Presence of NGAL/MMP-9 complexes in human abdominal aortic aneurysms

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Summary

It has been suggested that the intraluminal thrombus of abdominal aortic aneurysms (AAAs) predisposes for AAA enlargement and rupture. The growth of the AAA is dependent on proteolytic degradation of elastin. Here, we analysed whether the neutrophil gelatinase-associated lipocalin (NGAL) is expressed within the thrombus and the aneurysm wall. NGAL can bind to metalloproteinase-9 (MMP-9) and inhibit its degradation, thereby preserving enzymatic activity. Biopsies were obtained from thrombus-free and thrombus-covered aneurysm wall and the intraluminal thrombus from patients undergoing elective surgery for AAA. Immunohistochemistry and real-time PCR were used to study NGAL and MMP-9 expression. Immunoprecipitation, gel zymography, Western blot and ELISA were used to detect and quantify NGAL/MMP-9 complexes. NGAL was detected in the thrombus, the interface between the thrombus and the underlying wall and in the wall itself. Double staining showed that neutrophils are the major source of NGAL expression. Immunoprecipitation of MMP-9 with antibody against NGAL showed that complexes of NGAL and active MMP-9 were present in thrombus, the interface fluid and the aneurysm wall. Western blot analyses using non-reducing conditions and gel zymography demonstrated that high-molecular-weight complexes of NGAL/MMP-9 were present within the different regions. The concentration of the NGAL/MMP-9 complex was highest in the luminal part of the thrombus. In conclusion, NGAL in complex with activated MMP-9 is present in AAA wall and thrombus. Neutrophil-derived NGAL could enhance the proteolytic activity associated with AAA, but the importance of this mechanism for aneurysm growth remains to be shown.

Keywords

Abdominal aortic aneurysm, NGAL, matrix metalloproteinases-9, thrombus

Introduction

Abdominal aortic aneurysms (AAA) evolve through chronic degradation of extracellular matrix components leading to dilatation of the aortic wall. Matrix metalloproteinases (MMPs) are suggested to play a major role in the pathological process leading to AAA growth and rupture, since they can degrade all macromolecules present in the connective tissue matrix (1).

The molecular mechanisms responsible for the induction and modulation of protease expression in the aneurysm wall are not fully known. Neutrophil gelatinase-associated lipocalin (NGAL) (for review see [2]) has been suggested to influence the activity of MMP-9 (3). NGAL binds covalently to MMP-9 (3) and inhibits its degradation, thereby preserving MMP-9 enzymatic activity (4). NGAL was initially identified as 24p3 in SV40-infected mouse kidney cells (5). In humans it was detected in granules of neutrophils, hence its name (3, 6).

AAAs are filled to a varying extent with a laminated thrombus (7), and both aneurysm growth and rupture have been associated with growth of the thrombus (8–10). The wall covered by thrombus is thinner and contains less elastin and fewer smooth muscle cells (11). Recent gene expression analyses in wall seg-

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ments with and without thrombus from patients undergoing elective AAA repair indicated that MMP-9 (gelatinase B) derived from macrophages present in the interface between the intraluminal thrombus and the underlying vessel wall contribute to the enhanced proteolytic activity detected within this region (12). However, other cells trapped within the intraluminal thrombus could also secrete proteolytic enzymes. Accordingly, leukocytes contained within the luminal part of the thrombus have been shown to secrete MMP-9 and elastase (13, 14), and polymorphonuclear leukocytes were shown to be the main source of MMP-9 release during clot formation (13).

Since MMP-9 expression and activity is present in the interface between the intraluminal thrombus and the underlying wall and leukocytes are trapped within the thrombus, it could be hypothesized that neutrophil-derived NGAL plays an important role for the proteolytic activity responsible for the degradation of the thrombus-covered wall. A further support for this hypothesis is that c-Jun N-terminal kinase (JNK), of importance for the development of AAA in animal models, regulates lipocalin-2 (NGAL). Consequently inhibition of JNK leads to down regulation of NGAL and attenuation of aneurysm formation (15).

In the present work, we have analysed whether NGAL and NGAL/MMP-9 complexes are present in different regions of AAAs with a focus on the thrombus-covered wall and within the intraluminal thrombus itself.

