From Atherosclerosis Research Unit,
King Gustaf V Research Institute, Department of Medicine
Karolinska Institutet, Stockholm, Sweden

CELLULAR AND MOLECULAR MECHANISMS IN ABDOMINAL AORTIC ANEURYSM GROWTH AND RUPTURE

MONSUR KAZI

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ABSTRACT

Fragmentation of elastin, loss of smooth muscle cells (SMCs) and accumulation of inflammatory cells are the histological landmarks of abdominal aortic aneurysms (AAA). In larger aneurysms, an intraluminal thrombus is almost always present. It has been suggested that the thrombus predisposes for AAA rupture. In order to study the structure and composition of the thrombus-free and the thrombus-covered aneurysm wall, we collected biopsies from patients selected for elective operation, where CT scan showed an eccentrically located thrombus with both thrombus-free and thrombus-covered parts of the AAA wall.

In paper I it was shown that there was a significant difference in wall thickness between the two wall segments. The wall underneath the thrombus was thinner compared to the wall without thrombus. Furthermore, the thrombus-covered wall contained less and degraded elastin fibers, less SMCs but more inflammatory T- and B-cells. The decreased levels of SMCs within the thrombus-covered wall may be a result of increased SMC apoptosis, which was supported by the results of electron microscopy analyses and TUNEL assay.

In paper II we examined the possibility that the intraluminal thrombus influences expression and activity of matrix-degrading proteases in the AAA wall. Gene arrays and quantitative real-time PCR showed that matrix metalloproteinase (MMP)-1, -7, -9 and -12 mRNA expressions were upregulated in the thrombus-free wall compared with the thrombus-covered wall. Immunohistochemistry confirmed the differential expression of MMP-9 but also localized MMP-9 to the interface between the thrombus and the underlying vessel wall. MMP-9 expression was co-localized with the presence of macrophages. Gelatinase activity was detected in the same regions as MMP-9 protein expression, i.e. within the thrombus-free wall and in the interface between the thrombus and the underlying wall.

Neutrophil gelatinase-associated lipocalin (NGAL) has been suggested to influence the activity of MMP-9 by binding to MMP-9 and inhibit its degradation, thus preserving its enzymatic activity. In paper III it was demonstrated that NGAL/MMP-9 complexes were present in different regions of the AAA. NGAL expression was co-localized with CD66b positive neutrophils. Importantly, NGAL/MMP-9 complexes were present in the thrombus itself and in the interface fluid between the thrombus and the underlying vessel wall. These findings support the concept that the wall underlying the thrombus is degraded from within the lumen by enzymatic activity related to the thrombus. This could lead to AAA rupture after blood has entered the thrombus.

In paper IV we studied the presence of mast cells (MCs) in AAA. Immunostaining of MC tryptase in AAA specimens showed that MCs, in contrast to normal aorta, were absent in the intimal layer of AAAs, and that MCs associate with medial and adventitial neovessels. Furthermore, MCs reside partly in the same areas with macrophages, CD3 positive T-cells and neutrophils. Cathepsin G and tryptase double immunostaining showed that MCs positive both for cathepsin G and tryptase are more numerous than cells with only tryptase or cathepsin G. Cathepsin G and chymase expressions did not differ significantly between thrombus-covered AAA wall and AAA wall without intraluminal thrombus when evaluated by RT-PCR.

In summary, the present thesis provides evidence that the intraluminal thrombus is of importance for the pathogenesis of aneurysm growth and possibly ruptures.

To My Parents

If you shut the door to all errors,

truth will be shut out.

Rabindranath Tagore

Contents

LIST OF PUBLICATIONS

This thesis is based on the following original papers and manuscripts:

I. **Kazi M**, Thyberg J, Religa P, Roy J, Eriksson P, Hedin U, Swedenborg J. Influence of intraluminal thrombus on the structural and cellular composition of the abdominal aortic aneurysm wall.
J Vasc Surg. 2003;38:1283-92

II. **Kazi M**, Zhu C, Roy J, Paulsson-Berne G, Hamsten A, Swedenborg J, Hedin U, Eriksson P. Difference in matrix-degrading protease expression and activity in thrombus- free and thrombus-covered wall of abdominal aortic aneurysm.
Arterioscler Thromb Vasc Biol. 2005;25:1341-6

III. **Kazi M**, Folkesson M, Zhu C, Hemdahl A.-L, Hamsten A, Hedin U, Swedenborg J, Eriksson P. Presence of NGAL/MMP-9 complexes in human abdominal aortic aneurysms.
Submitted

IV. Mäyränpää M, **Kazi M**, Trosien J, Paulsson-Berne G, Kovanen PT, Eriksson P, Swedenborg J, Hedin U. Mast cells in abdominal aortic aneurysms associate with medial and adventitial neovessels.
Manuscript

LIST OF ABBREVIATIONS

AAA	Abdominal aortic aneurysm
AOD	Atherosclerotic occlusive disease
CT	Computed tomography
DNA	Deoxyribonucleic acid
EC	Endothelial cell
ECM	Extracellular matrix
EDP	Elastin degradation product
HRP	Horse- radish peroxidase
HSP60	Heat Shock Protein 60
IL	Interleukin
LDL	Low density lipoprotein
MCs	Mast cells
MMP	Matrix metalloproteinase
NGAL	Neutrophil gelatinase-associated lipocalin
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCF	Stem cell factor
SMC	Smooth muscle cell
SEM	Scanning electron microscopy
TIMP	Tissue inhibitor of matrix metalloproteinases
TNF	Tumor necrosis factor
tPA	Tissue type plasminogen activator
TUNEL	Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling
uPA	Urokinase type plasminogen activator
WBC	White blood cells

1 BACKGROUND

1.1 INTRODUCTION

Cardiovascular disease is a leading cause of mortality and morbidity of people both in developed and developing countries and the incidence seems to be increasing worldwide. Together with coronary artery disease, peripheral artery disease like extremity ischemia vasculitis and aneurysm, form the main bulk of cardiovascular disease. Aneurysm can be defined as an abnormal and persistent dilatation of a vessel. A common site of aneurysm formation is in the abdominal aorta below the renal arteries. Abdominal aortic aneurysm (AAA) primarily affects males over the age of 60. The ratio between men and women is 4:1. The prevalence is 5-10 % depending on the definition of aneurysm [1, 2]. AAA rupture is the 13th commonest cause of death in the western world [3]. The natural history of AAA is growth and ultimately rupture of the aneurysm. Surgery is the treatment of choice either by open surgery or endovascular methods. Even with a standard level of surgical activity, rupture stands for 1.5% of the total mortality in males over 55 years. Health awareness and easy access to health facilities causes a growing number of senior citizens. However, the increased number of elderly in the population will increase the number of AAA and subsequently increase health costs. Taking this issue into consideration, early detection of AAA and understanding its pathology leading to preventive strategies to inhibit the growth of AAA, will benefit both health and health related costs. There are two main pathological processes of AAA; dilatation and rupture. However, the molecular mechanisms are still not fully known. Moreover, there are no clinical diagnostic markers for the risk of AAA rupture except diameter.

1.2 THE VESSEL WALL

There are three types of vessels: arteries, veins and capillaries. Arteries are divided into large, medium and small ones, the latter continue as capillaries. Large- and medium sized arteries are also called elastic and muscular arteries. Both in arteries and veins, the wall is divided into 3 layers; the tunica intima, the tunica media and the tunica adventitia (Figure 1). Tunica intima is covered by a monolayer of endothelial cells, which acts as a semi-permeable barrier to the passage of substances from the blood into

the arterial wall. The endothelium, which is the cellular layer covering the intima towards the lumen, has anti-coagulant properties and is actively involved in the regulation of vascular tone. The intima is separated from the media by the internal elastic lamina. A network of elastic lamellae and layers of SMCs builds up tunica media. SMCs are present only in arteries and veins and are the exclusive cellular component in the media. Elastic lamellae support the recoil property of the vessel wall and the external elastic membrane separates the media from the tunica adventitia. The adventitia contains blood vessels (vasa vasorum) and nerves (nervi vascularis) embedded in a collagen-rich connective tissue with fibroblasts and fat cells. The vasa vasorum acts as a feeding vessel for large and medium sized arteries.

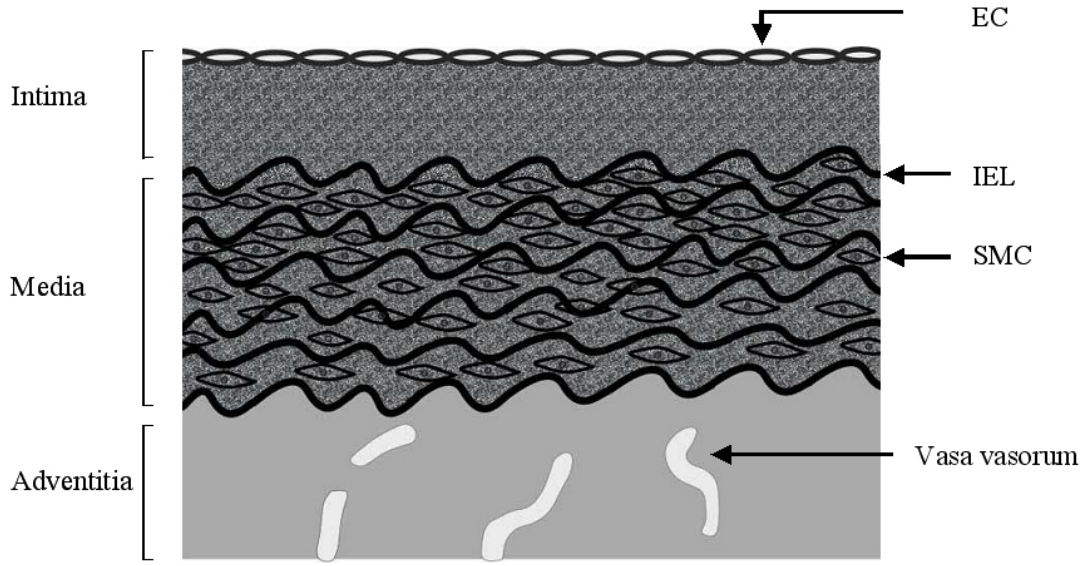

Fig 1. Schematic presentation of a large artery showing endothelial cells (EC) in the luminal part of intima, elastin fibers (IEL; internal elastic lamellae), SMCs in the media and vasa vasoruae in adventitia.

