A239	Mouse mAb	1:150	Quidel	IgG, IgM, CRP, fH, properdin, αSMA, Ki67	I, II, III
	Wu 7-2	Mouse mAb		(Würzner *et al.*, 1991)	-	I, II
Complement regulators						
Properdin	anti-properdin	Goat pAb	1:400	INCSTAR	TCC	II
Factor H	anti-factor H	Goat pAb	1:400	Calbiochem	TCC	III
C4bp	anti-C4bp	Rabbit pAb	1:1000	(Hallstrom *et al.*, 2007)	-	III
Protein S	anti-protein S	Rabbit pAb	1:400	Sigma[1]	-	III
Protectin (CD59)	BRIC 229	Mouse mAb	1:200	IBGRL	-	I, III
Complement activators						
CRP	anti-CRP	Goat pAb	1:200	Kallestad	TCC	II
IgG	anti-IgG (FITC)	Rabbit pAb	1:50	Sigma[2]	TCC	II
IgM	anti-IgM (FITC)	Rabbit pAb	1:50	Sigma[2]	TCC	II
ApoB-100	MB47	Mouse mAb	1:500	(Young *et al.*, 1986)	C1q	II
mmLDL	Ox4E6	Mouse mAb	1:200	(Holvoet *et al.*, 1996)	C1q	II
HNE	HNE7	Guinea pig pAb	1:1000	(Palinski *et al.*, 1990)	C1q	II

Other						
Ki67	MIB-1	Mouse mAb	1:250	DAKO	TCC	I
αSMA	1A4 (Cy3)	Mouse mAb	1:1000	Sigma[2]	TCC	I, II

C4bp, C4b binding protein; CRP, C-reactive protein; IgG, immunoglobulin G; IgM, immunoglobulin M; mmLDL, minimally modified low-density lipoprotein; HNE, hydroxynonenal; αSMA, alpha smooth muscle actin; FITC, fluorescein isothiocyanate; Cy3, Cyanine Dye 3; pAb, polyclonal antibody; mAb, monoclonal antibody; fH, factor H; DAKO, DakoCytomation, Glostrup, Denmark; Quidel, Quidel Corporation, San Diego, CA, USA; INCSTAR Corporation, Stillwater, MN, USA; Calbiochem, EMD Chemicals Inc., Darmstadt, Germany; Sigma[1], Sigma Bio Sciences, St. Louis, MO, USA; IBGRL, International Blood Group Reference Laboratory, Bristol, UK; Kallestad, Chaska, MI, USA; Sigma[2], Sigma-Aldrich, St. Louis, MO, USA

Table 2. Primary antibody targets and their functions in complement activation.

Antigen (staining for)	Role
Complement C1q	CP component
Complement C3c (C3b/iC3b)	AP components
Complement C3d	AP component
Complement C4c (C4b/iC4b)	CP components
Complement SC5b-9 (C5b-9)	Terminal complement complex
Properdin	C3bBb stabilizer; AP amplification
Factor H	Soluble decay accelerator of C3bBb; AP inhibition
C4b binding protein	Soluble decay accelerator of C4b2a; CP inhibition
Protein S	Anticoagulant protein; in complex with C4b binding protein
Protectin / CD59	Membrane-bound C5b-9 inhibitor; inhibition of membrane attack complex
C-reactive protein	CP activation
Immunoglobulin G	CP activation
Immunoglobulin M	CP activation
ApoB-100	Main LDL protein; potential CP activator
Minimally modified LDL	Oxidized LDL; potential CP activator
Hydroxynonenal	Oxidized LDL; potential CP activator
Ki67	Proliferation marker; potentially induced by C5b-9
Alpha smooth muscle actin	Smooth muscle cell cytoskeletal protein; potential target cell

CP, complement classical pathway; AP, complement alternative pathway; LDL, low-density lipoprotein

Immunohistochemistry

The tissue sections were fixed with 4% paraformaldehyde, and nonspecific binding was blocked with 1% bovine serum albumin (BSA; Sigma-Aldrich Inc., St. Louis, MO, USA) or 3-5% normal goat or horse serum (Vector Laboratories Inc., Burlingame, CA, USA) in phosphate-buffered saline (PBS). Some sections were additionally blocked with 0.5% cold fish skin gelatin (Sigma) in PBS containing 0.5% BSA (BSA/PBS). The primary antibodies were diluted in 1% BSA/PBS and incubated for 30 min at room temperature or

overnight at 4°C. The endogenous peroxidase was blocked with 0.3-0.7% hydrogen peroxide in PBS or methanol. The sections were incubated with biotinylated secondary antibodies (Vector) for 30 min at room temperature. The signal was enhanced by horseradish peroxidase or alkaline phosphatase-conjugated avidin-biotin complexes and visualized with diaminobenzidine (Sigma) or Vector Blue (Vector), respectively. The background was stained with Mayer's hematoxylin or Carmaleum.

Immunofluorescence stainings

For immunofluorescence stainings, the fixation, blocking, and incubation with the primary antibody were performed as described above. The primary antibody (Table 1), if unconjugated, was detected with fluorochrome-conjugated Alexa Fluor 488 (green) or 546 (red) secondary antibody (Molecular Probes Inc., Eugene, OR, USA) diluted to 1:100-1:200. For double-immunofluorescence stainings, the incubations were repeated with antibodies originating from different species. The slides were mounted in Vectashield with DAPI (Vector) to detect the nuclei.

Controls

Human tonsils and infarcted heart served as the positive controls for the stainings. For negative controls, the primary antibody was omitted or replaced with an irrelevant antibody of the same immunoglobulin isotype as the primary antibody.

2.3 TUNEL assay (I)

TUNEL (terminal deoxynucleotidyl transferase (dUTP) nick end labeling) assay was used to detect apoptotic cells. For immunofluorescence and TUNEL double stainings, an additional TUNEL reaction was performed using a Fluorescein In Situ Cell Death Detection kit (Roche Diagnostics GmbH, Penzberg, Germany) following the immunofluorescence staining (Kitagawa *et al.*, 1998).

2.4 Electron microscopy and immunoelectron microscopy (I)

The IA sample was immersion-fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA, USA). For the morphological analysis, specimens were further fixed in 2.5% glutaraldehyde (Fluka, Buchs, Switzerland), postfixed with 1% osmium tetroxide, dehydrated, and embedded in Epon (TAAB Laboratories Equipment Ltd., Berkshire, England). The diluent was 100 mmol/L natrium phosphate buffer, pH 7.4. For immunoelectron microscopy (IEM), paraformaldehyde-only-fixed specimens were low-temperature-embedded into Lowicryl (HM20; TAAB Laboratories Equipment Ltd.). Dehydration, resin infiltration, and ultraviolet polymerization were performed at -30°C.

The ultrathin sections (60 nm) were blocked with 1% BSA, 0.5% cold fish skin gelatin (Sigma), and 1% heat-inactivated fetal calf serum (BioWhittaker, Walkersville, MD). The primary antibody (A239) was detected with goat anti-mouse IgG and IgM conjugated with 10 nm colloidal gold (British Biocell, Cardiff, England). Both antibodies were diluted with 2% BSA, 0.1% Tween 20 (Electron Microscopy Sciences), and 0.1% cold fish skin gelatin. All specimens were poststained with uranyl acetate (Ultrostain I; Leica, Wetzlar, Germany), lead citrate (Ultrostain II; Leica).

2.5 Imaging

The immunohistochemical stainings were photomicrographed with an Axioplan 2 imaging microscope and AxioCam MRc (Carl Zeiss Vision GmbH, Aalen, Germany). The immunofluorescence stainings were viewed and photographed with Axioplan 2 and AxioCam (HR/MR) cameras, with appropriate filters to create pseudocolored images (Zeiss). The electron microscopy was performed with a Jeol EX 1200 II transmission electron microscope (Jeol Ltd., Tokyo, Japan) operating at 60 kV.

2.6 Histological evaluation

For semiquantitative and quantitative histological evaluations, the measurements were performed up to three times, and the mean values were used for calculating correlations or for comparisons.

Evaluation of MAC-positive areas (I)

The following two kinds of measurements of MAC-positive areas were performed from immunoperoxidase-stained histological samples with the KS300 3.0 image processing software (Carl Zeiss GmbH, Oberkochen, Germany): 1) The percentage of the MAC-positive area of the total surface area of one section of each histological sample was measured under x1.25 magnification. The mean values were used to correlate the area of complement activation with IA rupture status with the patient’s clinical parameters, IA rupture status, histological markers, and inflammatory cell infiltrations. 2) The percentages of the MAC-positive areas of the areas representing specific wall types were measured under x5 magnification. The areas were classified on the basis of the previously published grading system of four different histological, rupture-related IA wall types: (A) an endothelialized wall with linearly organized smooth muscle cells; (B) a thickened wall with disorganized smooth muscle cells; (C) a hypocellular wall with either myointimal hyperplasia or organized thrombus; and (D) an extremely thin thrombosis-lined hypocellular wall (Frösen *et al.*, 2004).

