The Matrix Metalloproteinase-9/Neutrophil Gelatinase-Associated Lipocalin Complex Plays a Role in Breast Tumor Growth and Is Present in the Urine of Breast Cancer Patients

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Abstract

Purpose: Having previously shown that the binding of neutrophil gelatinase-associated lipocalin (NGAL) to matrix metalloproteinase-9 (MMP-9) protects this extracellular matrix remodeling enzyme from autodegradation, we hypothesized that the addition of NGAL to breast cancer cells, which do not express this protein but do express MMP-9, might result in a more aggressive phenotype *in vivo*. Based on our previous reports that MMPs can be detected in the urine of cancer patients, we also asked whether MMP-9/NGAL could be detected in the urine of breast cancer patients and whether it might be predictive of disease status.

Experimental Design: Clones of MCF-7 human breast cancer cells differentially expressing NGAL were generated by stable transfection with human NGAL expression constructs. The established clones were then implanted s.c. in immunodeficient mice and tumor growth was monitored. In addition, we analyzed the urine of individuals with breast cancer and age-matched, sex-matched controls using gelatin zymography for the presence of MMP-9/NGAL.

Results: Increased NGAL expression resulted in significant stimulation of tumor growth. Immunohistochemical analysis of MCF-7 tumors revealed that the NGAL-overexpressing ones exhibited increased growth rates that were accompanied by increased levels of MMP-9, increased angiogenesis, and an increase in the tumor cell proliferative fraction. In addition, MMP-9/NGAL complex was detected in 86.36% of the urine samples from breast cancer patients but not in those from healthy age and sex-matched controls.

Conclusions: These findings suggest, for the first time, that NGAL may play an important role in breast cancer *in vivo* by protecting MMP-9 from degradation thereby enhancing its enzymatic activity and facilitating angiogenesis and tumor growth. Clinically, these data suggest that the urinary detection of MMP-9/NGAL may be useful in noninvasively predicting disease status of breast cancer patients.

Matrix metalloproteinases (MMP) are a family of zinc-dependent endopeptidases that collectively degrade most of the molecular components of the basement membrane. Disassembly of the basement membrane by MMPs represents one of the most important hallmarks of cancer progression, from angiogenesis to local growth, invasion, and distant metastasis formation (1). Endogenous inhibitors of MMPs, the tissue inhibitor of metalloproteinases, have been shown to suppress tumor growth and angiogenesis in a variety of *in vivo* systems, although they differ significantly with respect to the specific angiogenic processes that they inhibit (2, 3). The overproduction of MMPs at tumor sites by tumor cells or by the surrounding stromal cells has been associated with the metastatic phenotype and poor prognosis (4–7).

We have previously reported the detection of intact MMPs in the urine of cancer patients with a variety of cancers and have shown that these urinary MMPs serve as independent predictors of disease status (8, 9). MMPs detected in these urine samples included MMP-9 (gelatinase B, EC3.4.24.35) and MMP-2 (gelatinase A, EC3.4.24.24), as well as several other MMP activities of higher molecular masses (≥150 kDa). We have recently identified and characterized a distinct urinary MMP activity of ~125 kDa as being a complex of MMP-9 and human neutrophil gelatinase-associated lipocalin (NGAL). *In vitro*, the formation of MMP-9/NGAL complexes protected MMP-9 from autodegradation (10). The association of NGAL with cancer progression has recently been reported, with high levels of NGAL being detected in adenocarcinomas of lung, colon, and pancreas (11, 12). The rat NGAL homologue, *neu*-related lipocalin, was first identified as a protein whose expression

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Note: C.A. Fernández and L. Yan contributed equally to this study.

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was specifically induced in *neu* (HER2/c-erbB-2)–induced mammary tumors but not in *ras*- or chemically induced carcinomas (13). NGAL expression can increase during inflammation and has been shown to be regulated by various cytokines (14, 15). In human breast cancers, a significant correlation was detected between NGAL expression and several other markers of poor prognosis, such as estrogen receptor (ER)– and progesterone receptor–negative status and high proliferation (16). This association between high NGAL expression with ER-negative status was independently confirmed by a gene expression profiling study of 58 primary tumor nodules dissected from breast cancer patients (17).