While vasa vasorae are important to meet the nutritional need of the vessels, pathological neovessels which might transport inflammatory cells to the vessel wall are a feature of vascular disease. In aneurysm, dilatation and out-pouching affect all three layers of the vessel wall. Fragmentation of elastin, loss of SMCs and accumulation of inflammatory cells are the cardinal histological landmarks and neovessel is a consistent feature in AAA.

1.3 STRUCTURAL COMPONENT

Our body can make at least 100,000 different proteins. There are two general classes, functional proteins and structural proteins. The structural proteins take part in organizing the tissues and organs to give strength and provide flexibility and are likely to be involved in AAA. Some of the structural proteins are long and fibrous. The most common fibrous protein is collagen, which is most commonly found in tendons, ligaments, skin, blood vessels and lungs. Collagen fibers are responsible for the rigidity of tissues and organs.

Collagen fibers consist of a triple helix composed of three polypeptide chains. There are different types of collagen and the fibril forming collagens that provide tensile strength, type I and type III collagen, are found in vessels. Collagens are synthesized principally by fibroblasts and SMCs, and their biosynthesis is characterized by the presence of an extensive number of post-translational modifications of the polypeptide chains. Collagens can be degraded prior to, or after their secretion from the cell. Secreted collagen is degraded mainly by two different mechanisms, proteolytic and phagocytotic. Proteolytic degradation occurs mainly through the activity of matrix metalloproteinase (MMP) activity (see below). In addition, fibroblasts are able to phagocytose and degrade collagen fibrils. Phagocytosis of collagen fibrils by fibroblasts seems to be a continuous process in the remodeling of the ECM and after phagocytosis, collagen is digested in lysosomes by cysteine proteinases such as cathepsin B and/or L [4]. Collagen turnover is important for vessel wall repair and regeneration and its degradation is believed to be associated with the rupture of AAA [5, 6].

Elastin is a structural protein that gives elasticity to tissues. Elastin is found predominantly in arterial walls, lungs, intestines and skin. Elastin functions in consert with collagen in the vessel wall. It allows stretching of the large arteries and recoiling to their original position in a physiological range. However, in a pathological situation, loss of elastin gives rise to aneurysmal dilatation. Cross-linking makes elastin a relatively stable protein and elastin is not synthesized throughout our lives [7]. It is therefore very important to have intact elastin in the vessels wall. Elastin plays a major

role in the pathogenesis of some connective tissue disorders with cardiovascular pathology such as Marfan's Syndrome, in which elastin fibrillogenesis is disturbed because of a defect in the fribrillin gene and in Willams' Syndrome, in which elastin arteriopathy causes narrowing of artery [8].

1.4 CELLULAR COMPONENTS

Smooth muscle cells play an important role in the development of vasculature, provide structural integrity, the ability to dilate and constrict the vessels wall. SMCs can be defined as highly specialized cells, with a well-defined anatomical location mainly in muscular arteries. SMCs exhibit unique contractile properties, electrical activities and responsive to agonists that distinguish it from any other cell type in the body. SMCs in the adult vessel media are in a quiescent state with the primary function to contract in response to vasoactive factors. However, both in the embryo during vessel development and in certain disease states, such as restenosis and atherosclerosis, the cells are found in a fibroblast-like, synthetic phenotype, with the primary function to take part in vessel remodeling by proliferation and ECM production [9].

Numerous growth factors have been shown to stimulate proliferation of SMCs *in vitro* [10]. These include receptor tyrosine kinase (RTK) agonists such as FGF-2 and PDGF, G-protein coupled receptor (GPCR) agonists such as thrombin and angiotensin II. In addition to stimulating cell proliferation, most growth factors are able to prevent cell death by activating survival pathways [11]. SMCs produce ECM components such as collagen, elastin and fibronectin. In pathological conditions, excess proliferation of SMC is involved in restenosis whereas disappearance of SMC is one of the key features of AAA (see below). *In vitro* studies have shown that activated SMCs in AAA produce MMPs [12], suggesting that SMCs may take part in tissue remodeling. Apoptosis could be the mechanism of the disappearance of SMCs in AAA [13].

Endothelial cells (ECs) cover the inner surface of blood vessels in the entire circulatory system and provide an interface between circulating blood and the rest of the vessel wall. In small blood vessels and capillaries, ECs are often the only cell-type present. ECs are involved in many aspects of vascular biology, such as inflammation, formation

of new blood vessels, blood clotting, vasoconstriction, vasodilatation and atherosclerosis. Endothelial dysfunction, mostly defined clinically as impaired flow-mediated vasodilation, is a hallmark of vascular disease [14]. By being involved in the hemostatic balance, endothelial cells prevent thrombus formation. This is accomplished by binding of thrombin to thrombomodulin on ECs, which changes the specificity of thrombin from a fibrinogen-cleaving enzyme into an inhibitor of coagulation by inhibiting factors V and VII in the coagulation cascade [15, 16]. Ultrastructural changes of ECs in capillaries of AAA have been observed by transmission electron microscopy in ruptured aneurysms [17].

Red blood cells, leukocytes (white blood cells or WBC) and platelets are the cellular components of the blood. Leukocytes are responsible for the defense of the organism, and they are subdivided into granulocytes and agranulocytes. Granulocytes consist of neutrophils, eosinophils and basophils. Neutrophils are active in phagocytosis whereas eosinophils attack parasites and phagocyte antigen-antibody complexes. Basophils secrete anti-coagulant and vasodilator substances as histamine and serotonin. Although having phagocytic capability, the main function of basophils is secreting substances which mediate the hypersensitivity reaction. Aggranulocytes are lymphocytes (T-cells and B-cells) and monocytes. Most lymphocytes circulating in the blood are in a resting state. The lymphocytes of the lymphoid tissues and organs can be activated in a different amount following antigenic stimulation. These cells are the main constituents of the immune system and involved in the defense against pathogenic micro-organisms such as viruses, bacteria and fungi. Different types of lymphocytes possess diverse functions. When B-cells are activated, they become plasma cells and secrete antibodies thus forming the humoral response. T-cells contribute to cellular immunity and are divided into two major categories: cytotoxic T-cells, recognized by their expression of CD8, and helper T-cells recognized by CD4 expression. The cytotoxic T cells release perforin substances, which produce lesions in the membrane of the target cell and cause its death by osmotic lysis. The helper T cells activate both B and cytotoxic T cells. Normally, WBCs are not present in the vessel wall but they are evident in pathological situations.

Similar to basophils, mast cells (MC) contain granules and are found in connective tissue. MCs are involved in atherosclerosis [18] and have been suggested to be involved in AAA pathogenesis [19, 20]. MCs need to be de-granulated to perform their function. This can be achieved by physical destruction, such as high temperature, mechanical trauma, ionizing irradiation, or by chemical substances, such as toxins, venoms, and proteases. By releasing the potent inflammatory mediators, such as histamine, proteases and chemotactic factors, MCs play a central role in inflammatory and allergic reactions [21] and can be involved in antigen presentation [22], in activating collagenases [23] and possibly in remodeling [24]. Circulating precursor MCs can be differentiated *in vitro* into tryptase positive mast cells.

1.5 ABDOMINAL AORTIC ANEURYSM

Fragmentation of elastin, loss of SMCs and accumulation of inflammatory cells are the cardinal histological landmarks of AAA. One of the most common locations of the disease is the abdominal aorta below the renal arteries, where the aneurysm may extend into the iliac arteries (Figure 2). There are several definitions of the disease. AAA is commonly defined as an enlargement of the aorta exceeding 2.5 or 3 centimeters but several other definitions exist e.g. a diameter exceeding the suprarenal aorta by 50% [1]. AAA is characterized by ultrastructural changes of endothelial cells, synthetic SMCs or disappearance of SMCs, inflammatory infiltrate, elastin degradation, protease activation and neoangiogenesis [25, 26].

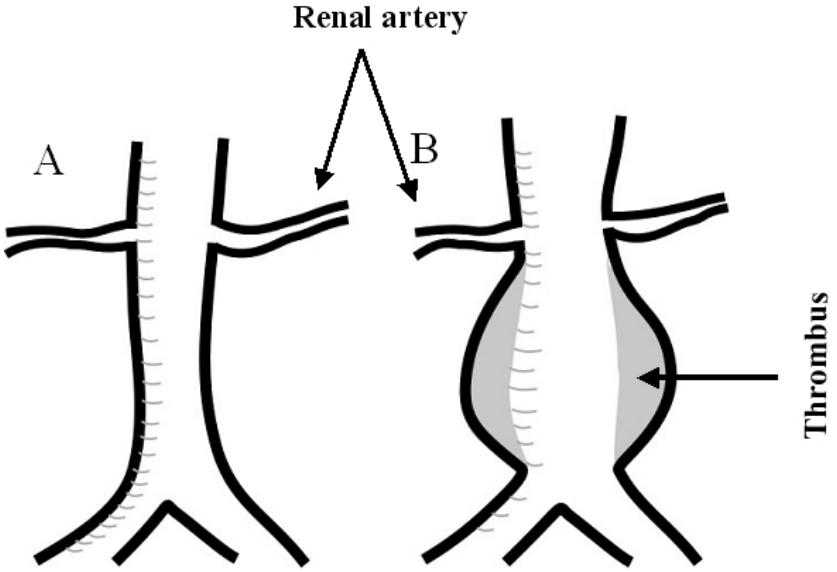

Fig 2. A schematic diagram of normal abdominal aorta (A) and infrarenal abdominal aortic aneurysm (B). Arrows showing renal arteries and intra aneurysmal thrombus.

