The High Molecular Weight Urinary Matrix Metalloproteinase (MMP) Activity Is a Complex of Gelatinase B/MMP-9 and Neutrophil Gelatinase-associated Lipocalin (NGAL)

MODULATION OF MMP-9 ACTIVITY BY NGAL*

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Detection of matrix metalloproteinase (MMP) activities in the urine from patients with a variety of cancers has been closely correlated to disease status. Among these activities, the presence of a group of high molecular weight (HMW) MMPs independently serves as a multivariate predictor of the metastatic phenotype (1). The identity of these HMW MMP activities has remained unknown despite their novelty and their potentially important applications in non-invasive cancer diagnosis and/or prognosis. Here, we report the identification of one of these HMW urinary MMPs of ~125-kDa as being a complex of gelatinase B (MMP-9) and neutrophil gelatinase-associated lipocalin (NGAL). Multiple biochemical approaches verified this identity. Analysis using substrate gel electrophoresis demonstrated that the 125-kDa urinary MMP activity co-migrates with purified human neutrophil MMP-9-NGAL complex. The 125-kDa urinary MMP-9-NGAL complex was recognized by a purified antibody against human NGAL as well as by a monospecific anti-human MMP-9 antibody. Furthermore, these same two antibodies were independently capable of specifically immunoprecipitating the 125-kDa urinary MMP activity in a dose-dependent manner. In addition, the complex of MMP-9-NGAL could be reconstituted in vitro by mixing MMP-9 and NGAL in gelatinase buffers with pH values in the range of urine and in normal urine as well. Finally, the biochemical consequences of the NGAL and MMP-9 interaction were investigated both in vitro using recombinant human NGAL and MMP-9 and in cell culture by overexpressing NGAL in human breast carcinoma cells. Our data demonstrate that NGAL is capable of protecting MMP-9 from degradation in a dose-dependent manner and thereby preserving MMP-9 enzymatic activity. In summary, this study identifies the 125-kDa urinary gelatinase as being a complex of MMP-9 and NGAL and provides evidence that NGAL modulates MMP-9 activity by protecting it from degradation.

Matrix metalloproteinases (MMPs) are a family of endopeptidases whose activities depend on metal ions, such as Zn$^{2+}$ and Ca$^{2+}$. Collectively, MMPs are capable of degrading all of the molecular components of extracellular matrix, the barrier separating the tumor cells from their normal surrounding tissues, which is disassembled as part of the metastatic process (2). MMPs have been shown to play critical roles in a variety of biological as well as pathological processes, especially in tumor cell invasion and metastasis, and the overproduction of MMPs by tumor cells or surrounding stromal cells has been correlated with the metastatic phenotype (3).

We have recently reported that intact and biologically active MMPs can be detected in the urine of cancer patients and are independent predictors of disease status (1). These urine samples were obtained from patients with a variety of cancers including prostate, renal, bladder, and breast carcinomas. The MMP activities detected in these urine samples included MMP-9 (gelatinase B, type IV collagenase, EC3.4.24.35) and MMP-2 (gelatinase A, type IV collagenase, EC3.4.24.24). Both of these urinary MMPs have been shown to be independent predictors of cancer. In addition to these two major gelatinase species, several MMP activities with molecular sizes greater than 100 kDa were detected and were shown to be predictive of metastatic diseases. Elevated MMP levels in biological fluids from animals bearing experimental tumors or from cancer patients have also been reported in several other studies (4–8).