In the current study, we investigated the role of NGAL in breast cancer growth. We engineered MCF-7 human breast cancer cell lines to express elevated levels of NGAL and show that NGAL overexpression leads to increased breast tumor growth that is accompanied by an increase in MMP-9 activity, tumor angiogenesis, and tumor cell proliferation. In addition, the enzymatic activity of urinary MMP-9/NGAL complex was detected in the urine of breast cancer patients but not in the age-matched, sex-matched, healthy control samples.

Taken together, our findings suggest, for the first time, that NGAL may contribute to breast cancer disease progression, at least in part, by protecting MMP-9 from degradation, thereby enhancing its enzymatic activity and facilitating tumor progression. The appearance of MMP-9/NGAL complexes in the urine of breast cancer patients suggests that urinary MMP-9/NGAL detection may serve as an independent predictor of disease status.

Materials and Methods

Cell culture. MCF-7 human breast carcinoma cells were purchased from American Type Culture Collection (Manassas, VA) and cultured in DMEM (Invitrogen, Carlsbad, CA) containing 10% calf serum, 1% L-Glutamine, Penicillin G Streptomycin Sulfate (Invitrogen), and 10 μg/mL bovine insulin (Invitrogen), as recommended by the vendor.

Generation of stable neutrophil gelatinase-associated lipocalin–overexpressing human breast cancer cells. pcDNA3.1/GS vector containing full-length human *NGAL* (GeneStorm Human Clones, Invitrogen) was transfected into MCF-7 human breast carcinoma cells using Effectene transfection reagent according to manufacturer's recommendations (Qiagen, Valencia, CA). NGAL-overexpressing clones were established by selecting zeocin-resistant colonies.

Neutrophil gelatinase-associated lipocalin ELISA. Stably transfected cells were cultured to subconfluency, washed with PBS, and then cultured in serum-free DMEM medium for 24 hours. Conditioned medium was collected and NGAL protein concentration was determined by ELISA as described by Kjeldsen et al. (18). Briefly, samples diluted in blocking buffer [0.5 mol/L NaCl, 3 mmol/L KCl, 8 mmol/L Na_2HPO_4/KH_2PO_4 (pH 7.2), 1% Triton X-100, 1% bovine serum albumin] were applied to Costar medium binding ELISA plates (Corning, NY) that had previously been coated with 15.8 ng of affinity-purified rabbit anti-NGAL antibodies (kindly provided by Drs. Niels Borregaard and Lars Kjeldsen, Department of Hematology, University Hospital, Rigshospitalet, DK2100 Copenhagen, Denmark) and allowed to react. Monomeric NGAL was used as the standard. Bound antigens were then detected using biotinylated affinity-purified polyclonal anti-NGAL antibodies (72 ng/mL) followed by peroxidase-conjugated Streptavidin (Pierce, Rockford, IL). Colorimetric analysis was done using a SpectraMax 190 MicroPlate Reader (Molecular Devices, Sunnyvale, CA) at 450 nm with background correction at 540 nm.

Western blot analysis. Immunoblot analyses were done as previously described (10). Briefly, conditioned medium was concentrated using Vivaspin Concentrators with molecular weight cutoff of 5 kDa (Vivascience, Hanover, Germany). Proteins normalized to cell number were separated by SDS-PAGE under nonreducing conditions and then electrophoretically transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Reactive proteins were visualized by enhanced chemiluminescence (Pierce).

Reverse transcription-PCR analysis of neutrophil gelatinase-associated lipocalin–overexpressing clones. Total RNA was isolated from cultured cells with NucleoSpin RNA II Kit (BD Biosciences Clontech, Palo Alto, CA) according to manufacturer's instructions. Total RNA was reverse-transcribed to cDNA with SuperScript III (Invitrogen) and amplified with Platinum PCR SuperMix (Invitrogen). The PCR primers for NGAL are forward 5′-AGACCAAAGACCCGCAAAAG-3′ and reverse 5′-TGGCAACCTGGAACAAAAG-3′. The PCR primers for MMP-9 are forward 5′-ACTACTGTGCCTTTGAGTCC-3′ and reverse 5′-AGAATCGCCAGTACTTCCC-3′. The PCR primers for glyceraldehyde-3-phosphate dehydrogenase are forward 5′-CAGCCTCAAGATCATCAGCA-3′ and reverse 5′-GTCTTCTGGGTGGCAGTGAT-3′.