Evaluation of cellular infiltrations (I)

The histological data for inflammatory cell infiltrations of the 43 previously studied IAs had been published by Frösen *et al.* (2004). The inflammatory cell infiltrations were quantified as the number of positive cells, and the proliferation was quantified as the proportion of Ki67-positive nuclei per standardized grid area (0.0625 mm^2). The presence or absence of proliferating cells, endothelial cells, remnants of elastic lamina, atherosclerotic calcifications, myointimal hyperplasia, and thrombosis lining the lumen was categorically scored (yes/no). The proportion of TUNEL-positive cells was quantified per field of view (0.0956 mm^2) and the colocalization of positive staining for MAC and positive TUNEL reaction was evaluated.

Evaluation of complement components (II)

Antigen deposition in the IA wall was classified as mild, moderate, or strong depending on the extent of the positively staining areas relative to the whole IA wall surface area (mild <1/3, moderate 1/3-2/3, strong >2/3). The presence or absence of complement components was screened in the areas of interest within parallel sections.

Evaluation of complement activators (II) and inhibitors (III)

Single- and double-stained IAs were screened for the location of the positive staining within cells or the extracellular matrix. The localizations of the antigens were compared with each other in serial sections.

3 Analysis of single-nucleotide polymorphisms (III)

DNA was extracted from blood mononuclear cells by the ethanol precipitation method (Gentra Puregene Genomic DNA Purification Kit, Gentra Systems, Minneapolis, MN, USA). 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’-ttagaaagacatgaacatgctagg-3’. PCR amplifications were performed in 50-µl volumes containing 80 ng of genomic DNA, 30 pM each primer, polymerase buffer, 10 nM each nucleotide (dNTP), and 0.8 U of the 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, USA). The reactions were run on an ABI 3730 capillary sequencer according to the manufacturer’s instructions.

4 Statistical analysis

For the statistical comparison of clinical and histological data on unruptured and ruptured aneurysm samples, the proportions were calculated for patient gender, multiple IAs, patients with prior aneurysmal SAHs, and binomially classified histological markers. Means, medians, and ranges were calculated for patient age, IA dimensions, and inflammatory cell infiltrations or proportions of TUNEL$^+$ cells, and Chi-Square (χ^2) and Mann-Whitney U tests were used when appropriate. For comparison of the proportion of MAC-positive area with clinical data and histological markers, Mann-Whitney U and Spearman-Rank correlation tests were used. For calculating the correlation between IA wall type and complement activation, the percentages of MAC-positive areas specific for each wall type were converged to square roots to achieve normal distribution. The differences were considered significant when the P-values were below 0.05.

A linear mixed model (LMM) was used to explore the association between the percentage of areas positive for MAC in order to account for variation between histological cuts with varying number of measurements. The LMM model (Laird and Ware, 1982) for percentage area positive for MAC in histological cut *i* at measurement *j* is

$$y_{ij} = \alpha_1 + \sum_{p=2}^{4} \alpha_p * x_{pij} + \beta_{1i} + \varepsilon_{ij}$$

Here, α_1 is the square root of the percentage of areas positive for MAC in wall type A adjusted for other effects, coefficients for fixed effects, and random effects. In all models, α_2, α_3, and α_4 are the regression coefficients for comparing three different IA wall types: the proliferating type B ($x2ij$=1, if wall type B, 0 otherwise), the decellularized type C ($x3ij$=1, if wall type C, 0 otherwise), and the thin, degenerated type D ($x4ij$=1, if wall type B, 0 otherwise) with the intact-appearing type A. To allow cut-specific variation in percentage area positive for MAC, we introduce a random intercept:

$$\beta_{1i} \overset{iid}{\sim} N(0, \sigma_I^2)$$

Basic univariate tests were obtained with NCSS 2000 (NCSS Statistical Software, Kaysville, UT, USA) or SPSS 16.0 (SPSS, Chicago, IL, USA), and a linear mixed model was fitted using R 2.1.0-A Language and Environment (public domain software) (R Development Core Team, 2004).

V RESULTS AND DISCUSSION

1 Complement activation in intracranial aneurysms

Complement becomes activated in the IA wall through the classical pathway with amplification via the alternative pathway. By immunohistochemical and immunofluorescence stainings, C1q was seen broadly in the IA wall and both C4b/iC4b and C3b/iC3b overlapped with the band-like area of the terminal pathway activation and C5b-9 formation at the less cellularized area at the outer part of the IA wall. The lack of properdin in the same area contradicts an independent activation of the alternative pathway. The luminal part of the IA wall is protected by fH and C4bp, possibly through an interaction with glycosaminoglycans. Bound fH and C4bp did not appear to be sufficient to prevent TCC activation in all IA areas because they partially overlapped with C5b-9 in the outer part of the IA wall. The limiting inhibitor for full assembly of C5b-9 seems to be protectin, which stained reciprocally to C5b-9. Complement may become activated by many activators, as IgG, IgM, CRP, and oxLDL were scattered throughout the IA wall without a reproducible pattern. However, neutral lipids seemed to colocalize with C5b-9, suggesting their role in TCC activation (Figure 9).

1.1 Role of complement in intracranial aneurysm inflammation and vascular damage

The most intense staining for TCC was located consistently as a distinct layer in the outer IA wall. This area showed loss of nuclei, as fewer nuclei were present in this layer than in the adjacent MAC-negative area in 28/35 samples (80%). Notably, this area would represent the outer media of a healthy arterial wall. Electron microscopy (EM) analysis of this area revealed dying cells featuring necrotic cell death as well as probable cell remnants (I). Some additional TCC was found occasionally in patches of myointimal hyperplasia, where cell nuclei were scattered normally in a majority of the samples (21/23; 91%). The relative proportion of TCC-positive areas within the samples varied (range 5-77%), suggesting differences in the depth of inflammation in IAs. The relationships between TCC activation, as a marker of complement activation intensity, and different histological and clinical parameters are referred to and discussed in the following sections.

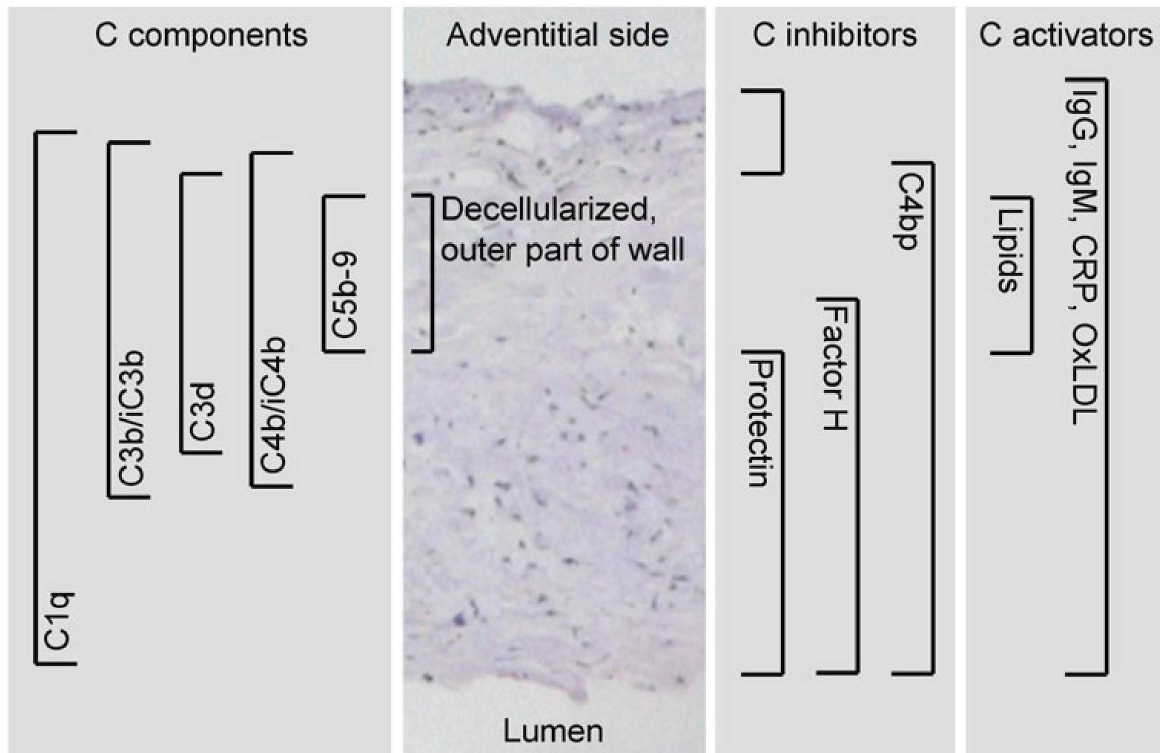

Figure 9. Presence of complement components, regulators, and potential activators in the intracranial aneurysm wall. The brackets represent the typical localizations of positive stainings for the different complement (C) components studied in the immunohistochemical, immunofluorescence, and histological stainings of the IA walls.