According to the law of Laplace, the strain on the wall increases with the diameter of the wall [27]. There is considerable variation between patients in the rate of expansion and the diameter at which rupture occurs [28]. Untreated AAA carries a high mortality rate, and a surveillance study of small aneurysms in UK indicated that infrarenal AAA of 5.5 and 6.5 cm in diameter have annual rupture rates of 11% and 26%, respectively [29]. The clinical presentation of AAA is usually asymptomatic until they rupture or threaten to rupture. At least 50% of patients with ruptured AAA die from sudden cardiovascular collapse without reaching hospital.

Conventional risk factors for AAA are male gender, age above 50 years, tobacco smoking, family history, and atherosclerosis [30, 31]. In addition, hypertension [32] and chronic obstructive pulmonary disease is often elicited in AAA [33]. Ultrasonography is the best method to obtain the diagnosis of AAA due to its cost effectiveness [34]. However, computed tomography (CT) is often used and is the method of choice in the preoperative evaluation of AAA. CT is also a sensitive method to diagnose an inflammatory aneurysm. One of the recent advances in CT technology is the introduction of helical CT with 3-dimensional reconstruction of images [35]. With this technique, it is now possible to more accurately assess the origin of renal and mesenteric arteries in relationship to the aneurysm, without arteriography. The technique is of particular use in detecting tortuosity of the aorta [36].

Treatment of AAA dates back to as early as 1684, when Dr Moore attempted to induce thrombosis of an aneurysm by introducing large masses of intraluminal wires ([37]. The treatment is now an elective surgical approach with a mortality rate less then 5% [38-40]. Percutaneous endovascular technique is an alternative to the open surgical approach. However, this technique needs a careful selection of patients.

AAA is associated with extensive vascular matrix remodeling. Collagen and elastin is required to maintain tensile strength and elasticity in the normal aortic wall and the fiber-forming collagens, type I and type III, are the predominant collagen isotypes in the aorta [5]. Histologically, aneurysm tissue has been shown to contain decreased

content of elastin and fragmentation of the elastic lamellae in the media [41, 42]. MMPs are a large group of proteases with the capacity to degrade all types of ECM. A number of MMPs have been identified in AAAs [43]. AAA has also been suggested to develop as a consequence of inflammatory reactions in the vessel wall, perhaps sharing features with inflammation in atherogenesis. In contrast to atherosclerosis, AAA has been reported to contain infiltrating macrophages and lymphocytes in the media and adventitia [44]. Although recruitment of inflammatory cells may be important in the healing response against e.g infectious pathogens, these cells may also contribute to the disease, e.g. by secreting proteolytic enzymes and thereby enhancing the dilatation of the vessel wall as a consequence of elastin degradation [45]. The exact identity of the subsets of inflammatory cells that contribute to the disease process is not known.

In larger aneurysms, an intraluminal thrombus is almost always present. The thrombus is either concentric or eccentric, leaving one segment of the vessel exposed to flowing blood [46, 47]. The most important concept of thrombus-formation is an initiating endothelial injury, turbulent blood flow and hypercoagulability. Small thrombi may be dissolved without any symptoms whereas a larger thrombus may only partially dissolve and then reform [48].

1.6 ATHEROSCLEROSIS AND AAA

Atherosclerosis is an inflammatory and degenerative disease of the intima in large and medium sized arteries (Figure 3) [49]. In most Western countries, atherosclerosis and its consequences such as myocardial infarction, stroke and extremity ischemia is the

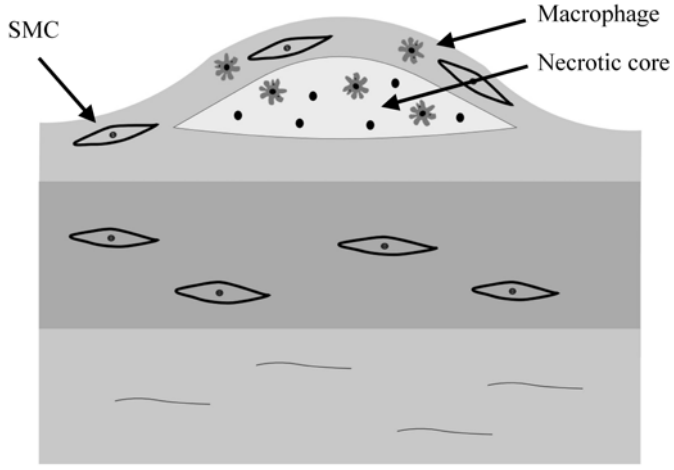

Fig 3. Schematic diagram of plaque showing, migrating SMC and macrophages in fibrous cap and necrotic core.

leading cause of illness and death. This slow and complex disease starts in childhood and progresses throughout age. According to current theories, the disease begins with damage to the innermost cellular layer of the artery, the endothelium. Early accumulation of lipids and inflammatory cells, mostly monocyte/macrophages, lead to the formation of a fatty streak. Lipids, such as low-density lipoprotein (LDL) become oxidized and are taken up by macrophages, which are transformed into lipid-filled foam cells. This discrete inflammatory lesion in the intima, initiates the migration of SMCs from the media which proliferate and deposit ECM in the intima. This process results in the formation of a fibrous 'cap' on top of the lesion and protects the accumulated lipids and foam cells from contact with the flowing blood, forming a fibrous plaque. Plaques that grow larger due to ongoing inflammation, fibrosis and lipid accumulation can eventually reduce blood flow through the artery. As the disease progresses, the central portion of the lesion, the so-called necrotic core, becomes filled with necrotic cells, debris, cholesterol crystals and calcified structures. These advanced lesions can eventually become fragile and rupture. Rupture usually takes place at the outer edges of the fibrous cap where inflammation is most prominent and involves the activity of matrix-degrading proteases leading to destruction of collagen. In addition, apoptosis and necrosis of SMCs in the cap reduce collagen synthesis. This process will expose a tissue factor (TF) -rich and prothrombotic necrotic core to the coagulation system in the blood and initiate thrombosis, either focal in the plaque itself or more general which may obliterate the vessel lumen. As a consequence, disturbances of the circulation to the main target organ, such as the heart, may evolve and cause clinically fatal end-organ ischemia such as myocardial infarction or stroke [49-52].

AAA shares some of the common predispositing factors with atherosclerosis. Moreover, atherosclerosis is a risk factor of AAA [53]. However, there are some fundamental features that make AAA different. In atherosclerosis, the intimal layer is mainly affected and macrophage accumulation is mainly restricted to the intima during lesion formation whereas all three layers of the vessels wall are involved in aneurysm. Differences of cultured SMCs obtained from AAA and AOD indicate that AAA is not the result of atherosclerosis [54]. In addition, diabetes mellitus is

associated with atherosclerosis but is a negative risk factor for AAA [55] and the male dominance is much more promounced in AAA compared to AOD. The term atherosclerotic aneurysm and non-specific aneurysm have been used to distinguish common forms of fusiform infrarenal AAA from rare hereditary, infected or traumatic aneurysms.

1.7 THROMBUS

The thrombus is the final product of blood coagulation. It consists of a complex structure of aggregated platelets and blood elements trapped by a fibrin network. According to 'Virchow's Triad', endothelial injury/damage, turbulent blood flow and hypercoagulable blood is responsible for thrombus formation. The thrombus is the immediate consequence of an injury, thus protecting further bleeding. However, when an atheroslerotic plaque ruptures, the formed thrombus can occlude the vessels and bring the patient to an acute stage. Furthermore, a dislodged thrombus causes embolism.

Evidence suggests that presence of a thrombus relates to poor prognosis in disease like Kawasaki disease, is rare childhood illness that causes inflammation of blood vessels [56]. Intraluminal thrombi are usually present in AAA (Figure 4) but whether the thrombi influence AAA growth and rupture is not clear. The presence of a thrombus in

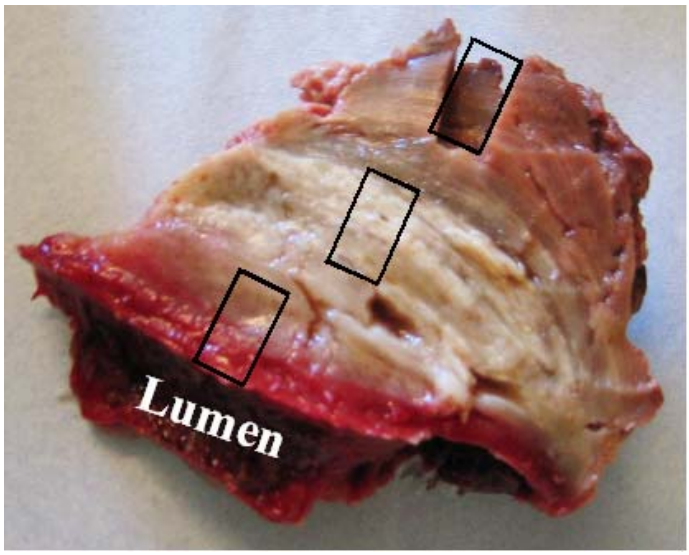

Fig 4. Photomicrograph of organised intraluminal thrombus shows different layers.

