Expression of Neutrophil Gelatinase–Associated Lipocalin in Atherosclerosis and Myocardial Infarction

Anne-Louise Hemdahl, Anders Gabrielsen, Chaoyong Zhu, Per Eriksson, Ulf Hedin, Jens Kastrup, Peter Thorén, Göran K. Hansson

Objective—Neutrophil gelatinase-associated lipocalin (NGAL) modulates the activity of matrix metalloproteinase (MMP) 9, an important mediator of vascular remodeling and plaque instability in atherosclerosis. This study aimed to analyze the expression of NGAL in atherosclerotic plaques and myocardial infarction (MI).

Methods and Results—Atherosclerotic apolipoprotein E (apoE) \(-/-\) \(\times\) low-density lipoprotein receptor (LDLR) \(-/-\) and C57BL/6J control mice were exposed to brief hypoxic stress (10 minutes of 10% oxygen). Expression of the mouse NGAL homolog (24p3) and MMP-9 was analyzed 48 hours later by quantitative RT-PCR, immunohistochemistry, and zymography. Hypoxic stress increased NGAL/24p3 mRNA in the cardiac vasculature. NGAL/24p3 was also increased in atherosclerotic plaques of apolipoprotein E \(-/-\) \(\times\) LDLR \(-/-\) mice compared with C57BL/6J mice. Mice developing MI exhibited the highest plaque mRNA expression of NGAL/24p3 and MMP-9. Zymography revealed strong proteolytic activity in areas rich in 24p3 and MMP-9 protein. Immunohistochemistry performed on human carotid endarterectomy specimens and control tissue from the internal mammary artery showed colocalization of MMP-9 and NGAL with macrophages in the atherosclerotic plaques.

Conclusions—NGAL/24p3 is increased in atherosclerotic plaques and MI. Colocalization with MMP-9 in areas with high-proteolytic activity suggests a role for NGAL/24p3 in modulating the MMP-9-mediated remodeling of plaques and infarcted hearts. (Arterioscler Thromb Vasc Biol. 2006;26:0-0.)

Key Words: atherosclerosis • myocardial infarction • ischemia • hypoxia • matrix metalloproteinase • remodeling

Complications to coronary atherosclerosis leading to myocardial infarction were previously believed to be because of the physical obstruction of the vessel lumen; however, 60% to 70% of myocardial infarctions (MIs) result from nonocclusive plaques.1–4 Current evidence instead suggest that physical disruption of atherosclerotic plaques triggers thrombus formation, which may lead to MI.5–7 The 2 major precipitating factors of thrombus formation are disruption of the plaque cap and erosion of its endothelial lining. Inflammation within the atherosclerotic plaque has been suggested to promote the progression toward plaque disruption by causing plaque instability.8 Inflammatory mediators found in the atheroma have been shown to inhibit smooth muscle growth and collagen production and to augment matrix metalloproteinase (MMP) activity.9–12 This can result in decreased collagen content and weakening of plaque structure, leaving the fibrous cap prone to rupture.

MMPs are a family of endopeptidases capable of degrading the molecular components of the extracellular matrix. They play important roles in a variety of pathological processes, such as atherosclerosis and tumor cell invasion. In particular, gelatinase B (MMP-9) is thought to be associated with diseases such as abdominal aortic aneurysm, atherosclerosis, and plaque rupture.13 In cancer patients, urinary high-molecular weight MMPs have been shown to be independent predictors of metastatic cancers,14 and the complex of MMP-9 and neutrophil gelatinase-associated lipocalin (NGAL)15 has been identified as such a high-molecular weight metalloproteinase.16 NGAL is a 25-kDa glycoprotein found in granules of human neutrophils.15 It is a proposed scavenger of bacterial products at sites of inflammation17 and a modulator of inflammation because of its binding to the chemotactic peptide fMLP.18,19 Its mouse homologue, 24p3, has been shown to be involved in apoptosis20 and is upregulated by lipopolysaccharide (LPS) in macrophages.21

It was demonstrated recently that NGAL inhibits MMP-9 inactivation leading to enhanced proteolytic activity with prolonged effects on collagen degradation.16 Elevated plasma...
concentrations of NGAL have been detected in patients experiencing from acute cerebral ischemia and in hypertensive women, but neither the expression of NGAL in atherosclerotic plaques nor its possible involvement in precipitation of MI has been investigated. We have, therefore, studied the expression of NGAL and MMP-9 in human atherosclerotic lesions and in mouse models of atherosclerosis and MI and found that NGAL and its mouse homologue 24p3 are highly expressed in atherosclerosis and especially in areas with high-proteolytic activity and MI.

