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Intracellular Signaling Pathways in Human Intracranial Aneurysms

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ACADEMIC DISSERTATION

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List of original publications

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

I. Laaksamo E, Tulamo R, Baumann M, Dashti R, Hernesniemi J, Juvela S, Niemelä M, Laakso A. Involvement of mitogen-activated protein kinase signaling in growth and rupture of human intracranial aneurysms. *Stroke* 39:886-892, 2008.

II. Laaksamo E, Ramachandran M, Frösen J, Tulamo R, Baumann M, Friedlander RM, Harbaugh RE, Hernesniemi J, Niemelä M, Raghavan ML, Laakso A. Intracellular signaling pathways and size, shape, and rupture history of human intracranial aneurysms. *Neurosurgery* 70:1565-1572, 2012.

III. Laaksamo E, Tulamo R, Liiman A, Baumann M, Friedlander RM, Hernesniemi J, Kangasniemi M, Niemelä M, Laakso A, Frösen J. Oxidative stress is associated with cell death, wall degradation, and increased risk of rupture of the intracranial aneurysm wall. *Neurosurgery* 72:109-117, 2013.

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Abbreviations

3D	Three-dimensional
α SMA	alpha-smooth muscle actin
AAA	Abdominal aortic aneurysm
ADPKD	Autosomal dominant polycystic kidney disease
Bad	Bcl-associated death protein
BSA	Bovine serum albumin
CFD	Computational fluid dynamics
CREB	Cyclic AMP response element binding protein
CT	Computer tomography
CTA	Computer tomography angiography
DAMP	Danger-associated molecular pattern
DSA	Digital subtraction angiography
ECM	Extracellular matrix
ERK	Extracellular signal-regulated kinase
FASL	FAS ligand
FKBP12	FK506 binding protein
GWAS	Genomic-wide association study
H ₂ O ₂	Hydrogen peroxide
Hb	Hemoglobin
HO-1	Heme oxygenase-1
Hp	Haptoglobin
IA	Intracranial aneurysm
IEL	Internal elastic lamina
ILT	Intraluminal thrombus
ISUIA	International Study of Unruptured Intracranial Aneurysms
JNK	c-Jun N-terminal kinase
LDL	Low-density lipoprotein
MAC	Membrane attack complex
MAPK	Mitogen-activated protein kinase
MCA	Middle cerebral artery

MMP	Matrix metalloproteinase
MOMP	Mitochondrial outer membrane permeabilization
MPO	Myeloperoxidase
MRA	Magnetic resonance angiography
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
mTORC	mTOR complex (e.g. mTORC1)
NGS	Normal goat serum
O ₂ ⁻	Superoxide radical
oxLDL	Oxidized low-density lipoprotein
p38	p38 mitogen-activated protein kinase
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PI	Phosphatidylinositol
PMN	Polymorphonuclear leukocyte
RBC	Red blood cell
ROS	Reactive oxygen species
RT	Room temperature
SAH	Subarachnoid hemorrhage
SDS	Sodium dodecyl sulphate
SMC	Smooth muscle cell
SNP	Single-nucleotide polymorphism
STA	Superficial temporal artery
TBST	Tris-buffered saline with Tween-20
TNF α	Tumor necrosis factor alpha
TUNEL	Terminal deoxynucleotidyl transferase (dUTP) nick end labeling
VEGF	Vascular endothelial growth factor
VMTK	Vascular Modeling Toolkit
VSMC	Vascular smooth muscle cell
WB	Western blotting
WSS	Wall shear stress

Abstract

Background and purpose

Intracranial aneurysm (IA) rupture causes subarachnoid hemorrhage (SAH), which has high mortality and morbidity. Treatment methods for IAs (microneurosurgical clipping and endovascular coiling) are invasive and may carry considerable risks. Some predisposing factors for IA rupture, such as smoking, hypertension, and large IA size, are known. However, these factors do not differentiate rupture-prone IAs sufficiently well. Better predictors for IA rupture and less invasive treatment methods for IAs are required. An IA wall shows signs of vascular remodeling, and the wall degeneration is associated with IA rupture. Loss of mural cells seems to be a crucial step in wall degeneration. Despite previous research on IA pathobiology, the cell signaling pathways involved in wall remodeling and the mechanisms of cell death in IA wall remain largely unknown. This study aimed to characterize cell signaling pathways associated with IA rupture and size and shape indices at the protein level as well as to determine possible cell death pathways in the IA wall.

Materials and methods

The localization of signaling molecules (c-Jun N-terminal kinase (JNK), p38, extracellular signal-regulated kinase (ERK), Akt, mammalian target of rapamycin (mTOR), cyclic AMP response element binding protein (CREB), and Bcl-associated death protein (Bad)) was studied using immunofluorescence staining in ruptured and unruptured IA samples, collected during microneurosurgical clipping. The levels of phosphorylated and total forms of signaling molecules were determined semi-quantitatively from IA tissue homogenates using Western blotting (WB). Altogether, 40 ruptured and 37 unruptured IA samples were investigated. IA size and shape indices were determined using 3D remodeling, and the associations between the levels of signaling molecules and IA rupture status and morphology were analyzed. The JNK signaling pathway was characterized further with c-Jun and pro-MMP-9 (MMP, matrix metalloproteinase) WB. Cell death in the IA wall was studied using terminal deoxynucleotidyl transferase (dUTP) nick end labeling (TUNEL) and cleaved caspase-3 immunostaining and caspase-3 WB. The activation of caspase cascades (caspase-9, intrinsic apoptosis; caspase-8, extrinsic

apoptosis) was investigated using WB. The role of oxidative stress in IA wall degeneration was studied using heme oxygenase-1 (HO-1). Potential sources of oxidative stress were revealed using endogenous peroxidase stainings.

Results

All signaling molecules (JNK, p38, ERK, Akt, mTOR, CREB, and Bad) were found in the IA wall in immunofluorescence staining. Stress-activated kinases (phospho-p38 and phospho-JNK) were associated with size and rupture, probably indicating that stress signaling contributes to IA growth and rupture. Pro-MMP-9 levels were increased in ruptured IAs. Of well-known growth-promoting factors, phospho-Akt was associated with size, but not with rupture, indicating active cell proliferation in large IAs. On the other hand, phospho-mTOR levels were downregulated in ruptured IAs suggesting that cell proliferation signaling is decreased in ruptured IAs. Phospho-CREB levels were associated with the non-sphericity and ellipticity indices, whereas a negative association between total CREB levels and the undulation index was observed. On the other hand, phosphorylated levels of p38 and Akt correlated negatively with the undulation index, and total levels of CREB correlated positively with size indices, suggesting a complex role of signaling molecules in IA remodeling. Furthermore, TUNEL staining was associated with rupture. A few cleaved caspase-3-positive cells were also detected. Intrinsic activation of apoptosis (cleaved caspase-9) was observed in WB, mainly in ruptured samples, but cleaved caspase-8 was not detected. Unphosphorylated Bad levels, a regulator of the intrinsic apoptotic pathway, were also associated with rupture. HO-1 was associated with wall degeneration and rupture, and endogenous peroxidase staining localized mainly in polymorphonuclear cells of the intraluminal thrombus.

Conclusions

This study revealed associations of several intracellular signaling molecules with IA morphology and rupture and also suggested oxidative stress as an important factor in the degeneration of an IA wall. Characterization of signaling pathways in the human IA wall may aid in finding biomarkers differentiating rupture-prone IAs and in developing less invasive treatment methods for IAs. However, the role of signaling pathways in IA growth and rupture must first be evaluated with appropriate experimental models.

1 Introduction

Intracranial aneurysms (IAs) are dilatations of cerebral arteries (Figure 1A). IA rupture causes subarachnoid hemorrhage (SAH), which is an arterial bleeding to the subarachnoid space (Figure 1B). Sometimes intracerebral hemorrhage may also occur. SAH is a life-threatening condition. Typical symptoms are sudden headache with nausea and vomiting and often loss of consciousness. SAH may lead to death or to severe neurological defects.

IAs mainly occur in the bifurcations of the vessels near the circle of Willis, which is an arterial circle supplying blood to the brain. IAs are divided into saccular and fusiform subtypes according to their shape and relation to the parent artery. This study deals with saccular IAs (Figure 1A).



Figure 1 *Saccular intracranial aneurysm (A) and subarachnoid hemorrhage (B). Computer tomography angiography image of a typical saccular intracranial aneurysm (A). In the computer tomography image (B), blood is seen in the subarachnoid space (black arrow) as a result of intracranial aneurysm rupture.*

IAs are regarded as acquired rather than congenital lesions. Risk factors for IA development and rupture are female gender, smoking, and hypertension. However, the pathobiology of IAs is poorly known. Wall degeneration, characterized by loss of mural

1 Introduction

cells and inflammatory cell infiltration, is associated with IA rupture (Kataoka et al. 1999, Frösen et al. 2004, Tulamo et al. 2006, Frösen et al. 2012).

Vascular remodeling is an important process in many vascular diseases. The trigger may be, for instance, abnormal hemodynamic stress or oxidative stress. In vascular remodeling, the cells may start to proliferate, change their phenotype, migrate, or die (Newby et al. 2000, Michel et al. 2007). Many intracellular pathways are associated with vascular remodeling. Stress-activated kinases (c-Jun N-terminal kinase (JNK) and p38) belonging to the family of mitogen-activated protein kinases (MAPKs) respond to cellular stress and inflammatory cytokines (Kyriakis et al. 2001). Akt transmits cell survival signaling (Morello et al. 2009). The mammalian target of rapamycin (mTOR) inhibitor, sirolimus, prevents the proliferation of smooth muscle cells (SMCs) and is used in coronary stents to inhibit restenosis (Poon et al. 2002). Cyclic AMP response element binding protein (CREB) regulates several important functions of the cell such as proliferation and differentiation (Ichiki 2006). Cell death is divided into apoptosis and necrosis. Apoptosis is a programmed cell death that involves – in most instances – the activation of caspases. Nowadays, necrosis is also considered a “regulated” cell death (Galluzzi et al. 2012). However, the triggers and mediators of necrosis differ from those of apoptosis.

Apoptosis has been detected in the IA wall (Pentimalli et al. 2004). However, the intracellular signaling pathways mediating the vascular remodeling in the IA wall are unknown. This study focuses on intracellular signaling pathways and possible mechanisms of cell death in the IA wall. Knowledge of cell signaling mechanisms in the IA wall at the molecular level may help to uncover clues for differentiating rupture-prone IAs and for developing new pharmaceutical therapies.

2 Review of the literature

2.1 Intracranial aneurysms (IAs) and subarachnoid hemorrhage (SAH)

2.1.1 Epidemiology

IAs are saccular outbulgings of the cerebral artery. They are acquired – not congenital – lesions (Stehbens 1989). Based on radiological imaging and pathological examination, the prevalence of IAs has been estimated to be approximately 2% (0.4-6.0%) (Rinkel et al. 1998, Ronkainen et al. 1998, Vlak et al. 2011). In patients with IA, up to one-third have multiple IAs (Rinne et al. 1994). Rupture of IA causes SAH, and sometimes also bleeding into the brain parenchyma. The incidence of aneurysmal SAH is approximately 9-11 per 100 000 person-years in most Western populations, but is twofold in Finland (19.7-22.5 per 100 000) and in Japan (22.7 per 100 000) (Ingall et al. 2000, de Rooij et al. 2007). SAH accounts for about 5% of all strokes. Despite modern intensive care, SAH leads to significant mortality (30-40%) and morbidity (20% require help in daily activities) (Nieuwkamp et al. 2009). A significant proportion of SAH patients are of working age, which causes a loss of productive life-years comparable to that of ischemic stroke (Johnston et al. 1998, Nieuwkamp et al. 2009). The annual rupture rate of IAs has been reported to be 0.05-1.9% (ISUIA 1998, Rinkel et al. 1998, Juvela et al. 2000), being higher in patients with larger IAs and previous SAH (Wiebers et al. 2003, Ishibashi et al. 2009).

2.1.2 Risk factors for IA rupture

The relatively high prevalence of IAs as compared with the incidence of aneurysmal SAH suggests that not all IAs rupture during the lifetime of their carriers. This epidemiological finding has raised the question of which kinds of IAs are prone to rupture. The risk factors for having an IA partly differ from the risk factors for an SAH. On the other hand, the rupture risk of IAs varies between patients, but also between IAs of the same patient. Risk factors for both formation and rupture of IAs are discussed below.

2.1.2.1 Aneurysm-related risk factors

Location

The majority of IAs (80-90%) are located in the anterior circulation (Schievink 1997b, Rinkel et al. 1998), but posterior circulation IAs rupture more easily (Rinkel et al. 1998, Wiebers et al. 2003, Ishibashi et al. 2009).

Size

IA size is an important risk factor for rupture (ISUIA 1998, Wiebers et al. 2003). In the retrospective part of the International Study of Unruptured Intracranial Aneurysms (ISUIA), the cumulative rupture rate of IAs with diameter smaller than 10 mm was less than 0.05% per year in patients without previous SAH, whereas the rupture rate for IAs with diameter exceeding 10 mm approached 1% per year (ISUIA 1998). In the large prospective cohort of ISUIA, IAs smaller than 7 mm in the anterior circulation in patients with no previous SAH did not rupture in the 5-year follow-up (Wiebers et al. 2003). Rupture rate increased with increasing IA size (Wiebers et al. 2003). In the same study, the 5-year cumulative rupture rate for giant IAs (diameter 25 mm or greater) was 40% in the anterior circulation and 50% in the posterior circulation and the posterior communicating artery (Wiebers et al. 2003). Other studies have also reported a correlation between IA size and rupture risk (Rinkel et al. 1998, Juvela et al. 2000, Morita et al. 2005, Ishibashi et al. 2009). However, a significant proportion of ruptured IAs is smaller than 7 mm (Juvela et al. 2000, Forget et al. 2001).

Morphology

Because size and location do not predict IA rupture alone, other risk factors for rupture have been sought. Higher aspect ratio (IA fundus length/neck diameter) has been associated with rupture (Ujiie et al. 2001, Weir et al. 2003, Nader-Sepahi et al. 2004, Hoh et al. 2007, Sadatomo et al. 2008), with a few exceptions (Beck et al. 2003). It is not clear what the threshold value is for aspect ratio, but aneurysms with an aspect ratio > 3 are at a high risk of rupture, whereas an aspect ratio < 1.4 predicts low rupture risk (Lall et al. 2009).

2 Review of the literature

Irregular multilobular appearance and secondary pouches have also been associated with ruptured IAs (Crompton 1966, Sampei et al. 1991, Asari et al. 1993, Hademenos et al. 1998, Beck et al. 2003).

Raghavan et al. studied various size and shape indices in three-dimensional (3D) reconstructions to determine which best discriminates between ruptured and unruptured IAs. They found that quantified shape (especially non-sphericity index, undulation index, and ellipticity (ellipticity index and aspect ratio)) better differentiated ruptured IAs from unruptured lesions than size (Figure 2) (Raghavan et al. 2005).

The effect of relationship of the aneurysm to its parent artery as a predictor for rupture has also been studied. Sadatomo et al. investigated the relationship of IA neck, parent artery, and daughter branches and found that when IA neck is an extension of the midline of the parent artery and the diameters of daughter arteries are equal the IAs are more often ruptured (Sadatomo et al. 2008). Dhar et al. noted that aneurysm-to-vessel size ratio and aneurysm inclination angle with respect to the parent artery had the strongest correlation with rupture risk, but also aspect ratio, ellipticity index, non-sphericity index, and undulation index differed between ruptured and unruptured IAs (Dhar et al. 2008). Hassan et al. reported that IAs with an aspect ratio > 1.6 and either a sidewall or sidewall with branching vessel-type tend to be ruptured (Hassan et al. 2005).

Xiang et al. studied both morphological indices and hemodynamic parameters and found that size ratio, average wall shear stress (WSS), and oscillatory shear index differentiate IA rupture status (Xiang et al. 2011).

