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# Protease activity in the multi-layered intra-luminal thrombus of abdominal aortic aneurysms

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ABSTRACT

*Introduction:* Rupture of an abdominal aortic aneurysm (AAA) is the cause of death in approximately 2% of men above 65 years. Most AAAs contain an intra-luminal thrombus (ILT), which is a potential source of proteases capable of degrading the underlying aneurysm wall. The AAA wall covered by a thick ILT shows more signs of matrix degradation compared to the wall free from ILT. The purpose of the present study was to evaluate the presence of protease activity in the ILT.
*Materials and methods:* ILT and peripheral blood from 32 patients undergoing elective surgery were collected. The ILT was divided into abluminal, luminal, and a middle layer in between. Collagenases, gelatinases, elastase, and their inhibitors were measured using ELISA in protein extracts from these layers. Immunohistochemistry was used for identification of cells.
*Results:* Neutrophil leukocytes and platelets were mostly detected in the luminal layer of the ILT. MMP-9 and neutrophil elastase were also abundant in this layer but with low activity. High concentrations of TIMP-1 and PAI-1 were detected in the abluminal layer, while alpha 1 antitrypsin was mostly found in the luminal layer of the ILT.
*Conclusions:* In AAA thick ILTs with multiple layers contain substantial amounts of proteases, but their activity is limited to the luminal layer. Proteases in the abluminal layer are mostly inactive, probably due to excess amounts of inhibitors and are consequently unable to directly participate in the pathogenesis of AAA.



## 1. Introduction

Rupture of abdominal aortic aneurysm (AAA) is a common cause of death and the incidence of rupture has been reported to increase during recent years [1]. The main load bearing components of the aortic wall are elastin and collagen. Decreased elasticity as a result of elastin degradation is an early event in AAA development. Neutrophil derived elastase (NE) is the predominant elastase found in AAA tissue [2]. Collagen synthesis is initially increased compensating for loss of non-renewable elastin. As the AAA progresses, degradation of collagen overwhelms its production eventually leading to rupture [3]. Previous reports have demonstrated presence of metalloproteases (MMPs) in AAA tissue capable of degrading aortic wall matrix components [4]. In particular geltatinases (MMP-2 and 9), collagenases (MMP-1, 8 and 13), and stromelysins (MMP-3, 10 and 11) have been associated with AAA formation. MMP-9 and MMP-2 are the most studied proteases, shown to be present and active in the ILT [5]. Most MMPs are produced by inflammatory cells [6], but MMP-2 is a product of smooth muscle cells (SMCs) rather than inflammatory cells [7]. The result of elastin degradation is increased wall stiffness, elongation, and tortuosity of the aorta leading to areas of turbulent flow, which in combination with endothelial injury favors thrombus formation seen in most AAAs [8]. The volume of the ILT correlates with AAA diameter [9] and the risk of rupture has been related to growth of the ILT [10].

Turbulent flow with low shear stress, endothelial injury, and platelet accumulation favors thrombus formation in aneurysms [11]. Initially the ILT is thin and contains platelets, red blood cells and other hematopoietic cells. Eventually it is renewed resulting in a thicker thrombus with multiple layers, a luminal fresh layer and abluminal layers devoid of cells [12]. In contrast to the thick multilayered ILT the newly built thrombus is thin and lacks different layers, but is similar to the red luminal layer of the renewed multilayered ILT.

We have previously compared the AAA wall covered by an eccentrically located thick ILT with the wall without ILT, as judged by computer tomography (CT). We found that the thrombus covered wall is thinner and shows more signs of proteolytic activity

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with more degraded elastin [13]. Furthermore the AAA wall underlying a thick thrombus has decreased strength possibly increasing the risk of rupture [14]. These studies have led to the suspicion that the thick ILT could be the source of degrading enzymes influencing the wall beneath. On the other hand a recent study by Wiernicki et al. showed that active MMP-9 and NE are significantly higher in AAA wall segments covered by a thin (<10 mm) compared to a thick (>25 mm) ILT [15].

Recently it was reported that neutrophil leucocytes are actively recruited into the luminal layer of the ILT, activated to produce proteases that could contribute to the weakening of the already degraded AAA wall [16]. In a previous study we showed that MMP-9 is produced in the ILT by neutrophils and is bound to neutrophil gelatinase associated lipocalin (NGAL), a complex mostly found in the luminal layer of the ILT [17]. MMP-9 activity is preserved when bound to NGAL, a protein of 24 kDa, which binds to MMP-9 and prevents its degradation [18]. Together, these findings suggest a role of a multilayered ILT in the pathogenesis of AAA.

Since the aortic wall covered by a thick multilayered ILT shows increased signs of elastin degradation compared to wall segments without ILT, the purpose of the present study was to answer the following unresolved questions regarding the role of such an ILT in AAA development: (I) Which proteases and inhibitors are present in the different layers of thick ILTs? (II) Are the amounts of proteases and their inhibitors related to the plasma levels? (III) Which are the cellular sources of the proteases in the ILT? and finally (IV) Is there a relationship between protease activity and their inhibitors in each layer of the thick ILT?