Methods

Human Specimens

Atherosclerotic lesions were retrieved from patients undergoing endarterectomy for symptomatic carotid stenosis (n = 4). The specimens from the carotid artery were removed and washed in PBS, mounted in OCT medium (Sakura Finetek) for cryosectioning, snap-frozen on dry ice, and stored in −80°C. Control tissue was obtained from the mammary artery of patients undergoing coronary artery bypass surgery (n = 6) and treated in the same way. The patient studies were approved by the Ethical Committee of Copenhagen and Stockholm and were in agreement with institutional guidelines and the principles set forth in the Declaration of Helsinki.

Animals and Diet

Male apoe−/−×LDLR−/− mice23,24 on the C57BL/6J background were obtained from M&B A/S Ry after weaning. The mice were fed a “Western-type diet” (Lactamin AB, Kimstad) containing 0.15% cholesterol and 21% total fat (wt/wt) for 8 months.25 Age-matched C57BL/6J mice were fed a standard chow for an equally long time and treated in the same way. The patient and experimental procedures were in accordance with the principles set forth in the Declaration of Helsinki.

Hypoxic Stress

Fifteen apoe−/−×LDLR−/− and 6 C57BL/6J mice were anesthetized with 2% isoflurane and exposed to hypoxia by reducing oxygen supply from 21% to 16%, 14%, 12%, and finally 10%, each for 2 minutes. After the hypoxic stress, oxygen supply was returned to 21%, and the mice were allowed to regain consciousness and returning to the normal habitat for 48 hours. Control atherosclerotic (n = 7) and C57BL/6J mice (n = 9) were exposed to anesthesia for 30 minutes. Forty-eight hours after hypoxic stress, the mice were anesthetized, peripheral blood was collected from the jugular vein, and the mice were euthanized by cervical dislocation. Immediately thereafter, the right auricle was removed and washed in PBS, snap-frozen on dry ice, and stored in −80°C. Control tissue was returned to 21%, and the mice were allowed to regain consciousness and returning to the normal habitat for 48 hours. Control atherosclerotic (n = 7) and C57BL/6J mice (n = 6) were anesthetized and returned to the normal habitat for 48 hours. Control tissue was returned to 21%, and the mice were allowed to regain consciousness and returning to the normal habitat for 48 hours. Control atherosclerotic (n = 7) and C57BL/6J mice (n = 6) were anesthetized and returned to the normal habitat for 48 hours.

Hypoxic Stress

Fifteen apoe−/−×LDLR−/− and 6 C57BL/6J mice were anesthetized with 2% isoflurane and exposed to hypoxia by reducing oxygen supply from 21% to 16%, 14%, 12%, and finally 10%, each for 2 minutes. After the hypoxic stress, oxygen supply was returned to 21%, and the mice were allowed to regain consciousness and returning to the normal habitat for 48 hours. Control atherosclerotic (n = 7) and C57BL/6J mice (n = 9) were exposed to anesthesia for 30 minutes. Forty-eight hours after hypoxic stress, the mice were anesthetized, peripheral blood was collected from the jugular vein, and the mice were euthanized by cervical dislocation. Immediately thereafter, the right auricle was removed and washed in PBS, snap-frozen on dry ice, and stored in −80°C. Control tissue was returned to 21%, and the mice were allowed to regain consciousness and returning to the normal habitat for 48 hours. Control atherosclerotic (n = 7) and C57BL/6J mice (n = 6) were anesthetized and returned to the normal habitat for 48 hours.