Substrate gel electrophoresis. Substrate gel electrophoresis (zymography) was done as previously described (10, 19, 20). Briefly, proteins were resolved on a 10% SDS-PAGE gel containing 0.1% gelatin under nondenaturing conditions. The gels were washed in 2.5% Triton X-100 and then incubated overnight at 37°C in substrate buffer [50 mmol/L Tris (pH 8), 5 mmol/L $CaCl_2$, and 0.02% NaN_3]. Gels were then stained with 0.1% Coomassie blue and destained for 1 hour. Gelatinolytic activity was detected as clear bands on a blue background. MMP-9/NGAL complex was purchased from Calbiochem (San Diego, CA) and used as control.

Animal studies. Subconfluent cells were trypsinized and resuspended in 0.9% sodium chloride at a concentration of 2×10^7 cells/mL and then mixed with an equal volume of Matrigel (BD Biosciences Discovery Labware, Bedford, MA). One hundred microliters (1×10^6 cells) were injected s.c. into the right flank of 8-week-old female *nu/nu* nude mice (Charles River Laboratory, Wilmington, MA). At the same time, 30-day slow release estrogen pellets (Innovative Research of America, Sarasota, FL) were implanted s.c. Tumor growth was monitored twice weekly by measuring the tumor with calipers. Tumor volume was calculated based on the formula (length × width × width) / 2. Statistical analyses were done using unpaired *t* test analysis.

Immunohistochemical analysis. At the termination of the study, tumor nodules were dissected, rinsed in ice-cold PBS, and fixed in 4% paraformaldehyde overnight. Immunohistochemistry of tumor nodules was done using 5-μm-thick formalin-fixed, paraffin-embedded tissue sections. Slides were covered with Peroxidase Block (DAKO USA, Carpinteria, CA) for 5 minutes to quench endogenous peroxidase activity followed by a 1:5 dilution of goat serum in 50 mmol/L Tris-HCl (pH 7.4) for 20 minutes to block nonspecific binding sites. Primary murine anti-human MMP-9 (1:500 dilution; Chemicon International, Inc., Temecula, CA), murine anti-human MMP-2 (1:250 dilution; Chemicon International), murine anti-human Ki-67 (MIB-1 clone, 1:250 dilution; DAKO USA), rabbit anti-human NGAL (1:100 dilution; kindly provided by Drs. Niels Borregaard and Lars Kjeldsen), or rat anti-murine CD34 (MEC 14.7, 1:150 dilution; Abcam, Cambridge, MA) was applied in 50 mmol/L Tris-HCl (pH 7.4) with 3% goat serum at room temperature for 1 hour. Slides were then washed in 50 mmol/L Tris-HCl (pH 7.4) and antigen-antibody binding was detected using horseradish peroxidase–conjugated secondary antibodies (Envision detection kit, DAKO USA). Hematoxylin was used as a counter stain. Chromogenic terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) staining (Intergen, Milford, MA) with methyl green counterstaining was done as per manufacturer's instructions.

Urine sample collection and analysis. Urine samples were collected from patients with breast cancer or healthy controls as previously reported (8, 21). Samples were aliquoted on ice and immediately frozen after collection and stored at −20°C until assay. Before analysis,

specimens containing blood or leukocytes were excluded by testing for the presence of blood and leukocytes using Multistix 9 Urinalysis Strips (Bayer, Elkhart, IN). The creatinine concentrations of urine samples were determined using a commercial kit (Sigma Chemical Co., St. Louis, MO) according to manufacturer's instructions. Immunoprecipitation of MMP-9/NGAL complexes from urine samples was done as previously described (10).