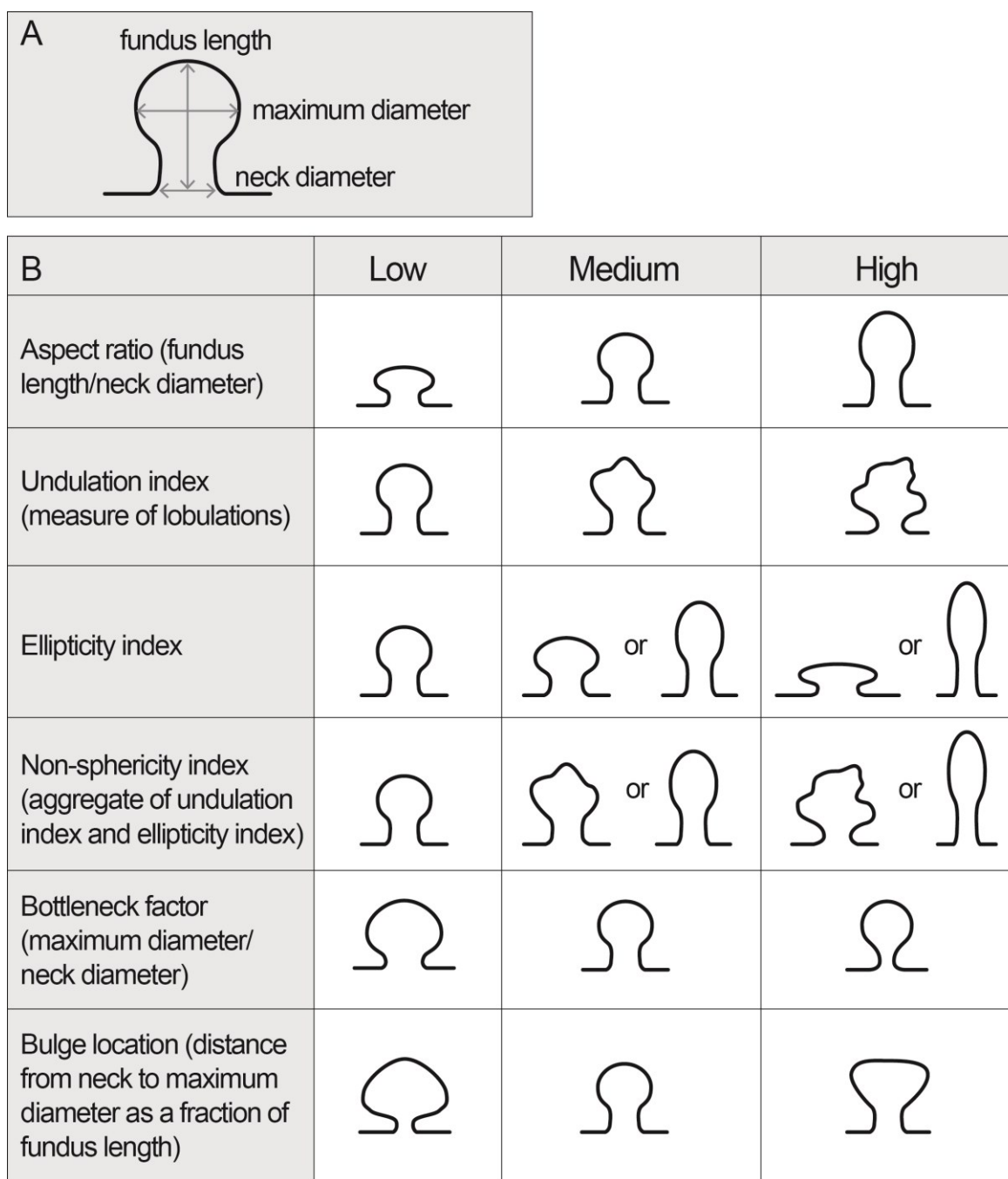


Figure 2 Selected size indices of intracranial aneurysm pouch (A) and schematic illustrations of shape indices, according to Raghavan et al. (2005) (B).

2.1.2.2 Patient-related risk factors

Age

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). The annual risk of IA rupture is approximately

1.3% (Juvela et al. 2000). Thus, the cumulative incidence of SAH is most significant for younger IA patients (Juvela et al. 2000).

Gender

Female gender is a risk factor for IA formation and growth (Juvela et al. 2001), but also for SAH, especially after the age of 55 years (Rinkel et al. 1998, de Rooij et al. 2007, Sandvei et al. 2009, Korja et al. 2011). Hormone replacement therapy has been associated with a lower risk of SAH (Longstreth et al. 1994, Mhurchu et al. 2001). The effects of hormonal factors on the vascular wall, especially the effect of estrogen, have been suggested to explain the gender differences (Harrod et al. 2006).

Smoking

Cigarette smoking increases the risk for both IA formation and growth as well as for IA rupture (Juvela et al. 2000, Juvela et al. 2001, Isaksen et al. 2002, Sandvei et al. 2009, Korja et al. 2011). Cigarette smoke has several possible harmful effects on the vascular wall (Pipe et al. 2010), although the exact mechanism of how smoking impairs the function of the vascular wall is unknown.

Hypertension

Hypertension is associated with the prevalence of IAs (Taylor et al. 1995) as well as with SAH (Bonita 1986, Knekt et al. 1991, Isaksen et al. 2002, Sandvei et al. 2009). Recently, in two prospective studies, the joint effect of current smoking and hypertension on the risk of SAH was stronger than the sum of the independent effects (Korja et al. 2011, Lindekleiv et al. 2012).

Other

Alcohol consumption does not seem to be associated with IA formation or growth (Juvela et al. 2000, Juvela et al. 2001). However, excessive drinking may cause the rupture of a pre-existing IA (Juvela et al. 1993). Drinking more than five cups of coffee a day was an independent risk factor for SAH in one case-control study (Isaksen et al. 2002). Hypercholesterolemia is a well-known cardiovascular risk factor (deGoma et al. 2012),

but serum cholesterol levels do not appear to be associated with SAH (Knekt et al. 1991, Isaksen et al. 2002).

2.1.2.3 Family history and genetics

The prevalence of IAs is higher in patients with heritable connective tissue disorders like autosomal dominant polycystic kidney disease (ADPKD), Ehlers-Danlos syndrome type IV, and neurofibromatosis type 1 (Schievink et al. 1994, Schievink 1997a, Rinkel et al. 1998). However, these heritable diseases are rare, comprising a minority of all IAs. Unlike previously thought, Marfan syndrome is not associated with increased prevalence of IAs (Conway et al. 1999).

Family history is a significant risk factor for both IAs and SAH. In a family with two or more affected members, the prevalence of carrying IA is as high as 8.7% (Ronkainen et al. 1997), and the risk of aneurysmal SAH is significantly increased (Bor et al. 2008).

The association of IAs with heritable diseases and the findings of epidemiological studies have raised the question about the role of genetics in the development and rupture of IAs. However, a large twin study showed only a moderate role of genetic factors in the etiology of SAH, suggesting that most of the familial SAHs are due to the familial clustering of such confounding risk factors as smoking and hypertension (Korja et al. 2010).

Nevertheless, the genetic background of IAs has been widely investigated. Genetic studies started with a functional candidate-gene approach (Ruigrok et al. 2005). This method requires knowledge of the disease mechanisms and it ignores genes that are potentially important in the disease process, but involved in unknown pathways (Ruigrok et al. 2005, Ruigrok et al. 2008). Genome-wide studies allow a hypothesis-free approach. The most promising loci of genome-wide linkage studies are 1p34.3-p36.13, 7q11, 19q13.3, and Xp22 because these findings have been replicated in different study populations (Ruigrok et al. 2008). The first genomic-wide association study (GWAS) of Finnish, Dutch, and Japanese cohorts identified common SNPs on chromosomes 2q, 8q, and 9p that are associated with IAs (Bilguvar et al. 2008). The follow-up GWAS identified three new loci, namely 18q11.2, 13q13.1, and 10q24.32, strongly associating with IAs, and confirmed the previous associations with 8q11.23-q12.1 and 9p21.3

(Yasuno et al. 2010). Further studies have shown a significant association of SNPs on chromosomes 4q31.23 and 4q31.22 near the endothelin receptor type A gene with IA (Yasuno et al. 2011, Low et al. 2012). Yasuno et al. also found a suggestive IA risk locus at 5q23.2 (Yasuno et al. 2011). Further, Gaál et al. described an association between Putative histone-lysine N-methyltransferase PRDM6 gene at 5q23.2 and systolic blood pressure (Gaál et al. 2012).

2.1.3 Diagnostics and current treatment options

2.1.3.1 Ruptured IAs and SAH

SAH is often characterized by sudden headache. Other symptoms of IA rupture may be vomiting, seizures, depressed consciousness, neck stiffness, and/or focal neurological defects. SAH is diagnosed by computer tomography (CT), magnetic resonance imaging (MRI), or lumbar puncture. IA is identified using computer tomography angiography (CTA), magnetic resonance angiography (MRA), or digital subtraction angiography (DSA). The anatomical configuration of IA and the adjoining vessels are also visualized using angiographic imaging, which facilitates choosing the optimal treatment method for the IA (van Gijn et al. 2007). Without intervention, the cumulative risk of rebleeding is approximately 40% in the first three weeks (Brilstra et al. 2002). The current treatment methods to prevent re-bleeding are microneurosurgical clipping and endovascular occlusion of IA using detachable coils. Both methods are invasive and have a risk of morbidity and even mortality (Vanninen et al. 1999, Molyneux et al. 2005, van der Schaaf et al. 2005, Raja et al. 2008).

2.1.3.2 Unruptured IAs

Nowadays, a growing number of unruptured IAs are incidentally found in patients imaged for reasons other than IAs or when families with a known history of SAH are screened. The current intervention options for unruptured IAs to prevent IA bleeding are the same as for ruptured IAs (clipping and coiling) – both invasive and somewhat risky (Wiebers et al. 2003, Niskanen et al. 2005, Raja et al. 2008). However, it is known that not all IAs rupture

during the life-time of their carrier, and neither the safety nor the effectiveness of the prophylactic treatment of unruptured IAs has been tested in randomized controlled trials (Raymond et al. 2011). Better predictors are needed to differentiate rupture-prone IAs in order to concentrate prophylactic treatment efforts on those patients most at risk. One part of this endeavor can be research on IA pathogenesis in the hope of finding biomarkers predicting a high risk of rupture. IA wall research could also aid to develop less invasive, and thus, less risky, treatment methods for IAs in the future.

2.2 Pathobiology of IA

2.2.1 Normal cerebral artery wall

Like extracranial arteries, the cerebral artery wall consists of three layers. The intima is lined by endothelial cells, and a small amount of connective tissue is located beneath the endothelium (Sawabe 2010). The media consists of regular layers of SMCs surrounded by collagen fibers and sparsely occurring elastic fibers (Nyström 1963). The adventitia, the main cellular component of which is fibroblasts, is the outer layer of the vessel wall (Ruigrok et al. 2005). The adventitia in cerebral arteries is only weakly developed (Nyström 1963). The intima is separated from the media by an internal elastic lamina (IEL), whereas the external elastic lamina seen in extracranial arteries between the media and the adventitia is absent (Ruigrok et al. 2005).

The existence of a vasa vasorum in intracranial arteries has been debated. Aydin et al. found vasa vasorum-like vessels only in the proximal segments of vertebral and internal carotid arteries with a medial thickness of more than 250 μm in adults (Aydin 1998). However, Connolly et al. detected vasa vasorum-like vessels also in the adventitia of proximal segments of middle and anterior cerebral arteries (M1 and A1 segments), in addition to proximal segments of the carotid artery (Connolly et al. 1996).

2.2.2. Vascular wall remodeling

Vascular remodeling is a process in which the vascular wall changes its shape and structure in response to surrounding environmental conditions. In some situations, the

cellular responses may lead to pathological end-points, for example, to stenosis, as in atherosclerosis, or to aneurysmal dilatation (Michel et al. 2007).

Early atherogenesis in humans begins with extracellular deposition of apolipoprotein B-containing lipids in the outer layer of the diffusely thickened intima (Nakashima et al. 2007, Nakashima et al. 2008) and endothelial activation (Badimon et al. 2011). Early atherosclerotic changes include also the modification of lipids, e.g. oxidation of LDL, and the infiltration of CD68-positive macrophages. After phagocytizing the deposited lipids, macrophages become foam cells. Macrophages are also found in the adventitia and outer media, suggesting that macrophages also infiltrate the arterial wall from outside (Nakashima et al. 2007, Nakashima et al. 2008). Phenotypic modulation of SMCs is also crucial for vascular remodeling. In the atherosclerotic process, SMCs migrate into the intima, proliferate, secrete molecules like proteoglycans, and transform to foam cells, but apoptosis and loss of SMCs have also been detected (Nakashima et al. 2008). Progenitor cells from blood and adventitia may also migrate into the intima and differentiate to neo-SMCs (Xu 2006).

Adventitial response also occurs in vascular remodeling. In atherosclerosis, adventitial angiogenesis has been detected (Michel et al. 2007). The vasa vasorum is more often seen in cerebral arteries with atherosclerosis or thrombosis, or in large IAs, than in the normal cerebral artery wall (Atkinson et al. 1991, Aydin 1998). Especially in mechanical vascular injuries, like hypertension and balloon injury, the adventitial response also includes proliferation and phenotypic modulation of fibroblasts, leading to perivascular fibrosis (Michel et al. 2007).

Abdominal aortic aneurysms (AAAs) are characterized by de-endothelialization and formation of intraluminal thrombus (ILT) (Michel et al. 2011). In AAAs, degradation of extracellular matrix proteins, such as elastin (Rizzo et al. 1989), and death of vascular smooth muscle cells (VSMCs) (Lopez-Candales et al. 1997) leads to medial destruction (Michel et al. 2011). ILT may have an important role in mediating medial destruction (Humphrey et al. 2008, Michel et al. 2011). The AAA wall covered with ILT is thinner, has more inflammatory cells, increased apoptosis of SMCs, and signs of increased extracellular matrix (ECM) degradation (Kazi et al. 2003). ILT is a source of proteinases due to trapped polymorphonuclear leukocytes (PMNs) (Fontaine et al. 2002). It also predisposes the AAA wall to hypoxic conditions that can lead to weakening of the wall

(Vorp et al. 2001). The pathogenesis of AAAs includes inflammation and fibrosis in the adventitia (Michel et al. 2007).

2.2.3 Hemodynamic stress in the vascular wall

Vessel walls are exposed to different hemodynamic forces as the heart pumps blood through the circulation. Hydrostatic forces and circumferential cyclic stretch cause a pressure that acts perpendicularly to the wall (Miller 2002). Movement of blood causes tangential shear stress (Miller 2002). In addition, axial wall stress is generated during the development of arteries due to elastin (Humphrey 2008). This longitudinal retractive force opposes vessel elongation (Dobrin et al. 1990).

WSS is the component of frictional forces developing between flowing blood and endothelial cells. It acts parallel to the luminal surface of the vessel wall (Chiu et al. 2011). Vessels with uniform geometry are exposed to laminar, undisturbed flow with physiological shear stress. Physiological flow conditions are essential for normal functioning of the endothelium. Near arterial bifurcations and curvatures, the flow becomes disturbed and oscillatory, with a varying shear stress gradient. These conditions have been shown to promote atheroma formation and intimal thickening (Cunningham et al. 2005, Cecchi et al. 2011). In areas of low shear stress, endothelial cells produce less nitric oxide and more vasoconstrictors and mitogens, like endothelin 1, angiotensin II, and platelet-derived growth factor B, activate circulating monocytes, and are themselves more susceptible to proapoptotic stimuli (Malek et al. 1999).

Another primary mechanical force is blood pressure-induced circumferential wall stress (Humphrey 2008, Lu et al. 2011). This circumferential cyclic stretch affecting the whole vascular wall (intima, media, and adventitia) causes mechanical stimulation of both endothelial cells and SMCs (Ando et al. 2011, Lu et al. 2011). Cyclic stretch is needed to maintain the differentiated, contractile VSMC phenotype, but it may also enhance proliferative response of VSMCs (Birukov et al. 1995). Later, cyclic stretch has been shown to activate SMC proliferation and migration (Li et al. 2000, Li et al. 2007) as well as SMC apoptosis (Mayr et al. 2000, Wernig et al. 2003, Li et al. 2007). The final outcome of mechanical stress, such as SMC response, may depend on the intensity of mechanical stress and the micro-environment of SMCs (Li et al. 2007).

Wall tension is directly proportional to the intramural pressure and the radius of a spherical balloon, and inversely proportional to the wall thickness, as determined by Laplace's law (Hall et al. 2000, Jeong et al. 2012). The magnitude of systemic blood pressure affects the intramural pressure of the vascular wall, thus having an impact on wall tension. A well-known fact is that "aneurysm rupture occurs when wall tension exceeds the mechanical strength of wall tissue" (Jeong et al. 2012). Therefore, factors affecting the structure of an IA wall as well as IA hemodynamics have been investigated.