1.1.1 Association with aneurysm wall degeneration and rupture (I, II)

The terminal complement complex (TCC) or complement membrane attack complex (MAC) was found in all studied IAs, totaling 26 unruptured and 32 ruptured IAs. The proportion of TCC-positive area within the whole IA wall surface area was associated with IA rupture (p=0.005; Mann-Whitney U test). The ruptured IAs showed wider relative areas of terminal complement activation (median 39%; range 13-60%) than the unruptured IAs (median 20%; range 5-77%) (I). In addition, the differences in the distribution of earlier complement components (C1q, C3b/iC3b, C3d, and C4b/iC4b) between unruptured and ruptured IAs were similar, as unruptured IAs showed less accumulation of all the studied earlier components (p=0.011 for C1q, p=0.001 for C4b/iC4b, p<0.001 for C3b/iC3b, p=0.048 for C3d; χ^2 test) (II). C5b-9 accumulation was located in subareas of the earlier components. This indicates that in the TCC-negative areas, TCC formation was inhibited, as was shown in Study III (Figure 9). The need for activation of the complement system for TCC formation was suggested by the presence of earlier complement components in the same areas. In atherosclerotic plaques, the magnitude of TCC deposition has been associated with plaque degeneration (Vlaicu *et al.*, 1985a) and increased susceptibility to plaque rupture (Meuwissen *et al.*, 2006). Thus, TCC activation seems to be related to mechanisms that weaken the vascular wall, making it more prone to

rupture. Whether the critical element is the complement activation itself or, for example, a consequent leukocyte influx that leads to wall degeneration cannot be determined at this point.

Further evidence for the association of complement activation and IA wall degeneration was obtained from the evaluation of the median percentages of TCC-positive areas within the different IA wall types (determined by Frösen *et al.* 2004; described in Section 1.5.3). The areas differed significantly between different wall types ($p<0.001$; LMM), except between types C and D. The median areas were 9% in A-type (n=12), 20.5% in B-type (n=12), 39% in C-type (n=33), and 40% in D-type (n=9) walls (I). As the degenerated and decellularized walls (types C and D (Frösen *et al.*, 2004); found more often in ruptured IAs) showed larger TCC-positive areas than the proliferative B-type and the quiescent, endothelialized A-type IA walls (found more often in unruptured IAs), complement activation seemed to be associated with wall degeneration. Furthermore, as complement activation was present also in all unruptured IAs, and as some IAs lacked inflammatory cell infiltrations (Crompton, 1966; Frösen *et al.*, 2004), complement activation seemed to precede the inflammatory cell infiltration into the IA wall.

1.1.2 Association with macrophage and T-cell infiltration (I)

Complement activation is chemotactic for inflammatory cells. Complement activation against foreign surfaces, e.g. those of microbes, is usually associated with an apparent inflammatory cell infiltration. However, the lack of a correlation between complement activation (TCC deposition) and inflammatory cells other than $CD3^+$ T-cells ($p=0.047$) and $CD163^+$ macrophages ($p=0.037$) suggested an absence of an infectious focus (I).

The alternatively activated $CD163^+$ macrophages are more likely linked to the dampening of the inflammatory response and resolution of inflammation than to an acute inflammatory reaction associated with direct killing of pathogens (Moestrup and Moller, 2004). CD163 is a macrophage scavenger receptor that recognizes, for instance, cell-free hemoglobin (Fabriek *et al.*, 2005). The IA wall has been reported to show occasional staining for hemosiderin (Schlote and Gaus, 1994; Rajesh *et al.*, 2004; Holling *et al.*, 2009) as well as for intact erythrocytes (Nyström, 1963; Scanarini *et al.*, 1978a; Kataoka *et al.*, 1999), indicating clinically silent intramural bleeding. Aged erythrocytes are known to activate complement (Peterson *et al.*, 1989). Free iron from lysed erythrocytes causes oxidative stress, leading to oxidative modification of surrounding particles, e.g. of LDL, also shown to be present in IA walls (II). The oxidatively modified lipids and proteins are capable of binding immunoglobulins, acute phase proteins, and C1q (II).

In addition to the possible hemoglobin-mediated link between complement activation and the presence of $CD163^+$ macrophages, other factors might also be responsible for both complement activation and recruitment of $CD163^+$ cells.

T-cells are the key players of acquired immunity. Complement activation seems to favor a regulatory T-cell response, as complement anaphylatoxins promote IL-4, -5, and -10 production and suppress IL-12 production from leukocytes. Also cross-linking of CD46 strongly induces development of IL-10-producing regulatory T-cells, and cross-linking of CD59 enhances T-cell proliferation and IL-2 -production (see also Figure 6) (Kemper and Atkinson, 2007).

The T-cell profile in human IAs has not been confirmed to date. However, the lack of IL-10 (Jayaraman *et al.*, 2008) in ruptured IAs and the presence of TNF-α (Jayaraman *et al.*, 2005) hint at the dominance of the T_h1 direction or a cytotoxic T-cell response. At a transcriptional level, the receptors for IFN-γ, produced by T_h1-cells, T_c-cells, and activated macrophages, are upregulated in IA walls (Krischek *et al.*, 2008; Shi *et al.*, 2009). In atherosclerosis, the T_h1-type response has been found to dominate (Hansson and Libby, 2006). The discrepancy between the theoretical direction of T-cells in a regulatory rather than an effector direction and the suggestion of T_h1-typed response in practice could be explained by the complicated mechanisms that regulate inflammatory reactions *in vivo*.

1.1.3 Endothelial dysfunction may potentiate complement activation and accumulation in the aneurysm wall (I, II)

De-endothelialization occurs during IA progression and has been seen in both human and experimental IAs (Crompton, 1966; Kataoka *et al.*, 1999; Frösen *et al.*, 2004; Jamous *et al.*, 2007). In experimental IAs, endothelial dysfunction has been the earliest change in IA progression (Jamous *et al.*, 2007). Heavier C5b-9 accumulations were observed in de-endothelialized IAs, and the relative TCC-positive area correlated significantly with de-endothelialization ($p<0.001$; Mann-Whitney U test) (I). Also, a gradually decreasing staining for IgG and IgM in the lumen-to-adventitia direction, accumulation of IgG and IgM in the ECM, and accumulation of neutral lipids in the outer part of the wall were noted. These indicate the possibility of an increased flow of plasma proteins to the IA wall (II). No IgG and only small amounts of IgM have been detected in the walls of normal basilar arteries (Chyatte *et al.*, 1999). The normal centrifugal mass transport of micro- and macromolecules through the vascular wall due to hemodynamic forces can become disturbed in pathological conditions (Michel *et al.*, 2007). Thus, several plasma factors, including complement components, are hypothesized to leak and accumulate in the IA wall because of endothelial dysfunction.

Under normal circumstances, molecules pass through the vascular wall. Vascular stress may disturb these normal clearance mechanisms. Because of anatomical and physiological abnormalities, the IA wall suffers from chemical and mechanical stresses. The complement system helps in the clearance of altered host structures and particles by

opsonizing them. Thus, complement accumulation may indicate disturbed tissue homeostasis and emergence of altered structures that need clearance.

1.1.4 Relationship between complement activation and cell death (I, II)

Important structural and functional components of the IA wall are SMCs, which disappear from the IA wall upon its degeneration. The finding of a decreased number of nuclei in the TCC-positive layers in the outer IA walls indicates a major cell loss in these regions (I). A probable association between TCC activation and cell loss was also seen within B-typed and C-typed walls, both of which are thick, but in which the C-type wall is decellularized. The relative TCC-positive area was found to be almost twice as large in C-type (39%) than in B-type (20.5%) IA walls (I).

An increase in apoptotic cell death in IAs (Hara *et al.*, 1998; Guo *et al.*, 2007) and its relation to IA rupture (Sakaki *et al.*, 1997; Frösen *et al.*, 2004; Pentimalli *et al.*, 2004) have been described. Also, gene expression for downstream regulators of apoptotic pathway, caspases 1 and 3, is upregulated in IAs (Guo *et al.*, 2007; Shi *et al.*, 2009). As complement can kill cells by direct lysis and by apoptosis (Cole and Morgan, 2003; Bohana-Kashtan *et al.*, 2004), double stainings for TUNEL and MAC were performed. $TUNEL^+$ cells were found in 54% (12/22) of unruptured and 77% (24/31) of ruptured IA walls. The proportion of $TUNEL^+$ cells of total cell count was slightly more in ruptured (median 26%, range 0-89%) than in unruptured (median 11%, range 0-65%) samples ($p=0.052$). The presence of apoptosis was verified in electron microscopy (I). Although $TUNEL^+$ cells colocalized with MAC-positive areas in some ruptured samples (4/7; and in 0/7 unruptured samples) and complement depositions were found adjacent to the $TUNEL^+$ nuclei in 5/14 samples at larger magnification, no double-positive cells were detected. In addition, the statistically weak correlation between $TUNEL^+$ cells and complement activation suggested that apoptosis was not the major mechanism behind complement activation and cell loss in the IA wall. Although a massive attack against SMCs by the MAC appears an unlikely explanation for the loss of cells in the IA wall, in one of the series, we managed to detect C5b-9-positive SMCs in one unruptured IA and a few C5b-9-positive cells in 4/10 other unruptured IAs (II).