AAA was initially thought to be protective as thrombus reduced transmitted wall pressure [57] and a mathematical model of AAA showed that the intraluminal thrombus may be protective by decreasing wall stress [58]. These findings are also supported by another mathematical model showing that thicker thrombi can reduce the wall stress significantly [59]. However, a morphological study of small aneurysms observed no impact of thrombus thickness on AAA rupture [60]. Furthermore, the intraluminal thrombus did not reduce arterial pulse pressure when studied during operation indicating that the presence of thrombus does not reduce the risk of rupture [61]. AAA is a multifactorial disease with a heterogeneous wall structure and it is likely that the human situation is much more complicated than the mathematical models. Emerging data now sees the thrombus as being a culprit for AAA pathology [62, 63] and growth of the aneurysm has been associated with growth of the thrombus [64]. Furthermore, aneurysm rupture has been associated with the growth of thrombus [65]. When comparing the volume and morphology of intraluminal thrombus in intact and ruptured abdominal aortic aneurysms from CT findings, it has been observed that ruptured AAAs are larger in diameter and have a greater volume of thrombus when compared to intact AAAs [66]. The information on the influence of the thrombus on the structural and cellular content of the underlying aneurysm wall is limited. However, it has been suggested that the thrombus may cause ischemia in the underlying vessel wall, which may be a possible mechanisms behind a focal weakening of this tissue [63]. The impact of the thrombus on the vessel wall is the focus of this thesis (see Papers I to IV).

1.8 APOPTOSIS

Apoptosis is a physiological process of cell death, in which caspases become activated in the early stages of the process (Figure 5). Activated caspases break down essential

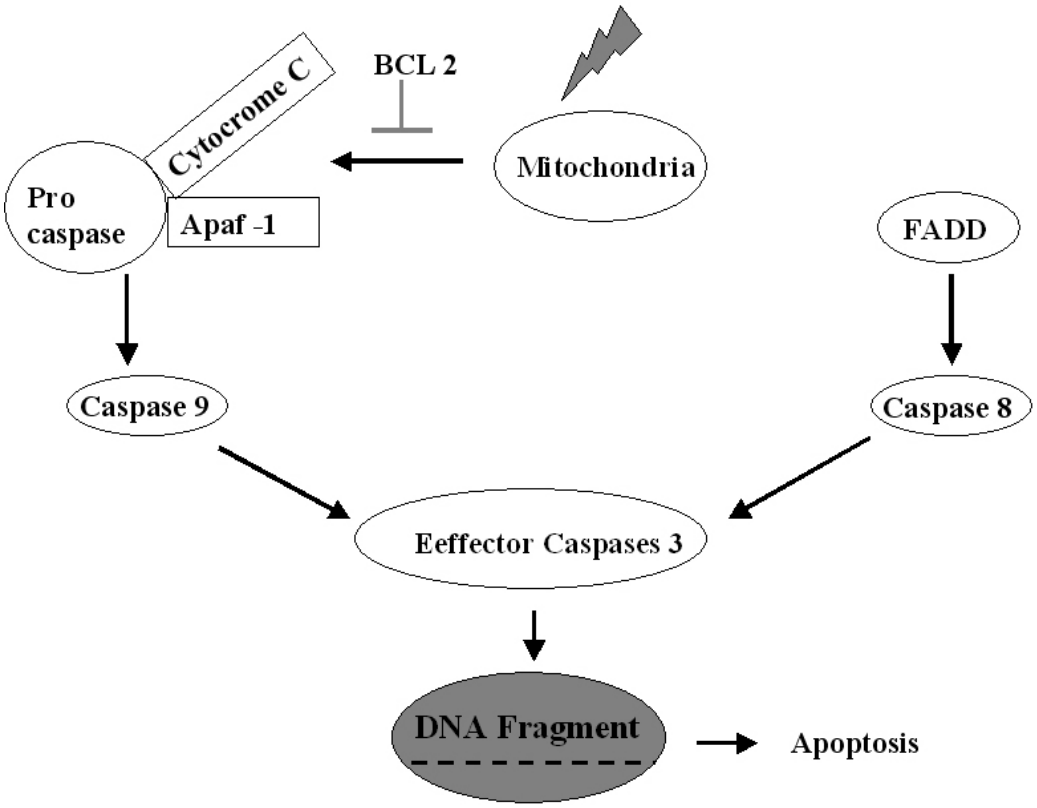

Fig 5. Schematic illustration of apoptotic pathway, illustration modified from Mayr et al [67]

components in normal cellular function including structural proteins and nuclear proteins such as DNA repair enzymes. Caspases can also activate other enzymes such as DNases, which cleave DNA [68]. The net result of the actions of caspases is a number of morphological and structural changes in the cell. Such changes can be examined by electron microscopy, or as fragmentation of DNA, detected by TUNEL assay. Apoptosis of SMCs plays an important role in normal and pathological remodeling of the vessel wall. It has previously been concluded that apoptosis in the fibrous cap of atherosclerotic plaques is detrimental since it will reduce SMC content

and overall collagen synthesis and thereby lead to plaque rupture and thrombosis [51]. In aneurysms, the synthetic capacity of SMCs is important to maintain vessel wall integrity. SMC depletion in human aneurysm tissues is accompanied by biochemical, morphological and molecular changes consistent with SMC apoptosis [13]. In addition, SMCs have also been demonstrated to prevent aneurysm formation in a xenograft model [69].

1.9 PROTEASES IN THE VESSELS WALL

Proteases are involved in various physiological as well as pathological processes such as infection, inflammation, allergic reaction, fertilization, cell growth and death, blood clotting, tumor growth and remodeling processes. Proteases are secreted by cells into the surrounding tissues and cause the destruction of proteins in the ECM. According to the structure of their active sites, proteases are classified as serine, cysteine, aspartic and metalloproteinases (MMPs). Most proteases are secreted and active outside cells.

Table 1 Showing member of MMP expressed in vascular tissues. Modified from Brauer et al[70]

Group	MMPs	Name
Collagenases	MMP-1	Interstitial collagen
	MMP-8	Neutrophil collagenase
	MMP-13	Collagenase-3
Gelatinases	MMP-2	Gelatinase-A
	MMP-9	Gelatinase-B
Stromelysins	MMP-3	Stromelysin-1
	MMP-10	Stromelysin-2
	MMP-7	Matrilysin
Membrane-type MMPs (MT-MMP)	MMP-14	MT1-MMP
	MMP-15	MT2-MMP
	MMP-16	MT3-MMP
	MMP-17	MT4-MMP
Others	MMP-11	Stromelysin-3
	MMP-12	Macrophase metalloelastase

MMP is a large group of proteases with the capacity to degrade all types of ECM (see Table). MMPs play an important role in many normal physiological processes such as embryonic development, morphogenesis, reproduction and tissue remodeling but also in many pathological processes such as arthritis, cancer and cardiovascular disease. MMPs are synthesized as inactive zymogens that require activation [71]. A variety of MMPs have been involved in regulating and remodeling of ECM [72]. Furthermore, MMPs can induce cell apoptosis [73]. Degradation of the extracellular matrix is important for vascular adaptation and repair in response to changes in hemodynamics, arterial injury, inflammation, and oxidative stress [74]. There is mounting evidence that MMPs are the predominant proteases in AAA [75] and that MMPs play a principal role in AAA development [76, 77]. The expression of several MMPs has been identified in AAAs, including MMP-1, MMP-2, MMP-3, MMP-9, and MMP-13 [43]. Targeted gene disruption of MMP-9 can suppress experimental AAA growth [78]. In a rat model of AAA, aneurysm growth was inhibited after seeding of SMCs overexpressing TIMP [79]. Furthermore, inhibition of MMPs by the non-specific MMP inhibitor doxycycline, suppressed connective tissue degradation in AAA [80]. In addition, suppression of AAA growth has also been achieved by tetracycline derivatives [81]. Indomethacin, a nonsteroidal anti-inflammatory drug (NSAID), prevented the development of elastase-induced AAA possibly by inhibiting MMP-synthesis by macrophages [82].

As reflected by their names, elastases are enzymes with their main substrate being elastin. Elastases [83] can be liberated from different cells such as granulocytes, monocytes/macrophages, lymphocytes and fibroblasts. Degradation of elastin can be estimated by measuring serum levels of elastin degradation peptides (EDP). Increased serum levels of EDP have been observed in patients with AAA [84]. Elastases can also degrade collagen type III and procollagen type IV of blood vessels and elastin-derived peptides can act as chemotactic agents for cells in the vessel wall (Hance, 2002 #246). It has been demonstrated that EDP is asssociated with the induction of monocyte and neutrophil chemotaxis (Cohen, 1991 #264). The chemoattractant property of EDP may facilitate Th1 cell differentiation in the vessel wall, and thereby contribute to cellular immunity [85].

1.10 REGULATION OF PROTEASES

MMPs are controlled at different levels: gene transcription, activation of the latent forms, inhibition by their endogenous inhibitors and by their degradation and clearance (Figure 6).