2.2.4 IA formation

Saccular IAs typically form at the bifurcation of cerebral arteries. Tears in IEL are found at arterial bifurcation sites more frequently in patients with IAs (Hassler 1961). IEL disruption has been thought to precede IA formation and is associated with intimal thickenings (Stehbens 1960). However, the early process of IA formation is still largely unknown. Degeneration of the cerebral vessel wall and hemodynamic factors are proposed to have important roles in the initiation of IA formation (Stehbens 1989).

In experimental animal models, the combination of hypertension and disrupted collagen synthesis induces the formation of aneurysms in cerebral arteries (Hashimoto et al. 1978, Hashimoto et al. 1987, Morimoto et al. 2002). Endothelial changes have been detected just distal to the apical intimal pad, leading to the formation of an inflammatory zone. Expansion of this inflammatory zone results in saccular dilatation of the arterial bifurcation (Jamous et al. 2007). Disruption of elastic laminae and death of medial SMCs are also first steps in the formation of induced aneurysms (Kim et al. 1993, Kondo et al. 1998). However, these induced aneurysms do not spontaneously rupture (Aoki et al. 2011).

2.2.5 IA wall degeneration and rupture

2.2.5.1 IA wall structure

The IA wall is characterized by the loss of IEL and absent normal intima-media-adventitia layers (Hassler 1961, Scanarini et al. 1978). Unruptured IAs are usually characterized by a

linearly organized endothelium and regularly organized mural cells (VSMCs, myofibroblasts, fibroblasts) (Kataoka et al. 1999, Frösen et al. 2004). In addition, myointimal hyperplasia can be found in unruptured IAs, but it is also seen in some ruptured IAs (Frösen et al. 2004). In contrast, degenerative changes of the IA wall, like loss of endothelium, loss of mural cells, partial hyalinization of the wall, and disorganization of the collagen matrix, are associated with ruptured IA walls (Stehbens 1963, Scanarini et al. 1978, Sakaki et al. 1997, Kataoka et al. 1999, Frösen et al. 2004). Thrombus formation is associated with wall degeneration and rupture (Frösen et al. 2004). In the adventitial side of some ruptured IAs, the vasa vasorum with partially occluded lumen is seen (Scanarini et al. 1978).

2.2.5.2 Mechanisms of IA wall degeneration and rupture

The loss of endothelial cells, adhesion of blood cells, and subsequent thrombus formation seen on the luminal side of the IA wall and are associated with IA rupture and wall degeneration (Scanarini et al. 1978, Kataoka et al. 1999, Frösen et al. 2004). In vascular pathologies, injury to the endothelium or to the medial layer leads to intimal response and myointimal hyperplasia (Newby et al. 2000). Signs of myointimal hyperplasia, proliferation of mural cells, synthesis of a new matrix, and organization of a mural thrombus by SMCs are seen in unruptured IAs, but also in some ruptured IA walls, and this may be a protective “healing” response of the vascular wall (Frösen et al. 2004, Frösen et al. 2012). Linearly organized collagen fibers maintain the strength of the vascular wall against hemodynamic stress, and they need to be repaired and synthesized when the vascular wall is exposed to mechanical stress (Sawabe 2010). Myointimal hyperplasia and synthesis and repair of collagen fibers likely increase the tensile strength of the IA wall and protect against IA rupture (Frösen et al. 2012).

Unlike unruptured IAs, ruptured IA walls are often thin, hyalinized structures characterized by a loss of mural cells and breakdown of collagen fibers (Kataoka et al. 1999, Frösen et al. 2004). Myointimal hyperplasia does not strengthen the wall. Instead, the death of mural cells leads to decreased synthesis of collagen fibers. In addition, proteinases degrade the ECM (Bruno et al. 1998). Matrix metalloproteinases (MMPs), serine proteinases, and plasmin are enzymes capable of cleaving ECM structural proteins,

such as collagens, elastin, and fibronectin (Mignatti et al. 1993, Bäck et al. 2010), and thus, their activity weakens the vascular wall. MMP-9 is synthesized locally in the IA wall (Kim et al. 1997b), but increased pro-MMP-2 levels in serum samples of some IA patients have also been detected (Todor et al. 1998). Compared with normal cerebral arteries, increased MMP and serine proteinase activity and increased expression of plasmin, membrane type 1 MMP, MMP-2, and MMP-9 have been detected in the IA wall (Kim et al. 1997b, Bruno et al. 1998, Caird et al. 2006). Expression of MMP-2 and MMP-9 messenger RNA (mRNA) is also higher in ruptured IAs than in unruptured IAs (Jin et al. 2007).

Cell death in the IA wall

Mural cell loss has raised the question of possible cell death mechanisms in the IA wall. Sakaki et al. found that apoptosis is associated with IA rupture (Sakaki et al. 1997). They used TUNEL (terminal deoxynucleotidyl transferase (dUTP) nick end labeling) staining. Pentimalli et al. replicated the finding using TUNEL and electron microscopy (Pentimalli et al. 2004). In two Finnish studies, TUNEL-positivity did not reach statistical significance between ruptured and unruptured IAs (Frösen et al. 2004, Tulamo et al. 2006), but apoptotic cells were found using electron microscopy (Tulamo et al. 2006). Later, Guo et al. showed apoptotic cells in ruptured IA walls using electron microscopy (Guo et al. 2007). They also studied caspases, which are enzymes that mediate apoptotic signaling. They noted increased expression of caspase-3 mRNA in ruptured IA samples and AAAs, as compared with normal arteries, but they did not investigate the activity of caspases (Guo et al. 2007). Necrotic cell death in ruptured IA walls has also been reported (Holling et al. 2009). However, how cells in the IA wall die and what triggers cell death remain obscure.

Inflammation in the IA wall

Inflammation was observed in the IA wall already by Virchow (Virchow 1847), and after Virchow's report, inflammation has been suggested to be the cause of wall degradation and rupture by many authors. Inflammation is a response to tissue injury or irritants. The goal of the inflammatory response is to heal the injury or remove the irritant. If the irritant cannot be removed, the inflammatory reaction may become chronic. The immune system

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mediates the inflammatory reaction, and it can be divided into two different types of responses: innate and adaptive immunity. Natural antibodies, part of innate immunity, recognize pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). One class of DAMPs is oxidation-specific epitopes, including oxidatively modified lipids like oxidized low-density lipoprotein (oxLDL) and epitopes on apoptotic cells (Chou et al. 2008, Miller et al. 2011). Natural and acquired antibodies activate complement, which is an important part of innate immunity. Inflammatory cells are also recruited to the site of inflammation. Macrophages and dendritic cells, part of innate immunity, are able to present antigens and to activate the cells of adaptive immunity, e.g. T-cells and B-cells.

Inflammatory cells, including T-cells, macrophages, and PMNs, have been detected in the IA wall (Crompton 1966, Chyatte et al. 1999, Kataoka et al. 1999, Frösen et al. 2004). The complement system in the IA wall is activated by a classical pathway with an alternative pathway amplification (Tulamo et al. 2010b, Tulamo et al. 2010c), and complement activation may be one of the triggers of inflammatory cell infiltration into the degenerating IA wall (Tulamo 2010, Frösen et al. 2012). Antibodies are found in most IA walls, but complement activation does not correlate with the accumulation of antibodies (Chyatte et al. 1999, Tulamo et al. 2010b), suggesting that antibody-mediated inflammatory response is not the primary trigger of inflammation in the IA wall (Frösen et al. 2012). Terminal complement activation itself (the formation of membrane attack complex (MAC)) is associated with IA wall degeneration and rupture (Tulamo et al. 2006). MAC formation on the surface of cells causes cell damage and may lead to apoptosis or necrosis. However, in IA walls, MACs are detected in the matrix and cellular debris in decellularized regions, but rarely on the cell surface (Tulamo et al. 2006), suggesting that complement activation may be a reaction to necrotic cell death (Frösen et al. 2012).

Inflammatory cell infiltration is associated with ruptured IAs (Kataoka et al. 1999, Frösen et al. 2004). However, it has been suggested that inflammatory cell infiltration occurs before the rupture, as inflammatory cells are present in ruptured IAs that have been resected after a very short time interval from IA rupture, and they are also found in unruptured IAs (Kataoka et al. 1999, Frösen et al. 2004).

The majority of macrophages in the IA wall are CD163-positive (Frösen et al. 2004). CD163 is a hemoglobin scavenger receptor that is expressed in macrophages involved in “alternative activation” (Moestrup et al. 2004, Fabriek et al. 2005). “Alternatively activated” macrophages may dampen the inflammatory response; they scavenge the components of damaged cells and take part in anti-oxidative defense of the tissue (Moestrup et al. 2004, Fabriek et al. 2005, Nielsen et al. 2009). Macrophages may induce apoptosis using FAS ligand (FASL) and tumor necrosis factor alpha (TNF α) (Ueda et al. 2002, Boyle et al. 2003). TNF α and its downstream target, Fas-associated death domain protein (FADD), have been detected in ruptured IAs (Jayaraman et al. 2005). TNF α induces apoptosis via activation of caspase-8 (Alikhani et al. 2004), but the apoptotic pathways in the IA wall have not thus far been investigated.

Hence, inflammation is likely an important modulator in the degeneration process of the IA wall, but its significance as a cause of cell death remains unclear (Frösen et al. 2012).

Cell signaling in the IA wall

Only a few studies about signaling protein expression at the protein level in the human IA wall have been published to date. Kilic et al. evaluated 10 unruptured and 10 ruptured human IA samples collected perioperatively and compared them with autopsy samples of the circle of Willis using immunohistochemistry (Kilic et al. 2005). They found that transforming growth factor- α expression was lower in IA samples than in normal circle of Willis samples (Kilic et al. 2005). Frösen et al. noted in their immunohistochemical series of 21 unruptured and 35 ruptured human IA samples that vascular endothelial growth factor (VEGF), transforming growth factor- β , and basic fibroblast growth factor receptors were associated with IA wall remodeling (Frösen et al. 2006). Takagi et al. assessed 12 human IA samples obtained at surgery and compared them with 5 control middle cerebral artery (MCA) samples obtained at autopsy (Takagi et al. 2002). Using immunohistochemistry, they found that phosphorylated JNK and phosphorylated c-Jun expression was increased in IA samples relative to control vessels (Takagi et al. 2002).

2.2.5.3 IA wall degeneration and rupture – results from gene expression studies

In recent years, several genome-wide microarray studies investigating IA wall gene expression have been published. The first microarray study comparing unruptured and ruptured IAs found an association between antigen presenting inflammatory cells and IA rupture (Krischek et al. 2008). A study examining unruptured and ruptured IAs and the superficial temporal artery (STA) observed pathways involved in apoptosis, inflammation, focal adhesion, extracellular matrix-receptor interaction, and cell communication to be differentially expressed (Shi et al. 2009). Genes associated with both intrinsic and extrinsic apoptotic pathways were differentially expressed in IA samples, but differential expression of caspase-9 or caspase-8 mRNA was not observed (Shi et al. 2009). A microarray study comparing unruptured and ruptured MCA aneurysms revealed that genes related to inflammatory cell infiltration, oxidative stress, disturbed cell homeostasis, and dysfunctional endothelium were upregulated in ruptured IAs (Kurki et al. 2011). The largest microarray study to date compared 10 unruptured and 12 ruptured IAs with middle meningeal artery and STA samples and found overexpression of collagenases (MMP-2 and MMP-9), proapoptotic genes (Fas, Bax, and Bid), and inducible nitric oxide synthase (iNOS) in ruptured IAs, whereas antiapoptotic genes (Bcl-X(L) and Bcl-2) were downregulated (Marchese et al. 2010). In contrast to other studies, one microarray study reported decreased inflammatory process in ruptured IAs (Pera et al. 2010). A microarray study comparing 3 unruptured IAs with 3 STA samples found ECM-related genes to be upregulated and inflammation-related genes to be downregulated (Li et al. 2009).

2.2.5.4 Hemodynamic stress in IA development

Hemodynamic stress has been suggested to have an important role in IA formation (Stehbens 1989). However, studying IA hemodynamics is complicated because many different factors, such as parent vessel and IA geometry, flow dynamics, pressures, and IA wall structure, should be taken into account (Gobin et al. 1994). Hemodynamics in the circle of Willis and inside the IA pouch has earlier been investigated using glass and silicone models (Ferguson 1972, Roach et al. 1972, Stehbens 1975, Steiger et al. 1987, Steiger et al. 1988, Gobin et al. 1994) and computational models (Perktold et al. 1988,

Gonzalez et al. 1992, Low et al. 1993). These studies have concluded that turbulent flow occurs inside IAs. In addition, they suggest that the flow is slower inside the IA pouch than in the parent vessel and even stagnation of flow may occur at the fundus, whereas the flow rate is higher at the neck region (Ujiie et al. 1999).

Ujiie et al. studied the relationship between aneurysm morphology and flow conditions using a rabbit model. They found that a high aspect ratio (>1.6) (fundus length/neck diameter) resulted in low-flow conditions near the IA dome and suggested that low shear stress near the dome predisposes to IA rupture (Ujiie et al. 1999).

In recent years, the development of medical imaging technology and computer equipment has enabled computational fluid dynamics (CFD) analysis. CFD enables more accurate prediction of the hemodynamics of IAs (Jeong et al. 2012). Due to the well-known effects of WSS and flow type (laminar vs. turbulent) on the endothelium, WSS and local flow conditions in IA pathophysiology have been actively studied (Nixon et al. 2010). Simple stable flow patterns have been observed in unruptured IAs, whereas complex or unstable flow patterns have been found in ruptured IAs (Cebal et al. 2005, Xiang et al. 2011). Low WSS has been noted especially in ruptured IAs (Shojima et al. 2004, Xiang et al. 2011). Boussel et al. demonstrated that low WSS correlates with IA growth (Boussel et al. 2008). In addition to WSS, IA wall tension and possible pressure changes inside the IA pouch have been investigated using CFD. Isaksen et al. showed that high wall tension and wall displacement localize on the same regions where IAs usually rupture (Isaksen et al. 2008).

However, the results of CFD analysis depend highly on material properties and chosen boundary conditions (Jeong et al. 2012). For example, local wall properties, possible effects of intraluminal thrombus, and outflow boundary conditions should be taken into account to improve the reliability of CFD results (Jeong et al. 2012). All in all, CFD analysis is a new, developing approach, but much effort is needed before this method can be applied to clinical work, e.g. to predict IA rupture risk of individual patients.

2.2.6 Summary of putative mechanisms of IA wall degeneration and rupture

Current data suggest that degeneration of the IA wall is a dynamic process, which probably starts with endothelium dysfunction. Hemodynamic factors affect the

functioning of the endothelium and mural cells. As the IA grows, the hemodynamics inside the IA pouch change from the normal laminar flow. Aberrant flow conditions activate pathological cell mechanisms, possibly leading to the loss of mural cells, inflammation, and degradation of the matrix. These mechanisms have the potential to weaken the vascular wall and change its structure, potentially resulting in outpouchings of the IA wall and other morphological variations from the spherical shape. These new morphological features lead to a more complex hemodynamic environment, which may, in turn, lead to activation of mechanisms that weaken the IA wall. Hence, the IA wall gradually degenerates. Finally, it is too fragile to resist hemodynamic stress leading to IA rupture (Frösen 2006, Tulamo et al. 2010a, Tulamo 2010, Frösen et al. 2012).

2.3 Intracellular signaling pathways in vascular wall pathobiology

2.3.1 Mitogen-activated protein kinases (MAPKs)

MAPK signal transduction pathways are a widespread mechanism in eukaryotic cell regulation. MAPKs are a family of intracellular signaling proteins that are activated by phosphorylation by upstream kinases. They are activated by a large number of different stimuli, such as growth factors, inflammatory cytokines, and environmental stressors, including ischemic injury, ionizing radiation, osmotic shock, and mechanical stretch (Kyriakis et al. 2001). MAPKs control the activation of gene transcription, protein synthesis, cell cycle machinery, cell death, and differentiation (Kyriakis et al. 2001). In the following section, three major MAPKs, JNK, p38, and extracellular signal-regulated kinase (ERK), are described in more detail, focusing on their role in vascular pathobiology. ERK responds to mitogenic stimuli, whereas JNK and p38 are mainly activated by stress signaling (Kyriakis et al. 2001).