Occasional findings of MAC near $TUNEL^+$ cells could be indicative of a role of complement in the clearance of cellular debris in the IA wall. It is well known that complement can become activated as a clearance mechanism to remove cells (Cole and Morgan, 2003; Bohana-Kashtan *et al.*, 2004). In fact, immunoelectron microscopy revealed that structures in the area of MAC accumulation probably represent cellular debris. MAC was also found on remnants of cell membranes. In light microscopy, the majority of MAC localized to ECM (I). A recent study on a series of perioperatively resected ruptured IAs revealed necrotic cell death in a remarkably large proportion of IA

samples (Holling *et al.*, 2009). This supports also the finding of necrotic cells in electron microscopy (I). The rarity of colocalization of MAC and $TUNEL^+$ cells suggests that complement-mediated cell death and apoptosis both have their own distinct roles in cell loss. However, it should be noted that although TUNEL assay is widely used to detect apoptotic cell death, the assay also detects cells that have DNA fragmentation for other reasons, including proliferation, necrotic cell death, or tissue preparation (e.g. cryosectioning).

1.1.5 Complement activation and other clinical and histological parameters

In general, TCC activation was not associated with other clinical (patient age, sex, multiplicity of IAs, prior SAHs, Fisher's grade, or IA dimensions) or histological parameters (presence of remnants of IEL, organizing thrombus, atherosclerotic calcifications, pads of intimal hyperplasia, or cell proliferation; amounts of $CD11b^+$ or $CD68^+$ macrophages, $CD45^+$ leukocyte infiltrations or proportion of TUNEL+ cells) ($p>0.05$; Mann-Whitney U, Kruskal-Wallis, and Spearman-Rank correlation tests). The lack of these associations indicates that complement activation is more specifically related to cell loss, as discussed above.

1.2 Complement activation pathways and potential complement activators

Both the classical and alternative pathway components stained positive in the IA walls, the maximum positive staining being in the outer part of the wall in the same area as C5b-9. Many of the potential complement activators studied were distributed in wide areas in the IA wall. The neutral lipids were an exception, as they colocalized with C5b-9 (Figure 9).

1.2.1 Activation via the classical pathway and recruitment of alternative pathway amplification (II)

The presence of C1q together with C4b/iC4b and C5b-9 clearly indicates that the classical pathway of complement has become activated in IA walls. Properdin, a specific marker for alternative pathway activation, was seen only weakly in IA walls. Complement C3b/iC3b deposits were also observed in the same areas and usually in greater abundance than C4b/iC4b, whereas the area positive for C3d was more restricted. The presence of C4b/iC4b in the same areas as C3b/iC3b and C3d may indicate that classical pathway activation has recruited alternative pathway amplification in the IA wall. The deposition of C3d, a covalently bound long-lived breakdown product of C3b, suggests that a more prolonged inflammation and complement accumulation in the IA wall has occurred (Davis *et al.*, 1984; Nakagawa *et al.*, 2000).

The classical pathway has been suggested to be active also in atherosclerotic plaques (Yasojima *et al.*, 2001a; Oksjoki *et al.*, 2003a). In AAAs, the presence of increased amounts of several IgG subtypes together with C3 degradation products has been suggested to indicate complement activation by the classical pathway (Capella *et al.*, 1996). Interestingly, many chronic inflammatory diseases, i.e. rheumatoid arthritis, atherosclerosis, and membranous nephropathy, show signs of parallel involvement of the classical and the alternative pathways (Cunningham and Quigg, 2005; Theroux and Martel, 2006; Okroj *et al.*, 2007). The alternative pathway may become recruited in the inflamed tissue following persistent classical pathway activation due to the action of the C3/C5 convertases and alternative pathway amplification (Sim and Tsiftsoglou, 2004; Sjöberg *et al.*, 2009). However, in IAs the virtual lack of properdin underlines the role of classical pathway activation, and thereby, the potential importance of the classical pathway activators in IA pathobiology.

1.2.2 Several known complement activators are present in the aneurysm wall (II)

IgG as well as IgM were seen in the IA wall, mostly bound to the ECM. IgG was detected in 61/90 (68%) and IgM in 8/10 IAs. Of the IgG-positive and -negative samples, 62% (38/61) and 55% (16/29) were ruptured, respectively (p=0.520, χ^2 test). This finding is consistent with previous results of immunoglobulin accumulation in abnormal vascular tissues. Abdominal aortic aneurysms show strongly increased amounts of IgG together with complement C3d (Capella *et al.*, 1996). IgG was located diffusely in the ECM in a majority (93%; 57/61) of the samples, whereas IgM stained more in a patch-like manner.

IgM and antigen-bound IgG subclasses IgG1 and IgG3 are potent complement classical pathway activators. The majority of IgMs are naturally occurring antibodies (NAbs) which have been proposed to activate the classical pathway in, for example, myocardial infarction (Zhang *et al.*, 2006). Oxidized epitopes, present also in cells and in the ECM of IA walls, have recently been found to be the main targets for naturally occurring antibodies (Chou *et al.*, 2009). NAbs have a low affinity to multiple ‘nonself’ and ‘self’ antigens, e.g. negatively charged phospholipids and other antigens exposed during apoptosis (Ochsenbein and Zinkernagel, 2000; Fleming, 2006). In addition to IgM, also IgG3 and IgA isotypes occur among NAbs (Ochsenbein and Zinkernagel, 2000; Fleming, 2006).

As hypothesized, immunoglobulins may leak and accumulate in the IA wall because of endothelial dysfunction. Thus, any antibodies with natural or acquired specificity for structures in the ECM would be expected to become deposited in the IA wall. Novel antigen exposure could occur via pressure-induced damage, membrane damage, and/or proteolysis of components in the ECM. Immunoglobulins have been

detected earlier in unruptured IA walls in similar proportions as those seen here in both unruptured and ruptured IAs (II), but not in basilar artery samples used as controls (Chyatte *et al.*, 1999). In atherosclerotic coronary arteries, IgM has been shown to accumulate and colocalize with the classical pathway activation products C4b/iC4b in the superficial intima (Oksjoki *et al.*, 2007).

In normal cerebral arteries, lipids accumulate subendothelially with aging, and IEL seems to function as a barrier limiting lipid accumulation within the IEL and intima (Zugibe and Brown, 1961). In IAs, accumulation of neutral triglycerides and lipids was detected in the same areas where C5b-9 was deposited. In an experimental setting, C5b-9 has been found to colocalize with extracellular lipids upon progressive lipid accumulation in rabbit atherosclerotic aortas (Seifert *et al.*, 1989). Native and oxidized lipids accumulate in stressed vascular walls, and oxidized lipids may activate the classical pathway *in vivo* through C1q in the presence of CRP (Frösen, 2006; Biró *et al.*, 2007).

In IAs, the ECM stained positively in all samples (10/10) for the main LDL protein ApoB-100 and oxLDLs. The positive staining for cells was seen with ApoB-100, minimally modified LDL, and hydroxynonenal in 6/10, 8/10, and 10/10 samples, respectively. OxLDLs were detected in areas positive for CRP, similarly as seen in atherosclerotic plaques (Chang *et al.*, 2002; Meuwissen *et al.*, 2006), and in experimental ischemic myocardium (Griselli *et al.*, 1999). The detection of oxLDL in both the ECM and cells probably indicates a generalized imbalance in oxidative and antioxidative potential.

Peroxidation of lipids is a common event in healthy individuals, e.g. in apoptotic cell death. In proinflammatory diseases, it becomes accelerated. The vascular wall carries multiple oxidative mechanisms to produce reactive oxygen species (ROS). They can modify a wide variety of extracellular and cellular proteins and lipids (Hartvigsen *et al.*, 2009). The formed oxidized phospholipids as well as oxidized lipoproteins are proinflammatory, as they are recognized by natural antibodies (Baumgarth *et al.*, 2005), phagocyte scavenger receptors (Goldstein *et al.*, 1979), and innate soluble proteins like CRP (Chang *et al.*, 2002) and complement C1q (Torzewski *et al.*, 1998; Meuwissen *et al.*, 2006). The interaction between ROS and antibodies may also increase the affinity of the antibodies to a wider range of autoantigens, as reviewed in Dimitrov *et al.* (2008).

Native CRP is present widely in inflamed tissues, but has been found only in a few healthy aortas (Reynolds and Vance, 1987; Diehl *et al.*, 2000; Ji *et al.*, 2006). In IAs, C1q was mostly bound to ECM similarly as oxLDL and CRP, suggesting that classical pathway activation through C1q in IA walls could be induced by oxLDL and/or CRP. ECM showed also a faint double positivity for CRP and C5b-9, and CRP was found in the band-like area of heavy C5b-9 staining in the outer IA wall. However, C1q is also capable of interacting with several matrix proteins (Sjoberg *et al.*, 2009).

