Induction of Neutrophil Gelatinase-Associated Lipocalin in Vascular Injury via Activation of Nuclear Factor- κ B

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Neutrophil gelatinase-associated lipocalin (NGAL) has recently emerged as an important modulator of cell homeostasis. Elevated plasma NGAL levels, possibly because of activation of blood leukocytes, are associated with atherosclerosis. However, little is known about induction of NGAL expression in blood vessels. Using a rat carotid artery injury model, we found that NGAL was highly induced in the intima after angioplasty but was attenuated by adenovirus-mediated expression of a dominant-negative mutant of inhibitor of nuclear factor (NF)- κ B kinase β (dnIKK β). Expression of NGAL mRNA and protein was also up-regulated in an NF- κ B-dependent manner in rat and human vascular smooth muscle cells (SMCs) in response to interleukin-1 β stimulation. Rat SMC-produced NGAL was present as mono- and homomeric forms in the cytosol and in a complex containing matrix metalloproteinase-9 (MMP-9) after secretion. In agreement with levels of NGAL, proteolytic activity of MMP-9 was markedly high in the intima of injured vessels and in the culture supernatant of activated intimal SMCs but was reduced in the vessels transduced with dnIKK β . The present study reveals a previously unrecognized vascular response to angioplastic injury, characterized by NF- κ B-dependent expression of NGAL in vascular SMCs. Furthermore, SMC-produced NGAL interacts with MMP-9, a mechanism by which NGAL may modulate MMP-9 proteolytic activity in the vascular repair process. (*Am J Pathol* 2006, 169:2245–2253; DOI: 10.2353/ajpath.2006.050706)

Human neutrophil gelatinase-associated lipocalin (NGAL), also known as lipocalin 2, belongs to the lipocalin superfamily. NGAL is a 25-kd glycoprotein that was initially identified as 24p3 in SV-40-infected primary mouse kidney cells.¹ Subsequently, the human homologous protein was found in specific granules of neutrophils,^{2,3} involved in the allosteric activation of matrix metalloproteinase (MMP)-9, and protection of the latter from degradation.^{4,5} Previous studies indicated that NGAL was able to bind small lipophilic substances including bacterial-derived formylpeptides, lipopolysaccharides,^{6,7} and catecholate-type ferric siderophores.⁸ Therefore it may function as an effector molecule of the innate immune system. Recent studies suggest that NGAL also plays an important role in cell homeostasis.^{9,10}

The relevance of NGAL to cardiovascular diseases is primarily unknown. Recently, elevated plasma NGAL levels, possibly because of activation of blood leukocytes, were associated with atherosclerosis and implicated as a predictor for cardiovascular mortality after cerebrovascular ischemia.^{11–13} We have recently reported the presence of NGAL in atherosclerotic plaques,¹⁴ raising the possibility that expression of NGAL can be induced in vascular cells during atherogenesis. However, the underlying mechanisms for the induction of NGAL in vascular cells remain unknown.

Transcription factor nuclear factor (NF)- κ B plays a pivotal role in regulation of vascular inflammatory response.^{15–17} Activation of NF- κ B is mediated essentially by I κ B kinase (IKK) complex, which contains two catalytic subunits, IKK α and IKK β , as well as the regulatory subunit IKK γ . IKK β is important for I κ B phosphorylation and degradation and implicated in inflammatory signaling,¹⁸ whereas IKK α is required for phosphorylation-induced p100 processing and activation of the alternative path-

Supported in part by the Swedish Research Council (grant 12660), the Swedish Heart and Lung Foundation, and the AFA Health Fund.

Accepted for publication September 6, 2006.

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way, mainly activating genes involved in development and maintenance of secondary lymphoid organs.¹⁹ Recently, NF- κ B activation has also been implicated in regulation of NGAL expression in macrophages and epithelial cells.^{20–22} Using a rat model of vascular injury, we investigated the expression of NGAL in vascular smooth muscle cells (SMCs). Here we report that angioplastic injury induces NGAL expression primarily in intimal SMCs via activation of IKK β -mediated NF- κ B signaling.