Results

Characterization of neutrophil gelatinase-associated lipocalin–transfected MCF-7 human breast cancer cells. To determine whether NGAL alone can alter breast tumor growth, clones of NGAL-overexpressing MCF-7 cells, designated N1 and N2, were generated by stable transfection using the pcDNA3.1/GS vector containing full-length human *NGAL* (GeneStorm Human Clones, Invitrogen). The NGAL concentration in the conditioned medium of cells transfected with *NGAL* and in wild-type cells was determined by ELISA according to the methods of Kjeldsen et al. (18). A substantial increase in NGAL secreted into medium was detected in the *NGAL*-transfected clones, N1 and N2 (58.9 ng NGAL/mg protein and 213.1 ng NGAL/mg protein, respectively) compared with their wild-type counterparts (19.4 ng NGAL/mg protein; Fig. 1A). The presence of NGAL was also confirmed by Western analysis using monospecific antibodies (Fig. 1B). Protein bands detected at ~25 and 21 kDa are consistent with the identification of glycosylated and unglycosylated forms of NGAL (22). NGAL overexpression did not result in increased expression of MMP-9 in these clones (Fig. 1C). However, analysis of the conditioned media from each clone by zymography showed that high levels of NGAL expression result in the formation of MMP-9/NGAL complexes (Fig. 1D) migrating at the molecular mass consistent with their identification as MMP-9 complexed with NGAL monomers or dimers (10, 22). In N1 clones, where NGAL expression is low, levels of complex formation as detected by zymography were below the limits of detection in this assay. Consistent with our previous findings (10), NGAL secreted into the medium protected both MCF-7-secreted and exogenously added MMP-9 from autodegradation as assessed by zymography (data not shown).

In vivo *tumor growth of neutrophil gelatinase-associated lipocalin–overexpressing breast cancer cells.* Independent experiments using different clones of *NGAL*-transfected MCF-7 human breast cancer cells were done to determine the effects of NGAL expression on tumor growth in nude mice. A positive correlation between the growth rate of s.c. implanted tumors and their NGAL expression level was observed. Four weeks after tumor implantation, the tumor volumes (mean ± SE) were 24.85 ± 3.75 mm^3 for wild-type tumors, 38.27 ± 4.05 mm^3 for N1 tumors ($P = 0.0038$), and 69.42 ± 5.80 mm^3 for N2 tumors ($P = 0.000049$; Fig. 2A). These results show that in this xenograft model, NGAL expression significantly stimulated tumor growth *in vivo* in a dose-dependent fashion and suggest that elevated NGAL expression in tumor cells is associated with enhanced *in vivo* tumor growth potential.

Histologic analysis of tumors expressing elevated levels of neutrophil gelatinase-associated lipocalin. To assess the *in vivo* effects of NGAL expression on MMP levels in tumor tissues, immunohistochemical studies were done to determine the expression pattern of MMP-2 and MMP-9 in tumors derived from wild-type and NGAL-overexpressing tumor cells. Higher levels of NGAL and MMP-9 were detected in the N1 and N2 tumors compared with low levels seen in wild-type tumors, with the most prominent staining being observed in N2 tumors which express the highest levels of NGAL protein, as determined by ELISA (Fig. 2C). These results suggest a strong correlation between NGAL expression and MMP-9 levels in tumor tissues. Relatively low levels of MMP-2 staining were observed in both control and NGAL-overexpressing tumors (data not shown).

As with the conditioned medium, tissue extracts obtained from wild-type and NGAL-overexpressing tumors confirm the formation of the MMP-9/NGAL complex in the N2 tumors (Fig. 2B), which express high levels of NGAL, suggesting that elevated NGAL expression results in protected MMP-9 activity *in vivo*.