Surface-bound CRP itself is a potent complement classical pathway activator. In 5/10 unruptured IAs, only a few CRP-positive cells were seen and in double-stainings with C5b-9, only occasional double-positive cells were detected. However, in one unruptured IA, many CRP-positive mural cells without C5b-9 accumulation were detected, which might be explained by CRP's ability to bind complement inhibitors, e.g. factor H, to protect the target structures from further complement activation (Jarva *et al.*, 1999).

1.2.3 Impaired clearance mechanisms and complement activation

Only the accumulation of neutral lipids in the outer IA wall was found to colocalize with C5b-9, as also seen in experimental studies (Seifert *et al.*, 1989). Based on the otherwise lacking one-to-one colocalization of complement components and potential complement activators, complement seems to have become activated in the IA wall in response to more than one specific activator. As the IA wall is exposed to both mechanical hemodynamic stresses and humoral inflammatory stresses, complement activation might be caused by the combined effect of traditional activators plus mechanochemical stress, leading to a disturbance in the local tissue homeostasis. Increased cellular death and associated accumulation of cellular debris may trigger and maintain prolonged complement activation, especially in the outer part of the IA wall. Thus, disturbances and possible overloading in the wall clearance mechanisms may lead to the accumulation of molecules and material in the vascular wall that would normally be transported through it (Michel *et al.*, 2007). Many of these molecules and exposed structures can also activate complement.

1.3 Complement regulation (III)

1.3.1 Factor H and C4bp protect the luminal part of the aneurysm wall from complement membrane attack complex

The main complement classical pathway inhibitor C4b binding protein and the alternative pathway inhibitor factor H were detected mostly in the ECM of the luminal parts of IA walls, whereas C5b-9 staining remained mostly negative in these areas. The luminal part stained also with Alcian Blue, indicating the presence of sulfated glycosaminoglycans. C4bp interacts directly with small glycoproteins of the ECM (Happonen *et al.*, 2009). C4bp as well as factor H have been shown to bind to arterial proteoglycans and to colocalize with them in atherosclerotic lesions (Oksjoki *et al.*, 2003a; Oksjoki *et al.*, 2007). Factor H has been demonstrated to bind to glycosaminoglycans, thereby having a pivotal role in discriminating between complement activators and nonactivators (Meri and Pangburn, 1990b; 1994). The finding of factor H in similar areas with

glycosaminoglycans, but without MAC, indicates the importance of their role in complement inhibition in the IA wall. Thus, the distribution of glycosaminoglycans and their modulation by as-yet-unknown factors have important roles in regulating complement activation in the IA wall.

C4bp and some factor H were also found in the area of strong C5b-9 accumulation in the outer part of the IA wall. As expected, protein S was seen in identical areas with C4bp in parallel sections in all IAs (18/18; 9 unruptured and 9 ruptured IAs), although C4bp was sometimes detected in larger areas than protein S. The staining for C4bp was especially prominent in colocalization with C5b-9, whereas factor H deposition was less intense, only partially overlapping with C5b-9. Similarly as in IAs, in atherosclerotic lesions, C4bp is widely expressed luminal to C5b-9 accumulation, but without an overlap with C5b-9 (Oksjoki *et al.*, 2007).

The partial colocalization of C4bp with C5b-9 suggests that in IAs a stronger inbalance is present between complement regulation and activation than in atherosclerotic lesions. This could be due to either stronger complement activation potential because of wider vascular wall damage or weaker regulatory activity in this region.

In the IA wall, the area positive for C5b-9 has lipid-containing particles (II), a decreased number of viable cells, apoptotic and necrotic cell death, and marked amounts of probable cell debris (I). C4bp has an inherent capacity to bind apoptotic cells through interaction of protein S and phosphatidylserine of apoptotic blebs (Blom *et al.*, 2004). As the area of heavy C4bp and C5b-9 accumulation at the outer part of the IA wall presents only a few nuclei, the local synthesis of such amounts of C4bp only by cells in the arterial wall seems unlikely (Yasojima *et al.*, 2001a). Rather, C4bp is suggested to accumulate in this area because of passive diffusion or physiological mass transport through the IA wall (Michel *et al.*, 2007). Why factor H did not represent similar heavy accumulation as the C4bp, but rather a scarcer distribution, could be explained by differences in the affinity of factor H and C4bp for the extracellular matrix components. Nevertheless, the lack of factor H from this region may be the key factor for activation of alternative and terminal pathways to occur.

1.3.2 CFH Y402H is not associated with intracranial aneurysms or aneurysm rupture

The factor H polymorphic variant Y402H has been strongly associated with age-related macular degeneration, a disease characterized by impaired eye pigment and debris clearance (Thakkinstian *et al.*, 2006). The role of Y402H polymorphism in cardiovascular diseases remains controversial. One study has supported an association of the 402H variant with myocardial infarction (Kardys *et al.*, 2006), but further studies have failed to confirm this finding (Zee *et al.*, 2006; Nicaud *et al.*, 2007; Stark *et al.*, 2007). The factor H

variant with histidine in position 402 has a reduced affinity for CRP (Laine *et al.*, 2007; Sjoberg *et al.*, 2007). CRP is an acute-phase reactant associated with tissue damage and inflammation, also in IA walls (II). Thus, we were interested in investigating Y402 polymorphism in patients with IAs.

However, no significant difference was seen in Y402H distribution between IA patients and healthy controls (97 IA patients and 100 individuals representing the normal Finnish population). Of the IA patients, 36/97 (37%) had the TT genotype, 53/97 (55%) the heterozygous CT genotype, and 8/97 (8%) the most strongly AMD-associated CC genotype. Of the aneurysm patients with TT, CT, and CC genotypes, 26/36 (72%), 35/53 (66%), and 3/8 (38%) had had a ruptured IA (aSAH), the distribution differing nonsignificantly from the IA patients carrying only unruptured IAs (p=0.188; Table 3). The CFH genotype did not correlate with recorded hypertension (p=0.101; χ^2 test), gender (p=0.777, χ^2 test), IA diameter (p>0.173; Mann-Whitney U -test), or multiplicity of IAs (p=0.197, χ^2 test). The genotypes of controls did not differ significantly from those of IA patients: TT=41/100 (41%), CT=42/100 (42%), CC=17/100 (17%) (p=0.088; χ^2 test). Although the latter comparison was based on a relatively small series, the 402H variant (CC genotype) does not appear to have a role in IAs in general or in IA pathobiology and rupture.

1.3.3 Protectin limits C5b-9 formation in the aneurysm wall

Protectin is a membrane-bound GPI-anchored complement regulator that inhibits the formation of MAC. Protectin stained positive in all 44 (24 unruptured and 20 ruptured) studied IAs. In general, the staining seen was the reverse of that of the C5b-9 complex; the areas of intensive staining for protectin were virtually negative for C5b-9. Only faint or no staining for protectin was observed on the matrix in the areas of strong positive staining for C5b-9 in the outer band-like part of the IA wall. In the 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. The staining was located mainly on cell membranes, but some protectin staining was also present diffusely in the ECM.

In general, SMCs are protected against complement attack by expressing protectin, but loss of protectin expression may occur in, for instance, myocardial infarction (Väkevä *et al.*, 1992). Cultured rat aortic SMCs are sensitive to loss of expression of protectin when grown overconfluently or for prolonged periods. Thereby, the cells become sensitive to complement attack (Capey *et al.*, 2007). In addition, cultured porcine aortic SMCs have been found to be extremely sensitive to complement-mediated lysis due to a low level of expression of protectin (Capey and van den Berg, 2005). Whilst ischemic cells lose protectin (Väkevä *et al.*, 1992), the possible role of hypoxia in the IA wall pathobiology remains hypothetical (Inci and Spetzler, 2000). Also the presence of macrophages in the

IA wall (Kosierkiewicz *et al.*, 1994; Kataoka *et al.*, 1999; Frösen *et al.*, 2004) may contribute to the loss of protectin, as the majority (95%) of macrophages in atherosclerotic lesions have been found to express GPI-specific phospholipase D, which is capable of hydrolyzing the GPI anchor of protectin (O'Brien *et al.*, 1999).

The areas of strongest C5b-9 accumulation were mostly in areas lacking protectin and with only a few cells, underlining the importance of cell-mediated complement inhibition in IAs. However, whether the lack of protectin is a cause or consequence of the cell loss in the same area, needs to be elucidated. Either could lead to increased C5b-9 formation.

In some IA walls, the staining for protectin partially overlapped with that of C5b-9 in areas between the strongest protectin expression and the strongest C5b-9 accumulation. A similar phenomenon has also been seen in atherosclerotic lesions (Seifert *et al.*, 1992). This may indicate an imbalance of complement inhibition and activation in the IA tissue. If the changes in the IA wall are considered a continuous degenerative process ultimately leading to critical thinning and rupture of the IA wall, this area of partially decreased inhibitory capacity of complement activation might represent a transitional zone in the process. Protectin has a role in preventing inflammation related to tissue debris clearance, as indicated by studies with atherosclerosis-prone ApoE-/- mice, in which protectin-deficient mice showed signs of accelerated atherosclerosis (Wu *et al.*, 2009). In Study I, the areas of intensive C5b-9 accumulation also showed lipid accumulation as well as cellular debris. Thus, protectin may play a critical role in the IA wall homeostasis by preventing MAC attack.