Materials and Methods

Carotid Artery Injury Model and Adenoviral Gene Transfer

All animal experiments were approved by the Regional Ethics Committee for Animal Research at the Karolinska Institute. Male Sprague-Dawley rats (average body weight, 350 g; B&R, Sollentuna, Sweden), anesthetized with 2 mg/kg pentobarbital plus 50 mg/kg Hypnorm (Janssen Pharmaceutica, Beerse, Belgium), were subjected to balloon injury of the left common carotid artery, as described previously.²³ Subsequently, 50 μ l of recombinant, replication-deficient, adenoviral vectors expressing *Escherichia coli* β -galactosidase (β -gal) or dominant-negative IKK β (dnIKK β) (kindly provided by Dr. R. de Martin, Vienna, Austria) at 4×10^{10} plaque-forming units (pfu)/ml was instilled into the common carotid artery via the external carotid and allowed to dwell for 40 minutes. Rats were provided with standard chow and water *ad libitum*. Animals were sacrificed by overdosing with pentobarbital at day 3 and day 14 after injury and perfused with 100 ml of phosphate-buffered saline via the left ventricle before carotid arteries were harvested.

Immunohistochemistry

Immunostaining was performed on frozen sections fixed in 4% formaldehyde. Goat polyclonal anti-human NGAL antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was incubated with sections at 4°C overnight, followed with biotinylated horse anti-goat IgG antibody (Vector Laboratories, Burlingame, CA) and an avidin-biotin-complex (Vector Laboratories). Subsequently, the sections were developed with diaminobenzidine and counterstained with hematoxylin. The specificity of NGAL antibody was assessed by neutralizing the primary antibody with blocking peptide (Santa Cruz Biotechnology, Inc.).

Cell Culture and Adenoviral Infection

SMCs were isolated from the media and intima of adult Sprague-Dawley rats as described²⁴ and grown in Dulbecco's modified Eagle's medium (Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal calf serum and 100 U/ml penicillin/streptomycin (Invitrogen). Cells were serum starved with Dulbecco's modified Eagle's medium-F12 (Invitrogen), supplemented with 1% fetal

calf serum for 24 hours before infection with β -gal or dnIKK β at a multiplicity of infection from 50:1 to 200:1 for 1 hour, the supernatants were removed and replaced by Dulbecco's modified Eagle's medium with 10% fetal calf serum for 48 hours, and thereafter the cells were again exposed to serum starvation for 6 hours before treated with 10 ng/ml murine recombinant IL-1 β (Genzyme, Cambridge, MA) for the indicated times. Subsequently, the cell lysates and supernatants were harvested for different analysis.

Human coronary artery SMCs (hCASMCs; Cambrex Bio Science, Baltimore, MD) of passages five to seven were cultured using SmGM2 kit medium (Cambrex Bio Science). The cells were serum-starved for 24 hours before the experiment and subsequently treated with 10 ng/ml human IL-1 β (Pepro Tech) for the indicated times.

Quantitative Real Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was prepared from tissue samples or cells using the RNeasy kit (Qiagen, Valencia, CA) with an additional DNase digestion step, analyzed by BioAnalyzer (Agilent Technologies, Palo Alto, CA). Subsequently, 1 μ g of RNA was used in a 40- μ l cDNA synthesis using hexanucleotides and Superscript II reverse transcriptase (Invitrogen). Real time RT-PCR on 3 μ l of cDNA was performed in an ABI 7700 sequence detector (Applied Biosystems, Foster City, CA) using Assay-on-Demand (Applied Biosystems) primers and probes for rat NGAL, MMP-9, mouse hypoxanthine guanine ribonucleosyltransferase, human NGAL, and cyclophilin A, as well as primers and probe for rat tumor necrosis factor (TNF)- α (the sequences of TNF- α primers are forward 5'-GACCTCAGCTCAGATCATCCTTCT-3', reverse 5'-ACGCTGGCTCAGCCACTC-3', and probe 5'-TAGCCACGTCGTAGCAAACCAACCA-3'). Levels of transcripts were expressed as the ratio versus hypoxanthine guanine ribonucleosyltransferase in rat samples and cyclophilin A in human samples.

Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared from both rat SMCs and hCASMCs as previously described.²⁵ In brief, 10 μ g of nuclear extract from rat SMCs or from hCASMCs were incubated with the ³²P-labeled NF- κ B consensus oligonucleotide (Promega, Madison, WI) or with the ³²P-labeled NF- κ B consensus oligonucleotide in the human NGAL promoter region,²¹ respectively. DNA-protein complexes were separated by electrophoresis on 4% polyacrylamide gel (Promega), dried for 2 hours, and thereafter analyzed by autoradiography. Specificity of the NF- κ B signal was verified by addition of excess (50-fold) nonradioactive AP-1 and NF- κ B oligonucleotide.

Western Blotting and Immunoprecipitation (IP)

Protein extracts were prepared by solubilizing cells in RIPA buffer supplemented with the following protease

inhibitors (final concentration): phenylmethyl sulfonyl fluoride (1 mmol/L), pepstatin A (10 μ mol/L), ethylenediaminetetraacetic acid (1 mmol/L), and E64 (10 μ mol/L) (all from Sigma, St. Louis, MO). Total protein concentration was determined using the MicroBCA method (Pierce, Rockford, IL). Twenty μ g of protein lysates were separated on 12% sodium dodecyl sulfate-polyacrylamide gels under either reducing condition or nonreducing condition⁵ and transblotted onto polyvinylidene difluoride Hybond P membrane (Amersham Biosciences). NGAL and MMP-9 were detected using goat polyclonal anti-mouse (or human) NGAL and MMP-9 antibody (Santa Cruz Biotechnology, Inc.), respectively, followed by rabbit anti-goat immunoglobulins/horseradish peroxidase-conjugated antibody (DakoCytomation), and visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech). Recombinant human NGAL (rNGAL; 0.5 μ g), a gift from Niels Borregaard (Copenhagen, Denmark) was used as positive control. β -Actin levels were used to assure equal loading.

For IP, protein extracts were precleaned with protein A/G-agarose before IP. Thereafter, the culture supernatants or cell lysates were incubated with 2 μ g of NGAL antibody (Sc-186898; Santa Cruz Biotechnology, Inc.) and 40 μ l of protein A/G-agarose at 4°C for 1 hour. After precipitation, the pellets were resuspended in 60 μ l of sample buffer and subjected to electrophoresis on a 4 to 15% gel, and the proteins were transferred to nitrocellulose membrane. The membrane was further evaluated by Western blotting with MMP-9 antibody (Sc-681; Santa Cruz Biotechnology, Inc.). In reverse, IP was also performed by pulling down the proteins from the supernatants with MMP-9 antibody and then probing with NGAL antibody.

Assessment of Gelatin Activity

For *in situ* zymography analysis, unfixed cryostat sections were obtained from the rat carotid artery. The procedure was modified from the technique described by Galis and colleagues.²⁶ In brief, fluorescein-labeled gelatin (1 mg/ml, DQ gelatin from pig skin; Molecular Probes, Eugene, OR) and 20 mmol/L MMP-2 inhibitor, OA-Hy *cis*-9-octadecanoyl-*N*-hydroxylamide (Calbiochem, San Diego, CA) were mixed (1:1) with agarose melted in buffer (50 mmol/L Tris-HCl, pH 7.4, 10 mmol/L CaCl₂, and 0.05% Brij 35). The fluorescein-labeled gelatin-agarose was spread on slides and incubated in a light-protected and humidified chamber at 37°C for 24 hours. The specificity of MMP-9-related fluorescent signal was verified by addition of the MMP inhibitors phenanthroline and ethylenediaminetetraacetic acid. Gelatin activity was also analyzed using a 10% zymogram gel (Invitrogen) as described by the manufacturer.