Hypoxygenation was performed before Western blot. To first separate off all of the MMP-9 containing proteins (monomers, dimers, and heterodimers of MMP-9), cell medium and total protein from cell supernatant were precleared with IgG/IgA-agarose beads. Beads were washed 3 times with 1 mL of radioimmunoprecipitation assay buffer containing β-mercaptoethanol and boiled for 10 minutes before loading. A total of 30 μg of protein was loaded and separated on SDS-PAGE (15%) and transferred onto PVDF (Hybond P) membrane (Amersham Biosciences). Proteins were detected with goat polyclonal antimouse NGAL antibody (sc-18698, Santa Cruz Biotechnology) and rabbit antigoat immunoglobulins/horseradish peroxidase-conjugated antibody (DakoCytomation), visualized by enhanced chemilumines-

LPS Stimulation

For the dose-response study, mouse J774 macrophages were stimulated with LPS (Sigma Chemicals) in concentrations of 1, 10, 100, or 1000 ng/mL or medium only and incubated for 3, 6, and 12 hours. At all of the time points, the cells were harvested for RNA extraction, and, at 12 hours, the cell medium was collected for immunoprecipitation (IP) and Western blot analysis. For time-course study, mouse macrophages were incubated with 10 ng/mL of LPS or medium only and harvested at 6, 12, 24, 48, and 72 hours. At all of the time points the cell lysates and medium were collected for IP and Western blot. In parallel, cells were stimulated with 10 ng/mL of LPS at the same time points as above (6 to 72 hours) and harvested for quantitative real-time RT-PCR. Three separately repeated experiments were performed for each study.

RNA Extraction, cDNA Synthesis, and Quantitative Real-Time RT-PCR

Total RNA extraction, cDNA synthesis, and quantitative real-time RT-PCR was performed as described previously. The probe for the mouse homologue of human NGAL, 24p3, and the forward and reverse primers were designed by using Primer Express software (Perkin Elmer/Applied Biosystems). Sequence of the 24p3 probe was CGCGCTAAACATTGGTCT, and forward and reverse primer was TGCAACTGTGTTGTTGTA and CGCGCTAAACATTGGTCT, respectively. The mouse MMP-9 and hypoxanthine guanine phosphoribosyl transferase (hprt) probe and primer sets (Mm00630016_1_m1 and Mm0046906_1_m1) were obtained by Assay-on-demand from Applied Biosystems. Threshold cycle values were normalized to permit comparisons between the groups. Thus, data from animal experiments were normalized to the mean value for untreated C57BL/6J mice, and cell culture studies were normalized to the amount of hprt gene expression in each sample.

IP and Western Blot

Cells were lysed with 200 μL of radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mmol/L Tris (pH 8.0)] containing proteinase inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 10 μmol/L EDTA, and 1 μg/mL pepstatin A), incubated 30 minutes on ice, centrifuged 15 minutes at 14,000 rpm in 4°C, and supernatants collected. To ensure that analyzed NGAL/24p3 protein belonged to the complex of 24p3/MMP-9, IP was performed before Western blot. To first separate off all of the MMP-9 containing proteins (monomers, dimers, and heterodimers of MMP-9), cell medium and total protein from cell supernatant were precleared with IgG/IgA-agarose beads and incubated with MMP-9 goat polyclonal antibody (sc-6841, Santa Cruz Biotechnology) followed by precipitation with IgG/IgA-agarose beads. Beads were washed 3 times with 1 mL of radioimmunoprecipitation assay buffer containing β-mercaptoethanol and boiled for 10 minutes before loading. A total of 30 μg of protein was loaded and separated on SDS-PAGE (15%) and transflected onto PVDF (Hybond P) membrane (Amersham Biosciences). Proteins were detected with goat polyclonal antimouse NGAL antibody (sc-18698, Santa Cruz Biotechnology) and rabbit antigoat immunoglobulins/horseradish peroxidase-conjugated antibody (DakoCytomation), visualized by enhanced chemilumines-

Hyponxia

Hyponxia condition was created by flushing 5% CO2 and 95% N2 through a humidified chamber at 37°C until atmosphere containing 1% O2 was achieved and measured with a MiniOXI oxygen meter (Mine Safety Appliances Company). Cells were exposed to hypoxia for 60 minutes and, thereafter, cultured 2 hours in fresh medium before being harvested. Culture medium for hypoxia condition was preequilibrated 3 hours before the experiment at an indicated oxygen level. Control cells were maintained in normal oxygen condition at all times.
cence reagent (Amersham Pharmacia Biotech) followed by autoradiography for 30 s to 60 minutes.