**Material and methods**

**Biochemical reagents**

Phosphate-buffered saline (PBS) was purchased from Life Technologies (Paisley, UK). Hydrogen peroxide (H₂O₂), methanol and xylene were from Merck (Darmstadt, Germany), mounting media (Mountex) from Histolab (Göteborg, Sweden) and bovine serum albumin from Sigma Diagnostics (St. Louis, MO, USA). Vectastain ABC kit, 3,3-diaminobenzidine (DAB-) peroxidase substrate kit, biotinylated anti-mouse secondary antibodies were purchased from Vector laboratories, Inc (Burlingame, CA, USA). The primary antibodies against NGAL used for immunohistochemistry and western blotting were from Antibody shop (Gentofte, Denmark) and MMP 9 from R&D systems (Minneapolis, MN, USA). Ready gels 4–15% were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Nitrocellulose membrane (Hybond™-C super), secondary antibody, and ECL Advanced TM Western Blotting Detection Kit were from Amersham Life Science (Buckinghamshire, UK). Human MMP-9/NGAL Complex Quantikine ELISA Kit was from R&D system (Abingdon, UK).

**Sample collection**

Patients undergoing elective surgery for infrarenal AAA with a preoperative computed tomography (CT) scan demonstrating an eccentric intraluminal thrombus with both thrombus-free and thrombus covered wall segments, were selected for the study as described (12). Biopsies were taken during surgery from the anterior or lateral wall depending on the presence and absence of thrombus and on the thrombus itself. Added risk to the surgical procedure prevented collection of biopsies from the posterior wall. Tissue sections were obtained from the thrombus-free and thrombus-covered aneurysm wall. Organized thrombi were dissected, and luminal, middle and abluminal parts were immediately preserved in liquid nitrogen. The adventitia was dissected free of excess perversacular fat, and the sections were immediately fixed in 4% paraformaldehyde for light microscopy or snap frozen in liquid nitrogen for RNA isolation and protein extraction. Biopsies from nine male patients without severe comorbidities and multiple medications were used for RNA preparation.

None of them were on statins or ACE inhibitors. Biopsies from 14 patients were used for immunostaining, from two patients for Western blot analysis and from two patients for gel zymography. Thrombus preparations from 27 patients and paired samples of thrombus-covered and thrombus-free wall from 10 patients were used for the ELISA quantification of NGAL/MMP-9 complex. When present, fluid from the interface between the thrombus and the underlying wall was also sampled with a syringe. All patients approved the intraoperative retrieval of tissue from the aneurysm wall according to informed consent procedures and approval by the local ethics committee.

**Sample preparation and Western blot analysis**

AAA tissue and thrombus stored at −70°C were placed on Petri dishes on dry ice and dissected into small pieces. Then, 1 ml of modified RIPA buffer (Non reducing: 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM NaF and 1 µg/ml each of aprotonin, leupeptin and pepstatin) was added and the tissue was homogenised in a dismembranator (B. Braun Melsungen AG, Germany). Samples were kept at −70°C for Western blotting, gel zymography and immunoprecipitation analyses. Protein concentration was determined using the Bradford method (Biorad).

The supernatant and interface fluid were mixed with non-reducing Laemli sample buffer and run on a 4–15% gradient gel under non-reducing condition. The gel was transferred to nitrocellulose membrane and blocked with 5% milk in TBPS (0.1% Tween in PBS) for 1 hour at room temperature. The membrane was then incubated with NGAL (1:1,500) antibodies in 3% milk in TBPS at 4°C overnight with subsequent washes with TBPS in 1% milk (6 times for 5 minutes [min]) and incubation with horse-radish labelled secondary antibody (1:20,000) in 3% milk in TBPS. The membrane was washed with TBPS (3 times for 5 min) and developed with ECL kit according to the manufacturer’s protocol. After stripping the membrane, it was incubated with MMP-9 (1:1,000) antibodies following the same procedure as described above.