2.3.1.1 Stress-activated kinases

c-Jun N-terminal kinase (JNK)

Three genes encode the JNK family members JNK1, JNK2, and JNK3. There are four splice variants of JNK1, four splice variants of JNK2, and four splice variants of JNK3.

These splice variants lead to the production of 12 JNK polypeptides, the molecular weights of which are either 46 or 54 kilodaltons (kDa) (Muslin 2008, Kyriakis et al. 2012). The functional significance of these two isoforms, p46-JNK and p54-JNK, is unclear (Kyriakis et al. 2012). JNK is activated by mitogens, heat shock, ionizing radiation, oxidative stress, DNA-damaging chemicals, ischemic reperfusion injury, mechanical shear stress, proinflammatory cytokines of the TNF family, vasoactive peptides like endothelin and angiotensin II, PAMPs, DAMPs, and protein synthesis inhibitors (Kyriakis et al. 2001, Kyriakis et al. 2012). Depending on the cellular context, JNK may regulate either apoptosis or survival signaling (Davis 2000). Transcription factor c-Jun is a downstream target of JNK (Kyriakis et al. 2001). c-Jun enhances the expression of MMP-9 (Shin et al. 2002).

JNK is activated in balloon-injury models and in hypertrophic cardiac and hypertensive vascular tissue (Hu et al. 1997, Kim et al. 1997a, Izumi et al. 1998, Kim et al. 1998). JNK activation leads to SMC proliferation and to neointimal formation in balloon-injury model (Izumi et al. 2001). Mechanical strain activates JNK in rat aortic VSMCs, producing induction of alpha-smooth muscle actin (α SMA) expression (Tock et al. 2003). c-Jun regulates VSMC proliferation and balloon injury-induced intimal hyperplasia (Yasumoto et al. 2001). In macrophages, JNK regulates foam cell formation after exposure to oxLDL (Rahaman et al. 2006). In cultured bovine endothelial cells, a similar activation pattern for JNK was observed as a result of cyclic strain and shear stress (Azuma et al. 2000). JNK is involved in AAA pathogenesis, with JNK enhancing the degradation of ECM and suppressing genes that encode ECM biosynthetic enzymes (Yoshimura et al. 2005). An immunohistochemistry study comparing surgically obtained unruptured and ruptured human IAs with MCA autopsy samples suggested increased expression of phospho-JNK and phospho-c-Jun in IA samples (Takagi et al. 2002).

p38 MAPK

Four different genes encode p38 MAPKs, p38 α - δ (Kyriakis et al. 2012). Like JNK, p38 is activated by environmental stresses, inflammatory cytokines, PAMPs, and DAMPs (Kyriakis et al. 2001, Kyriakis et al. 2012). p38 has a central role in inflammation (Schieven 2005), but it also participates in several other biological processes, e.g. cell cycle control in embryogenesis (Kyriakis et al. 2001).

p38 was activated in cerebral arteries in an experimental SAH model, leading to the development of vasospasm (Sasaki et al. 2004). Like JNK, p38 activates strain-induced α SMA expression in rat aortic VSMCs (Tock et al. 2003) and promotes neointimal formation after balloon injury (Ohashi et al. 2000, Proctor et al. 2008). p38 has also been found to regulate cyclic strain stress-induced SMC proliferation and migration in cultured rat aortic VSMCs (Li et al. 2000, Li et al. 2007), but also cyclic stretch-induced β 1-integrin-mediated VSMC apoptosis (Wernig et al. 2003, Li et al. 2007). In endothelial cells, shear stress induced greater activation of p38 in vitro than cyclic strain (Azuma et al. 2000). p38 has also been shown to be activated by oxLDL and may regulate foam cell formation (Zhao et al. 2002).

2.3.1.2 Extracellular signal-regulated kinase (ERK)

ERK has two isoforms, ERK1 and ERK2, the alternative names of which are p44-MAPK and p42-MAPK, respectively (Davis 1993, Kyriakis et al. 2012). ERK is known as insulin- and mitogen-activated MAPK (Kyriakis et al. 2012). The most familiar activator of ERK is the Ras proto-oncoprotein (Kyriakis et al. 2012). However, in some instances, ERK can also be activated by proinflammatory cytokines, by PAMPs like lipopolysaccharide produced by microbes, and by DAMPs like oxLDL in atherosclerosis (Kyriakis et al. 2012).

ERK has been shown to be activated in a balloon-injury model, in cardiac hypertrophy, and in hypertensive vascular tissue (Hu et al. 1997, Kim et al. 1997a, Izumi et al. 1998, Kim et al. 1998, Koyama et al. 1998). ERK activation triggers SMC proliferation, leading to neointimal formation (Izumi et al. 2001). In rat aortic VSMCs, ERK is activated in vitro in response to both shear stress and cyclic strain (Hu et al. 1998, Li et al. 1999). In cultured endothelial cells, ERK activation is greater for shear stress than for cyclic strain (Azuma et al. 2000).

2.3.2 Protein kinase B (PKB/Akt)

Akt is a serine-threonine kinase with three isoforms, Akt1, Akt2, and Akt3. It has important roles in cell survival, cell growth, and glucose metabolism (Coffer et al. 1998).

In the vascular system, Akt signaling has specific functions in normal physiology and disease, e.g. in the endothelium, VSMCs, and atherosclerotic plaques (Morello et al. 2009). In the endothelium, Akt regulates vascular tone by acting as a positive regulator of endothelial nitric oxide synthase (Luo et al. 2000). Akt also mediates VEGF-stimulated angiogenesis (Ahmad et al. 2006). In VSMCs, Akt participates in medial hyperplasia and vascular remodeling (Nemenoff et al. 2008, Morello et al. 2009). Akt signaling may promote the atherosclerotic process (Chang et al. 2007, Fougerat et al. 2008, Morello et al. 2009, Eisenreich et al. 2011). Treatment with a PI3K γ inhibitor (AS605240) decreased Akt phosphorylation in leukocytes and in an atherosclerotic lesion and reduced the development of atherosclerotic lesions (Fougerat et al. 2008). On the other hand, Akt1 has been suggested to have an atheroprotective role (Fernandez-Hernando et al. 2007, Morello et al. 2009). Loss of Akt1 in the vessel wall increases inflammatory mediators, reduces endothelial nitric oxide synthase phosphorylation, and enhances the apoptosis of endothelial cells and macrophages in a murine model of atherosclerosis (Fernandez-Hernando et al. 2007).

2.3.3 Mammalian target of rapamycin (mTOR)

mTOR affects a wide variety of cellular functions, including metabolism, cell growth, survival, aging, synaptic plasticity, immunity, and memory. It is a serine-threonine kinase and is found in two complexes, mTOR complex 1 (mTORC1) and mTORC2 (Yang et al. 2007). Rapamycin, also known as sirolimus, is an mTOR inhibitor. Rapamycin acts by binding to its cytosolic receptor, FK506 binding protein (FKBP12) (Poon et al. 2002). The target of rapamycin-FKBP12 complex is mTOR (Sabatini et al. 1994).

mTOR promotes cell-cycle progression in VSMCs and regulates VSMC differentiation (Rzucidlo et al. 2007). Rapamycin inhibits VSMC proliferation and migration (Marx et al. 1995, Poon et al. 1996). It has been successfully used in coronary stents to inhibit restenosis (Sousa et al. 2001, Poon et al. 2002, Caixeta et al. 2009). Depending on the complex (mTORC1 or mTORC2), rapamycin has different effects on mTOR signaling and its subcellular localization in fibroblasts (Rosner et al. 2008).

2.3.4 Cyclic AMP response element binding protein (CREB)

CREB is a phosphorylation-dependent transcription factor that controls cell proliferation, differentiation, metabolism, and survival (Reusch et al. 2004, Ichiki 2006, Wen et al. 2010). It also has a role in the regulation of immune responses. Several kinases phosphorylate CREB at serine 133 (Wen et al. 2010). CREB has a wide range of target genes, e.g. cyclins, antiapoptotic Bcl-2 gene, growth factors, structural proteins like fibronectin, and heme oxygenase-1 (HO-1) (Mayr et al. 2001, Krönke et al. 2003, Ichiki 2006).

CREB maintains the normal function and structure of the heart, but in the vascular vessel wall CREB may have both positive and negative roles in the remodeling process (Ichiki 2006). CREB has been shown to support quiescent and healthy phenotypes in VSMCs and in endothelial cells (Klemm et al. 2001, Reusch et al. 2004). However, another study found that CREB was activated in the neointima after balloon injury of the rat carotid artery, and transduction of dominant-negative CREB by an adenoviral vector decreased the neointimal formation (Tokunou et al. 2003). Thus, Tokunou et al. concluded that CREB has an important role in the survival and proliferation of neointimal VSMCs and that CREB increases neointimal formation (Tokunou et al. 2003). After acute exposure to vascular toxins, like oxidative stress, high glucose, or cytokines, CREB is also phosphorylated and activated (Reusch et al. 2004). This has been interpreted as a protective response, possibly intended to decrease oxidant injury (Reusch et al. 2004, Schauer et al. 2010). A further study has shown that in rodent models of aging, hypertension, and insulin resistance, CREB expression was decreased (Schauer et al. 2010). In the same study, the exposure of rat aortic VSMCs to LDL and oxLDL led to a rapid CREB activation (Schauer et al. 2010). However, longer exposure to oxLDL decreased expression of CREB protein (Schauer et al. 2010). The observed loss of VSMC CREB expression was suggested to be a common response to a vascular injury, probably to oxidative stress, making VSMC more susceptible to proliferation, migration, and apoptosis (Schauer et al. 2010).

2.3.5 Pathways of cellular death

Cell death mechanisms are classically divided into apoptosis, autophagic cell death, and necrosis. Nowadays, it is suggested that cell death is “regulated” in most instances and “accidental” cell death is triggered only in extremely harsh physical conditions (Galluzzi et al. 2012). In recent years, several new cell death mechanisms have been described (Galluzzi et al. 2012). The most well-known mechanisms are still apoptosis and necrosis. Apoptosis has been divided into caspase-dependent and caspase-independent pathways as well as into extrinsic and intrinsic apoptosis (Galluzzi et al. 2012). Increasing evidence shows that necrosis is also “regulated” in many settings (Vandenabeele et al. 2010). Cell death by regulated necrosis can be triggered by alkylating DNA damage, excitotoxins, and ligation of death receptors (Vandenabeele et al. 2010, Galluzzi et al. 2012).

2.3.5.1 Caspases

Caspases are a family of enzymes that take part in programmed cell death. Caspases are activated by cleavage. Classically, apoptotic pathways are divided into “extrinsic” and “intrinsic”. A simplification of the activation of these pathways is represented in Figure 3.

In extrinsic apoptosis, also known as the death-receptor pathway, cytokines like FASL, TNF α , and TNF-related apoptosis-inducing ligand bind to death receptors, including FAS, TNF α receptor 1, and TNF-related apoptosis-inducing ligand receptor 1-2, which results in activation of initiator caspase-8 (Figure 3) (Cohen 1997, Galluzzi et al. 2012). Especially inflammatory cells produce these cytokines (Aggarwal 2003), and inflammation is one important activator of extrinsic apoptosis. Recently, mitochondrial contribution to extrinsic apoptosis has been detected in some cell types, e.g. hepatocytes and pancreatic β cells, and another form of extrinsic apoptosis, apoptosis by dependence receptors, has been described (Galluzzi et al. 2012).

Intrinsic apoptosis is activated by cellular stress, including hypoxia, oxidative stress, irradiation, DNA damage, and cytosolic Ca²⁺ overload (Kuida 2000, Ueda et al. 2002, Galluzzi et al. 2012). All of these triggers are able to cause a metabolic catastrophe that includes mitochondrial outer membrane permeabilization (MOMP). MOMP leads to the dissipation of mitochondrial transmembrane potential and arrest of mitochondrial ATP synthesis (Galluzzi et al. 2012). Reactive oxygen species (ROS) are overgenerated as the

respiratory chain gets uncoupled and proteins like cytochrome c are released into the cytosol (Galluzzi et al. 2012). Initiator caspase-9 is activated (Figure 3). Caspase-independent intrinsic apoptosis has also been described (Galluzzi et al. 2012).

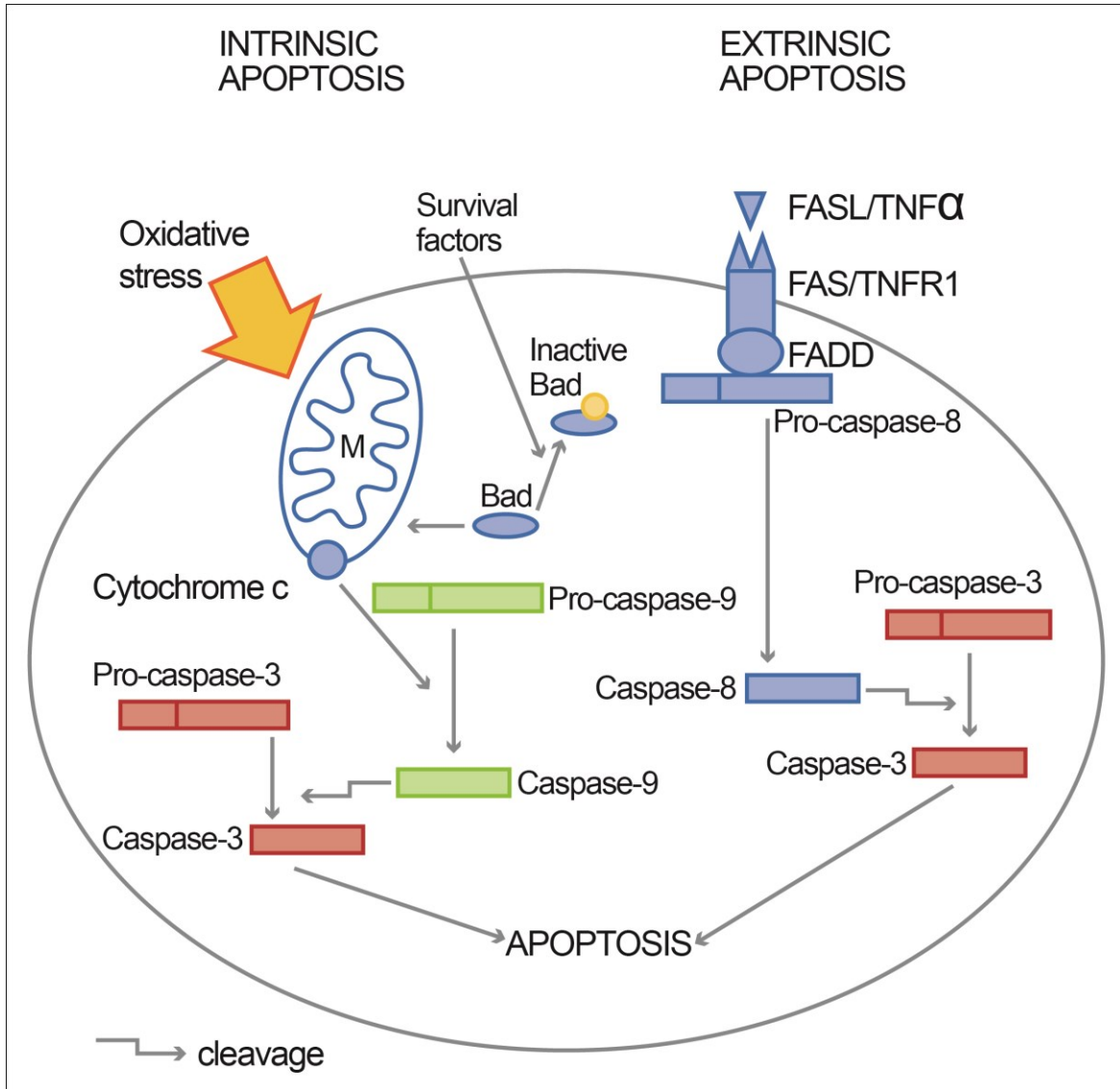


Figure 3 *Simplification of intrinsic and extrinsic apoptotic pathways.* Cellular stress like oxidative stress activates the intrinsic apoptotic pathway with caspase-9 activation. Bad protein is one of the regulators of the intrinsic apoptotic pathway, and it can be inactivated by phosphorylation by survival factors (yellow circle indicates the phosphorylated form of Bad). The extrinsic apoptotic pathway is activated by inflammatory cytokines such as tumor necrosis factor alpha. Both of these pathways converge in the cleavage and activation of caspase-3 and apoptosis of the cell. Bad, Bcl-associated death protein; FADD, Fas-associated death domain protein; FASL, Fas ligand; M, mitochondrion; TNF α , tumor necrosis factor alpha; TNFR1, tumor necrosis factor receptor 1

Both extrinsic and intrinsic apoptotic pathways converge in the cleavage and activation of caspase-3, which is an executioner caspase. Activated caspase-3 causes apoptosis of the cell (Figure 3).