Statistical Analysis

Results are reported as mean $\pm$ SEM. Student's *t*-test and one-way analysis of variance were used to evaluate

differences between groups, and values of $P < 0.05$ were considered statistically significant.

Results

Angioplastic Injury Induces Expression of NGAL and MMP-9 in Artery

Using quantitative real-time RT-PCR, transcription of NGAL and MMP-9 was investigated in the balloon-injured rat carotid arteries. As shown in Figure 1, our results indicate there is no constitutive expression of NGAL in the normal artery. Angioplastic injury to the artery, however, induced high expression of NGAL mRNA at day 14 but not yet at day 3. In addition to NGAL, a marked up-regulation of MMP-9 was observed in the vessels shortly after injury (Figure 1A). To explore the regulatory mechanism for the injury-induced vascular expression of NGAL, NF- κ B signaling was blocked in the injured vessel by transduction with dnIKK β . This resulted in virtually complete suppression of NGAL and MMP-9 expression at day 14 (Figure 1A), implying a pivotal role for NF- κ B signaling in their transcriptional regulation. However, the induction of MMP-9 was not abolished by dnIKK β at day 3, in agreement with our previous finding that NF- κ B activity was not inhibited by dnIKK β at this time point.²⁷ Furthermore, immunohistochemical analysis revealed that NGAL protein predominantly located in the intima area of injured vessels at day 14 (Figure 1B, bottom left) but was markedly reduced in the vessel transfected with dnIKK β (Figure 1B, bottom right).

Differential Activation of NF- κ B in Intimal and Medial SMCs in Response to IL-1 β Stimulation

We investigated NF- κ B activation in SMCs derived from the media and intima. Electrophoretic mobility shift assay revealed that both intimal and medial SMCs have basal NF- κ B-DNA binding activity in culture without stimulation (Figure 2A, lanes 1 and 5). IL-1 β , however, triggered a stronger NF- κ B activation in intimal cells than in medial SMCs (Figure 2A), indicating a difference in the response to proinflammatory cytokine stimulation between the two types of SMCs. IL-1 β -induced NF- κ B activation in SMCs seems to be IKK β -dependent because it was blocked by dnIKK β at a multiplicity of infection of 200:1, a concentration without induction of cell death according to a dose-response study (data not shown) (Figure 2A).

IKK β Mediates IL-1 β -Induced NGAL and MMP-9 Expression in SMCs

Induction of NGAL in SMCs was further verified *in vitro*. Intimal cells, but not medial SMCs, constitutively expressed noticeable levels of NGAL and TNF- α (Figure 2B). Stimulation of SMCs with IL-1 β resulted in up-regulation of NGAL mRNA in both types of cells, whereas IL-1 β induced a compatible level of TNF- α expression in both types of SMCs, it triggered stronger

Figure 1. Expression of NGAL and MMP-9 in the injured vessels. **A:** Transcripts of NGAL and MMP-9 were determined by real-time quantitative RT-PCR at the indicated times in the injured carotid arteries transduced with recombinant adenoviral vector expressing β -galactosidase (β -gal) or dominant-negative IKK β (dnIKK β). Levels of transcripts are expressed in relative units normalized to hypoxanthine guanine ribonucleosyltransferase (HPRT) mRNA. Data are presented as mean $\pm$ SEM from six to eight rats for each time point. $^*P < 0.05$, versus β -gal group at day 14; $^{\#}P < 0.05$, β -gal group at day 14 versus the uninjured group; $^{\&}P < 0.05$, β -gal group at day 3 versus the uninjured group. **B:** Immunostaining for NGAL in the carotid arteries transduced with β -gal or dnIKK β at day 3 and day 14 after injury and in an uninjured vessel. Control for the NGAL immunostaining was conducted by applying excess blocking peptide in a β -gal-transduced vessel at day 14 (top right). The arrows indicate internal elastic lamina, and the arrowheads indicate cells showing representative positive signals for NGAL (brown color). All of the sections are counterstained with hematoxylin. Original magnifications, $\times 400$.