In Situ Zymography
From hypoxia-exposed C57BL/6J and apoE<sup>−/−</sup>×LDLR<sup>−/−</sup> and apoE<sup>−/−</sup>×LDLR<sup>−/−</sup> mice with MI, 5-μm unfixed cryostat sections were obtained from the aortic root. The procedure for in situ zymography was modified from the technique described by Galis et al. In short, fluorescein-labeled gelatin (1 mg/mL, DQ gelatin from pig skin, Molecular Probes) and 20 mmol/L of MMP-2 inhibitor, OA-Hy cis-9-Octadeconyl-N-hydroxylamide (Calbiochem, Merck) were mixed (1:1) with agarose melted in buffer [50 mmol/L Tris-HCl (pH 7.4), 10 mmol/L CaCl<sub>2</sub>, and 0.05% Brij 35]. The gelatin mixture was spread on slides and thereafter incubated in a light-protected and humidified chamber at 37°C for 24 hours. The fluorescent area produced by proteolytic digestion of quenched fluorescein-labeled gelatin was recognized as MMP-9 activity and imaged in a fluorescence microscope. Generation of proteolytic activity was prevented in controls by the addition of phenan-throlnine and EDTA.

Immunohistochemistry

Mice
A 5-mm-thick cross-section of the heart from the middle of the ventricles to the atrium was fixed in 4% formaldehyde in PBS for 24 hours, dehydrated, and paraffin embedded. Aortas were collected in OCT medium and snap-frozen in liquid nitrogen. From the paraffin-embedded mouse hearts, 5-μm-thick sections were collected. The sections were deparaffinized and endogenous peroxidase quenched for 30 minutes in 3% H<sub>2</sub>O<sub>2</sub>, washed 3 times with PBS for 5 minutes each, and blocked for 30 minutes with horse serum. Goat polyclonal anti-24p3 (sc-18698) and anti-MMP-9 (sc-6841) antibodies (Santa Cruz Biotechnology) were diluted 1:50, as determined by titration, in 1% BSA and allowed to incubate on sections overnight in 4°C. Sections were washed 3 times with PBS and biotinylated horse-antigoat IgG antibody (Vector Laboratories) diluted 1:200 in 1% BSA (σ-Aldrich) incubated for 30 minutes. Avidin-biotin-peroxidase complex (Vector Laboratories) was added for 30 minutes and developed with 3,3-diaminobenzidine tetrahydrochloride for 3 minutes. Between reactions, sections were washed with PBS 3 times for 5 minutes. The same procedure was performed on 10-μm acetone-fixed cryostat sections of aortas. The specificity of the antimouse 24p3 and MMP-9 antibodies were ascertained with blocking peptides to the primary antibodies.

Humans
Specimens were cut into 10-μm cryosections and fixed for 10 minutes in acetone. Sections were stained according to the above-described protocol with polyclonal goat antihuman NGAL IgG antibody (sc-18694; Santa Cruz Bioscience) with horse-antigoat IgG as a secondary antibody or with monoclonal mouse-antimouse MMP-9 antibody (R&D Systems) using a biotinylated horse-antimouse IgG secondary antibody. Antibodies were titrated as described above. Cellular localization of NGAL and MMP-9 was performed in adjacent sections stained with the goat-antihuman macrophage marker CD68 (DAKO) using biotinylated horse-antigoat IgG as secondary antibody or with mouse-antimouse smooth muscle cell α-actin and mouse-antihuman von Willebrand Factor with biotinylated horse-antimouse IgG as secondary antibody. Avidin-biotin-peroxidase was used for visualization as described above.

Statistical Analysis
Results are presented as mean±SEM. Data were analyzed by Mann–Whitney U test, and differences between groups were considered significant if P<0.05. Statview 5.0.1 (Abacus Concept) was used for statistical analysis.