2.3.5.2 Cellular death in vascular diseases

In the vascular system, cells are constantly exposed to various environmental stresses such as different types of hemodynamic forces and harmful metabolic byproducts. When the pathological disease process starts, the cells are exposed to new, harmful insults like abnormal hemodynamic stress. These changing environmental circumstances may trigger cell death (Zheng et al. 2011).

Apoptosis of endothelial cells, VSMCs, and macrophages is an important step in atherosclerosis and has been widely studied (Kockx et al. 2000, Martinet et al. 2011, Zheng et al. 2011). Both intrinsic and extrinsic apoptotic pathways are activated in the atherosclerotic process (Kutuk et al. 2006). Necrotic cell death in atherosclerosis is poorly investigated. One reason for this is that reliable methods to detect necrosis in situ are not available; morphological analysis is mostly used (Martinet et al. 2011). Morphological analysis of human carotid plaques with transmission electron microscopy has shown that most of the dying VSMCs and macrophages have an ultrastructure typical of necrotic rather than apoptotic cells (Crisby et al. 1997). In atherosclerotic plaques, oxidative stress (high ROS levels), toxic levels of mildly oxidized LDL and highly oxidized LDL, and increased intracellular Ca^{2+} may act as triggers for necrosis (Martinet et al. 2011). It has been speculated that “a truly silent cell death” in vascular lesions could lead to regression of the lesions (Zheng et al. 2011). However, impaired phagocytic clearance of apoptotic cells and necrotic cell death stimulate inflammation, and this downstream response may accelerate the progression of the atherosclerotic plaque (Martinet et al. 2011, Zheng et al. 2011).

Arterial aneurysms are characterized by a loss of VSMC relative to normal arteries. In human AAAs, signs of inflammation-mediated extrinsic apoptosis have been observed (Henderson et al. 1999). In a rodent model of elastase-induced experimental AAAs, activation of intrinsic apoptotic pathway has been reported (Sinha et al. 2005), and oxidative stress has also been speculated to play a role in the apoptosis of VSMC in

human AAA (McCormick et al. 2007). In elastase-induced experimental saccular aneurysms, the intrinsic apoptotic pathway was activated, whereas no activation of extrinsic apoptotic pathway was detected (Kadirvel et al. 2010).

2.3.5.3 Bcl-associated death protein (Bad)

Bad protein belongs to the BCL-2 family of cell death regulators. It is a proapoptotic BH3-only protein that is inactivated by phosphorylation by prosurvival signaling factors (Figure 3, page 36) (Danial 2009). Bad has three phosphorylation sites: Ser112, Ser136, and Ser155. Bad is a well-known apoptosis regulator acting in the intrinsic apoptotic pathway. Other roles of Bad have also been revealed, e.g. participation in glucose metabolism (Danial 2009).

In the vascular system, exposure to mildly oxidized LDL resulted in increased levels of Bad and induced apoptosis of coronary artery endothelial cells and SMCs (Napoli et al. 2000). Phosphatidylinositol (PI) 3-kinase-dependent pathway inactivates Bad and prevents VSMC death (Bai et al. 1999).

2.3.6 Role of oxidative stress

Increased formation or decreased destruction of ROS, including superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2), leads to redox imbalance and increased oxidative stress (McCormick et al. 2007). In a vascular lesion, leukocytes are a potential source of ROS (Martin-Ventura et al. 2012). Hemolysis of red blood cells (RBCs) releases the pro-oxidant hemoglobin (Hb), and heme-iron potentiates the toxicity of ROS (Martin-Ventura et al. 2012).

Oxidative stress may cause tissue damage in the vascular wall (McCormick et al. 2007), and it is involved in many vascular diseases such as hypertension, diabetes, ischemia/reperfusion injury, and atherosclerosis (Peterson et al. 2009).

In AAAs, O_2^- levels are increased relative to nonaneurysmal aortic segments (Miller et al. 2002), and increased H_2O_2 levels have been observed in intraluminal thrombus of AAA (Ramos-Mozo et al. 2011). Oxidative stress has been speculated to have an important role in the formation of AAAs (McCormick et al. 2007), and the intraluminal thrombus with

RBCs, leukocytes, and platelets has been proposed to be the main source of oxidative stress in AAAs (Michel et al. 2011, Martin-Ventura et al. 2012).

ROS are also able to initiate the peroxidation of lipids, which results in the formation of oxLDL (Mitra et al. 2011). OxLDL itself is able to enhance ROS formation, and it also has other potentially harmful effects on the vascular wall, including induction of endothelial cell and VSMC apoptosis (Nishio et al. 1996, Li et al. 2000, Vergnani et al. 2000, Mitra et al. 2011). The proatherogenic role of oxLDL in the atherosclerotic process is well established (Mitra et al. 2011).

To reduce harmful effects of oxidative stress, the vascular wall has several antioxidant mechanisms (Martin-Ventura et al. 2012). Free Hb and haptoglobin (Hp) form a complex, which is endocytosed by scavenger receptor CD163 (Martin-Ventura et al. 2012). CD163 is expressed on the surface of “alternatively activated” macrophages, which have anti-inflammatory and anti-oxidative functions (Gordon 2003, Moestrup et al. 2004, Fabriek et al. 2005). After endocytosis, heme is degraded to less toxic compounds by HO-1 (Martin-Ventura et al. 2012).

2.3.6.1 Heme oxygenase-1 (HO-1)

HO has three isoenzymes: HO-1, HO-2, and HO-3. HO-3 is not expressed in humans. HO-2 is a constitutive isoform, whereas HO-1 is an inducible isoform. HO-1 and HO-2 play an important role in heme breakdown. HO-1 is induced by oxidative stress, and the heme degradation products produced by HO-1, like biliverdin and bilirubin, have antioxidant properties (Peterson et al. 2009). In addition to antioxidant effects, HO-1 has anti-inflammatory and antiapoptotic properties (Dulak et al. 2008b). Genetic polymorphisms (a (GT)_n dinucleotide length polymorphism, and two single-nucleotide polymorphisms (SNPs), G(-1135)A and T(-413)A), in the promoter region of human HO-1 gene affect the HO-1 response (Exner et al. 2004).

HO-1 has been detected in the endothelium, foam cells/macrophages, and VSMCs in human atherosclerotic lesions (Wang et al. 1998). OxLDL acts as an inducer of HO-1 in human vascular cells (Ishikawa et al. 1997), and HO-1 has a protective role against atherosclerosis (Ishikawa et al. 2001). In an experimental AAA model, flow loading (causing increased wall shear stress and relative wall strain) increased HO-1 expression

(Nakahashi et al. 2002). In macrophages, HO-1 expression promotes antioxidant and anti-inflammatory effects, thereby resulting in an antiatherogenic effect in atherosclerotic lesions (Orozco et al. 2007). HO-1 is also proangiogenic by inducing VEGF in VSMCs, endothelial cells, and macrophages (Dulak et al. 2008a). GT dinucleotide polymorphism is associated with the formation of AAA (Schillinger et al. 2002) and with aneurysmal SAH (Morgan et al. 2005).

2.3.7 Summary of molecular pathways in vascular wall pathobiology

Cell differentiation, proliferation, migration, and apoptosis have a crucial role in vascular pathologies. Hence, knowledge of the signaling pathways regulating these cellular functions is important to elucidate the disease processes. Activation of a signaling pathway may either initiate pathological remodeling or act as a protective response against harmful insults. In recent years, signaling pathways in different vascular pathologies, especially in atherosclerotic plaques and in the restenosis after balloon injury, have been investigated (Ichiki 2006, Muslin 2008, Morello et al. 2009, Peterson et al. 2009). However, signaling molecules, like MAPKs, often have different biological roles depending on cell type and on pathophysiological process (Muslin 2008). Thus, it is important to investigate the role of different signaling molecules in IA pathobiology.

3 Aims of the study

The role of different intracellular signaling pathways has been unraveled in many vascular diseases. However, their role in IA wall pathobiology is poorly known.

The objectives of this study were the following:

1. To investigate the localization and activation of different signaling proteins (MAPKs (JNK, p38, ERK), Akt, mTOR, CREB, and Bad) in the IA wall in relation to IA rupture.
2. To compare the activation of different signaling proteins (MAPKs (JNK, p38, ERK), Akt, mTOR, CREB, and Bad) with IA morphology to better understand the mechanisms of IA growth, wall degeneration, and rupture.
3. To evaluate the possible mechanisms of cell death in the IA wall by analyzing the activation of caspase-3 in the IA wall, whether the caspase activation involves the intrinsic (caspase-9) or extrinsic (caspase-8) pathway, and the role of oxidative stress.

4 Patients, materials, and methods

4.1 Patients and samples

Ruptured and unruptured human IA samples were collected after microsurgical clipping of the aneurysm by cutting a part of the IA dome. The samples were immediately snap-frozen and stored at -80°C . Clinical data were obtained from medical records. The study protocol was approved by the local ethics committee of the Departments of Neurology, Neurosurgery, Otorhinolaryngology, and Ophthalmology at Helsinki University Central Hospital. The patients or their relatives gave informed consent. A total of 77 IA (40 ruptured and 37 unruptured) samples were included in the study.

IA dimensions (fundus length, fundus width, and neck diameter) and Fisher grading were obtained from preoperative vascular imaging studies (CTA or DSA) and CT scans. Reconstruction of lesion geometry from computed tomography angiograms was separately performed for 26 aneurysms to calculate five size indices (fundus length, maximum diameter, neck diameter, volume, surface area) and six shape indices (aspect ratio, bottleneck factor, bulge location, undulation index, ellipticity index, non-sphericity index) from 3D models (Figure 2, page 16) (II). Shape indices were defined as follows. Aspect ratio is the ratio of fundus length to neck diameter. Bottleneck factor is the ratio of maximum diameter to neck diameter. Bulge location is the distance from the neck plane to the largest cross-section as a fraction of fundus length. Undulation index is the measure of lobulations on the aneurysm surface. Ellipticity index is the measure of ellipticity of the aneurysm. Non-sphericity index is the aggregate measure of deviation from spherical shape due to ellipticity and undulation.

4.1.1 3D remodeling (II)

For reconstruction of the IA geometry ($n = 26$), the IAs containing significant intraluminal thrombus based on imaging or intraoperative findings were not included in the study. Minor intraluminal thrombus visible in the histological analysis is a frequent finding in IA, and it was not considered an exclusion criterion. The analyzer was blinded to IA rupture status.

3D segmentation

Computed tomography angiograms were scanned at 1.25-mm slice thickness in all but one case, which had a thickness of 0.625 mm. The 3D models were reconstructed from the source data using level-set segmentation techniques implemented in the Vascular Modeling Toolkit (VMTK). The parent vasculature and the aneurysm were segmented using the level-set initialization methods, referred to as colliding fronts and fast marching, respectively (Piccinelli et al. 2009). The deformable models were created by applying evolution parameters within a set range – number of iterations (200-300), propagation scaling (0-1), curvature scaling (0-1), and advection scaling (0-1). VMTK developers have published detailed reviews of these methods earlier (Antiga et al. 2002, Antiga et al. 2003, Piccinelli et al. 2009). The surfaces were smoothed using Taubin's non-shrinking algorithm with consistent parameters implemented in VMTK to remove artifacts like unrealistic sharp edges (Taubin et al. 1996, Antiga et al. 2003, Ma et al. 2004).

Aneurysm isolation

For quantification of IA morphology, IAs were isolated from their parent vasculature by defining a single plane in 3D space (Ma et al. 2004). The plane was chosen with manual discretion using the 3D visualization software TECPLOT 360 (2009 Release, Tecplot Inc., Bellevue, WA, USA), and the aneurysm was isolated using isolation algorithm implemented in the computing software program MATLAB (version 7.8, The Mathworks Inc., Natick, MA, USA). To be consistent when choosing a plane for IA isolation, a clinical criterion was defined (i.e. the plane where a surgeon would place a clip while operating on the IA during microneurosurgical clipping).

Five size indices and six shape indices were then calculated (Figure 2, page 16).

4.2 Histological studies (I-III)

The snap-frozen tissue samples were embedded in OCT Tissue Tek compound (Sakura Finetek Europe B.V., Zoeterwoude, Netherlands) and cryosectioned at 4 μ m.

Controls and antibodies

Human tonsils and rat balloon-injured aorta served as positive controls. For negative controls, the primary antibody was omitted. Primary antibodies used in immunofluorescence and immunohistochemical stainings and in Western blotting are listed in Table 1.

Table 1. *Primary antibodies used in Western blotting and immunohistochemical and immunofluorescence stainings*

Antibody target	Antibody type	Clone / Catalog number	Dilution in WB	Dilution in IHC	Dilution in IF	Source	Study
Phospho-proteins							
p-JNK (Thr183/Tyr185)	rabbit pAb	9251	1:1000	-	1:20	1	I, II
JNK	rabbit pAb	9252	1:1000	-	-	1	I, II
p-p38 (Thr180/Tyr182)	rabbit pAb	9211	1:1000	-	1:20	1	I, II
p38	rabbit pAb	9212	1:1000	-	-	1	I, II
p-p44/42 MAPK (Thr202/Tyr204)	mouse mAb	E10/ 9106	1:2000	-	1:100	1	I, II
p44/42 MAPK	rabbit pAb	9102	1:1000	-	-	1	I, II
p-c-Jun (Ser73)	rabbit pAb	9164	1:1000	-	-	1	I, II
c-Jun	rabbit mAb	60A8/ 9165	1:500	-	-	1	I, II
p-Bad (Ser136)	rabbit pAb	9295	1:500	-	-	1	II
Bad	rabbit pAb	9292	1:500	-	1:20	1	II
p-Akt (Ser473)	rabbit mAb	D9E/ 4060	1:2000	-	-	1	II
p-Akt (Ser473)	rabbit pAb	9271	-	-	1:20	1	II
Akt	rabbit pAb	9272	1:1000	-	-	1	II
p-CREB (Ser133)	rabbit pAb	9191	1:1000	-	1:50	1	II
CREB	rabbit mAb	48H2/ 9197	1:500	-	-	1	II
p-mTOR (Ser2448)	rabbit pAb	2971	1:1000	-	1:20	1	II
mTOR	rabbit pAb	2972	1:500	-	-	1	II

Caspases							
Cl. caspase-3	rabbit pAb	9661	-	-	1:50	1	III
Caspase-3	rabbit pAb	9662	1:1000	-	-	1	III
Caspase-8	mouse mAb	1C12/ 9746	1:1000	-	-	1	III
Caspase-9	rabbit pAb	9502	1:1000	-	-	1	III
Other							
α -SMA	mouse mAb	1A4	-	-	1:400	2	II, III
α -SMA	mouse mAb (Cy3- conjugated)	1A4	-	-	1:1000	2	I, II
actin	mouse mAb	C4	1:6000	-	-	3	I-III
CD45	mouse mAb	2B11+P D7/26	-	1:100	-	4	III
HO-1	rabbit pAb	SPA- 896	1:1000	1:1000	1:1000	5	III
MMP-9	mouse		1:500	-	-	6	I

α -SMA, alpha-smooth muscle actin; Cl. caspase-3, cleaved caspase-3; HO-1, heme oxygenase-1; IF, immunofluorescence; IHC, immunohistochemistry; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; MMP-9, matrix metalloproteinase 9; p-, phospho; pAb, polyclonal antibody; WB, Western blotting; 1. Cell Signaling Technology Inc., Danvers, MA, USA; 2. Sigma, St. Louis, MO, USA; 3. Millipore, Temecula, CA, USA; 4. DAKOCytomation, Glostrup, Denmark; 5. Assay Designs, Stressgen, Ann Arbor, MI, USA; 6. Serotec Ltd., Oxford, UK

4.2.1 Histological stainings (I-III)

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

4.2.2 Immunofluorescence stainings (I-III)

The sections were fixed with 4% formaldehyde and blocked with 5% normal goat serum (NGS) (Vector Laboratories Inc., Burlingame, CA, USA) diluted in 0.3% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) in phosphate-buffered saline (PBS/Triton). After fixation, some sections were incubated in 0.1% Triton X-100 in PBS for 10 min, followed by blocking with 3% NGS, 3% bovine serum albumin (BSA) (Sigma-Aldrich) in PBS or

5% NGS in PBS. Primary antibodies (see Table 1) were diluted in 5% NGS in PBS/Triton or in 1.5% NGS/PBS, incubated for 30 min at room temperature (RT) and overnight at 4°C, and detected with fluorochrome-conjugated Alexa Fluor 488 (green) secondary antibody (Molecular Probes Inc., Eugene, OR, USA) diluted 1:200 in 5% NGS in PBS/Triton. For double-stainings with α -SMA, the sections were incubated with Cy3-conjugated anti- α -SMA antibody (in 1.5% normal horse serum/PBS) or with anti- α -SMA antibody (Table 1) in 1.5% NGS in PBS, followed by detection with Alexa Fluor 546 (red) or 555 (red) secondary antibody (Molecular Probes Inc., dilution 1:200). The sections were mounted in Vectashield medium with DAPI (Vector Laboratories Inc.) to detect the nuclei.