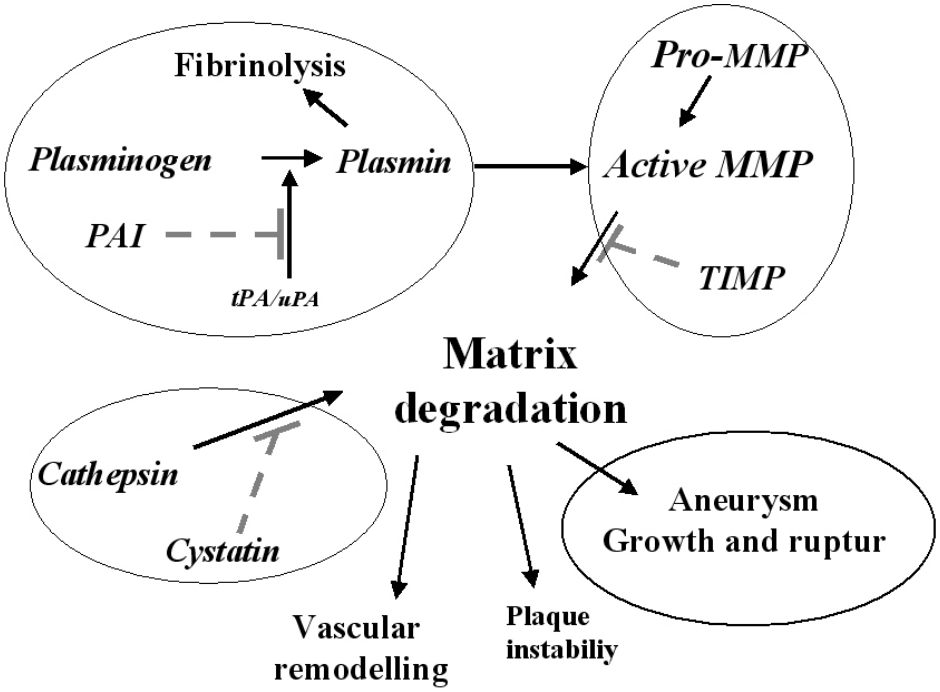

Fig 6. Diagram shows MMP activation and possible effects on vasculature.

MMPs are expressed as inactive zymogens or proMMPs. Induction of MMPs at the transcriptional level is mediated by a variety of inflammatory cytokines and growth factors such as interleukin-1 (IL-1), IL-6, tumour necrosis factor-α (TNF-α), and epidermal growth factor [86, 87].

Recent studies suggest that cell-matrix and cell-cell interactions via adhesion molecules and integrin families influence gene expression of MMPs [88]. Activated T-cells play a pivotal role in the induction of MMP-1, MMP-3, MMP-9, and MMP-11 expression in macrophages and VSMCs [89, 90]. Furthermore, oxidized LDL has been shown to

upregulate MMP activity by inducing MMP-9 expression while reducing TIMP-1 expression in macrophages [91].

ProMMPs secreted as inactive zymogens can be activated by proteinases or by nonproteolytic agents. Activation by proteinases is mediated by cleavage of the bait region. The proteinases involved include plasmin, trypsin, chymase, elastase, and kallikrein, with plasmin suggested to be the main physiological activator of most MMPs [92]. The chemical activation depends on modification of the cysteine switch sulfhydryl, resulting in partial activation of the MMPs and intramolecular cleavage of the propeptide. Full activation is achieved by complete removal of the propeptide as a result of intermolecular processing. The nonproteolytic agents are thiol-modifying agents, oxidized glutathion, chaotropic agent, and reactive oxygen species (ROS). ROS strongly modulate vascular MMP activity [93].

Activated MMPs are inhibited through the binding of natural inhibitors named tissue inhibitors of metalloproteinases (TIMPs). Four TIMPs (TIMP-1 to 4) have been identified. When binding to MMPs, they form noncovalent complexes with the active form of MMP as well as with some proMMPs, thereby inhibiting active MMPs or impairing the activation of proMMPs [94].

1.11 NEOANGIOGENESIS

Medium and large size arteries receive part of oxygen and nutrients from the main lumen but partly also from the vasa vasorum. However, in atherosclerosis, microvessels originating from vasa vasorum penetrate into the media and intima, and the newly formed microvessels provide a route for inflammatory cells and plasma constituents into the plaque [95]. If the neovessels rupture, an intraplaque heamorrhage ensues and leads to increasing plaque volume, increasing plaque cholesterol content and plaque destabilization [96]. However, neoangiogenesis is important in hypoxic tissues, such as ischemic myocardium, to maintain its normal function which is best achieved by collateral formation. Interestingly, human infrarenal aortas normally do not have vasa vasorae in the vessel wall media. Therefore, enrichment with microvessels is an important feature of neovascularization of the aortic wall during AAA development

[44, 97-99]. However, the significance of AAA neovascularization is still unclear. Herron et al. [100] hypothesized that new vessels may have a causal role in the pathophysiology of AAA by secreting proteinases which destabilize matrix proteins. Paik et al. [101] addressed the question whether neovascularization is an ongoing process in well-developed aneurysms. By detection of αvβ3 integrin and e-NOS, this group observed the presence of active angiogenesis in the adventitia. Anti-eNOS staining served as a marker for microvessels since the antibody detects the endothelial layer of both small and large microvessels. Recently, it has been shown that the presence of MCs is associated with neovessel formation [102, 103] and that the concentration of activated MCs was significantly higher in aneurysm than in both atherosclerostic tissue and in normal aortas [20].

2 AIMS OF THE STUDY

It has been suggested that rupture is associated with the growth of the thrombus in the aneurysm and case reports have suggested that the rupture is initiated by blood entering the thrombus. Furthermore, the growth of the aneurysm has been associated with the growth of the thrombus. However, relatively few studies have addressed the pathogenetic influence of the thrombus in AAA. Furthermore, morphological, biochemical and molecular biological studies of specimens from AAA do not usually specify whether the specimen is obtained from a wall segment covered by thrombus or from a segment exposed to flowing blood. The purpose of the present thesis was to make morphological and gene expression comparisons between these two types of wall segments in order to evaluate if the thrombus-covered wall could be more prone to rupture or dilatation.

Hypothesis: The intraluminal thrombus plays a major role in AAA pathogenesis that could lead to an increased risk of AAA rupture.

Specifically the present thesis aimed at:
1, studying potential morphological differences between the thrombus-covered and the thrombus-free AAA wall

2, analyze the expression and activity of matrix-degrading proteases in the thrombus-covered and the thrombus-free AAA wall

3, investigating the presence of MCs in AAA. Mast cells have been less studied than other inflammatory cells in vascular disease. However, MCs have been shown to have a potential role in protease secretion.

3 MATERIALS AND METHODS

3.1 STUDY DESIGN

To characterize human AAA tissue and to study the influence of thrombus on AAA wall. The study was approved by the local ethical committee (Dnr: 00-337). Intra operative retrieval of AAA was used to study wall structure and cellular components. Furthermore, gene expressions were analyzed in the study materials.

3.2 COLLECTION OF SAMPLES

Patients undergoing elective surgery for infrarenal AAA with preoperative CT scan demonstrating excentric intraluminal thrombus and with a thrombus free aneurysm wall segment were selected for the study. Two 2x4 cm tissue sections were cut transversely from the thrombus free and thrombus covered aneurysm wall (Figure 7).

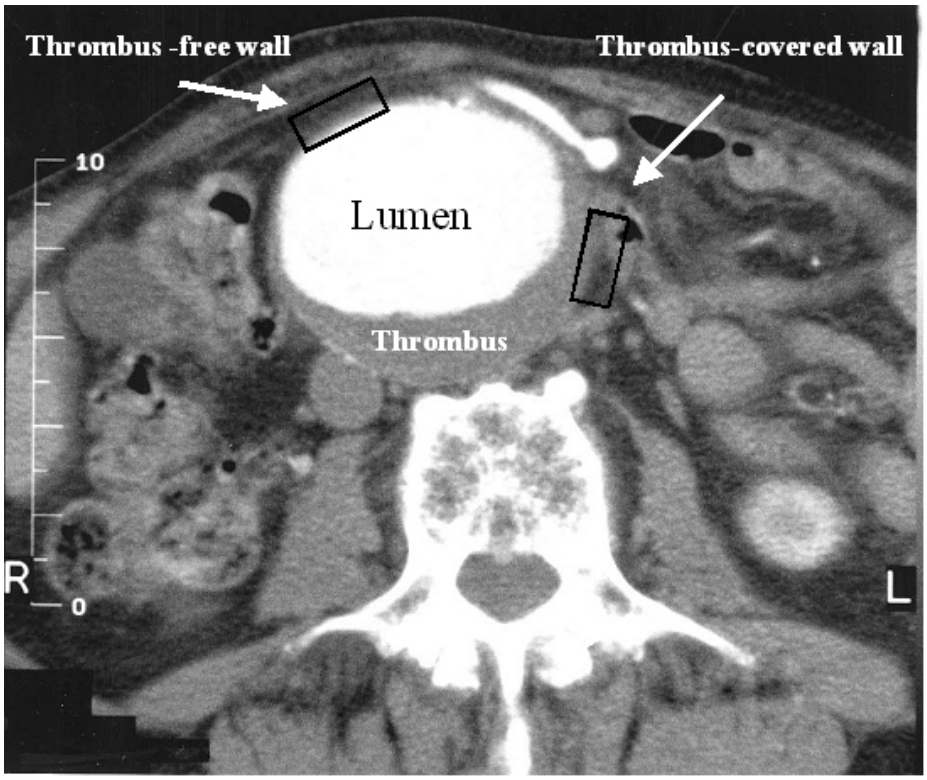

Fig 7. A CT scan representing the areas of sample collection of thrombus-covered and thrombus-free wall of AAA.

The adventitia was dissected free of excess perivascular fat and debris for RNA isolation and the sections immediately fixed in 4% paraformaldehyde for light microscopy or 3% glutaraldehyde in 0.1 M sodium cacodylate-HCl buffer (pH 7.3) containing 0.05 M sucrose for electron microscopy. For scanning electron microscopy (SEM), the samples were flushed gently with Tyrode's solution supplemented with heparin in order to remove blood. The AAA specimens were carefully dissected into ~ 7 x 7 mm pieces suitable for scanning electron microscopy, and processed for scanning electron microscopy as described previously [104]. The entire luminal surface of each AAA sample was examined with a DSM 962 scanning electron microscope (from Carl Zeiss, Oberkochen, Germany) operating at an acceleration voltage of 10kV. All patients approved the intra-operative retrieval of tissue from the aneurysm wall according to informed consent procedures and approval by the local ethical committee.