Results

Figure 1. Quantitative real-time RT-PCR analysis of 24p3 and MMP-9 expression in heart and aorta after hypoxic stress and MI. A, 24p3 mRNA in the heart was significantly increased after exposure to hypoxia, both in normal C57BL/6J and atherosclerotic apoE<sup>−/−</sup>×LDLR<sup>−/−</sup> mice (DOKO) (P=0.002, #P=0.006). Atherosclerotic mice that developed MI exhibited the highest increase in 24p3 mRNA expression (P=0.035). There was no difference between normal and atherosclerotic mice in the absence of hypoxic stress. B, Hearts from atherosclerotic mice (DOKO) expressed more MMP-9 mRNA than hearts of normal C57BL/6J mice (#P=0.015). Stress-exposed C57BL/6J mice exhibited increased MMP-9 mRNA expression compared with nonstressed ones (P=0.030). Although MMP-9 was slightly increased in apoE<sup>−/−</sup>×LDLR<sup>−/−</sup> mice after hypoxic stress and in the apoE<sup>−/−</sup>×LDLR<sup>−/−</sup> mice with infarction, the change was not significant. C, Aortas of atherosclerotic apoE<sup>−/−</sup>×LDLR<sup>−/−</sup> mice displayed a significant increase in the 24p3 gene as compared with normal mice (P=0.016). ApoE<sup>−/−</sup>×LDLR<sup>−/−</sup> mice with MI expressed the largest increase in 24p3 transcript (#P=0.037). D, In aortas of normal mice, MMP-9 mRNA was significantly increased after hypoxic stress (P=0.037). Aortas of apoE<sup>−/−</sup>×LDLR<sup>−/−</sup> mice with MI exhibited the largest increase in MMP-9 mRNA levels (#P=0.010).

MI in Mice
Atherosclerotic mice were exposed to hypoxic stress (n=15); 47% of them developed MI (n=7) after 48 hours as determined by elevated serum troponin T levels (0.16±0.05 μg/L, mean±SEM).

24p3 and MMP-9 Expression in the Mouse Heart

mRNA Expression
Hypoxic stress significantly increased 24p3 gene mRNA in the myocardium both in normal and in atherosclerotic mice (Figure 1A); however, there was no difference in the basal expression level between normal and atherosclerotic mice. The highest increase in myocardial 24p3 mRNA was observed in atherosclerotic mice developing MI after hypoxic stress (Figure 1A). MMP-9 expression was higher in non-stressed apoE<sup>−/−</sup>×LDLR<sup>−/−</sup> as compared with nonstressed C57BL/6J mice (Figure 1B). In response to hypoxic stress, however, mRNA levels of MMP-9 were significantly increased in normal mice but not in atherosclerotic mice. There was a trend toward increased MMP-9 gene expression in infarcted apoE<sup>−/−</sup>×LDLR<sup>−/−</sup> mice as compared with un-
touched apoE<sup>−/−</sup> × LDLR<sup>−/−</sup> mice, but this change was not statistically significant (Figure 1B).

**Immunohistochemistry**

Evaluation of 24p3 and MMP-9 expression demonstrated colocalization of the 2 proteins in cardiomyocytes from apoE<sup>−/−</sup> × LDLR<sup>−/−</sup> mice (Figure 1A and IB, available online at http://atvb.ahajournals.org). No 24p3 or MMP-9 protein was detectable in cardiomyocytes of C57BL/6J mice (Figure IC and ID).

**NGAL/24p3 and MMP-9 in Mouse Aortic Atherosclerotic Lesions**

**mRNA Expression**

Aortas from atherosclerotic mice exhibited higher levels of 24p3 mRNA as compared with aortas from normal C57BL/6J mice (Figure 1C). In the group of mice with MI, 24p3 expression was increased 6-fold compared with normal mice and almost twice as high as in apoE<sup>−/−</sup> × LDLR<sup>−/−</sup> mice exposed to stress but without infarction. Hypoxic stress increased 24p3 expression only in atherosclerotic mice that developed MI, suggesting that 24p3 was upregulated as a result of infarction or possibly that 24p3 expression was necessary for MI development (Figure 1C). MMP-9 mRNA levels were increased in C57BL/6J mice after hypoxic stress and increased several-fold (≈2.5-fold) in aortas of apoE<sup>−/−</sup> × LDLR<sup>−/−</sup> mice with MI as compared with apoE<sup>−/−</sup> × LDLR<sup>−/−</sup> mice exposed to hypoxia but without MI (Figure 1D).

**Immunohistochemistry**

Abundant 24p3 protein was demonstrated in the atherosclerotic plaques of apoE<sup>−/−</sup> × LDLR<sup>−/−</sup> mice (Figure IE) that colocalized with MMP-9 expression (Figure IF). Normal aortas of nonatherosclerotic C57BL/6J mice did not display either 24p3 or MMP-9 protein (Figure IG and IH).