2 Possible role of complement activation in intracranial aneurysms

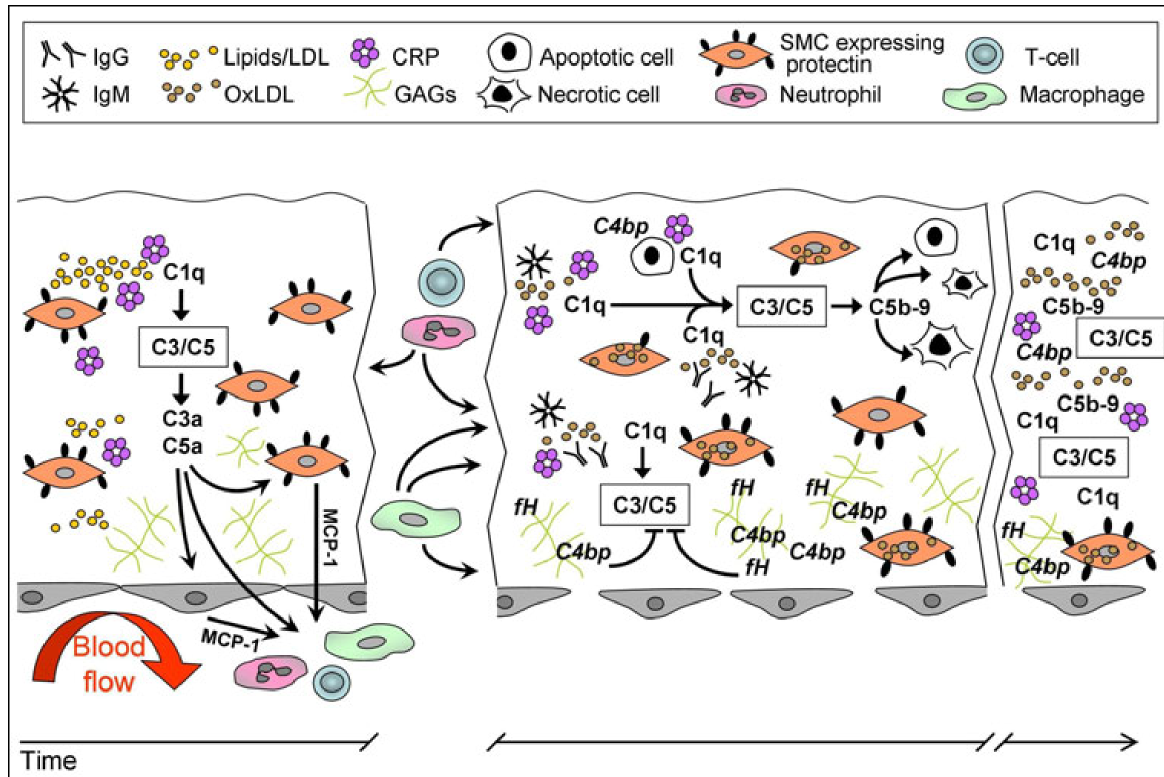

Figure 10. Schematic representation of a possible role of complement activation in the induction of intracranial aneurysm wall inflammation and degeneration. The shear stress from blood flow leads to endothelial dysfunction, allowing increased plasma material to diffuse into the vascular/aneurysmal wall. The lipids are recognized by C-reactive protein (CRP), leading to complement activation, formation of anaphylatoxins, and chemotaxis for inflammatory cells. These will affect IA wall remodeling and result in formation of proinflammatory epitopes and cell death. The subsequent loss of complement inhibitors and associated cell death at the outer part of the wall enables full complement activation and C5b-9 formation, further enhancing cell death. The luminal part of the IA wall is protected from complement activation by extracellular matrix and glycosaminoglycan (GAG)-bound complement inhibitors, C4b binding protein (C4bp) and factor H (fH), as well as protectin, expressed by smooth muscle cells. Immunoglobulins IgG and IgM, capable of activating complement, accumulate in the IA wall because of endothelial dysfunction. As C4bp binds apoptotic cells, it may accumulate in the outer part of the IA wall together with C5b-9.

IA formation is characterized by the breakdown and loss of IEL (Crompton, 1966; Stehbens, 1989). In atherosclerotic extracranial arteries, IEL seems to function as a barrier that restricts lipid accumulation and associated complement activation in the intima (Ostergaard and Hog, 1985; Oksjoki *et al.*, 2003a; Oksjoki *et al.*, 2007). In IAs, the missing IEL allows the penetration of plasma proteins and lipid particles deeper into the IA wall, seen as lipid and C5b-9 accumulations in the outer part of the IA wall (I-III). Usually, the substances are transported through the vascular wall (Michel *et al.*, 2007). In

endothelial dysfunction, however, the accumulation of lipids and protein aggregates might exceed the capacity of normal clearance mechanisms. Furthermore, due to the function of IEL in extracranial atherosclerotic lesions, the cells exposed to complement activation are predominantly intimal, whereas in IAs, the exposed cells are mainly representative of medial SMCs.

As complement activation becomes more persistent, the relative amount of anaphylatoxins, produced during early complement activation, increases. Anaphylatoxins themselves are chemotactic to inflammatory cells (Klos *et al.*, 2009) and may induce both endothelial cells (Laudes *et al.*, 2002) and myofibroblasts (Helske *et al.*, 2008) to produce MCP-1, which is chemotactic to monocytic inflammatory cells. Anaphylatoxins also induce endothelial cells to express cellular adhesion molecules (Foreman *et al.*, 1996; Albrecht *et al.*, 2004; DiScipio and Schraufstatter, 2007) that aid the inflammatory cells to migrate to the IA wall. Among inflammatory cells, anaphylatoxins can also attract neutrophils. They are short-lived and may after their death, even within hours, release hydrolytic enzymes, proteases, and phospholipases with a tissue-destructive effect. However, probably at this point, the SMCs are still viable enough to express protectin (Meri *et al.*, 1991; Seifert *et al.*, 1992) and DAF (Seifert and Hansson, 1989b) to inhibit terminal pathway complement activation and MAC formation.

Inflammatory cells secrete several proinflammatory cytokines and growth factors that probably induce the histological changes (see Figures 3 and 6), e.g. myointimal hyperplasia and fibrosis, observed in the IA wall. As inflammatory cells express several complement receptors (Morgan and Gasque, 1997), the inflammatory cell responses may be in part be induced by anaphylatoxins and other complement components. The inflammatory cells may also enhance the inflammatory response themselves by expressing MCP-1 and synthesizing complement components (Andrews *et al.*, 1995; Morgan and Gasque, 1997). Migrating monocytes and macrophages also phagocytose the PMNs that have come to the end of their mission.

As IA wall and associated myointimal hyperplasia proceed, the SMCs change their phenotype from the contractile to the synthetic type (Nakajima *et al.*, 2000). They increase the synthesis of matrix (collagens), indicated by the expression of prolyl-4-hydroxylase (Frösen *et al.*, 2004). In fact, complement activation may also be responsible for myointimal hyperplasia (Bohana-Kashtan *et al.*, 2004; Shagdarsuren *et al.*, 2008). The SMCs within the areas of myointimal hyperplasia are probably responsible for the synthesis of glycosaminoglycans seen in the luminal parts of IA walls (III). The arterial glycosaminoglycans bind the soluble complement inhibitors C4bp and factor H, which protect from complement activation in the luminal parts of IAs, similarly as observed in atherosclerotic lesions (Oksjoki *et al.*, 2003a; Oksjoki *et al.*, 2007). For the most part,

C4bp and factor H originate from the circulation, although some local synthesis of complement components may also occur.

At this later stage, the IA wall has probably accumulated many potential complement activators, including immunoglobulins, CRP, and lipids from the circulation (II). In addition, proteins and lipids (II) oxidatively modified by the action of ROS and RNS by macrophages, the main targets of NAbs, can deposit in the IA walls (Chou *et al.*, 2009). All of these factors can activate the classical complement pathway (II). This leads to alternative pathway activation (II) and ultimately to MAC formation (I-III) in areas with limited complement inhibitory capacity (III). This is probably a key factor maintaining inflammation in the IA wall. Ongoing inflammation always increases the probability of formation of PAMPs and epitopes recognized specifically by antibodies formed in response to a prior exposure to an antigen (Chou *et al.*, 2008). Ongoing inflammation and complement activation may predispose to the development of an acquired immune response against a newly formed epitope (Carroll, 2004b; Weyand *et al.*, 2008).