MMP-9 expression in medial SMCs than that in intimal SMCs (Figure 2B). The underlying molecular mechanism for the enhanced MMP-9 expression observed in the medial SMCs remains elusive. To clarify further the role of NF- κ B in NGAL and MMP-9 expression observed *in vivo*, the SMCs were infected with β -gal or dnIKK β before the stimulation with IL-1 β . Results showed that dnIKK β notably inhibited IL-1 β -induced expression of TNF- α , NGAL, and MMP-9 in intimal SMCs ($P < 0.05$) (Figure 2B).

By Western blot analysis under reducing condition, a 25-kd immunoreactive protein was detected in lysates of resting rat SMCs, which is consistent with human NGAL previously identified in neutrophils and with the deduced molecular weight from rat cDNA (Figure 3).² In agreement with the level of mRNA expression, the NGAL protein level was higher in intimal cells than in medial SMCs under resting condition and was further elevated at 24 and 48 hours after IL-1 β stimulation (Figure 3). However, levels of NGAL protein were reduced in both types of SMCs after infection with dnIKK β (Figure 2C). In addition, MMP-9 protein was detected on the same membrane of NGAL protein analysis. Our data show that levels of MMP-9 protein were reduced in the SMCs infected with dnIKK β and that MMP-9 protein was more abundant in medial SMCs than that in intimal SMCs (Figure 3), which

is also consistent with the pattern of MMP-9 mRNA expression in the two types of SMCs.

NGAL-MMP-9 Complex Formation and Interaction

Under nonreducing conditions, NGAL could be detected in various forms in the cell lysates corresponding to monomer (25 kd), homodimer (46 kd), and homotrimer (70 kd) by Western blotting (Figure 4A, top). Moreover, a band ~ 150 kd was detected. MMP-9 was detected mainly as monomer (82 kd) and homodimer (220 kd) (Figure 4A, middle) by using the same membrane. Therefore, this suggests that NGAL and MMP-9 cannot form complex within the cells. In addition, IP was performed on both cell lysates and supernatants of cultured intimal SMCs before and after IL-1 β stimulation. Results of IP using NGAL antibody revealed the presence of NGAL/MMP-9 complex exclusively in the culture supernatants of cells exposed to IL-1 β but not in the cell lysates (Figure 4B, top), indicating NGAL and MMP-9 could form complex once they are secreted out of the cells. Presence of the complex in the supernatants of SMCs was also sustained by IP using anti-NGAL antibody (Figure 4B, bottom).

Figure 2. Effect of dnIKK β on NF- κ B activation and the expression of TNF- α , NGAL, and MMP-9 in the medial and intimal SMCs. **A:** NF- κ B activation was assessed by electrophoretic mobility shift assay in both medial and intimal SMCs. Cells are stimulated with IL-1 β for 30 minutes with or without preinfection with β -gal or dnIKK β for 1 hour (h). The specificity of binding was verified by using excess amounts of unlabeled NF- κ B or AP-1 probes. The **arrow** indicates NF- κ B-specific DNA-protein complex. **B:** Cells are stimulated with IL-1 β for 6 hours with or without preinfection with β -gal or dnIKK β . Transcripts of TNF- α , NGAL, and MMP-9 in SMCs were determined by real-time quantitative RT-PCR and normalized to hypoxanthine guanine ribonucleosyltransferase (HPRT) mRNA. Data are presented as mean $\pm$ SEM from three independent experiments. * P < 0.05, versus β -gal group treated with IL-1 β ; $^{\#}P$ < 0.05 versus the untouched intimal SMCs; $^{\&}P$ < 0.05, versus the untouched medial SMCs.

To study whether NGAL affects MMP-9 activity on complex formation, gelatinase zymography was performed. NGAL/MMP-9 complexes were detected as gelatinolytic activities of ~125- and/or ~115-kD proteins, consistent with their identity being MMP-9 complexed with NGAL monomer and dimer⁵ from the IL-1 β stimulated or nonstimulated intimal and medial SMC supernatants (Figure 4C). In addition, stronger free MMP-9 proteolytic activity was observed in supernatants from intimal SMCs than that from medial SMCs after 72 hours of IL-1 β stimulation.