4.2.3 Immunohistochemical stainings (III)

For immunohistochemical stainings, the fixation, blocking, and incubation with the primary antibody were performed as described above. The endogenous peroxidase was blocked with 3% H₂O₂ in PBS. Next, the sections were incubated with biotinylated secondary antibodies (Vector) diluted 1:200 in 1.5% NGS or normal horse serum in PBS for 30 min at RT. Sections were then incubated with horseradish peroxidase-conjugated avidin-biotin complexes and visualized with diaminobenzidine (Vector). Finally, the background was stained with Mayer's hematoxylin.

For the detection of endogenous peroxidase activity, the snap-frozen samples were directly incubated with diaminobenzidine. In addition, the sections were counterstained with hematoxylin and mounted.

4.2.4 TUNEL assay (III)

TUNEL assay was used to detect cell death. TUNEL reaction was performed using a Fluorescein In Situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions, followed by immunofluorescence staining.

4.2.5 Imaging

Immunofluorescence stainings were microphotographed with an Axioplan2 fluorescence microscope and camera (Carl Zeiss Vision GmbH, Aalen, Germany) with appropriate filters. Pseudo-colored images were created with Axiovision 4.8 (Carl Zeiss) and Image J (NIH software, Bethesda, MD, USA). Immunohistochemical stainings were microphotographed with an Axioplan 2 light microscope and camera (Carl Zeiss). The aneurysm wall was reconstructed from serial microphotographs using Image J software.

4.2.6 Histological evaluation

Evaluation of localization of phospho-proteins (I, II)

To study the existence and localization of active forms of signaling proteins in IA samples, the localization of phosphorylated forms of signaling proteins (Table 1, except the unphosphorylated form of Bad) in the IA wall (luminal / medial / outer side of the wall) was evaluated categorically. Localization inside the cell (cytoplasm / perinuclear region / nucleus) and colocalization with α SMA-positive cells were scored.

Evaluation of TUNEL stainings

For quantification of TUNEL stainings, the percentage of TUNEL-positive cells of the total number of cells per view (0.3621 mm^2) was calculated. One to three “hot spot” fields were analyzed per sample. The measurements were performed three times, and the mean values were calculated. The colocalization of TUNEL-positive nuclei with α SMA-positive cells was evaluated from double-stainings.

Evaluation of cleaved caspase-3 stainings

The amount of cleaved caspase-3-positive cells was scored as follows: completely negative sample, 1 to 5 positive cells, more than 5 positive cells per view. The presence or absence of nuclear staining, positively staining matrix, positively staining cell remnants without nuclei, and colocalization of cleaved caspase-3 staining with α SMA-positive cells was categorically scored (yes / no).

Evaluation of HO-1 expression

Localization of the HO-1 signal in the IA wall (luminal surface / intramural / adventitial / thrombus) was scored. Cell populations (endothelium / mural cells / inflammatory hot spots / thrombus) positive for HO-1 were also scored. The number of HO-1-positive cells per total number of cells and per sample area was counted. In addition, the amount of HO-1-positive staining was also categorically scored (none / one spot / several spots / ubiquitous). The association of HO-1 expression with specific wall type was analyzed. The previously published grading system was applied to classify the IA wall type: A, endothelialized wall with linearly organized SMCs; B, thickened wall with disorganized SMCs; C, hypocellular wall with either myointimal hyperplasia or organized thrombus; D, extremely thin thrombosis-lined hypocellular wall (Frösen et al. 2004). The number of CD45-positive cells was also counted, and the correlation of HO-1 expression and CD45-positive cells was analyzed. Using immunofluorescence stainings, the colocalization of HO-1-positive cells with α SMA-positive cells was evaluated.

4.3 Western blotting (I-III)

IA samples were pulverized in liquid nitrogen using a mortar and pestle, followed by homogenization in hot 1% sodium dodecyl sulphate (SDS) in PBS buffer, and sonication. Samples were kept at 100°C for 10 min and immediately frozen on dry ice. The mixture was centrifuged at 14 000 g for 15 min at 4°C. The supernatant was then separated. Using the DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA), the protein concentrations were determined according to the manufacturer's instruction. Samples were mixed (5:1) with sample buffer (0.3 mol/L Tris, pH 6.8, 10% SDS, 25% β -mercaptoethanol, 50% glycerol, and bromophenol blue) for gel electrophoresis. Proteins were separated in 7.5-12% SDS polyacrylamide gel with Tris-glycine-SDS running buffer. With Tris-glycine transfer buffer and wet blotter (Bio-Rad Laboratories), proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories). Then, 5% nonfat dry milk in 0.1% Tween-20 (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) in Tris-buffered saline (TBST) was used to block the membrane for 1 hour at RT. The blots were then incubated with primary antibodies (Table 1) diluted in 5% BSA (Sigma-Aldrich) in TBST or 5% nonfat dry milk in TBST. Next, the blots were incubated with

peroxidase-conjugated goat anti-mouse/anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) diluted 1:50 000 in milk/TBST. For visualization of the antibody-antigen complexes by chemiluminescence, ECL Plus Western Blotting Detection Reagents (Amersham Biosciences/GE Healthcare, Piscataway, NJ, USA) and Typhoon scanner (Amersham) were used according to the manufacturer's instructions.

For actin Western blot, 20 μg of protein was loaded. Actin, which is a ubiquitous intracellular protein, served as an internal loading control to standardize the amount of intracellular protein for each IA sample. For caspase analysis, the gels with about 60 μg (50 to 79 μg) of total protein per sample (4 unruptured and 6 ruptured IAs) were run first to determine whether cleaved forms of caspases exist in IAs.

Quantification of Western blotting (WB) scans was conducted using Scion Image software (www.scioncorp.com). Quantified optical densities on Western blots were standardized to actin levels of the same samples. Results are expressed as arbitrary units, where the mean of the unruptured group is set at 1.00. Phosphorylation state was calculated as the ratio between phosphorylated form and total protein level. In the case of Bad, the ratio between phosphorylated and unphosphorylated protein was calculated.

In caspase WB, cytochrome c-treated Jurkat cell extracts (Cell Signaling Technology Inc., Danvers, MA, USA) served as controls.

4.4 Statistical analysis

For statistical analysis, SPSS for Windows (13.0, 17.0 (SPSS Inc., Chicago, IL, USA) and 19.0.0.1 (IBM Corporation, Somers, NY, USA)) was used. Proportions, means, standard deviations, medians, and ranges were calculated for categorical and continuous variables, and Fisher's exact, Pearson's χ^2 , non-parametric Mann-Whitney U, and Spearman's correlation test were used as appropriate. Phospho-proteins that were associated significantly with either rupture or morphometric indices were further tested for interactions between rupture status, morphometric indices (each index tested individually), and protein levels with two-way analysis of variance, using the Univariate General Linear Model module of SPSS. For variables with inequality of variances between rupture status

4 Patients, materials, and methods

groups (Levene's test), logarithmic transformation before two-way ANOVA was performed. Statistical significance was set at $P < 0.05$.

5 Results and discussion

5.1 Intracellular signaling pathways in IA wall degeneration and rupture (I, II)

Localization of signaling proteins in the IA wall (I, II)

Active (phosphorylated form, except for Bad, the unphosphorylated form) forms of all studied intracellular signaling proteins (JNK, p38, ERK, Bad, mTOR, CREB, and Akt) were found in the ruptured and unruptured IA wall in immunofluorescence staining. Double-staining with α SMA was performed.

Phospho-JNK was detected in the nuclei of α SMA-positive cells, and the cytoplasm was also weakly positive. In the outer IA wall, some α SMA-negative cells were positive for phospho-JNK with or without positive nuclei. Phospho-p38 was detected in the cytoplasm of most and the nuclei of some α SMA-positive cells. A few phospho-p38 positive cells were also localized in the luminal and outer sides of the IA wall. Phospho-ERK was detected in the cytoplasm of α SMA-positive cells and in some α SMA-negative cells in the outer and luminal side of the wall. Bad localized mainly in the cytoplasm and the perinuclear region of α SMA-positive cells. Some nuclei were also positive for Bad, as were some α SMA-negative cells. Phospho-mTOR was detected in all samples in the cytoplasm of α SMA-positive and α SMA-negative cells. In some samples, phospho-mTOR was also found in the nuclei of some α SMA-positive and some α SMA-negative cells. Phospho-CREB was mainly detected in the nuclei of α SMA-positive and α SMA-negative cells in the IA wall, but in a few samples phospho-CREB was also found in the luminal thrombus. Phospho-Akt staining localized mostly in the nuclei and in the cytoplasm of α SMA-positive cells. The nuclei and cytoplasm were also positive for phospho-Akt in some α SMA-negative cells.

5.1.1 Intracellular signaling and IA rupture (I, II)

The phosphorylation of p54 JNK was increased 1.5-fold in ruptured IAs compared with unruptured ones ($P = 0.028$, Student's independent samples t-test) in the univariate analysis. However, in the covariance analysis the phosphorylation of p54 JNK was

associated with IA size, but not with rupture status ($P = 0.023$ and $P = 0.296$, respectively). The phosphorylated and total levels of transcription factor c-Jun correlated with the levels of phosphorylated p54 and p46 JNK. Statistically nearly significant increase in c-Jun levels was observed in ruptured IAs.

In 3D remodeling, p38 levels were not associated with rupture in univariate analysis. However, phospho-p38 levels associated significantly with both rupture and size indices (volume, surface area, fundus length, maximum diameter) in covariance analysis. This result may indicate that cellular stress and inflammatory signaling are involved in both IA growth and rupture.

Phospho-Bad (Ser136) levels and the ratio between phosphorylated and unphosphorylated Bad were decreased 5-fold in ruptured IAs ($P < 0.001$ and $P = 0.001$, respectively). This association was also shown in covariance analysis. Bad is able to promote apoptosis when unphosphorylated. Therefore, our finding suggests the activation of proapoptotic Bad signaling and the intrinsic apoptotic pathway in ruptured IAs.

Phospho-mTOR levels and mTOR phosphorylation state were decreased in ruptured IAs compared with unruptured IAs in univariate analysis (0.39 ± 0.23 vs. 1.00 ± 0.44 , $P = 0.001$ and 0.63 ± 0.35 vs. 1.00 ± 0.40 , $P = 0.039$, respectively). In the covariance analysis, phospho-mTOR levels were significantly associated with rupture, whereas only nearly significant association between mTOR phosphorylation state and rupture was observed. mTOR regulates cell growth and proliferation. In the IA wall, mTOR may have a protective role in maintaining cell growth. This seems to be compromised in ruptured IAs.

ERK, CREB, and Akt were not associated with rupture.

MMP-9 expression (I)

Because the JNK pathway was active in large and ruptured IAs, we studied potential mechanisms affecting IA size and wall degeneration. By regulating MMP expression (Shin et al. 2002), JNK and c-Jun may affect matrix remodeling, thus affecting IA wall degeneration and growth. The regulatory role of JNK in the production of MMP-9 in AAA has been shown (Yoshimura et al. 2005). Thus, we evaluated the levels of pro-MMP-9 in the IA wall. Pro-MMP-9 levels were increased 4.3-fold in ruptured IAs ($P < 0.001$, Student's t-test). Pro-MMP-9 levels did not correlate with IA size. When comparing large IAs (>8.0 mm in diameter) with small IAs, pro-MMP-9 levels were 2.6-fold higher in

large IAs ($P = 0.014$). In covariance analysis, pro-MMP-9 levels were associated with IA rupture, but not with IA size ($P < 0.001$ and $P = 0.385$, respectively). A correlation between pro-MMP-9 levels and phosphorylation of p54 JNK was found in unruptured IAs, but not in ruptured ones ($r = 0.797$, $P = 0.002$ and $r = -0.266$, $P = 0.404$, respectively), suggesting that JNK may be one of the regulators of MMP-9. WB samples were highly denatured to preserve the phosphorylation state of the proteins. Thus, we could not investigate the enzymatic activity of MMP-9 using e.g. zymography.

5.1.2 Intracellular signaling and IA size (I, II)

The levels of phospho-p54 JNK and its phosphorylation state correlated with IA diameter ($r = 0.490$, $P = 0.015$ and $r = 0.501$, $P = 0.013$, respectively). In 3D remodeling, phospho-p54 JNK level was associated positively with three size indices (volume, surface area, maximum diameter). A correlation between c-Jun levels and IA size was not observed, but a nearly significant increase was found in phospho-c-Jun levels in large IAs (>8.0 mm in diameter) compared with small IAs.

The levels of phospho-p38 and total p38 correlated with IA size ($r = 0.506$, $P = 0.012$ and $r = 0.592$, $P = 0.002$, respectively). In 3D remodeling, p38 levels were associated positively with several size indices (volume, surface area, fundus length, maximum diameter). The levels of ERK did not correlate with IA size or shape indices.

JNK and p38 are stress-activated kinases. Their association with size indices may indicate that they have important roles in regulating IA wall remodeling. Their activation may be a pathological response to hemodynamic stress, leading to increased IA size, or they may have a protective role by maintaining cell proliferation and wall repair mechanisms. JNK and p38 were also associated with rupture, which could indicate that their activation results in a pathological cellular response leading to IA rupture. In AAAs, treatment with JNK inhibitor SP600125 resulted in regression of induced AAAs in mice and in diminished secretion of MMP-9 and MMP-2 in an ex vivo culture of the human AAA wall (Yoshimura et al. 2005, Yoshimura et al. 2012). p38 inhibitor (SB203580) has reduced the size of a atherosclerotic lesion in mice (Seeger et al. 2010), and preliminary clinical trials with p38 inhibitors suggest that p38 inhibition attenuates vascular inflammation and improves vascular function (Sarov-Blat et al. 2010, Cheriyan et al.

2011, Denise Martin et al. 2012, Elkhawad et al. 2012). In the future, stress-activated kinases should be studied with an appropriate experimental IA model to further elucidate their role in IA pathogenesis.

The levels of the well-known cell survival and growth promoter Akt (phospho-Akt(Ser473)) were associated with volume, surface area, fundus length, and maximum diameter in covariance analysis. Akt levels were not associated with rupture. Akt signaling probably maintains cell growth and proliferation in the IA wall during the remodeling process.

Phospho-Bad levels were not associated with morphometric indices. A positive correlation between unphosphorylated Bad levels and maximum diameter was found in univariate and covariance analyses.

Total CREB levels were associated with maximum diameter and neck diameter, which may indicate protective or growth-promoting signaling in large IAs as discussed further in the following chapter.

5.1.3 Intracellular signaling and IA shape (I, II)

The phosphorylation state of CREB correlated positively with nonsphericity index and ellipticity index in both univariate and covariance analyses. Total CREB was associated negatively with undulation index. CREB may have a protective role in vascular tissue or it may increase the neointimal formation (Tokunou et al. 2003, Reusch et al. 2004), and CREB activation has been suggested to be an acute, protective response against harmful insults in the vascular wall (Schauer et al. 2010). Our result suggests that CREB activation in an irregular IA wall may be a protective response to extracellular stress, but it is also possible that CREB promotes vascular remodeling, like myointimal hyperplasia, in the IA wall. The association between total CREB levels and maximum diameter of IA fundus may implicate that CREB is a positive regulator of cell proliferation in the IA wall. However, as the active, phosphorylated form of CREB was not associated with size indices, this interpretation is only tentative. On the other hand, the decreased expression of total CREB in the irregular IA wall is in line with the observation that the expression of CREB is decreased in chronic vascular pathologies (Schauer et al. 2010), suggesting that the protective CREB response may have failed in the irregular IA wall. All in all, the

associations between CREB and IA size and shape indices are complex. The possible activation or downregulation of CREB in the IA wall should be studied using a larger number of samples and experimental animal models or tissue cultures before definitive conclusions of the role of CREB in IA pathobiology can be drawn.