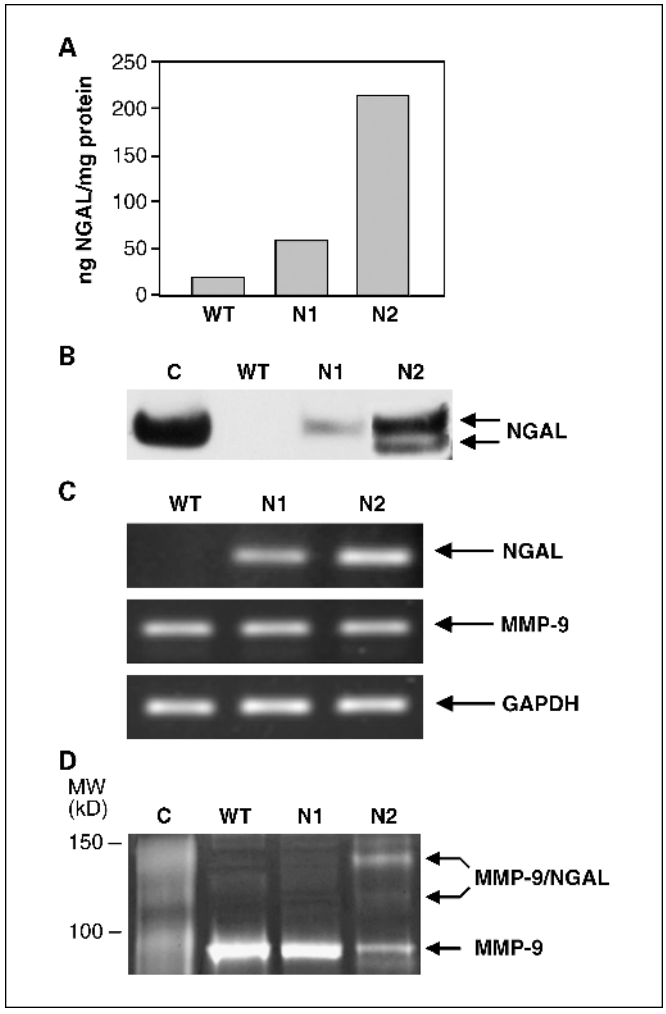


Fig. 1. NGAL overexpression in MCF-7 cells and MMP-9/NGAL complex formation *in vitro*. *A*, NGAL protein in conditioned medium of wild-type (*WT*) and NGAL-transfected clones (N1 and N2) was quantitated by ELISA. *B*, presence of NGAL in transfected clones was verified by Western analysis of conditioned medium. Purified NGAL was used as a control (*C*). *C*, MMP-9 expression was not altered by NGAL overexpression. *D*, high levels of NGAL expression led to the formation of MMP-9/NGAL complex in the N2 clones, as shown by zymographic analysis of the conditioned medium of the wild-type and NGAL-overexpressing MCF-7 clones. Commercially obtained MMP-9/NGAL complex was used as a control (*C*).