## 2. Materials and methods

### 2.1. Sample collection

Patients with an eccentrically located ILT providing a segment exceeding 10 mm were included. ILTs were collected from 32 patients (27 men and 5 women) undergoing elective surgery. Mean age was 70.3 ± 8.3, mean AAA diameter 61 ± 1.1 mm and mean thickness of the ILT 27 ± 10.8 mm (mean ± SD). AAA diameter and ILT volume and thickness were measured in three dimensional reformatted CT images obtained with multislice technique. The ILT was divided into three different layers, luminal, intermediate and abluminal. The specimens were frozen in dry ice and part of the fresh specimen was prepared for immuno-histochemistry. An ILT <10 mm was also obtained from one additional patient. Blood anticoagulated with citrate and EDTA was collected from each patient prior to surgery, centrifuged 20 min at 2500 × *g* followed by 30 min at 20,000 × *g* to obtain platelet free plasma. The study was approved by the regional ethics committee. Informed consent for collection of samples was acquired prior to surgery.

### 2.2. Protein extraction

Tissue was homogenized in RIPA lysis buffer containing 150 mM NaCl, 1.0% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, and a pH of 8. Protease inhibitors (complete mini EDTA-free from ROCHE) were added to the RIPA buffer prior to protein extraction, except for protein preparations aimed for activity measurements of neutrophil elastase (NE). The tissue was weighed, homogenized, and centrifuged at 16,100 × *g* for 15 min at 4 °C. Each extract was analyzed for total protein content using BCA protein assay kit (Thermo Scientific) according to the manufacturer's guidelines.

### 2.3. Enzyme-linked immunosorbent assay, ELISA

The amounts of candidate proteases and their inhibitors were quantified in plasma and extracted proteins from the ILT samples by ELISAs, as listed in supplementary Table 2. Concentrations of proteins in the ILT were normalized using weight of the tissue and presented as ng/g wet weight. Alpha 1-antitrypsin is given as mg/g wet weight.

### 2.4. Immunohistochemistry

Staining material was obtained from Histolab, Sweden. DIVA, peroxidase, Sniper and Mach 2 and 3 kit together with staining agents Vulcan Red, Romulin red chromogen kit was used according to the manufacturer's protocol. Antibodies are listed in supplementary Table 1.

### 2.5. Western blot

In order to explore whether TIMP-1 is in complex with its substrates or exists as free protein, a non reducing western blot analysis of ILT tissue immunoblotting for TIMP-1 was performed. Sample buffer Laemmli and precast gels Bis–Tris Mini gels 4–20% from Bio Rad were used according to the manufacturer's recommendations.

### 2.6. Protease activity measurements

Enzymatic activity of MMP-9 and NE in ILT and plasma was measured with kits stated in supplementary Table 2 according to the manufacturer's guidelines. Protein extracts from different layers of ILT and plasma from the same patients were analyzed. Patients with three complete layers in the ILT and available plasma samples were subjected to assay of MMP-9 activity. For measurements of NE-activity we used homogenized tissue at 4 °C without protease inhibitor and followed the manufacturer's instructions.

### 2.7. Statistics

Analysis of data from the three layers of the ILT was done by non-parametric methods for dependent variables. Wilcoxon matched pairs test and Spearman rank test were performed using the Statistica program.

## 3. Results

As seen in a representative specimen an ILT examined in the present study is a non homogenous structure consisting of three layers (Fig. 1A). Cells are readily found in the luminal but not in the abluminal layer (Fig. 1C–E). In contrast to this multilayered thrombus an ILT less than 1 cm thick is depicted, demonstrating presence of only one single layer (Fig. 1F) containing numerous neutrophil leucocytes (Fig. 1I).

Presence of various haematopoietic cells and proteins with inflammatory attributes in the ILT with multiple layers has been shown [19,20]. Here we confirmed presence of neutrophil leucocytes, CD163 positive macrophages/monocytes and platelets (Fig. 2). We also observed tryptase positive cells in the luminal layer, but to a lesser extent. MMP-1, 2 and 13 were present with very low amounts in all layers. In contrast to MMP-9 and NE the amounts of TIMP-1 (Fig. 3B) and PAI-1 were present with their highest concentrations in the abluminal layer (Table 1).

In order to find out whether levels of MMP-9 and NE in the luminal layer of the ILT are a reflection of their plasma levels or a result of protease expressing cells within the ILT, we compared protease levels in the luminal layer of the thick multilayered ILT with corresponding proteins in plasma. There were no significant correlations between investigated protein levels in plasma and in the luminal layer of the ILT except for TIMP-1 (Table 2). In fact, the