**Immunoprecipitation and gel zymography**

Homogenised tissue extracts were precleared with protein A/G-agarose prior to immunoprecipitation. Then, 200 µg of each sample were incubated at 4°C overnight with 2 µg NGAL antibody. Protein A/G-agarose (1/10 of the initial volume) was mixed with the sample and incubated at 4°C on a rotating device overnight with end-to-end mixing. The following morning the samples were centrifuged at 10,000xg for 2 min at 4°C, and the supernatant was aspirated and discarded. The pellet was gently washed 2–4 times with 1 ml of ice-cold RIPA buffer, and the centrifugation was repeated as above. In order to disrupt covalently bound MMP-9/NGAL complexes the pellets were resuspended in 42 µl of reducing Laemli sample buffer (3). The samples were...
subsequently heated for 5 min at 80°C, subjected to electrophoresis on a 4–15% gel and the protein transferred to nitrocellulose membrane. The membrane was evaluated by western blotting under reducing conditions to identify co-precipitated MMP-9 protein. Purified active MMP-9 (83 kDa, Calbiochem) was included in the electrophoresis analyses as a size marker. Purified NGAL/MMP-9 complex (Calbiochem) was included in the immunoprecipitation as a positive control.

Gelatinase activity was analyzed using a 10% Zymogram gel (Novex Invitrogen) under non-reducing conditions as described by the manufacturer.

**Immunohistochemistry**

Paraffin sections were cleared in xylene, rehydrated in graded ethanol (100–70%), immersed in water for 5–10 min, and incubated in 0.3% hydrogen peroxide (H₂O₂) in 70% methanol for 20 min to inhibit endogenous peroxidase activity. The specimens were then rinsed three times for 5 min each in PBS. Epitopes were unmasked by boiling in citrate buffer (pH 6.0) for 10–15 min when necessary. After rinsing in PBS, the sections were blocked for 30–60 min in PBS/3% bovine serum albumin (BSA) and then incubated with and without primary antibodies (1:100 in PBS/0.1% BSA) overnight at 4°C in a humidified chamber. The samples were then rinsed in PBS and incubated with 7.5 µg/ml biotinylated secondary antibody in PBS/0.1% BSA for 1 hour at room temperature followed by avidin-biotin amplification (ABC Elite) for 30 min, and developed with DAB substrate. Sections were counterstained with Mayer's hematoxylin for 3–5 min and mounted. Negative controls were obtained by substituting the primary antibody with IgG1 control antibody or with PBS.

Double staining of NGAL/MMP-9 and NGAL/neutrophil was performed according the manufacturers protocol (Biocare Medical, Concord, CA, USA). Primary antibodies used were: MMP-9 (sc-6841, Santa Cruz), NGAL (HYB 211–02, Antibody Shop), CD66b (MCA216, AB Serotec, Oxford, UK) and CD66ac (MCA1147G, AB Serotec). The chromogen systems were Ferangi blue for MMP-9 and CD66b/acd, and Vulcan Fast Red for NGAL. Staining was then followed by washing and mounting using Aqua Pertex® (Histolab Products, Gothenburg, Sweden). Co-localisation of the two antibodies appears purple. Nuclear staining with hematoxylin was omitted in the double staining experiments. Renuoir Red instead of primary antibodies for MMP-9 or CD66b/acd, and mouse IgG instead of specific antibodies for NGAL served as negative controls.

**Real-time RT-PCR**

Frozen samples were homogenised in a dismembranator. Lysis buffer was added to the homogenate and RNA isolated using RNeasy mini kit (Qiagen, West Sussex, UK). Quality and quantity of RNA were analyzed by an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). As primers and probes, Assay on Demand Kits from Applied Biosystem was used. Ribosomal protein large P0 (RPLP0) was used as a housekeeping gene to normalize for RNA loading. One µM of each primer and 0.25 µM of probe were used. The primers for RPLP0 were as described (16): RPLP0-FW: 5-CCATTCTATCATCAGGGTACAA-3 and RPLP0-RW: 5-AGCAAGTGGGAAGGTGTAATCC-3 and the probe was: RPLP0-TM: 6FAM5´-TCTCACGACAAAGCGACGACAGCTCGT-3 TAMRA. All TaqMan assays were cDNA specific. Each sample was analyzed in duplicate using ABI prism 7000 (Applied Biosystem). The PCR amplification was related to a standard curve.