3.3 IMMUNOHISTOCHEMISTRY

The principle of this method is to raise an antibody (primary antibody) against a specific antigen and use it as a tool to search for the antigen in tissue sections. The antibody can either be directly conjugated to a marker molecule to allow immediate detection, or it can be detected by another antibody (secondary antibody), which recognizes the primary antibody. In this study, most primary antibodies were non-conjugated and detected with appropriate pre-conjugated secondary antibodies (papers I-III) and co-localization of overlapping cellular markers was studied with an immunofluorescence double staining method (paper IV). In brief, immunostaining was performed on 4% buffered-formalin-fixed paraffin-embedded sections. The sections were deparaffinized in xylene, rehydrated in water, soaked in 0.03% H_2O_2 in 70% methanol in order to block the background activity of tissues enzymes. Thereafter, boiling in citrate buffer was used to unmask hidden epitopes and non specific interactions were blocked with serum diluted in phosphate-buffered saline (PBS). The sections were incubated with primary antibodies overnight followed by washing in PBS and thereafter incubated with secondary antibodies labeled with biotin. Biotinylated antibodies were visualized with ABC-Elite Vector horseradish peroxidase (HRP) and

developed with DAB as substrate for HRP. After rinsing, the sections were counterstained with Mayer's hematoxyline. For fluorescence microscopy the sections were counterstained with 4',6-Diamidino-2-phenylindole (DAPI). Immunohistochemistry is not ideal for quantitative analysis and selection of antibodies, as well as optimization of staining for every antibody makes immunohistochemistry a time-consuming method. However, it is a powerful method to detect and localize protein in tissues.

3.4 TUNEL ASSAY

During apoptosis, genomic DNA is partially cleaved into double-stranded, low-molecular weight DNA fragments (mono- and oligonucleosomes) as well as single-stranded breaks (nicks) in high-molecular weight DNA. Labelling free 3' OH termini with fluorescent nucleotides, e.g. fluorescent dUTP, can identify the DNA strand breaks. This polymerization reaction is accomplished by TdT (terminal deoxynucleotidyl transferase) which catalyses polymerization of nucleotides to free 3'OH DNA ends in a template-independent manner.

Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) is an *in situ* method for detection of the 3'-OH ends of DNA exposed during the internucleosomal cleavage of DNA that takes place in the process of apoptosis. Incorporation of labeled dUTP allows detection by immunohistochemical procedures and the labeled apoptotic cells may be visualized by light or fluorescence microscopy. In paper I, paraffin embedded sections were rehydrated and digested with proteinase K for 30 minutes at room temperature in order to unmask the DNA. The specimens were then washed and incubated with td-transferase and dUTP mixture labeled with biotin (Boehringer Mannheim, Mannheim, Germany) for 30 minutes at 37°C. The incorporation of biotinylated dUTP was visualized with Cy-2 labeled streptavidin (KPL, Gaithersburg, MD) using fluorescence microscopy. Propidium iodide was used for nuclear counterstaining.

3.5 ELECTRON MICROSCOPY

Electron microscopy was used to analyze the ultrastructure of the AAA wall with respect to cellular composition and morphology. Tissue preparation for electron microscopy involves fixation, dehydration and embedding in low-viscosity epoxy resins that polymerize to give a material suitable for the preparation of ultrathin sections. The main goal of this process is to stabilize and preserve the fine structural details of the fixed tissue as close to that of tissue in the living tissues. Glutaraldehyde and osmium tetroxide (OsO_4) were employed as the most effective fixatives for electron microscopy. Glutaraldehyde stabilizes tissues by cross-linking proteins. Osmium tetroxide reacts with lipids and certain proteins but also provides electron density to the tissue. Block staining with uranyl acetate was used to further enhance the electron density of the material. Thin sections were cut with diamond knives on an ultramicrotome. SMCs were analyzed and classified as contractile or synthetic in phenotype according to described standards [105]. During the phenotypic modulation process, contractile SMCs loose contractile myofilaments whereas the amount of endoplasmatic reticulum and Golgi apparatus (synthetic organelles) increases. Ultrastructurally, apoptosis is characterized by reduced cell size, condensation of nuclear chromatin, fragmentation into small membrane-bound structures called apoptotic bodies, destroyed organelles in the cytoplasm, but enclosed by an intact cell membrane [106].

3.6 MICRO ARRAY AND REAL TIME PCR

For the screening and analysis of gene expression profiles in human AAA tissue, Affymetrix U95A microarray chip was chosen for its broad-spectrum of approximately 12000 genes. Isolated RNA from AAA were checked for its quality in 1% agarose gel, thereafter RNA from different patients were pooled in two groups, i.e group from the wall under thrombus and group from the wall without thrombus, and used for microarray analysis. The limitation of this experimental approach was cost and lack of comparable standard, for example normal aorta. However, comparisons of gene expression profiles in different AAA wall segments were possible in each individual patient.

To confirm the expression data generated by a microarray analysis, we performed real time PCR for genes of interest. Real-time PCR is based on the detection and quantification of a fluorescent reporter where the signal increases in direct proportion to the amount of PCR product in the reaction. The method quantitates the initial amount of the template specifically, sensitively and reproducibly, and is a preferred method over standard PCR that detects the amount of final amplified product by gel electrophoresis at the end-point. Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle.

Real-time PCR was used to study gene expression in individual patients separately and gave similar results as microarray analysis from pooled samples. Expression of chosen genes was compared to expression of the house-keeping genes β-actin and RPLP0 in all experiments. However, while both the array analyses and the real-time PCR are quantitative methods, none of these methods provide information about the localization of gene expression.

3.7 WESTERN BLOTTING AND IMMUNOPRECIPITATION

Western blotting, also referred to as immunobloting, was used to study protein expression of NGAL and NGAl/MMP-9 complexes in AAA tissue, thrombus and the fluid in between the wall and thrombus in paper III. We immunoprecipitated NGAL/MMP-9 complex by using NGAL antibody. The precipitate was resuspended in non-reducing sample buffer and subjected to electrophoresis on a 4-15% gradient gel. The protein was transferred to a nitrocellulose membrane, followed by staining with Ponceau solution to verify equal protein loading. MMP-9 and NGAL/MMP-9 complex was detected by specific primary antibody against MMP-9 and visualized by horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescence substrate kit. The images were captured on light-sensitive films, and the band densities were scanned.

3.8 IN SITU ZYMOGRAPHY

Zymography is a method to detect enzymatic activity and is useful for demonstrating the activity of gelatin-degrading proteases (mainly MMP-2 and MMP-9). The presence and localization of MMPs were detected by microarray analysis, real time RT-PCR and immunohistochemistry. Therefore, the logical step was to detect the area of protease activity using *in situ* zymography in the samples. Frozen tissue sections were place on commercially available slides coated with a gelatin substrate and the slides incubated at 37°C in a humid chamber for 6-18 hours. Amido black was thereafter used to stain the tissue and gelatinolytic activity was detected in areas where precoated gelatin was degraded and appeared pale or white. Controls were obtained by inhibiting activity of MMPs with phenanthroline and EDTA. *In situ* zymography is not a method to quantitate the gelatinolytic activity in a organ or tissue but is very useful to localize gelatinolysis and can be helpful to predict the area of degradation i.e in AAA.

4 RESULTS AND DISCUSSION

4.1 STRUCTURAL AND CELLULAR DIFFERENCES IN THROMBUS COVERED AND THROMBUS FREE ABDOMINAL AORTIC ANEURYSM WALL (PAPER I)

In order to study the structure and composition of the thrombus-free and the thrombus-covered aneurysm wall, we collected biopsies from patients selected for elective operation where CT scan showed thrombus and thrombus-free parts within the AAA. The samples were preserved with glutaraldehyde fixatives for EM and with formalin for paraffin embedded sections.

There was a significant difference in wall thickness between the two wall segments within individual patients. The wall underneath the thrombus was thinner compared to the wall without thrombus. Furthermore, the thrombus-covered wall contained less elastin fibers and those found were degraded as evaluated by light microscopy. Fewer SMCs but more inflammatory T and B-cells were found in the thrombus-covered segments of the AAA wall.

SMCs are the main cells in the media of normal arteries and it has been suggested that disappearance of SMCs is a consistent phenomenon in AAA [107]. SMCs are the main producers of the vessel wall ECM [108] and the lack of SMCs in the aneurysm wall under the thrombus demonstrated in the present study is consistent with the idea that depletion of SMCs in the media is a feature of aneurysm disease. Electron microscopy demonstrated that the remaining SMCs under the thrombus were mainly of a synthetic phenotype. Such SMCs may be a significant source of ECM production but also matrix-degrading proteases participating in remodelling and the destruction of the elastin in the aneurysm vessel wall, even though the production of MMPs by synthetic SMCs is still a topic of debate [12, 109]. In human AAA and atheroma, other proteases with the capacity to degrade ECM have been localized to SMCs such as cathepsin L, a potent elastase and collagenase within the cysteine protease family [110].

The decreased number of SMCs within the thrombus-covered wall may be a result of increased SMC apoptosis, which is supported by the results of the electron microscopy analyses and the TUNEL assay. However, we could not confirm if the TUNEL activity was a result of apoptotic SMCs or due to the presence of increased apoptosis of inflammatory cells. Apoptosis is a physiological process of cell death that occurs in both normal and pathological remodelling of the vessel wall. Apoptosis has previously been demonstrated in human AAA tissue [13, 107] and AOD [111]. Interestingly, SMC apoptosis may be a specific feature of aneurysms since increased SMC apoptosis has been detected in AAA wall in comparison with femoral, poplitieal, iliac and carotid arteries [112].