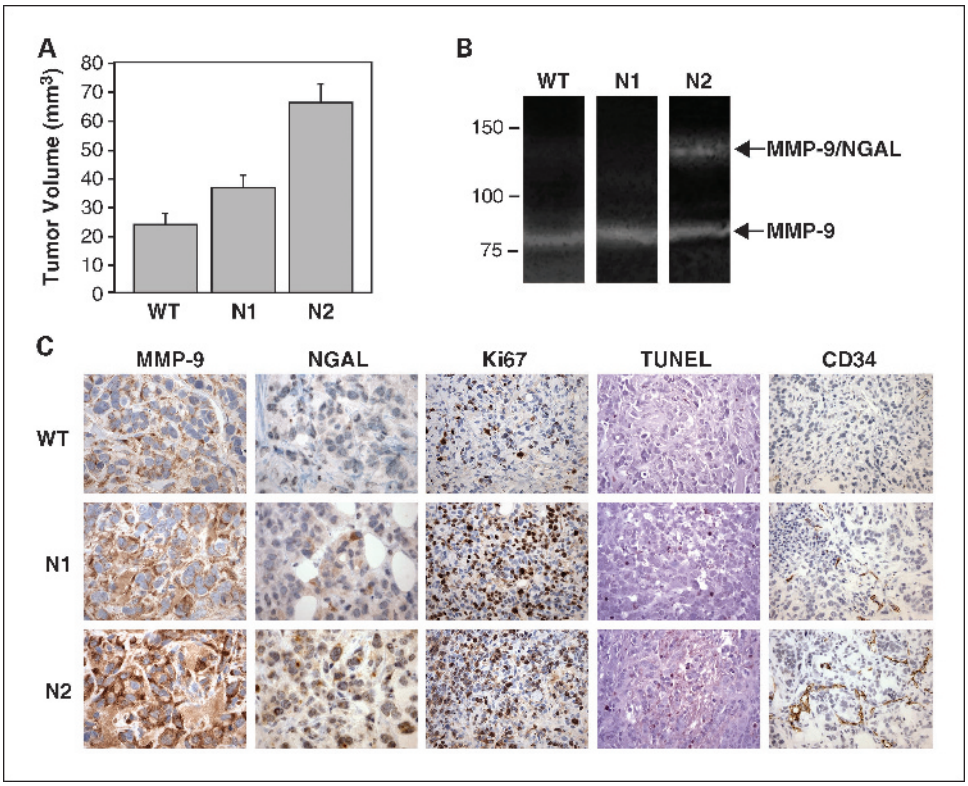


Fig. 2. *In vivo* growth property of tumors derived from wild-type (*WT*) or NGAL-transfected human breast cancer cells in nude mice. *A,* wild-type or NGAL-transfected MCF-7 human breast cancer cells were implanted s.c. in nude mice. Four weeks after tumor implantation, tumor volumes were 24.85 ± 3.75 mm^3 for wild-type tumors (mean ± SE), 38.27 ± 4.05 mm^3 for N1 tumors ($P = 0.0038$ compared with wild-type tumors), and 69.42 ± 5.80 mm^3 for N2 tumors ($P = 0.000049$ compared with wild-type tumors). *B,* zymographic analysis of tumor extracts showed the appearance of MMP-9/NGAL complex in N2 tumors but not in N1 and wild-type tumors. *C,* immunohistochemical analysis of tumor nodules derived from wild-type, N1 and N2 clones showed increased levels of MMP-9 and NGAL in NGAL-overexpressing tumors compared with controls (×400). Increased proliferation was observed in the NGAL-overexpressing tumors as determined by Ki67 immunohistochemistry and a modest increase in apoptosis as determined by terminal deoxynucleotidyl transferase–mediated nick-end labeling (*TUNEL*) staining (×200). Additionally, anti-CD34 staining of tumor nodules showed increased microvascular density in NGAL-overexpressing tumors compared with controls (×200).

Because MMP activity has been associated with tumor angiogenesis (6, 23, 24), the potential role of NGAL in modulating tumor angiogenesis was investigated. As determined by CD34 staining, NGAL-overexpressing tumors showed increased microvessel density when compared with controls (Fig. 2C). This was accompanied by increased tumor cell proliferation as assessed by Ki-67 immunoreactivity (~30% in wild-type tumors versus ~70-80% in N1 and N2 tumors), and an increase in the apoptotic rate as assessed by terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) staining (<1% in wild-type tumors versus 5% in N1 and N2 tumors; Fig. 2C).

Correlation of the urinary matrix metalloproteinase-9/neutrophil gelatinase-associated lipocalin activity and disease status in patients with breast cancer. In light of the fact that urinary MMP-9 has been reported to predict disease status in women with breast and other cancers (8), we next asked whether the MMP-9/NGAL complex could be detected in the urine of breast cancer patients as well. Urine samples obtained from patients with breast cancer ($n = 22$) or age-matched healthy controls ($n = 27$) were analyzed by zymography and MMP-9/NGAL complexes were detected as gelatinase enzymatic activities of ~125 and/or ~140 kDa, consistent with their identity being MMP-9 complexed with NGAL monomers and dimers (10, 22). To determine whether these species were indeed complexes of MMP-9 and NGAL, NGAL-specific antibodies were used to immunodeplete complexes from the urine. Anti-NGAL antibodies efficiently and specifically immunoprecipitated the gelatinase activities of NGAL-containing complexes in the breast cancer patient urine (Fig. 3A). The MMP-9/NGAL complex was detected in 86.36% (19 of 22) of urine samples from patients with breast cancer but not in those from healthy controls (Fig. 3B).

Discussion

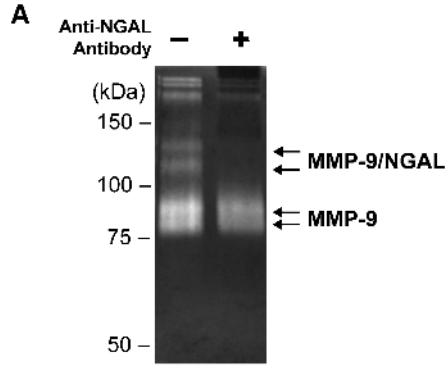
Human NGAL was originally identified as a 25-kDa protein associated with purified human neutrophil gelatinase (22). Along with its murine and rat counterparts, NGAL has been implicated in tumor growth and progression (11–13, 16, 17, 26). Known as mouse gene *24p3* product, murine NGAL protein expression is induced substantially (14- to 20-fold) after SV40 infection (25). Interestingly, the rat NGAL homologue, *neu*-related lipocalin, was discovered as a gene product that is significantly stimulated during cellular transformation when this was induced by the potent oncogene *neu* but not by weaker oncogenes such as *ras* or by chemical-induced transformation (13). In human cancers, elevated levels of NGAL expression have been detected in human breast tumors (16), in adenocarcinomas of lung, colon, liver, and pancreas (11, 12), as well as in various malignant human tumor cell lines, including those of pancreatic (12) and ovarian origin (26).