**Results**

**NGAL is expressed in the intraluminal thrombus and the interface between the thrombus and the underlying AAA wall**

NGAL protein expression was investigated by immunohistochemistry in thrombus-free and thrombus-covered AAA wall. In the latter, NGAL was mainly detected in the interface between the thrombus and the underlying wall, although some staining could be detected in the wall itself and in the thrombus. NGAL expression was seen as a diffuse staining not associated with cells in the thrombus indicating that it had been secreted (Fig. 1A). Cell-associated NGAL was seen in the media of thrombus-free wall (Fig. 1B). NGAL mRNA was measured by quantitative real-time PCR in RNA preparations from the thrombus-free and thrombus-covered walls of nine patients. There was no significant difference in mRNA expression between the two wall segments (p=0.75, paired t-test). Overall, NGAL mRNA expression was low (mean C, of 38 ± 2, mean ± SD), thus preventing firm conclusions regarding comparisons between the two wall segments.

In order to identify the cellular source of NGAL, the presence of neutrophils and macrophages was stained by applying with

![A](Thrombus-covered)  ![B](Thrombus-free)  

**Figure 1:** Immunohistochemical demonstration of NGAL protein expression in AAA tissue (brown staining). Representative pictures are shown of AAA wall covered with thrombus (A) and thrombus-free AAA wall (B). Dotted line outlines the abluminal part of the thrombus. Arrows outline minor NGAL positive areas within the thrombus covered wall.
CD66b (neutrophils) and CD68 (macrophages) of serial sections from cell rich areas of the thrombus-free aneurysm wall (Fig. 2). Strong staining of CD68 was detected in the media of thrombus-free wall (Fig. 2A). Neutrophils were detected in relatively few regions that overlapped with CD68 expression (Fig. 2B). NGAL expression (Fig. 2C) co-localised with areas of both CD66b and CD68 staining but not with the larger areas of only CD68 staining, suggesting that neutrophils (CD66b staining) may be the source of NGAL. To further analyze whether neutrophils may be a source of NGAL, double staining of NGAL and neutrophils was performed (Fig. 3). Two markers of neutrophils were used; CD66b and CD66acd. Co-localization of NGAL (red) and CD66acd (blue) expression was detected in the aneurysm wall (Fig. 3A-D) and in the thrombus (Fig. 3E). Similar results were obtained using CD66b instead of CD66acd (data not shown).

The luminal part of the thrombus stained positive for NGAL, MMP-9 and CD66b (Fig. 4). Double staining of NGAL and MMP-9 showed a co-localization of the proteins within the thrombus (Fig. 5). However, single staining of NGAL (red) and MMP-9 (blue) could also be detected.

**Complexes of NGAL and active MMP-9 are present in the two wall segments and in the thrombus**

The presence of NGAL/MMP-9 complexes was analyzed by immunoprecipitation and Western blot. Immunoprecipitation with antibodies against NGAL and hybridizing with antibodies against MMP-9 in the following reducing Western blot showed that MMP-9 originated from the NGAL/MMP-9 complexes in the interface fluid between the thrombus and the underlying vessel wall, within the abluminal and luminal parts of the thrombus as well as the thrombus-free and thrombus-covered wall (Fig. 6A). Two hundred µg of proteins were used in these lanes. In all investigated regions, the size of the MMP-9 protein corresponded to the activated form (compare Fig. 6A; lane 1 containing purified 83 kDa active MMP-9).

Western blot analyses using non-reducing conditions demonstrated that high-molecular-weight complexes between NGAL and MMP-9 were present within the different regions (Fig. 6B). Both proMMP-9 free of NGAL and MMP-9 in complex with NGAL were detected. ProMMP-9 monomer could be detected in the thrombus-free wall, the interface fluid, and in the thrombus itself (Fig. 6B). In agreement with the results of the immunopre-
Precipitation analyses, NGAL/MMP-9 complexes were detected in all analysed regions. A larger 220 kDa complex was also abundant in all regions. This complex may contain two MMP-9 molecules and one NGAL molecule (3). Furthermore, small amounts of a 135 kDa complex, probably reflecting a MMP-9 monomer/NGAL monomer complex, could be detected in the thrombus-free wall, the interface fluid and in the luminal region of the thrombus. In addition, a small amount of a >250 kDa complex containing both MMP-9 and NGAL protein was detected in all regions. The exact composition of this complex is unknown. Gelatin gel zymography demonstrated that a 135 kDa complex and a >250 kDa complex contained gelatinase activity (Fig. 6C).

Quantification of the NGAL/MMP-9 complex using an ELISA confirmed the presence of the complex within all analyzed sections of the thrombus and the vessel wall (Fig. 7). The highest concentration of the complex was found in the luminal part of the thrombus. There was no significant difference in the amount of complex between the two wall regions (paired t-test).