T cells and B cells are abundant in AAA tissue [113, 114]. The proportion of activated T and B lymphocytes was higher in AAA tissues when compared with peripheral blood lymphocytes of AAA patients [115]. In our study, we investigated the presence of these cells in the two wall regions. Both B-cells and T-cells were in excess in the wall under the thrombus as judged by staining with CD20 and CD3, respectively. We also found that cytotoxic T cells and helper T cells were more abundant in the thrombus-covered wall. The reason why these cells accumulate within the thrombus-covered wall is not known. Recently, a subtype of helper T cells (TH2) has been discussed in the context of AAA [116, 117]. In addition, chlamydia pneumoniae reactive T-lymphocytes have been identified in AAA [118], and there is evidence that chlamydia heat shock protein 60 (HSP60) can induce MMP expression by macrophages [119]. Whether the thrombus-covered wall or the thrombus is a site for bacterial infection remains to be shown. In addition to T-cells, macrophage infiltration plays a major role in chronic aortic wall inflammation and may account for the destruction of medial elastin [120]. In the present study, macrophages were identified with CD68 positive staining but we could not detect any differences in CD68 staining between the two wall regions. Interestingly, a recent study showed that macrophage migration inhibitory factor (MIF) expression was significantly increased in ruptured AAA wall compared to non-ruptured AAA wall, suggesting an important role of macrophages in AAA rupture [121].

Fibrilar collagen provides tensile strength of the aorta and the degradation of collagen has been suggested to be associated with AAA rupture, whereas elastin degradation has

been associated with AAA dilatation in a canine model [5]. Collagen type I and III are the principal types of collagen in aorta. In a necropsy study, increased collagenase activity was found in infrarenal AAA rupture [123]. We were not able to detect differences in collagen content in AAA tissues by Masson-Tricrome staining. Possibly, this method is not sensitive enough and further studies may be required to establish such differences and to identify the specific collagen subtypes in AAA.

As described above, degradation of elastin has been proposed to be the cause of arterial dilatation [5, 124]. Elastin degradation products (EDP) are chemotactic for inflammatory cells [125]. Increased blood flow may also cause elastin degradation leading to arterial dilatation [126]. In response to EDP, SMCs from AAA secrete high amounts of elastase compared to SMCs from AOD and control aortas [127]. In our work, elastin was more fragmented in the thrombus-covered wall compared to the wall without thrombus and EDPs could therefore serve as chemoattractants for the immune cells detected in the thrombus-covered wall.

In summary, the study showed that the thrombus-covered wall was thinner, contained less and degraded elastin, less SMCs and an accumulation of inflammatory T and B-cells. Together, this indicates that the thrombus-covered wall is at risk of rupture if bleeding into the thrombus occurs. However, whether the thrombus itself influences the integrity of the underlying vessel wall is not clear.

4.2 DIFFERENCES OF PROTEASES AND GENE EXPRESSION IN THROMBUS COVERED AND THROMBUS FREE WALL (PAPER II)

Increased expression of MMPs and other proteases has been demonstrated in AAA [43, 128] and is thought to play a major role in aneurysm development and rupture [75]. The intraluminal thrombus has been associated with structural properties indicating local wall weakness (Paper I) and an increased risk of rupture [65]. The objective of Paper II was to study if different expression of proteases between the thrombus covered and thrombus free wall of AAA could explain the structural differences detected in Paper I.

Patients with CT showing thrombus covered and thrombus free areas were selected for sampling. Biopsies taken from the two wall regions were dissected and quick-frozen in liquid nitrogen for RNA preparation and *in situ* zymography. Tissues were also preserved in formalin for immunohistochemistry. A microarray analysis on pooled RNA was used for screening of protease mRNA expression in the two regions of the AAA wall and real-time PCR analyses were used to quantify mRNA expression in individual patients.

In Paper I, we demonstrated that elastin fibers were less abundant and fragmented in the thrombus-covered wall whereas intact elastin could be detected in the thrombus free wall. Therefore, we hypothesized that matrix-degrading proteases would mainly be expressed in the thrombus-covered wall. The results, however, showed the opposite to be true. Gene array analysis on pooled RNA samples as well as quantitative real-time PCR on individual specimens demonstrated a consistent upregulation of MMPs in the media of the thrombus-free wall compared with thrombus-covered wall. However, immunohistochemistry localized MMP-9 protein expression and gelatinase activity also to the border region between the thrombus and the underlying vessel wall. Furthermore, *in situ* zymography analyses co-localized gelatinase activity with MMP-9 expression in both wall regions. The production and secretion of MMPs can be regulated by several different mechanisms. First, the expression of proMMPs is regulated by transcriptional regulation. Secondly, proMMPs are activated to obtain proteolytic activity by cleavage of the N-terminal domain. Thirdly, the proteolytic activity can be quenched by binding to specific inhibitors, TIMPs [73]. The exact mechanism responsible for the degradation of elastin of the thrombus-covered wall is not clear but it is possible that this proccess is an early event in AAA formation and thus not detectable in manifest lesions.

A correlation between mRNA levels of several MMPs and CD68 mRNA, as well as co-localized immunostaining of MMP-9 and CD68 was observed, which suggests that macrophages may be the main source of protease expression and thus, an integrated part of an ongoing inflammatory process in the interface between the vessel wall and the thrombus. Chronic inflammatory cells have been shown to be a major source of proteinases such as serine elastases [127], plasminogen activators [99] and MMPs

[129], of which MMPs are the most investigated group of proteases and believed to be the main gene family involved in the destruction of the ECM of the AAA wall [75]. Correlation between elastolytic activity and the number of inflammatory cells has previously been observed in AAA [130, 131].

The plasminogen system is required for the conversion of proMMPs to active MMPs. tPA, uPA and PAI-I mRNA expression has previously been detected in the vicinity of inflammatory infiltrate of AAAs [99]. In our study, uPA, UPAR and tPA mRNA were increased in the wall without thrombus. However, similarly to MMP-9, UPAR mRNA and protein could also be detected in the interface between the thrombus and the underlying vessel wall.

MMPs have been suggested as a potential therapeutic target in the prevention and treatment of AAA, and their inhibition using novel pharmacological interventions [80]. Suppression of aneurysmal degeneration has been observed in animals treated with nonspecific MMP inhibitors [132, 133]. However, specific inhibitors are probably needed since different MMPs have different biological functions and are active at different stages of the disease. All MMPs do not react similarly to specific stimuli [134] and a modified tetracycline has potential anti-inflammatory and anti-metastatic effect through MMP inhibition [122]. Furthermore, a recent study showed that cultured rat aortic SMCs treated with IL-1β expressed more MMP-9 mRNA and protein compared with SMCs from female rats suggesting that there are gender-specific differences in MMP-9 expression. It has also been shown that estrogen inhibits macrophage production of MMP-9 [135]. However, our materials were mainly from male patients. Of note, AAA occurs predominantely in males and we have not made any efforts to compare gender differences in a small study material. Whether gender specific expression of MMP-9 contributes to the fact that AAA is predominantly a male disease remains to be shown.

Taken together, protease expression and MMPs activity differs in different parts of AAA and it could be hypothesised that proteases derived from the thrombus may be involved in processes related to accelerated aneurysm growth and rupture.

4.3 REGULATION OF MMP-9 IN AAA (PAPER III)

In Paper II we demonstrated expression and activity of MMP-9 in the interface between the thrombus and the underlying wall. In addition to the classical regulation of MMPs, an additional regulatory mechanism has recently been proposed that could substantially enhance the proteolytic activity of MMP-9. Neutrophil gelatinase-associated lipocalin (NGAL) [136] has been suggested to influence the activity of MMP-9 by binding to MMP-9 and inhibit its degradation thus preserving its enzymatic activity [137]. In Paper III it was demonstrated that NGAL/MMP-9 complexes were present in different regions of the AAA. NGAL expression was co-localized with CD66b positive neutrophils. Importantly, NGAL/MMP-9 complexes were present in the thrombus itself and in the interface fluid between the thrombus and the underlying vessel wall. These findings support the concept that the wall underlying the thrombus is degraded from within the lumen by enzymatic activity related to the thrombus. This could lead to AAA rupture after blood has entered the thrombus [138]. These data suggest an important role of neutrophils in AAA as suggested by Fontaine et al. [139]

In addition to binding and stabilization of MMP-9, several other properties have been attributed to NGAL. Lipocalins are a functionally diverse family of proteins that bind small hydrophobic ligands [140]. It has been proposed that NGAL has immunomodulatory activity by binding and clearing lipophilic inflammatory mediators [141]. Furthermore, NGAL has been demonstrated to bind bacterial catecholate-type ferric siderophores with high affinity [141]. It could therefore be hypothesized that NGAL participates in the antibacterial iron depletion strategy of the innate immune system. Chlamydia pneumoniae reactive T-lymphocytes have been identified in AAA [118] and chlamydia has been localized to AAA tissue [142]. Whether the increased expression of NGAL in the thrombus is a result of bacterial infection is not known, and further studies are needed to define the specific role of NGAL in AAA growth and rupture.

In Paper III we detected CD66b-expressing neutrophils in the vessel wall and in the thrombus itself. Several recent studies have highlighted the importance of neutrophils in AAA. Fontaine et al, demonstrated that trapped leukocytes in the intraluminal

thrombus can be a source of protease production. Co-localization of MMP-9 and leukocytes in the luminal part of the thrombus has been shown by immunostaining [139]. This group also demonstrated that leukocyte elastase could be stored and released by the luminal part of the thrombus [143]. Recently, the role of neutrophils was studied in an elastase perfusion experimental AAA model in mice [144]. Neutrophil depletion limited both AAA size and incidence. Interestingly, AAA development in this model was independent of detectable changes in MMP-2 and MMP-9 levels, whereas aneurysm suppression was associated with a lower MMP-8 expression (neutrophil collagenase). MMP-8 deficiency, however, did not inhibit AAA formation, which suggests that other neutrophil-derived mediators are important for AAA development. Other proteases, such as cathepsins or serine proteases are obvious candidates. The results of the present work suggests that NGAL is an important factor, influencing the activity of a steady state level of MMP-9.