IL-1 β Induces NGAL Expression in Human SMCs

In accordance with our results in rat SMCs, IL-1 β (10 ng/ml) stimulation resulted in potent up-regulation of NGAL expression in hCASCs on both transcriptional

Figure 3. Western blot analysis of NGAL and MMP-9. Proteins extracted from medial and intimal SMCs stimulated with IL-1 β for 24 and 48 hours with or without preinfection of β -gal or dnIKK β were immunoblotted with NGAL antibody, reprobed with MMP-9 antibody after stripping and β -actin antibody for loading control under reducing conditions. Human recombinant NGAL (rNGAL) with a molecular mass of 20.5 kD was loaded as positive control. The identities of all proteins are indicated on the right. Molecular mass markers are indicated on the left. A representative from three independent experiments is shown.

and protein levels (Figure 5, A and B). Furthermore, IL-1 β induced the binding of NF- κ B to a putative responsive element in the human NGAL promoter using nuclear extract derived from hCASCs (Figure 5C).

dnIKK β Dampens Proteolytic Activity in the Vessel Wall

To understand the functional relevance of NF- κ B regulation of NGAL and MMP-9 expression, gelatinolytic activity was analyzed by *in situ* zymography. Little proteolytic activity was detected in the uninjured vessels. However, in accordance with the up-regulation of NGAL and MMP-9 expression, a significant increase in gelatinolytic activity was observed in the intima of the injured vessel at day 14. Moreover, the down-regulation of NGAL and MMP-9 observed in the injured vessels transduced with dnIKK β , was also associated with a drastic reduction of the *in situ* proteolytic activity (Figure 6).

Discussion

We investigated the expression and transcriptional regulation of NGAL in arteries after angioplastic injury. The main findings of the present study demonstrate that NGAL is highly expressed in the intima of the rat carotid artery after angioplastic injury and can be rapidly induced in rat and human vascular SMCs by IL-1 β . NGAL expression by SMCs is dependent on the activation of IKK β -mediated NF- κ B signaling.

Neutrophils have been known as the main source of NGAL.^{3,6} Increases in serum NGAL resulting from activation of neutrophils may reflect an acute systemic inflammatory response to events such as stroke, renal failure, or on infection^{12,28-30} but are also linked with the presence of chronic inflammatory diseases such as atherosclerosis.¹¹ Besides neutrophils, NGAL is also expressed by epithelial cells, renal tubular cells, and hepatocytes during inflammation or injury.^{10,21,31} In addition, we recently found that endothelial cells, SMCs, and macrophages in atherosclerotic plaques can express NGAL.¹⁴ Using a rat model of carotid injury and the isolated vascular SMCs, we now show that angioplastic

Figure 4. NGAL-MMP-9 complex formation and interaction. **A:** Western blot analysis of NGAL and MMP-9 under nonreducing condition in the medial and intimal SMCs stimulated with IL-1 β for 24 hours in the presence of β -gal or dnIKK β (see Materials and Methods). Proteins were immunoblotted with NGAL antibody and reprobed with MMP-9 antibody after stripping; rNGAL was loaded as a positive control. β -Actin antibody was used for loading control. **B:** IP of intimal cell lysates and supernatants with NGAL antibody and Western blot analysis with MMP-9 antibody (top) and reverse IP of supernatants from medial and intimal SMCs with MMP-9 antibody and Western blotting with NGAL antibody (bottom). SMCs were stimulated with or without IL-1 β for indicated times. The first lane both in top and bottom panels is the control without extract but immunoprecipitated as other lanes. **C:** Gelatinase zymographies were analyzed for MMP activities. Three major gelatinase activities were detected with apparent molecular masses of ~125/115, ~86, and ~68 kD from SMC supernatants. SMCs were stimulated with or without IL-1 β for 24 and 72 hours. The identities of all proteins are indicated by arrows. Molecular mass markers are shown on the left. A representative result from three independent experiments is shown.