In primary human breast cancer, the expression of NGAL significantly correlates with several markers of poor prognosis, including ER- and progesterone receptor–negative status and high proliferation (S-phase fraction; ref. 16). In a recent study of gene expression profiles of 58 node-negative breast carcinomas, higher levels of NGAL expression significantly associated with cancer cell ER-negative status and may represent one of the critical molecular changes underlying breast cancer disease progression from ER-positive to ER-negative status (17). The association of NGAL with ER-negative status and its elevated expression in tumor tissues suggested to us that NGAL overexpression may functionally contribute to disease progression.

The importance of MMP activity in tumor growth and progression is well established (4–7, 23, 24). Previous work from our laboratory has shown that MMP-9/NGAL complex formation protects MMP-9 from autodegradation, resulting in increased MMP-9 activity (10). Based on these findings, we hypothesized that overexpression of NGAL in tumor cells would result in an increase in tumor associated MMP-9 activity and increased tumor growth *in vivo*. We further hypothesized that this increase in tumor mass might be due, at least in part, to the ability of NGAL to protect MMP-9 from degradation. To test these hypotheses and to better understand the role of NGAL in breast tumor growth and progression, we transfected an ER-positive human breast cancer cell line, MCF-7, which expresses low levels of endogenous NGAL, with *NGAL* expression constructs. These cells were engineered to express elevated levels of NGAL, but not other genes often associated with ER-negative status (17) and are particularly useful in dissecting the functional involvement of NGAL in this transition. When implanted in nude mice, NGAL-overexpressing MCF-7 cells gave rise to significantly larger tumors that exhibited higher levels of MMP-9 protein compared with wild type (Fig. 2A). Interestingly, in a set of similar experiments in which NGAL was overexpressed in the more aggressive, ER-negative breast cancer cell line, MDA-MB-231, no significant difference in tumor growth was observed (data not shown). Because MDA-MB-231 already express elevated levels of NGAL (16), it is possible that further increases in NGAL expression have no additive effect on tumor growth. In fact, NGAL overexpression did not result in increased expression of MMP-9 in either cell type (this study and ref. 10) suggesting that the available MMP-9 in the tumor microenvironment might already be in complex with NGAL such that further increases in NGAL levels in MDA-MB-231 cells do not result in additional increases in MMP-9 activity.

We have previously reported that the formation of MMP-9/NGAL complexes *in vitro* can protect MMP-9 from autodegradation thereby stabilizing its activity (10). Although MCF-7 cells secrete very little MMP-9, both MMP-9 monomer and MMP-9 complexed with NGAL could be detected in the conditioned medium of the N2 NGAL-overexpressing clones (Fig. 1D). Consistent with our previous findings (10), complex formation in the conditioned media of N2 cells, which express high levels of NGAL, resulted in the protection of MMP-9 from autodegradation when compared with conditioned medium from wild-type controls. Although the complex could not be detected in the conditioned media of N1 clones, due to the low levels of MMP-9 expressed by MCF-7 being at the detection limits of zymography, incubation of the conditioned medium from both NGAL-overexpressing clones with exogenously added, purified MMP-9 also resulted in the protection of MMP-9 from autodegradation. These results suggest that although MCF-7 cells make little MMP-9, secretion of NGAL may result in increased stability of MMP-9 secreted by surrounding stromal cells. In fact, in a recent study in which MMP-9 was overexpressed in MCF-7 cells, secretion of MMP-9 was shown to result in increased tumor size (27). Together, these results led us to hypothesize that the protection of MMP-9 protein from autodegradation by NGAL *in vitro* might also result in increased levels of MMP-9 in NGAL-overexpressing tumors *in vivo* and increased tumor volume. In fact, NGAL-overexpressing tumors were significantly larger than controls (Fig. 2A) and immunohistochemical analysis revealed a significant increase in MMP-9 protein in the NGAL-overexpressing tumors compared with wild-type tumors (Fig. 2C). The most abundant MMP-9 levels were detected in the N2 tumors, which are derived from the MCF-7 clones that express the highest levels of NGAL. MMP-9 was detected in association with tumor and stromal cells although the majority of the staining was observed in the extracellular spaces, suggesting that secreted NGAL may result in increased stability of MMP-9 in the tumor microenvironment independent of the source. This also suggests that this increased proteolytic activity is not limited to the cancer cells and may, in fact, facilitate endothelial cell recruitment during angiogenesis.