**Activation of 24p3 in Mouse J774 Macrophages**

Hypoxia significantly increased 24p3 transcripts in isolated mouse macrophages already after 2 hours (Figure 2A). The gradual increase in LPS concentration augmented 24p3 gene and protein expression in a dose-dependent manner (graph in Figure 2B). On a transcriptional level, 24p3 mRNA was dose related to the concentration of LPS at all of the times (3, 6, and 12 hours), and an increase in expression was seen already at the lowest dose (1 ng/mL) and shortest time (3 hours; *P*< 0.05). The amount of 24p3 protein (25 kDa) originating from the secreted 24p3/MMP-9 complex in the medium was also dose-related to the LPS concentration (Figure 2B). At 10 ng/mL of LPS, the time course study (Figure 2C) exhibited significantly high mRNA levels of 24p3 (*P*< 0.05) at 6 hours, which peaked at 12 hours. Western blot (Figure 2C) of the cell lysate and medium demonstrated maximal protein concentrations at 48 hours that were reduced after 72 hours. LPS stimulation has been shown previously to increase expression of the 24p3 monomer in mouse macrophages. However, to our knowledge, our data show for the first time that the 24p3/MMP-9 complex is indeed present in mice and can be secreted from macrophages.

**Proteolytic Activity in Atherosclerotic Plaque**

Specific MMP-9 activity, ascertained by including an MMP-2 inhibitor to the reaction buffer, was detected in the border region of the atherosclerotic plaque facing the lumen (Figure 3C, small arrows) and in the lipid core (Figure 3C, long arrows). This area also displayed high-protein content of 24p3 (Figure 3A) and MMP-9 (Figure 3B) as determined by immunohistochemistry. In control samples, MMP inhibitors abolished proteolytic activity (Figure 3D).

**NGAL and MMP-9 Expression in Human Atherosclerotic Plaques**

Sections stained with cell type-specific markers for endothelial cells (von Willebrand factor; Figure 4A) and smooth muscle cells (αSMA; Figure 4C) were compared with adjacent sections stained for NGAL (Figure 4B and 4D) demonstrated that NGAL is present in these cell types. NGAL has been shown previously to be expressed by macrophages. Therefore, macrophages were detected by CD68 staining (Figure 5A). Adherent sections showed that MMP-9 (Figure 5B) and NGAL (Figure 5C) colocalized with macrophages in the lipid core of the atherosclerotic plaques. NGAL was also present in the normal mammary artery, whereas neither MMP-9 nor CD68 were detected (Figure IIA, IIB and IIC, available online at http://atvb.ahajournals.org). No positive staining was detected on control sections (Figure IID).
Discussion

Sudden alterations in the atherosclerotic plaque structure, such as cap rupture and intraplaque hemorrhage, frequently precede ischemic cardiovascular events, and MMPs have been found to play an important role in these processes. The present study shows that MMP-9 is increased in atherosclerotic mice with MI, that MMP-9 is expressed in areas with high-proteolytic activity, and that it colocalizes with NGAL/24p3, which has been shown previously to protect MMP-9 from inactivation.

Under normal conditions, the activity of MMP-9 is precisely regulated on the transcriptional level by conversion from pro-MMP-9 to its active form and through inhibition by endogenous inhibitors (tissue inhibitor of metalloproteinase; TIMP). However, in a study with unstable carotid plaques, spontaneous embolization, and histological evidence of stability, MMP-9 was highly increased and with a significant ratio of MMP-9:TIMP-1, suggesting an imbalance between proteolysis and inhibition in coronary artery disease patients as compared with healthy volunteers. Further- more, high-plasma levels of MMP-9 have been found to be an independent predictor of cardiovascular mortality. Our present data show increased MMP-9 mRNA expression in hypoxia (wild-type mice) and hypoxia-induced infarction (atherosclerotic mice). Although no increased MMP-9 protein staining was detectable in the former group, increased numbers of MMP-9-positive cells were observed in atherosclerotic mice that developed infarction. The lack of detectable protein response in the wild-type mice could be attributed to differences in sensitivity between the mRNA (real-time RT-PCR) and protein analysis (immunohistochemistry).