injury and proinflammatory cytokine IL-1 β can induce the expression of NGAL in vascular SMCs in a rapid manner similar to epithelial cells and hepatocytes.^{21,31} Importantly, our data demonstrate that IL-1 β , one of the prevalent proinflammatory cytokines implicated in atherosclerosis,³² can induce NGAL in human SMCs. Furthermore, our preliminary gene array analysis indicated marked up-regulation of NGAL in SMCs derived from human atherosclerotic lesions (unpublished data). Taken to-

Figure 5. Kinetics of the IL-1 β -induced NGAL in human coronary artery SMCs (hCASCs). **A:** Transcripts of NGAL in hCASCs were determined by real-time quantitative RT-PCR. Cells were stimulated with IL-1 β for the indicated times. Levels of transcripts are expressed in relative units normalized to cyclophilin A mRNA. Data are presented as mean $\pm$ SEM from three independent experiments. * P < 0.05, versus control hCASCs. **B:** Western blot analysis with polyclonal goat NGAL antibody on protein extracts from hCASCs stimulated with IL-1 β for indicated times (top); the same membranes were reprobed with β -actin antibody for loading control (bottom). A representative from three independent experiments is shown. **C:** NF- κ B activation in hCASCs was assessed by electrophoretic mobility shift assay using the NF- κ B sequence of the human NGAL promoter as probe. Nuclear extracts are from cells treated with or without IL-1 β for 2 hours. The specificity of binding was verified by using excess amounts of unlabeled NF- κ B or AP-1 probes. The arrow indicates NF- κ B-specific DNA-protein complex.

gether, these data suggest that expression of NGAL by vascular SMCs can be induced in the acute phase of inflammation provoked by mechanic injury but also in chronic inflammatory settings such as atherosclerosis.

In addition, two more points revealed by our studies appear noteworthy. The first is that intimal SMCs are dominant over medial SMCs in expression of NGAL. This notion is sustained by the findings that levels of induced NGAL transcript and protein are twofold higher in the intimal cells compared with medial SMCs. *In vivo*, NGAL was highly expressed and confined to the intima of arteries at day 14 but not yet detected in the media early after injury (day 3). Therefore, a mechanistic explanation for the absence of NGAL in the media at the early phase after vascular injury remains to be elucidated.

Figure 6. Gelatinolytic activity in the angioplastic injured carotid arteries by *in situ* zymography. Frozen sections were prepared at day 14 from the uninjured artery (left) and the injured artery transduced with β -gal (middle) or dnIKK β (right). A representative result is shown from four of the uninjured, seven of the injured transduced with β -gal, and five of the dnIKK β transduced. The **arrows** denote the internal elastic lamina, and the **arrowheads** indicate external elastic lamina, defining the borders of intima, media, and adventitia.

A second noteworthy finding is that injury-induced NGAL expression by vascular SMCs is NF- κ B-dependent. Activation of NF- κ B is a pivotal mechanism underlying the induction of genes implicated in vascular inflammation, cell proliferation, and death.¹⁸ Our recent study demonstrated that in contrast to the resolved NF- κ B activation in the media, the intima exhibited a persistent NF- κ B activation coupled with high levels of IKK activity at the late stage of vascular injury.²⁷ NGAL expression in the injured vessel seems consistent with the spatial pattern of NF- κ B activation. Moreover, analysis of the NGAL gene promoter identified NF- κ B binding sites potentially critical for the transcriptional regulation of NGAL expression.²⁰ The present study in human SMCs also demonstrates that IL-1 β triggers NF- κ B binding activity to DNA in the NGAL promoter region. Based on these findings, we hypothesized that the expression of NGAL might be modulated by NF- κ B signaling in SMCs subjected to injury. By transfecting the injured vessels with dnIKK β , which has been shown to effectively block injury-induced NF- κ B signal in the intima,²⁷ both NGAL transcripts and protein were significantly suppressed. Our data thus advanced the previous understanding of regulation of NGAL expression *in vitro*, implicating a critical role for IKK β -mediated NF- κ B signaling in NGAL expression within the injured artery. The direct function of NF- κ B signaling in mediating NGAL expression by SMCs was further ascertained by infecting the medial and intimal SMCs with dnIKK β , indicating that up-regulation of NGAL in SMCs is dependent on NF- κ B activation.