The IA wall is supplied by oxygen, probably through direct diffusion from the luminal side, and from vasa vasori (Takaba *et al.*, 1998) on the adventitial side. As the IA wall grows thicker, the blood supply to the wall by vasa vasori becomes even more important. An experimental occlusion of the vasa vasori leads to necrosis of the outer medial layer (Stefanadis *et al.*, 1995). Interestingly, in a small series reported by Scanarini (Scanarini *et al.*, 1978a), the vasa vasori of ruptured IAs often had partial occlusions of the lumen by probable thrombotic material. The study by Stefanadis suggested that the outer part of the wall, “the watershed area”, was most likely the first to suffer from diminished oxygen and nutrient supply. The distension caused by blood pressure can also weaken the microcirculation of the arterial wall and lead to further local ischemia (Marcus *et al.*, 1985). Ischemia can induce downregulation of complement inhibitors and promote complement activation, as shown in autopsy studies of heart and brain infarctions (Rus *et al.*, 1987; Lindsberg *et al.*, 1996; Väkevä and Meri, 1998). Cell loss may occur in the outer part of the IA wall because of both apoptotic and necrotic cell death (I) and accumulation of cellular debris (I). Thus, the accumulation of C5b-9 in the outer part of the IA wall might in part be secondary to cellular death. Thereby, local hypoxia may be an additional factor leading to cell loss (Inci and Spetzler, 2000) and associated terminal complement activation.

The outer part of the IA wall also lacked expression of protectin (III). SMCs are sensitive to loss of expression of protectin under stress (Capey *et al.*, 2007) or due to secretion of GPI-specific phospholipase D by macrophages (O'Brien *et al.*, 1999). The loss of protectin makes SMCs sensitive to complement-mediated lysis (Capey and van den Berg, 2005; Capey *et al.*, 2007). The formation of MAC has multiple consequences, including an increased Ca^{++} influx and a membrane-destabilizating detergent-like effect,

both of which can contribute to cell activation and death. Besides C4bp and factor H, protectin is also expressed in the luminal parts of the IA wall, limiting terminal complement activation (III). In addition, the accumulation of complement-activating substances in the outer part of the IA wall due to impaired clearance mechanisms (Michel *et al.*, 2007) may amplify complement activation. This may increase the susceptibility of SMCs to death. As C4bp binds to apoptotic cells by protein S, both of these factors are detected in the outer part of the wall (III). NAbs may also have a function in the clearance of apoptotic and cellular debris (Grabar, 1983; Fleming, 2006). In conclusion, complement activation, inflammatory cell infiltration, and cellular death together can create a vicious circle, promoting and sustaining inflammation in the IA wall.

Increased inflammation and cellular death can lead to an imbalance between matrix degradation and synthesis. As a consequence, the IA may rupture when the luminal pressure overcomes the tensile strength of the IA wall. In unruptured IAs, the inflammatory degenerative mechanisms may be compensated to reach a steady state or IA wall degeneration may progress undetectably slowly.

3 Limitations of the study

Investigating the pathobiology of IAs is inherently limited because of the unique nature of sample materials. As IAs are heterogeneous in histology, parallel studies should be performed with the same samples for the most comparable results. However, since the majority of IAs are inherently small, and the size of resected samples is even smaller, sample size restricts the number of serial sections from a single sample. In addition, confirming the results by parallel methods is in many cases impossible because of the limited quantity of the sample. Due to the low number of samples available, the ideal case-control type of study, where aneurysms could be matched by, for instance, size, patient characteristics, and/or present risk factors, is inconceivable.

As IA wall samples are surgical specimens, the IA neck left under the clip is never investigated. Moreover, the smallest IAs are never evaluated, as the size does not allow a sample resection. The sample resection itself causes mechanical injury that may be seen in histology as an artifact at the cutting edge. Having normal cerebral artery as a control tissue would answer many methodological and interpretation-related questions. However, obtaining an adequate control tissue as a surgical specimen is ethically impossible. The use of extracranial arteries as a control tissue is not the best option because of inherent histological differences.

Although a case-control study allows relationships to be evaluated, it does not provide data on the causality and timing of certain phenomena. Demonstrating causalities

would necessitate an animal study. However, no animal model for experimental IA where the IA degenerates and ruptures due to chronic inflammation (as in humans) is yet available. Unfortunately, surgically created or elastase-induced saccular arterial pouch models do not provide an environment sufficiently similar to human IAs. Hypertension-induced experimental IAs have some potential and similarities, but are too small for applications other than systemic pharmaceutical manipulations.

Immunohistochemistry is a great method when adequately controlled. It can demonstrate the presence of a given molecule and its location within the tissue sample. However the exact binding site or localization within the mesh of structural proteins, glycoproteins, and cells cannot be analyzed. In addition, with a preceding low-grade complement activation, whether the formed complex is a fully assembled MAC with polymeric C9 or a terminal complex without poly-C9 cannot be assessed.

Subarachnoidal hemorrhage as a potential source of artifact

Rupture of the IA itself and the subsequent SAH may induce complement activation, and complement activation has been found to be associated with IA rupture. Thus, the question arises whether complement activation is a cause or a consequence of IA rupture. That complement activation products accumulate already in unruptured IAs suggests that complement activation might contribute to ruptures.

The following findings indicate that the complement activation seen in ruptured IAs has already occurred prior to the rupture. First, the unruptured IAs also show relatively heavy accumulation of C3b/iC3b and C3d, suggesting prolonged inflammation. Second, the magnitude of C5b-9 accumulation in the IA wall is independent of the time from rupture to sample collection, and complement activation has also been observed in all unruptured IAs (I-III). Third, the heavier inflammatory cell infiltrations into the ruptured IA walls occur before the rupture (Kataoka *et al.*, 1999; Frösen *et al.*, 2004). Fourth, MAC and the preceding C5b-8 complex are such large protein aggregates (molecular weight approximately one million) (Bhakdi and Tranum-Jensen, 1981) that their diffusion into the IA wall from the cerebrospinal fluid or circulation seems unlikely. Fifth, no difference has been seen in complement activation between samples collected within or after 48 hours of the aneurysm rupture (Peterson *et al.*, 1989; Park *et al.*, 1997) (I). Collectively, these facts indicate that complement activation is part of an inherent process involved in IA progression, tissue weakening, and rupture.

4 Future perspectives

Unruptured IAs may remain intact for years and most may not rupture at all (Juvela, 2002). To direct the prophylactic but invasive treatment to rupture-prone IAs, they must first be identified. Since the presence of inflammation or other histopathological features is related to the probability of IA rupture, the ability to image inflammation (molecular imaging) in unruptured IAs could aid the clinical decision of IA closure and rupture-prevention by invasive treatment methods. In addition, as targeted pharmaceutical therapies become more feasible, the improvements may provide mechanisms to treat IAs in order to strengthen the wall and to slow down IA wall degeneration. These would stabilize the IA wall and support the intrinsic mechanisms to improve healing of the aneurysmal lesion.

4.1 Imaging inflammation

The clinical appearance of IA can already to some extent be correlated with histological changes of the IA wall (Asari and Ohmoto, 1994). However, as the inflammatory mechanisms in IAs are delicate, the imaging should be able to reach a resolution where differentiation of the exact phenotype of the cellular infiltration is possible.

No reports of imaging of inflammation in human IAs have thus far been published. However, DeLeo *et al.*, (2009) managed to image bacterial lipopolysaccahride-induced inflammation in an elastase-induced rabbit experimental IA model. They were able to detect macrophages and neutrophils in the IA wall using a myeloperoxidase-specific magnetic resonance contrast agent that has previously been used for the visualization of myocardial I/R injury in mice (Nahrendorf *et al.*, 2008) and atherosclerotic plaques in rabbits (Ronald *et al.*, 2006). Imaging of vascular inflammation has mainly been studied in humans in atherosclerotic plaques and vasculitis. Radiolabeled 18-F-fluorodeoxyglucose recognizes metabolically active macrophages and distributes the vulnerable and stable plaques (Rudd *et al.*, 2002) in positron emission tomography. The signal intensity correlates with the density of activated CD68-positive macrophages (Tawakol *et al.*, 2006) in carotid plaques and AAAs (Reeps *et al.*, 2008). The macrophage phagocytic activity can be detected and verified by ultrasmall superparamagnetic iron oxide particles (USPIOs) and imaged with T2-weighted magnetic resonance sequences for human carotid plaques (Trivedi *et al.*, 2004; Tang *et al.*, 2009). Radiolabeled Annexin V detects phosphatidylserine on apoptotic cells and has been successfully used to image the extent of apoptosis in experimental aortic atherosclerotic plaques in rabbits (Kolodgie *et al.*, 2003) as well as in human carotid arteries (Kietselaer *et al.*, 2004) in single photon emission tomography. As the imaging is strongly dependent on the technology,

advancements in imaging technology and utilization of new technologies in clinical use, e.g. MRI with higher Teslas, will improve imaging possibilities in the future.