NGAL has been shown to protect MMP-9 by forming a stable dimeric complex, which makes MMP-9 less susceptible to inactivation by TIMP-1. Furthermore, a study by Tschesche et al demonstrated that MMP-9 activation by HgCl2 or plasma kallikrein was additionally enhanced by human NGAL. This supports the notion that NGAL/24p3 is an important modulator of MMP-9, which augments its proteolytic effect. In the atherosclerotic plaque, where de novo collagen synthesis is reduced and collagen degradation by MMPs is high, NGAL may, therefore, promote increased and prolonged proteinase activity. This would contribute to decreased stability of the collagen cap, rendering the plaque more susceptible to erosion and rupture with subsequent thrombosis and infarction. In support of this notion, plaques with high-proteolytic activity displayed a high prevalence of 24p3 and MMP-9 in mice undergoing MI.

In response to injury, the heart undergoes remodeling, that is, changes in extracellular protein synthesis, degradation, and composition leading to long-term changes in chamber geometry and pump function. It is well established that MMPs play an important role in remodeling. This is considered to be beneficial for wound healing and involution, but remodeling can promote the development of left ventricular dysfunction, aneurysm formation, and plaque rupture in pathological situations. Our data show that 24p3 is increased in the heart on MI and after hypoxic stress. The in vivo results were supported by in vitro studies showing upregulated 24p3 expression in mouse macrophages exposed to hypoxia. Together, these data suggest that 24p3 is involved in the myocardial response to hypoxia and infarction.

NGAL/24p3 has been detected previously in organs that are continuously exposed to microorganisms, such as the stomach and the lungs, and has been shown to bind to proinflammatory ligands, such as bacterial formyl peptides, leukotriene B4, and platelet-activating factor. We now show that LPS significantly increased the gene expression of 24p3 and the protein complex of 24p3/MMP-9 in mouse macrophages. Soluble NGAL/24p3 secreted from neutrophils and macrophages may participate in an innate immune response to scavenge bacteria and debris from infected or injured areas. NGAL has also been shown to be an acute phase...
protein,38,39 and it should be noted that the human artery specimens were derived from mammary arteries used for coronary artery bypass surgery, that is, they were derived from atherosclerotic patients, although the mammary arteries were free from atherosclerosis. This might contribute to the presence of NGAL protein in these arteries. In healthy control mice, NGAL/24p3 was not expressed in the aorta.

Earlier studies have shown that NGAL is increased in patients with symptomatic cardiovascular disease and correlates with risk factors of atherosclerosis.22,40 Elevated plasma levels of NGAL also predicted mortality in a 4-year follow-up study of patients experiencing from cerebral ischemia.41 In contrast, plasma NGAL did not correlate to asymptomatic atherosclerosis or progression of disease.22 These data correspond with our findings of unchanged NGAL/24p3 expression in hearts from nonstressed, resting normal, and atherosclerotic mice, whereas hypoxic stress and acute infarction increased NGAL/24p3 expression in both groups of mice.

In summary, this study extends previous knowledge by showing that NGAL/24p3 is present in blood vessels, increased in atherosclerosis, and upregulated by hypoxia and in MI. Both 24p3 and MMP-9 were increased in plaques of mice experiencing from MI and colocalized in areas with high proteolytic activity. The complex of 24p3/MMP-9 was increased by LPS and secreted from mouse macrophages. These data suggest that NGAL/24p3 together with MMP-9 may be involved in vascular inflammation and the response to ischemia. In view of its protective and activation function of MMP-9, NGAL may affect plaque stability and cardiac remodeling after infarction.

Acknowledgments

This study was supported by the Swedish Heart-Lung Foundation and Research Council (6816, 4764 and 12660), the Söderberg and Wallenberg foundations, AFA insurance fund, and by a Marie Curie Fellowship (to A.G.) from the European Union. We thank Dr Allan Sirsjo for kindly providing the mouse macrophage J774 cells and Ingrid Thörnberg for excellent technical assistance.

References


Expression of Neutrophil Gelatinase-Associated Lipocalin in Atherosclerosis and Myocardial Infarction
Anne-Louise Hemdahl, Anders Gabrielsen, Chaoyong Zhu, Per Eriksson, Ulf Hedin, Jens Kastrup, Peter Thorén and Göran K. Hansson

Arterioscler Thromb Vasc Biol. published online October 27, 2005;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2005/10/27/01.ATV.0000193567.88685.f4.citation

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2005/11/08/01.ATV.0000193567.88685.f4.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Figure I

 apoE-/- x LDLR -/-

C57BL/6J

Plaque

Normal aorta
Figure II