Phospho-p38 levels correlated negatively with undulation index. p38 phosphorylation state correlated negatively with ellipticity index. In covariance analysis, the association with undulation index was not seen, and there was only a nearly significant association with ellipticity index.

Phospho-Akt levels and Akt phosphorylation state correlated negatively with undulation index in the univariate analysis, but significant associations were not seen in the covariance analysis.

The negative correlation of p38 and Akt with undulation index may indicate that Akt and p38 are involved in protective signaling pathways that have failed in irregularly shaped IA walls. Thus, the IA wall may become weaker and more prone to rupture. On the other hand, the observations that increasing p38 levels were associated with increased IA size and rupture, but decreasing phospho-p38 levels were associated with increased irregularity index (undulation index), which is known to predispose to rupture, suggests a more complex role of p38 in IA wall pathobiology. These observations of p38 activation also suggest that although both increasing IA size and increasing IA irregularity increase the risk of IA rupture, their underlying pathological mechanisms may differ.

5.1.4 Summary of intracellular signaling pathways and IA size, shape, and rupture

The associations between the levels of signaling molecules and IA size, shape, and rupture are summarized in Figure 4. Both IA size and irregularity predict IA rupture. However, the mechanisms underlying the weakening of the IA wall may differ between large and irregular IAs, as the activation of signaling pathways differs between them as discussed above.

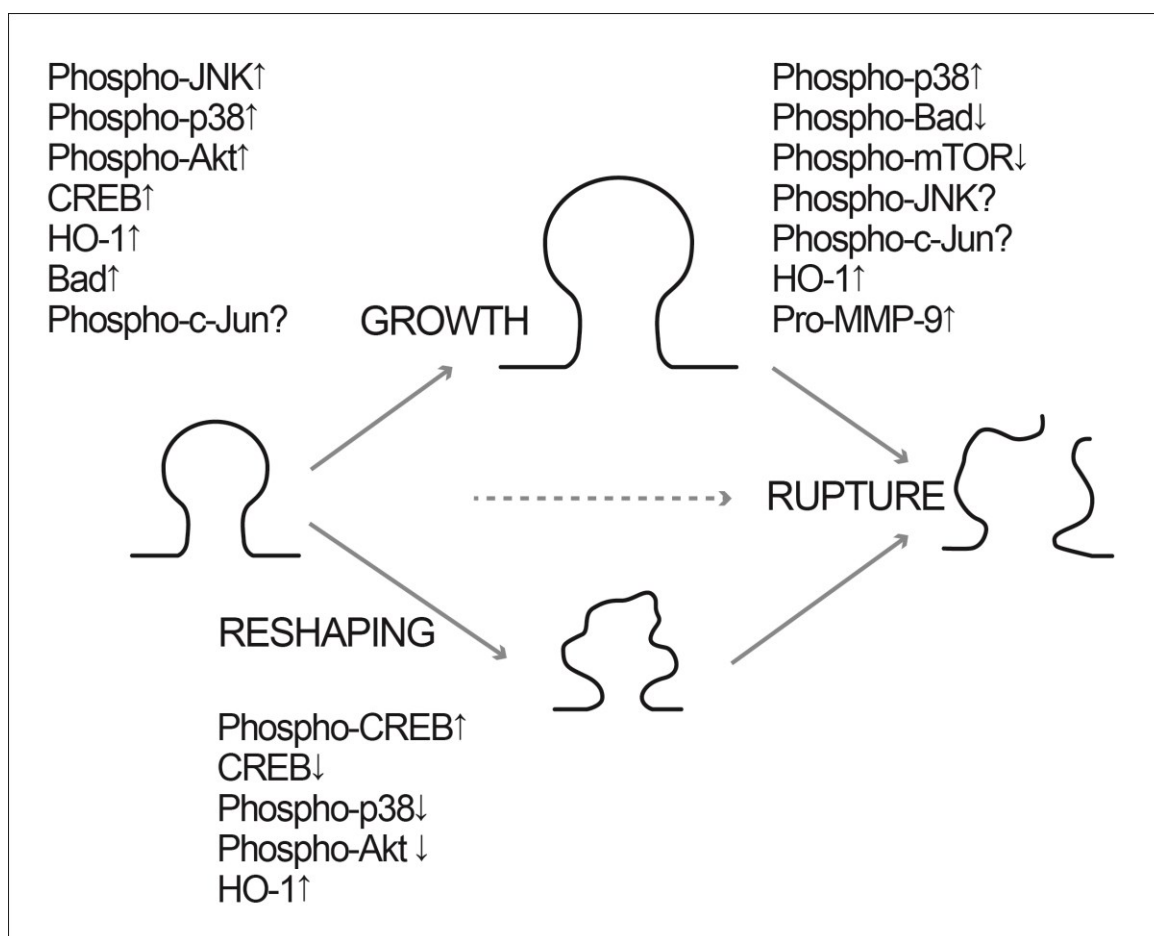


Figure 4 *Summary of the signaling pathways in the human intracranial aneurysm. Potential roles of signaling molecules in intracranial aneurysm growth, reshaping, and rupture as suggested by the associations of protein levels with aneurysm size and shape indices and aneurysm rupture are shown. Aneurysm growth and/or reshaping likely precede the rupture, but the exact mechanism leading to aneurysm rupture is not known. The dashed arrow indicates unknown mechanisms leading to rupture.*

5.2 Cellular death in IA wall (II, III)

Degeneration of the IA wall, including the loss of mural cells, is associated with IA wall rupture (Kataoka et al. 1999, Frösen et al. 2004, Tulamo et al. 2006). TUNEL-positive cells have been observed in several studies (Sakaki et al. 1997, Frösen et al. 2004, Pentimalli et al. 2004, Tulamo et al. 2006), and apoptotic cell death has been observed in IA walls also using electron microscopy (Pentimalli et al. 2004, Tulamo et al. 2006, Guo et al. 2007). Necrotic cell death has also been reported (Holling et al. 2009). However, the significance of different types of cell death in the IA wall is unknown. Also unknown are the triggers for cell loss and the cellular death pathways activated. In this series, the

percentage of TUNEL-positive cells was higher in ruptured samples (median 12%, range 0-45% in ruptured samples vs. 1%, range 0-12% in unruptured samples, $P < 0.001$, Mann-Whitney U-test). TUNEL-positive cells were mainly α SMA-negative.

5.2.1 Apoptosis vs. necrosis

To evaluate the role of apoptotic cell death, we investigated the activation of caspase-3 by WB and immunofluorescence staining. We found uncleaved caspase-3 (35 kDa) in all WB samples (12 unruptured and 12 ruptured), but cleaved caspase-3 (17 kDa and 19 kDa), the active form of caspase-3, was not detected in WB. We increased the amount of protein loaded to the gels to 60 μ g/well to enhance the sensitivity of the assay, but still could not detect cleaved caspase-3. However, using immunofluorescence staining, which is more sensitive in detecting random positive cells than WB, we found some cleaved caspase-3-positive cells in the IA wall. Cleaved caspase-3-positive cells were associated with IA rupture ($P = 0.01$, Chi-square test). The cells were mainly α SMA-negative.

The number of cleaved caspase-3-positive cells was extremely low relative to the number of TUNEL-positive cells. TUNEL stains not only apoptotic cells but also cells undergoing necrosis (Grasl-Kraupp et al. 1995, Huerta et al. 2007). The explanation for the discrepancy between the amount of TUNEL-positive and cleaved caspase-3-positive cells may be the different forms of cell death (apoptosis vs. necrosis) occurring in the IA wall or the short half-life of cleaved caspase-3. RNA synthesis, RNA splicing, mitosis, sample preparation, and fixation may also affect the pattern of TUNEL staining (Migheli et al. 1994, Davison et al. 1995, Lucassen et al. 1995, Sträter et al. 1995, Negoescu et al. 1996, Kockx et al. 1998, Saraste 1999).

In this series, only a few cells were double-positive for α SMA and TUNEL or cleaved caspase-3. This may indicate that the loss of the SMCs in the IA wall is a very slow process, whereas a larger number of α SMA-negative cells, which are most likely inflammatory cells, are dying. These dying inflammatory cells probably release agents that promote and maintain mural cell death.

5.2.2 Mechanisms of cell death in the IA wall

In all WB samples, full-length caspase-8 (57 kDa) and full-length caspase-9 (47 kDa) were detected. Cleaved caspase-9 (35 kDa and 37 kDa) was found in 5 (42%) of 12 ruptured samples, whereas unruptured samples were negative for cleaved caspase-9. When 60 µg of protein per well was used, cleaved caspase-9 was detected in all ruptured (6/6 samples) and one unruptured sample (1/4 samples). Cleaved caspase-8 (41 kDa and 43 kDa) was not detected in IA samples, even when 60 µg of protein per well was used.

Cleaved caspase-9 activation suggests that programmed cell death is activated by the intrinsic pathway in the IA wall. Increased levels of unphosphorylated, proapoptotic Bad protein in ruptured IAs also support this finding. No sign of extrinsic activation (cleaved caspase-8) of the apoptotic pathway was observed. Intrinsic activation of apoptosis has also been noted in elastase-induced aneurysms in rabbits (Kadirvel et al. 2010). Cell damage or stress, like oxidative stress, may induce this type of cell death.

5.3 Role of oxidative stress in IA wall degeneration and rupture (III)

5.3.1 HO-1 expression in the IA wall

Oxidative stress is involved in vascular lesions, including atherosclerotic plaque and AAAs (McCormick et al. 2007, Peterson et al. 2009). The important role of ROS in IA formation and growth has been shown in induced aneurysms in animal models (Aoki et al. 2009). Oxidized neoepitopes (Frösen 2006, Tulamo et al. 2010b) and CD163-positive macrophages (Frösen et al. 2004) are found in IA walls, suggesting that the cells in the IA wall are also exposed to oxidative stress. Oxidative stress is one of the activators of the intrinsic apoptotic pathway.

To study the possible involvement of oxidative stress in the IA wall, we investigated the levels and expression of HO-1. It has previously been shown that HO-1 polymorphisms are associated with aneurysmal SAH (Morgan et al. 2005) and HO-1 has various protective roles in vascular lesions (Peterson et al. 2009).

In WB, HO-1 protein expression was 8.7-fold higher in ruptured IAs than in unruptured IAs (mean 8.68 ± 11.16 vs. 1.00 ± 1.18 , $P = 0.002$, Mann-Whitney U-test) but HO-1 expression was not associated with the severity of SAH (Fisher score) or with the time from rupture to sample collection, suggesting that HO-1 expression is not just a reaction to rupture. Moreover, in immunohistochemical staining, HO-1 expression was associated with wall type ($P = 0.003$, Kruskal-Wallis test), and HO-1-positive cells were also found in unruptured IA samples, suggesting that oxidative stress has a role in the degeneration process of the IA wall. In WB, HO-1 levels were not associated with wall type, probably because thin, decellularized walls were not selected in WB.

The percentage of HO-1-positive cells was also associated with the percentage of TUNEL-positive cells and with the amount of cleaved caspase-3-positive cells. This result may indicate that the protective HO-1 response is activated due to high oxidative stress, but, in some cells, the protective signaling is insufficient and cell death is increased.

In immunohistochemical staining, HO-1-positive cells were mainly detected in the areas with inflammatory cell infiltration. Positive staining was especially localized in the areas where the lumen was lined by thrombus. The percentage of HO-1-positive cells was also associated with the number of CD45-positive cells ($P < 0.001$, Spearman correlation), and, in the immunofluorescence double-stainings, HO-1 expression was localized in α SMA-negative cells. Hence, in the IA wall, HO-1 is likely to be mostly expressed by inflammatory cells.

5.3.2 Thrombus as a source of oxidative stress

The thrombus itself had high endogenous peroxidase activity and the endogenous peroxidase staining localized into PMNs. Neutrophils, which belong to the PMN family, have azurophilic granules that contain myeloperoxidase (MPO) (Nicholls et al. 2005). Some phenotypes of macrophages also contain MPO (Sugiyama et al. 2001). MPO catalyzes the formation of ROS and is able to initiate lipid peroxidation (Klebanoff 1980, Zhang et al. 2002, Nicholls et al. 2005). Thus, MPO increases oxidative stress and has been shown to have detrimental effects on the progression of atherosclerosis (Nicholls et al. 2005).

In AAAs, ILT with leukocytes, RBCs, and platelets has been suggested as a main source of oxidative stress (Michel et al. 2011). Thrombus with PMNs is also likely to be a main source of oxidative stress in IA tissue. Another source of oxidative stress in the IA wall may be cells that are dying via necrosis because they may release free radicals and enzymes involved in oxidant pathways.

5.3.3 Oxidative stress in IA samples – association with SAH risk factors

In both WB and immunostaining series, HO-1 expression correlated with fundus length and aspect ratio. Large IA size and large aspect ratio favor the development of aberrant flow conditions in the IA fundus (Ujiie et al. 1999, Hashimoto et al. 2006). Nonphysiological WSS may promote de-endothelialization and thrombus formation, which again may increase oxidative stress and lead to degeneration of the IA wall.

Interestingly, HO-1 expression was downregulated in smokers in the immunostaining series. Cigarette smoke increases oxidative stress of the vascular wall (Pipe et al. 2010). In human macrophages, which are exposed to cigarette smoke condensate, HO-1 shows early induction (6 hours), but later (after 72 hours) HO-1 expression is decreased (Goven et al. 2009). Similarly, in human umbilical vein endothelial cells, HO-1 expression is decreased when the cells are exposed to smokers' serum (Fratta Pasini et al. 2012). Regulation of the protective HO-1 may have also failed in the IA wall of smokers, increasing oxidant injury.

In the WB series, HO-1 expression was downregulated in ruptured IAs in patients with hypertension. Hypertension also increases oxidative stress of the vascular wall (Peterson et al. 2009), but how hypertension affects the defense mechanism against oxidative stress in the IA wall remains to be elucidated.

Regardless of rupture status, HO-1 expression was increased in males in the WB series. The reason might be gender-related differences in the defense mechanisms against oxidative stress, but confirmation requires further studies. However, the association between decreased HO-1 expression and female gender is in line with the epidemiological observation that females are at higher risk of aneurysmal SAH than males.

The reason for the associations between HO-1 levels and clinical risk factors may be covariance with rupture status or other variables. Potential associations of HO-1 levels with clinical risk factors should be explored using a larger number of samples.

5.3.4 Possible role of oxidative stress in IA pathobiology

Our results show that signs of oxidative stress (HO-1 expression) are found in the human IA wall. HO-1 expression is associated with wall degeneration and cell death. Our results also show the intrinsic activation of caspases, suggesting that one activator of cell death may be oxidative stress. It is also possible that some cells exposed to oxidative stress die via necrosis. Our findings reveal that thrombus with PMN is one source of oxidative stress. Oxidative stress is also able to induce inflammatory response in the vascular wall (Ross 1999, Griendling et al. 2000, Nicholls et al. 2005), and inflammation itself has a role in the degeneration process of the IA wall.

5.4 IA pathobiology and cell signaling

Vascular remodeling has been characterized in the IA wall, and wall degeneration is associated with IA rupture (Kataoka et al. 1999, Frösen et al. 2004). The geometry of the IA wall affects the flow conditions inside the IA pouch, and hemodynamic stress inside the saccular IA differs from that of the normal cerebral artery (Hashimoto et al. 2006, Jeong et al. 2012). Cell signaling pathways mediating vascular remodeling are activated in response to abnormal hemodynamic stress. The ultimate goal of wall remodeling is likely to be adaptation to the changing hemodynamic environment (Hashimoto et al. 2006), and, based on our results, it seems that stress signaling and cell proliferation pathways are associated with IA growth. Some pathways are also associated with IA shape, and they may either enhance the irregular remodeling of the wall or mediate the protective response against pathological remodeling. Aberrant flow conditions inside the IA pouch are also likely to cause endothelial dysfunction and de-endothelialization of the IA wall, which predisposes to thrombus formation (Frösen et al. 2012). Thrombus with inflammatory cells seems to be an important source of oxidative stress and is likely to be an important modulator of IA wall degeneration. The imbalance between protective and harmful signaling pathways as well as the activation of cell death pathways probably lead to weakening of the wall and finally to IA rupture, as summarized in Figure 5.