4.2 Improvements in treatment methods

The only IA treatment methods currently available are clipping and endovascular therapy with coils or other embolization materials. However, these treatment methods are invasive and somewhat risky (Molyneux *et al.*, 2002). For patients in need of treatment of high-risk unruptured IAs or IAs, which have already ruptured, improvements in coil technology, e.g. coated coils, would enable more effective and permanent treatment results (Lanzino *et al.*, 2005). To date, coils releasing different growth factors have been tested in animal models with varying results. The local release of bFGF induces proliferation in the wall in the rat common carotid artery (Matsumoto *et al.*, 2003). bFGF also causes fibrosis and obliteration of rabbit venous-pouch and elastase-induced aneurysms (Hong *et al.*, 2001; Tsumoto *et al.*, 2007). The local release of VEGF enhances vascular wall thickness, endothelium formation, cell infiltration, lumen reduction, and fibrotization of ligated common carotid arteries in rats (Abrahams *et al.*, 2001; Ohyama *et al.*, 2004). TGF-β, by contrast, has not shown any improvement in long-term occlusion of rabbit elastase-induced aneurysms (de Gast *et al.*, 2001) or in a canine model (Ribourtout *et al.*, 2003).

However, it should be noted that although the surgically created aneurysm models commonly used in testing of different embolization materials may resemble human IAs in structure, the inflammatory milieu might differ. Also, as many human IAs are extensively decellularized, the basis for growth factor-aided occlusion of aneurysm sacks differs from animal models. Thus, the efficiency of such treatments warrants further investigations.

4.3 Systemic pharmacological therapy

Since inflammation can be restricted by several pharmaceuticals, an intriguing idea is to diminish progressive IA wall degeneration and transform it into a steady-state condition by pharmaceutical therapy. This may prevent IA rupture. Thus far, only a few experimental animal studies of the effect of systemic pharmaceutical treatment on the incidence and progression of IAs have been performed.

Aoki *et al.* (2008c; 2009a) have employed an experimental IA model in the rat. They showed that statins (simvastatin and pitavastatin) and HMG-CoA (3-hydroxy-3-methyl-glutaryl-coenzyme A) reductase inhibitors prevented IA progression (growth). MCP-1, VCAM-1, IL-1β, iNOS, and MMP-9 expressions were reduced through inhibition of NFκB activation by pitavastatin. On the other hand, doxycycline, a nonspecific MMP inhibitor, did not affect the rate of experimental IA formation in rats (Kaufmann *et al.*, 2006). Edavarone, a synthetic free-radical scavenger, inhibited experimental IA formation

and decreased MCP-1, VCAM-1, and MMP-2 expression (Aoki *et al.*, 2009c). Nifedipine, a calcium antagonist, inhibited DNA binding of NFκB, leading to a reduced size and thickened media of experimental IAs. It also prevented the progression of IA wall degeneration and enlargement of pre-existing IAs. Moreover, the expression of MCP-1 and MMP-2 was decreased (Aoki *et al.*, 2008b).

In addition to pharmaceutical treatment options, one could decrease chemotactic and proinflammatory signaling to prevent leukocyte infiltration. However, none of the therapies has prevented IA formation in general. As IA wall degeneration and the role of inflammation in the process are complicated, many possible treatment targets exist. Although the biology of human IA progression might be more complicated than the progression of experimental IAs, the results to date are promising and warrant further attempts to refine anti-inflammatory treatments.

Anti-complement therapy

As activation of the complement system has harmful effects in many diseases, strategies for systemic complement inhibition have been investigated widely in animal models (D'Ambrosio *et al.*, 2001; Acosta *et al.*, 2004; Arumugam *et al.*, 2009). Administration of C1-INH has yielded promising results in neuroprotection, whereas effects with soluble CR1 have been modest. The impact of specific antibodies against different complement components has also been investigated in rodents and humans (Fitch *et al.*, 1999). However, biological therapies are fairly expensive and likely would not offer a cost-effective option for long-term treatment. Moreover, since the complement system is an important defense mechanism against microbes, long-term complement depletion could lead to an increased risk of infections. However, as complement activation in IAs seems to be a local process, local administration of a complement inhibitory agent would probably be beneficial. Nevertheless, since complement might also have a role in the remodeling response, complement inhibitors cannot be considered a treatment option until the postulated positive effects of complement inhibition on IA degeneration have been carefully verified in experimental models.

VI SUMMARY AND CONCLUSIONS

Intracranial artery aneurysms (IAs) are estimated to be present in 2.3% of the population. A rupture of an IA causes subarachnoid hemorrhage, with up to 50% mortality. The annual low rupture risk of an IA indicates that most IAs never rupture. As IAs susceptible to rupture cannot be identified, unruptured IAs are also treated in order to decrease the risk of rupture. The current treatment options are invasive and somewhat risky.

The ultimate mechanisms behind the structural weakening and rupture of IA are unknown. The classical IA risk factors of smoking, hypertension, age, female gender, and alcohol abuse only partly explain the IA pathobiology. Inflammatory cell infiltrations have been found in IAs, indicating the role of inflammation in IA wall degeneration and rupture.

As the complement system is an important part of innate immunity, it may have a pivotal role in mediating the inflammatory reaction in the IA wall. Complement components have previously been shown in IA walls. In this study, the presence of complement activation in unruptured and ruptured IAs, complement activation mechanisms, and regulation of complement activation were analyzed. The association of complement activation with other histological and clinical parameters was also evaluated.

Terminal pathway complement activation was found universally in all IAs, both unruptured and ruptured ones. This indicates a role for complement activation already at an early phase of IA progression. Terminal complement activation was observed in larger areas in more degenerated IA walls and in ruptured IAs. This suggests a critical role for complement activation in IA wall degeneration and rupture.

The terminal complement complex was localized especially in the outer part of the wall in areas with cellular debris due to apoptotic and necrotic cell death. Thus, complement activation probably has a role in the induction of cellular death.

The complement system was observed to become activated by the classical pathway and to recruit alternative pathway amplification. This indicates the presence of specific complement activators in IA walls. Of the potential complement activators, immunoglobulins G and M, C-reactive protein (CRP), and oxidatively modified lipids were present in wide areas of the IA wall. This suggests that multiple activators contribute to complement activation. The occurrence and accumulation of activators could be due to endothelial dysfunction and impaired clearance mechanisms. The finding of lipids colocalizing with the terminal complement complex and CRP implicates their involvement in terminal complement activation, possibly due to oxidative modification.

The luminal part of the IA wall was protected from terminal complement activation by C4b binding protein and factor H, probably bound to arterial

glycosaminoglycans. Protectin (CD59), expressed by mural cells, has an important function in preventing the membrane attack complex (MAC) attack. The outer part of the IA wall lacked protectin, primary or secondary to cell death, allowing terminal pathway complement activation and MAC assembly. The outer part of the wall also lacked factor H, probably due to lack of factor H binding glycoproteins or glycosaminoglycans. Instead, the C4b binding protein, which binds to apoptotic cells through the anticoagulant protein S, was present.

Complement activation was associated with infiltrations of CD163-positive macrophages and T-lymphocytes, indicating a probable interaction of complement with these cells. The factor H single-nucleotide polymorphism Y402H was associated with neither the presence of IAs nor IA rupture, suggesting that it does not have a significant role in predisposition to IA wall pathobiology.

Our findings indicate that chronic inflammation is present in IA walls prior to IA rupture, in a manner comparable with other chronic inflammatory diseases. The accumulation of complement-activating molecules and material in the IA wall might be due to disturbances and possible overloading in wall clearance mechanisms. Since complement has an important role as a proinflammatory mediator when fully activated, it is strictly regulated in viable tissues by soluble and membrane-bound complement inhibitors.

Combining data from experimental IA studies in animals and studies of human IAs reveals that IAs seem to form and grow in response to hemodynamic stress and a proinflammatory milieu that recruits macrophages and other inflammatory cells. An underlying condition would be endothelial dysfunction. Based on the presence of complement activation in IAs, the activated complement system may actually mediate these reactions. The complement system may also have a more general role in IA wall remodeling. The modulation of IA wall structures by inflammatory cells may lead to the formation of immunogenes, which in turn can trigger complement-mediated inflammation.

An IA wall will rupture when the intraluminal pressure overcomes the wall tensile strength, a result of a degenerative net effect of cell death and decreased matrix turnover. This could be due to the insufficient compensatory mechanisms favored by inflammation. Among unruptured IAs, it is unknown whether the inflammatory degenerative mechanisms have been totally compensated to a steady-state situation or whether the IA wall degeneration is progressing undetectably slowly.

Complement activation may also have different functions in the IA wall at different time-points during IA progression. This was suggested by the complement system being differentially activated in unruptured and ruptured IAs.

Since all evidence points to a major role of inflammation, partly due to activation of the complement system, in the degenerative processes leading to IA rupture, anti-

inflammatory therapy could provide a tempting option for stabilizing the IA wall to prevent IA rupture. However, the new treatment options have been tested in experimental IAs only. Human IAs may have additional pathobiological mechanisms in IA formation, growth, and/or rupture, and caution is therefore needed in extrapolating these findings to human IAs. Nevertheless, advancements in (molecular) imaging and more sophisticated endovascular treatment methods (coated coils and stents) open new avenues for future IA diagnostics and treatment.

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