In summary, Paper III demonstrates that NGAL/MMP-9 complexes are present in AAA regions showing signs of matrix degradation. The findings suggest an important role for neutrophils in the enhanced proteolytic activity associated with AAA.

4.4 MAST CELLS IN ABDOMINAL AORTIC ANEURYSMS ASSOCIATED WITH MEDIAL AND ADVENTITIAL NEOVESSELS (PAPER IV)

Mast cells have been less studied than other inflammatory cells in vascular disease. However, MCs have been shown to have a potential role in protease secretion and in immunoregulation [145]. In Paper IV, we studied the presence of mast cells and their association with other cell types in AAA. Tissue from human AAAs and normal control arteries were used for microscopy, immunohistochemistry, and RNA analysis. Intraoperatively retrieved specimens were formalin fixed and paraffin embedded for immunohistochemistry or snap frozen for RNA preparation. Double immunostaining of endothelial cells and MC tryptase of AAA specimens showed that MCs were absent in the intimal layer of AAAs, in contrast to normal aorta, and MCs were associated with medial and adventitial neovessels. Furthermore, MCs reside partly in the same areas as macrophages, CD3 positive T-cells and neutrophils. Cathepsin G and tryptase double immunostaining showed that MCs positive both for cathepsin G and tryptase were

more numerous than cells with only tryptase or cathepsin G. Cathepsin G, tryptase and chymase expressions did not differ significantly between thrombus-covered AAA wall and AAA wall without intraluminal thrombus when evaluated by RT-PCR.

Inflammatory cells present in AAA are believed to play an important role in AAA development. MCs are usually found in connective tissue and do not circulate in blood. There are different types of MCs based on their protease content. Two examples are tryptase containing MCs and tryptase, chymase and carboxypeptidase containing MCs. By releasing potent inflammatory mediators, such as histamine, serotonin, proteases and chemotactic factors, MCs play a central role in inflammatory and allergic reactions. Furthermore, MCs are involved in activation of collagenase leading to pathological remodelling and presentation of antigens to T cells. MCs need to be degranulated, which is achieved by physical destruction or exposure to chemical substances and proteases, to perform their functions.

In contrast to a normal aorta, MCs could not be detected in the intima of the AAA wall but were instead localized to the media and the adventitia. MCs were colocalized with macrophages, T cells and neoangiogenesis. The colocalization of MCs with macrophages indicates that MCs may contribute to the protease expression detected within this region of the AAA wall. The presence of MCs in the vicinity of T cells suggests that MCs might be involved in immunoregulation in AAA disease.

The presence of MCs near neovessels suggests that they take part in neoangiogenesis which could contribute to AAA rupture. In humans, the infrarenal aorta is devoided of vasa vasorum. Inflammation and hypoxic insult are closely associated with neovessel formation [101, 146]. We detected neovessels in the adventitial and medial layer of AAA tissue but there were no differences between thrombus free and thrombus covered wall. The presence of neovessels in areas with destructed elastin and chronic inflammation was a consistent finding of AAA compared to normal aorta or occlusive aorta. Moreover, neovessels density has been shown to be three-fold higher in AAA compared to AOD [98].

Depending on the microenvironment, MCs are capable of producing proteases, thus potentially taking part in angionenesis and tumour progression [103]. In our study, presence of MCs in neovessels may indicate that in chronic inflammatory conditions, MCs in the vicinity of other inflammatory cells like macrophages and T cells may facilitate neovessel progression.

The presence of MCs in AAA does not explain the difference in morphology between AAA wall segments covered and not covered by thrombus. They may, however, be involved in an earlier stage of AAA development. The present study only studies the end-stage of the disease process and cannot explain the causes for neovessels formation in AAA tissue. Stem cell factor (SCF) is a chemotactic cytokine for MCs [147] and prevents apoptosis of MC thus promoting MC survival [148]. In AAA tissue we found SCF positive endothelium in neovessels particularly in the adventitial and medial layer, which suggests a close association of neovessels formation and presence of MCs in the outer layer of AAA. However, we can only hypothesize that hypoxia, inflammation and proteolytic imbalance orchestrating the degenerative multifactorial disease like AAA may be involved.

In summary, the results suggest participation of MCs in the pathogenesis of AAA, which might be associated with neovascularization in AAA but the functional role of MCs in AAA remains to be shown.

5 CONCLUDING REMARKS

Patients with AAA have manifestations of atherosclerosis and AAA shares many risk factors with AOD e.g. smoking and hyperlipidaemia. AAA has therefore been considered an atherosclerotic disease for a long time, but recently, entities separate from atherosclerosis have been recognized in the pathogenesis of AAA. The natural course of AAA is growth and rupture, but all patients with AAA do not die from rupture. Many succumb from the consequences of associated, mostly coronary, atherosclerotic disease. When rupture occurs, many patients never reach hospital and the surgical mortality for those that do so is high, consequently the total rupture mortality is even higher, approximately 75%.

A thrombus is almost always present in AAA [149] and its role has been discussed in relation to biomechanical properties, growth and rupture of AAA. In the present study, AAA wall segments covered by thrombus were compared with those without an overlying thrombus. The differences between these two wall segments were obvious. Degraded elastin, decreased wall thickness, increased inflammatory infiltrate and fewer SMCs were seen in the wall covered by thrombus. These findings, together with the clinical observation that bleeding into the thrombus is seen on computed tomography in cases of rupture, leads to the suspicion that that the thrombus-covered wall could be a potential place of and predispose for rupture.

Proteolytic degradation of matrix proteins is a key element in the pathogenesis of AAA. MMPs have been shown to play major role for this process, although the importance of other proteolytic systems has been less examined. Analysis of RNA expression in the two wall segments revealed an upregulation of MMPs in the wall without thrombus, which is in contrast to the findings of signs of proteolysis and inflammation in the thrombus covered wall. RNA levels, however, do not necessarily imply functional activity. The presence of gelatinase activity was demonstrated by zymography not only in the media of the wall without thrombus but also in the interface between the thrombus and the underlying wall. The function of MMP-9 can also be influenced by binding to NGAL, which prevents its degradation. The finding of MMP-9 in complex

with NGAL in the space between the thrombus and the underlying wall provides further evidence for MMP-9 activity affecting the thrombus-covered wall.

The present study examines a late stage of AAA and cannot identify early pathogenetic mechanisms with accuracy. MCs, not normally present in the media of the aorta, were demonstrated in the media of AAA independent of thrombus coverage. Co-localization of MCs with T cells and neovessels in the adventitial area could facilitate transport of proteases in AAA tissue and influence neovessel formation in the AAA wall. Migration of MCs to the aortic media could possibly represent one early mechanism in the pathogenesis of AAA. It could be hypothesized that the thrombus-free wall represents an earlier stage of aneurysm formation. A proposed model is presented in figure 8.

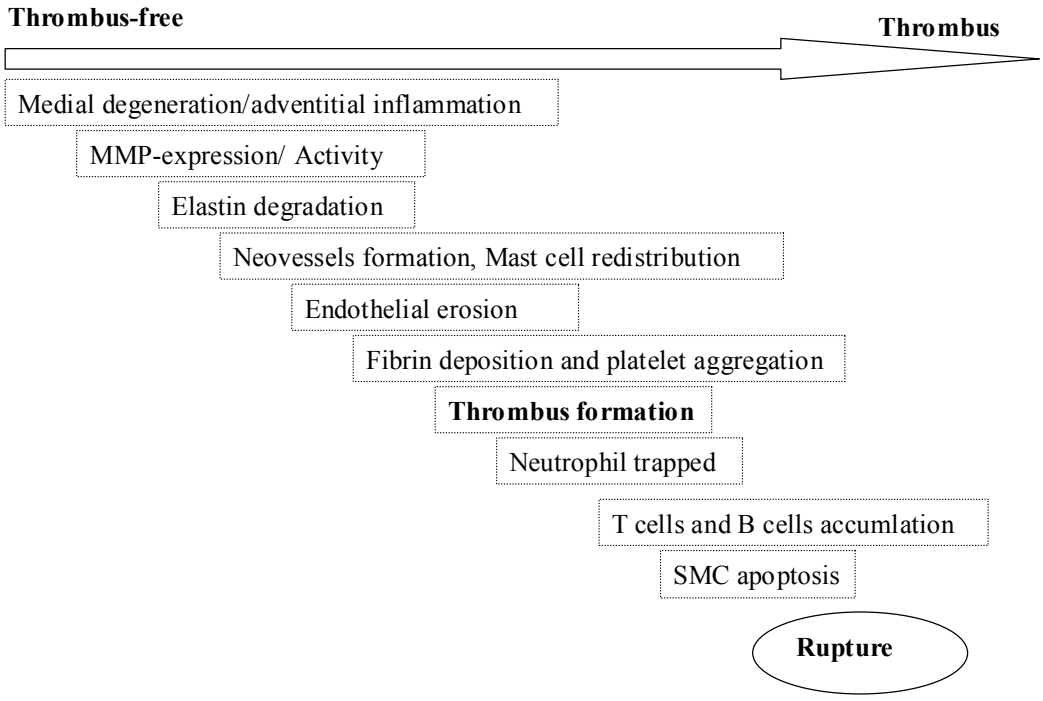

Fig 8. Proposed model of the different steps of AAA pathogenesis with the assumption that the thrombus-free wall represents an early stage of AAA development.

However, experimental animal models is the only way to study the initiation of aneurysmal disease and they also have the advantages of reproducibility and possibility to investigate pharmacological intervention to prevent development and growth of AAA. On the other hand most animal models of AAA lack an intraluminal thrombus [150]. Development of an appropriate animal model of AAA containing an intraluminal

thrombus could therefore be a desirable and valuable addition to the experimental research on AAA in order to resolve fundamental issues raised in this thesis.

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