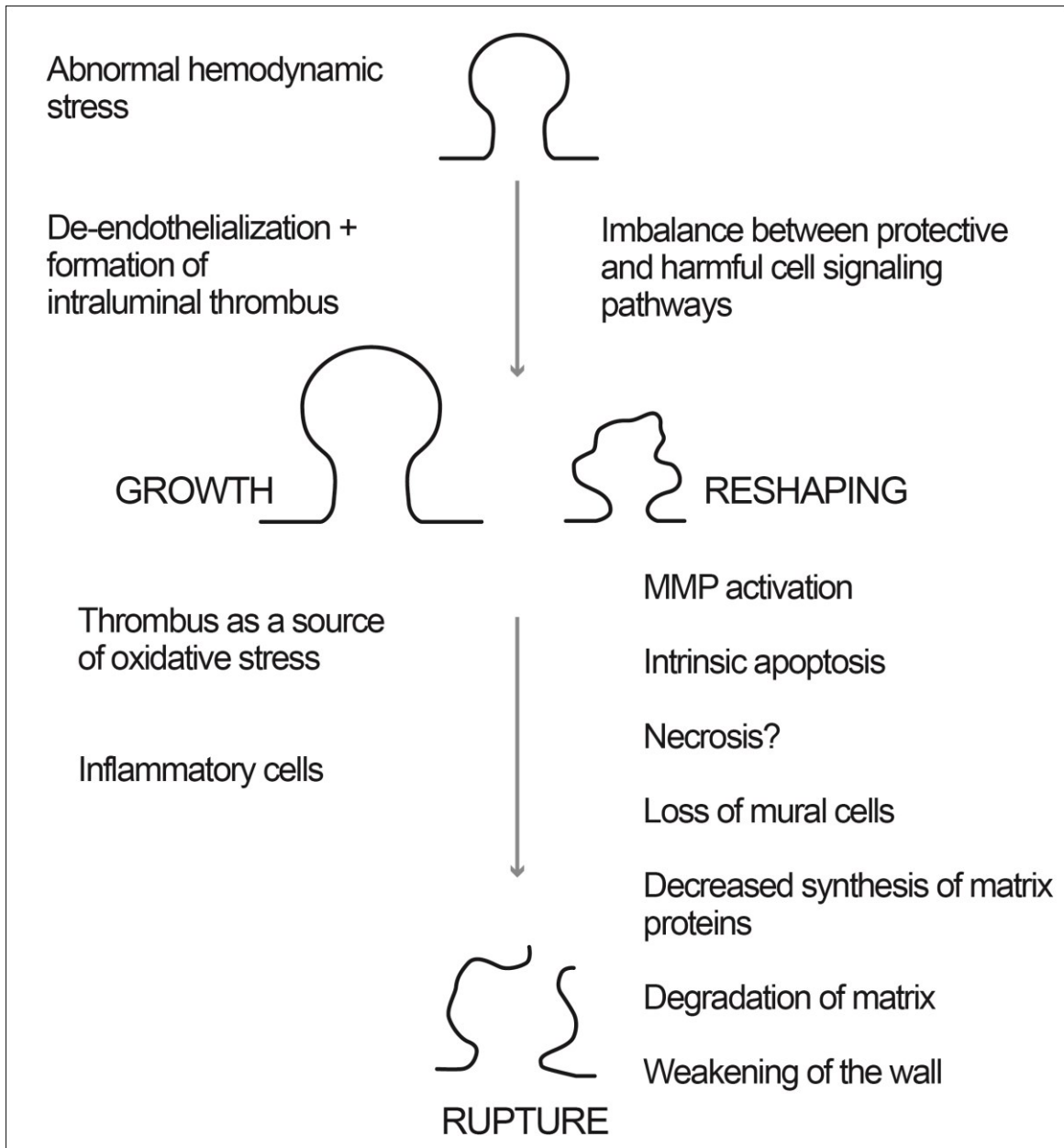


Figure 5 *Potential mechanisms of intracranial aneurysm wall degeneration and rupture.* Cell signaling pathways mediating, for example, cell proliferation and survival but also cell death and matrix degradation are activated probably due to abnormal hemodynamic stress. Thrombus with inflammatory cells is likely to be an important source of oxidative stress, thus modulating the remodeling process of the wall. The imbalance between “maintaining” and “degrading” pathways probably leads to enlargement of the IA dome as well as to wall irregularities, which in turn change the hemodynamic environment of the IA wall. The vicious circle of abnormal hemodynamics, wall irregularities, and an imbalance between protective and harmful signaling pathways likely leads to wall degeneration and IA rupture.

5.5 Limitations of the study

This study has certain methodological limitations. We used freshly frozen human IA samples. However, we did not have normal arterial tissue as a control sample. Fresh samples of normal intracranial arteries are extremely difficult to obtain, and the structure of extracranial arteries differs from that of intracranial arteries. On the other hand, postmortem samples are subject to dephosphorylation and autolysis/degradation of proteins. Thus, we did not consider postmortem samples to be reliable controls when studying phosphorylated proteins and caspases, which can be rapidly dephosphorylated, cleaved, and degraded. Although fresh samples were carefully stored and prepared for WB and immunostainings, the handling of tissue samples (like cryosectioning) is a possible source of artifacts.

Surgically obtained human IA samples are small. To obtain a sufficient amount of protein for WB homogenates, we were obliged to exclude the smallest samples, which were often unruptured. Thus, the unruptured samples in the WB series are larger than unruptured IAs on average. Acellular or highly thrombosed IA samples were excluded from the WB series because the aim was to study intracellular signaling proteins. IAs containing significant intraluminal thrombus based on imaging or intraoperative findings were excluded from 3D remodeling (II) because intraluminal thrombus prevents accurate angiographic determination of IA geometry. Because it is difficult to obtain fresh human IA samples, we had a relatively small number of samples, they were not equally distributed between males and females, and their localization in cerebral arteries was heterogeneous. Thus, the results may not be generalizable to all subtypes of IA. Because of the small sample size, we could not analyze the signaling pathway profiles in the subgroups of unruptured IAs, for instance, in relation to the wall type. Additionally, the reason for the associations between signaling proteins and clinical risk factors may be covariance with rupture status or other variables.

The IA neck, which is left under the clip in microneurosurgical clipping, cannot be taken as a sample, and thus, is unavailable for study.

Homogenized IA samples were used in WB, although the IA wall may have local structural differences and also the amounts of signaling proteins may differ in different parts of the wall. However, the quantitation of ubiquitously expressed signaling molecules using other methods, like immunohistochemistry, is practically impossible.

We used immunohistochemistry to study localization of the signaling proteins, cell death markers, and HO-1 in the IA wall. We performed the double-stainings using α SMA to determine whether the staining colocalizes with the mural SMCs. However, fibroblasts, endothelial cells, and different types of inflammatory cells are also found in the IA wall. In further studies, the types of α SMA-negative cells expressing the proteins of interest could be characterized in more detail using immunohistochemistry.

The presence of oxidative stress in IA wall was evaluated using HO-1, which is an anti-oxidant enzyme induced by pro-oxidant conditions, and using endogenous peroxidase staining. Further investigation of oxidative stress in IA wall could include the more detailed characterization of endogenous peroxidase activity in IA wall, for instance, if active MPO exist in IA wall or in ILT (Daugherty et al. 1994).

The differences between unruptured and ruptured IA samples may be due to SAH. Using this kind of experimental setting, it is impossible to definitely determine whether the association between a biochemical measure and rupture have a causal relationship and whether the observed changes predate the rupture or represent a reaction to it. TUNEL, the amount of cleaved caspase-3-positive cells, or the levels of HO-1 were not associated with the time between IA rupture and sample collection. Causality should be investigated using an appropriate experimental model – which to date does not exist.

All in all, IA formation and rupture is likely to be a heterogeneous disease process as supported by multifactorial etiology of IAs and varying histological appearance of IA wall. Homogenous experimental animal models may not represent the complex nature of IA disease sufficiently well, and the investigation of IA pathobiology would require a large series of unruptured and ruptured human IA samples with the analysis of subgroups of especially unruptured IAs to differentiate the characteristics of rupture-prone IAs.

5.6 Future perspectives

IA rupture followed by SAH is a life-threatening situation. The treatment options of IAs (clipping and coiling) also have risks of complications. Nowadays, a growing number of unruptured IAs are incidentally found when patients are imaged for other reasons or when families with aneurysmal SAH are screened. Not all IAs rupture during the lifetime of their carriers. The problem is how to differentiate rupture-prone IAs from IAs that would

never rupture. With the aid of basic research, IA pathobiology could be understood and biomarkers predicting rupture-prone IAs identified. Knowledge of IA pathobiology could also help to develop less invasive treatment methods for IAs to prevent rupture, and optimally, to prevent IA formation.

5.6.1 Further characterization of signaling pathways in the IA wall

Although in recent years research on IA pathobiology has increased, the mechanisms underlying IA formation, growth, and rupture warrant attention. We investigated cell signaling molecules in whole-tissue homogenates and found interesting associations between the expression of signaling molecules and IA size and shape indices and rupture. However, signaling molecules have different roles in different cells and tissues. Using laser capture microdissection techniques, each cell type and “the profile” of its cell signaling molecules could be studied separately from other IA tissue. In addition, the significance of associations detected in the levels of signaling molecules in human IA samples should be evaluated in experimental animal models or cell cultures. Endothelial cells and SMCs of the human IA wall have been successfully cultured (Boscolo et al. 2006, Bygglin et al. 2011). Moreover, experimental aneurysm models have been developed (Aoki et al. 2011), but an appropriate model of IA growth and rupture is still lacking.

5.6.2 Characterization of the intraluminal thrombus (ILT)

We found that ILT is a significant source of oxidative stress. In AAAs, ILT affects and modulates the remodeling process of the wall (Michel et al. 2011). Inflammatory cells, like T-cells and macrophages, have been detected in the areas of organizing thrombus/myointimal hyperplasia in IA tissue (Frösen et al. 2004), but further characterization of the structure of the thrombus and the cell types that it contains is needed to elucidate the degeneration process of the IA wall. In eroded and ruptured atherosclerotic plaques, the number of macrophages expressing MPO increases (Sugiyama et al. 2001). It would be interesting to determine whether the phenotypes of inflammatory

cells differ also between unruptured and ruptured IAs, potentially yielding a means of differentiating rupture-prone IAs.

5.6.3 Biomarkers

Prognostic biomarkers for cardiovascular diseases have been enthusiastically sought (Vivanco et al. 2005, Martin-Ventura et al. 2007, Urbonavicius et al. 2008, Michel et al. 2011). We found several interesting signaling molecules associated with IA morphology and rupture when studying IA tissue samples. When the aim is noninvasive detection of rupture-prone IAs, the focus is on identifying circulating biomarkers that can be analyzed from a blood sample, but this would require further studies. Modern proteomic methods, such as two-dimensional gel electrophoresis and mass spectrometry, could help to find biomarkers without an a priori hypothesis (Martin-Ventura et al. 2007). However, the special nature of IA disease, such as the small size of IAs and localization in the cerebral vasculature, makes the research on circulating biomarkers challenging.

5.6.4 Perspectives for pharmacological therapies

Investigating IA pathobiology could also aid to develop pharmacological therapy for IAs. In our study, the stress-activated kinases (JNK and p38) and HO-1 were associated with rupture and with size indices. Inhibitors of signaling molecules are available, but they may have toxic side-effects (Bogoyevitch et al. 2008, Davies et al. 2012). Some pharmacological inducers of HO-1 are known such as lipid-lowering drugs probucol and its analogs, statins, and fenofibrate (Peterson et al. 2009, Chan et al. 2011). However, probucol has adverse side-effects and the doses of statins used clinically may not have any effects on HO-1 expression (Chan et al. 2011). In addition, statins do not seem to affect IA formation in humans (Marbacher et al. 2012). The local delivery of drugs using coils could be beneficial to avoid systemic side-effects. However, whether signaling molecules have a pathological or protective role in IA wall degeneration should first be determined using an experimental model before testing them as a pharmacological therapy for IAs. The development of pharmacological therapy preventing IA formation would be most ideal but

5 Results and discussion

it would require further studies with focus on the differences between IAs and normal cerebral arteries.

6 Summary and conclusions

Significant mortality and morbidity are related to SAH. Approximately 2% of the population has IAs. However, not all of these IAs rupture. In addition, treatment methods (clipping and coiling) for ruptured and unruptured IAs have risks. Risk factors for IA formation are age, female gender, smoking, hypertension, and family history. Furthermore, risk factors for SAH are IA size, irregularity, location in posterior circulation, female gender, nationality (Finnish and Japanese people at higher risk), smoking, hypertension, family history, and probably excessive consumption of alcohol. However, even by combining all of these risk factors, one cannot definitely distinguish rupture-prone IAs from IAs that would not rupture. Better predictors for IA rupture risk are needed.

Studying IA pathobiology may yield clues to identifying rupture-prone IAs. Previously, degeneration and inflammation of the IA wall have been linked to rupture. However, these changes are already present in some unruptured IAs, suggesting that the IA wall continuously undergoes remodeling, and in some IAs, the remodeling process leads to pathological weakening of the wall and subsequent rupture.

In recent years, microarray analyses investigating the gene expression of IA walls have revealed associations between inflammation, apoptosis, and oxidative stress and IA rupture. However, to date, studies reporting on the expression of molecules at the protein level in human IA samples are scarce. This study aimed to characterize cell signaling pathways in the IA wall at the protein level.

We found that stress-activated kinases (JNK and p38) were associated with IA size and rupture. They may have protective roles in the growing IA wall. However, the association with IA rupture suggests that their activation may enhance apoptosis and other weakening mechanisms of the IA wall. When studying potential weakening mechanisms of the IA wall, we found elevated pro-MMP-9 levels in ruptured IAs. MMPs are able to degrade ECM, which weakens the vascular wall.

On the other hand, Akt activation was associated with size indices, but not with rupture, and mTOR levels were downregulated in ruptured IAs. Akt and mTOR are both well-known promoters of cell growth and cell proliferation. Their activity, which probably maintains and strengthens the IA wall, is compromised in ruptured IAs.

6 Summary and conclusions

The loss of mural cells is a crucial step in the degeneration and weakening of the IA wall. In addition to growth-promoting factors, we studied the mechanisms of cell death in the IA wall. Caspase-9 activation was observed mainly in ruptured IA samples, whereas no sign of cleaved caspase-8 was detected, suggesting that the intrinsic apoptotic pathway is activated in the IA wall. This finding was supported by the observation that the unphosphorylated, proapoptotic form of Bad, a member of the intrinsic pathway signaling proteins, prevailed in ruptured IAs. A few cells positive for cleaved caspase-3, which is an executioner caspase, were detected and they were associated with IA rupture. However, the number of cleaved caspase-3-positive cells was much lower than the number of TUNEL-positive cells. Thus, we speculated that cells in the IA wall may die via necrosis more often than via apoptosis.

We were also interested in investigating oxidative stress - a possible inducer of intrinsic apoptosis. We found that HO-1 levels, a detoxifying enzyme that is induced during oxidative stress, was associated with wall degeneration, IA rupture, IA size, and aspect ratio. HO-1 localized in the areas of inflammatory cells. Our results also showed that CREB, a signaling protein activated by for instance oxidative stress, was associated with IA irregularity.

The associations of HO-1 with IA size and aspect ratio and CREB with IA irregularity suggest an association between IA geometry and oxidative stress. Abnormal hemodynamic stress, such as nonphysiological WSS in an irregular IA wall, likely promotes de-endothelialization and thrombus formation. We noted that thrombus with PMN had a high endogenous peroxidase activity. Therefore, thrombus, formed especially in irregular IA domes, is likely to be an important source of oxidative stress, linking IA geometry with oxidative stress.

All in all, we found interesting signaling molecules associated with IA morphology and rupture and differences in signaling pathway profiles between unruptured and ruptured IAs. Further characterization of signaling pathways in appropriate experimental models could clarify their role in IA growth and rupture. This could help to identify markers differentiating rupture-prone IAs and, hopefully, also to develop less invasive treatment methods for IAs.

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7 Acknowledgments

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