The ability of NGAL to form a heterodimer complex with MMP-9 and to support allosteric activation and protection of MMP-9 from degradation^{4,5} endows NGAL with a potential role in the vascular remodeling process. Although our *in vivo* data show MMP-9 was rapidly induced in the media after vessel injury and remained highly expressed in the developing intima after 2 weeks, induction of this gene was not inhibited by dnIKK β at day 3. A possible explanation for this is that NF- κ B has not been suppressed by dnIKK β or the induction of MMP-9 is independent on NF- κ B activation at the early stage of vascular injury.^{33,34} Nevertheless, NGAL seems to be induced

later and predominantly expressed by intimal SMCs, suggesting that MMP-9 and NGAL are co-expressed during intimal formation. The co-expression of NGAL and MMP-9 by intimal SMCs implies a functional interaction between the two gene products. However, intimal SMCs produce much less MMP-9 both at the transcriptional and protein level compared with medial SMCs. Nevertheless, this may not correspond to its proteolytic activity, as discussed later.

Intriguingly, Western blot analysis performed under nonreducing conditions suggests that cytosolic NGAL exists as a monomer, homodimer, homotrimer in rat SMCs. IP analysis further showed that NGAL could be detected as two bands correspondent as ~20- and 25-kd molecules, dependent on whether it is glycosylated.^{3,35} Most importantly, IP analysis for cell supernatants and lysates demonstrates that NGAL and MMP-9 could form a complex only after they are secreted from the cells, and the amount of complexes is enhanced on IL-1 β stimulation. This finding implies a functional interaction between NGAL and MMP-9 produced by vascular SMCs. Indeed, gelatinase zymography of cell supernatants demonstrates that there is a considerable increase in MMP-9 activity accompanied with NGAL/MMP-9 complex. A previous study⁵ suggested that neutrophil-derived MMP-9 activity could be protected in the presence of NGAL, although precise molecular basis of the interaction remains to be elucidated. In the present study, the functional relevance of NGAL and MMP-9 in proteolytic activity was further examined *in vivo* by *in situ* zymography. Our data indicate a tight relationship between the proteolytic activity and expression of NGAL, as demonstrated by the substantially enhanced proteolytic activity in the intima. On the other hand, inhibition of NF- κ B results in dampened MMP activity in the injured vessels. These data support a plausible but yet-to-be-proven hypothesis that NGAL may contribute to be a novel mechanism in regulating the vascular repair-remodeling process by enhancing MMP-9 activity. However, the regulatory process of MMP-9 activity is rather complex, and several other molecules such as TIMPs could be involved.³⁶ Nevertheless, direct evidence for modulation

of MMP-9 activity by SMC-produced NGAL and the relevance to vascular remodeling need to be further elucidated using an animal model with tissue-specific knock-out or overexpression of NGAL.

NGAL produced in the process of renal tubule damage has recently been identified as an early biomarker for detecting acute renal failure.^{29,37} In addition to the drastic induction in vessels after mechanic injury, our previous data suggest that NGAL is strongly up-regulated in atherosclerotic lesions and also in the heart after ischemic injury.¹⁴ It is plausible that NGAL produced by vascular cells could be secreted into the circulation, thus affecting its serum level, suggesting exploration of its suitability as a marker of vascular injury.

In summary, we demonstrate here that the expression of NGAL occurs predominantly by vascular SMCs in the injured artery after NF- κ B activation. We hypothesize that induction of NGAL is a hitherto unrecognized mechanism in the regulation of proteolytic activity involved in the vascular repair process.

Acknowledgments

We thank Niels Borregaard for providing recombinant human NGAL and Norbert Gerdes for constructive discussion and critical comments on the manuscript.

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