Matrix metalloproteinase activity has been shown to promote tumor growth as well as angiogenesis in a number of studies of tumor-induced angiogenesis. Tumor cell–secreted MMP-9 promoted angiogenesis *in vivo* in the MCF-7 breast cancer model where the vascular endothelial growth factor/vascular endothelial growth factor receptor 2 association was shown to be dependent on MMP-9 activity (27). MMP-9 activity has also been shown to contribute to the metastatic and angiogenic phenotypes of human tumors. In an *in vivo* study of DU145 prostate tumors, treatment with antisense-MMP-9 oligos resulted in reduced tumor growth and angiogenesis (28). It has also been shown that antisense ablation of MMP-9 expression in DU145 and PC3 cells resulted in a concomitant inhibition of the gene expression of proangiogenic factors such



B

Urine Samples	Number of Patients	MMP-9/NGAL Positive	MMP-9/NGAL Negative
Breast cancer	22	19*	3
Healthy age-matched control	27	0*	27

Fig. 3. Detection of MMP-9/NGAL complex in urine samples of breast cancer patients. *A,* gelatinase activities of MMP-9/NGAL complexes were detected in urine samples from breast cancer patients. Fifty microliters of each urine sample were diluted 1:1 v/v with RIPA buffer and mixed with anti-NGAL antibodies. After a 30-minute incubation on ice, the antibody-antigen complexes were removed by immunoprecipitation. The supernatants were subjected to gelatin zymography to detect the remaining MMP activities. *B,* presence or absence of MMP-9/NGAL complexes in the urine samples of breast cancer patients or age-matched, sex-matched, healthy controls. MMP-9/NGAL complex was detected in 86.36% of urine samples from breast cancer patients but not in controls. *, $P < 0.00001$.

as vascular endothelial growth factor and intracellular adhesion molecule-1 (29). In a study of human glioma tumors, it was reported that MMP-9, which was localized to the cytoplasm of tumor cells, the endothelial cells, and the basement membrane, mediated the degradation of the extracellular matrix and promoted angiogenesis (30).

Given these findings and because MMPs, including MMP-9, have been implicated in multiple steps of cancer progression including tumor angiogenesis (4–7, 23, 24), we examined the effect of increased MMP-9 levels in NGAL-overexpressing tumors on tumor angiogenesis. Positive CD34 immunostaining, indicative of microvascular density, directly correlated with NGAL levels and was significantly increased in N2 tumors (Fig. 2C), suggesting that NGAL-associated increases in MMP-9 activity may contribute to MMP-9's positive role in angiogenesis. Increases in MMP-9 and angiogenesis in these tumors were accompanied by a significant increase in tumor cell proliferation and a modest increase in apoptotic nuclei, resulting in larger tumors (Fig. 2C). These results are consistent with studies that showed that MMP-9 overexpression in MCF-7 cells resulted in increased tumor growth and angiogenesis (27). Our results suggest that NGAL expression results in increased levels of MMP-9, enhanced tumor angiogenesis, and stimulation of tumor cell proliferation. Together, these changes significantly accelerate *in vivo* tumor growth and suggest that MMP-9/NGAL may represent a novel target for the treatment of human breast cancer.

In light of these findings, we asked whether MMP-9/NGAL complex might be present and detectable in the urine of breast cancer patients with greater frequency than in the urine from healthy controls. We have previously reported that MMP-2 and MMP-9 can be detected in urine samples of cancer patients and are independent predictors of disease status (8) and have also identified a gelatinase species of ~125 and/or 140 kDa in human urine as being a complex of MMP-9 and NGAL (10). In this latter biochemical study, the frequency of distribution of MMP-9/NGAL complex was not investigated. We now report that MMP-9/NGAL complexes were detected in ~90% of urine samples obtained from breast cancer patients (Fig. 3) but not in those from healthy controls. These results suggest that urinary MMP-9/NGAL may represent a new urinary biomarker for breast cancer and that this complex, together with MMP-2 and MMP-9, may represent a panel of MMPs and associated proteins that might be useful as novel, noninvasive cancer diagnostics and prognostics.

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