Discussion

The present work demonstrates that NGAL/MMP-9 complexes are present in different regions of the AAA. Importantly, NGAL/MMP-9 complexes are 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 by enzymatic activity related to the thrombus (12–14).

Our study suggests that neutrophils are the main source of the NGAL detected in AAs since NGAL expression co-localized with the presence of CD66b and CD66acd expressing cells. It cannot be excluded that macrophages localized in the same regions as neutrophils also contribute to NGAL expression. However, since NGAL could be detected in areas containing both CD66b and CD68 expressing cells but not in areas with only CD68 expressing cells, this argues against CD68 expressing cells being a major source of NGAL. Other cell types have been shown to express NGAL, such as rat myoblasts (17), bronchial epithelial cells (18) and BALB/c 3T3 mouse fibroblasts (19). In-
Interestingly, NGAL expression in human epithelial cells is induced by interleukin-1ß (18) and by lipopolysaccharide in murine PU5.1 macrophages (20). We have recently shown that NGAL is expressed in macrophages in the lipid core of human atherosclerotic plaques as well as in endothelial cells and SMC (21). Furthermore, NGAL was induced in SMC after vascular injury in rats (22). Thus, the expression pattern of NGAL could change during inflammation, which is important for the pathogenesis of AAA. Our previous findings show that the inflammatory component is more evident in the thrombus-covered walls (11).

The results of the immunoprecipitation suggest that the NGAL-bound MMP-9 is in its active state since the complex had similar size as the purified, cleaved, 83 kDa active MMP-9 used as marker. Interestingly, the gelatinase activity measurement indicates that free MMP-9 is mainly in its proform. The protease activity of the larger 92 kDa band in the zymography assay is a result of a well-known procedure-induced activation of the proform. Thus, although the amount of MMP-9/NGAL complexes appears to be low, the finding that all of the complexed MMP-9 appears to be of the smaller and active 83 kDa form may be of functional significance. Western blot under non-reducing condition indicated that NGAL and MMP-9 is also found in larger complexes than the 1:1 NGAL/MMP-9 complex with a molecular weight of 135 kDa. However, according to the gel zymography assay, only the complex with a molecular weight larger than 250 kDa harboured gelatinase activity. The reason why the 220 kDa complex, although containing MMP-9, does not harbour gelatinase activity is not known. The exact compositions of the 220 and the >250 kDa complexes are unknown.

Although our data clearly show that NGAL is expressed in AAA tissue and that NGAL forms complexes with MMP-9, the question of the role of NGAL for AAA growth and rupture persists. Several findings, however, indicate an important role for NGAL in AAA. We have previously localized a strong MMP-9 expression and activity to the interface between the thrombus and the underlying vessel wall (12). Furthermore, it has been
shown that neutrophils are trapped within the thrombus and may be of importance for the degradation of the underlying wall, thereby leading to aneurysm rupture (13, 14). These findings emphasize that not only presence or expression of MMP-9 but also its functional properties should be evaluated. In the present work, NGAL expression was localized to the interface between the thrombus and the underlying wall and to the thrombus itself, suggesting that it could be of importance for prolonging and enhancing MMP-9 activity within this region. It was recently shown that JNK regulates MMP activities, cellular infiltration and AAA development in mice. Interestingly, in addition to MMP-9, lipocalin-2 (NGAL) was identified as one of the most potent targets of JNK activity (15).

The role of neutrophils has been studied in an elastase perfusion experimental AAA model in mice (23). 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). However, MMP-8 deficiency 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, but the results of the present work suggest that NGAL could be an important factor, influencing the activity of a steady-state level of MMP-9.

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 (24). It has been proposed that NGAL has immunomodulatory activity by binding and clearing lipophlic inflammatory mediators (25). Furthermore, NGAL has been demonstrated to bind bacterial catecholate-type ferric siderophores with high affinity (26). It could therefore be hypothesized that NGAL participates in the antibacterial iron depletion strategy of the innate immune system. Interestingly, Chlamydia pneumoniae-reactive T-lymphocytes have been identified in AAA (27) and chlamydia has been localized to AAA tissue (28). However, 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 conclusion, NGAL/MMP-9 complexes are present in AAA tissue. Whether NGAL plays an important role in aneurysm development remains to be shown.

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References