Despite the potential importance of high molecular weight (HMW) MMPs in predicting disease status, the identity of these potentially novel MMPs remained to be determined. In the current study, we have identified and biochemically characterized one of these HMW MMPs in the urine of cancer patients. Using a combination of biochemical approaches, the 125-kDa urinary MMP activity was identified as a complex of gelatinase B (MMP-9) and human neutrophil gelatinase-associated lipocalin (NGAL), a small molecular weight lipocalin whose expression was found to be up-regulated in certain human neoplastic diseases (9–13) as well as in oncogene-transformed rodent cells (14, 15). The 125-kDa urinary MMP activity comigrates with the MMP-9-NGAL complex purified from human neutrophils. An ~125-kDa protein band was detected by both the purified antibody against human NGAL (16) and a monospecific anti-MMP-9 antibody in the urine samples that contain the 125-kDa MMP activity. These same antibodies also specifically immunoprecipitated the 125-kDa urinary MMP activity in a concentration-dependent manner. Furthermore, the...
formation of the MMP-9-NGAL complex was demonstrated by *in vitro* reconstitution experiments by mixing MMP-9 and NGAL. The MMP-9-NGAL complexes formed *in vitro* were also recognized by the purified anti-NGAL antibody and were specifically immunoprecipitated. Interestingly, degradation of MMP-9 was significantly inhibited in the presence of NGAL and resulted in the preservation of MMP-9 enzymatic activity. This protective effect of NGAL on MMP-9 activity was also observed in a cell culture system in which overexpression of NGAL in human breast carcinoma cells resulted in an increase in MMP-9 activity independent of changes in MMP-9 gene transcription.

Taken together, these data suggest a potential regulatory role for NGAL in modulating MMP-9 activity. Given the recent findings that NGAL expression levels are up-regulated in colorectal neoplasia (9) and several epithelial carcinomas (11–13), we hypothesize that NGAL may be involved in tumor progression via its interaction with MMP-9.

**MATERIALS AND METHODS**

**Urine Sample Collection and Preparation**—Urine sample collection was performed in urine bottles with a non-anticoagulant additive. Samples were collected at 24 h after collection and stored frozen at −20 °C until assayed. Prior to 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) according to the manufacturer’s instructions.

**Substrate Gel Electrophoresis**—Substrate gel electrophoresis was performed based on a previously described method with modifications (1). Original urine samples (50 μl) were mixed with non-reducing sample buffer (4% SDS, 0.15 M Tris, pH 6.8, 20% v/v glycerol, and 0.5% w/v bromphenol blue) and were separated on a 10% polyacrylamide gel containing 0.1% gelatin (Bio-Rad, Hercules, CA). After electrophoresis, gels were washed twice with 2.5% Triton X-100 (15 min/each wash). Substrate digestion was carried out by incubating the gel in 50 mM Tris-HCl, pH 7.6, containing 5 mM CaCl₂, 1 μM ZnCl₂, 1% Triton X-100, and 0.02% NaN₃ at 37 °C for 24 h. The gel was stained with 0.1% Coomassie Brilliant Blue R250 (Bio-Rad), and the location of gelatinolytic activity was detected as clear bands on the background of a uniform blue staining.

**Protein Electrophoresis and Western Blot Analysis**—The same urine samples used for zymography analysis were concentrated using an UltraFree-4 centrifugal filter device with molecular weight cut off of 50 kDa (Millipore, Bedford, MA). Protein concentrations of the concentrated urine samples were determined using the MicroBCA method (Pierce). Equal amounts of proteins (20 μg) were loaded onto 4–15% gradient gels and separated by SDS-polyacrylamide gel electrophoresis under non-reducing conditions. Resolved proteins were electrophoretically transferred to TransBlot nitrocellulose membranes (Bio-Rad). The membranes were blocked with 5% low fat dry milk in TBS-T (10 mM Tris, pH 7.2, 50 mM NaCl, 0.5% Tween 20) for 1 h at room temperature, followed by incubating with primary antibody at 4 °C for 18 h. Blots were washed eight times with TBS-T (5 min/wash) and incubated with 1:5000 dilution of horseradish peroxidase conjugated secondary antibody (Vector Laboratories, Burlingame, CA) diluted in TBS-T containing 3% bovine serum albumin for 1 h at room temperature. Labeled proteins were visualized with enhanced chemiluminescence (Amer sham Pharmacia Biotech). Purified antibodies against human NGAL were used at 1:100 dilution (16). Purified human neutrophil MMP-9/NGAL complex was used as positive control (Calbiochem).

**Immunoprecipitation**—Original urine samples containing the 125-kDa MMP activity were mixed with equal volumes of RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, 0.02% sodium azide). 50 μl of diluted urine samples were mixed with increasing amounts of the purified anti-human NGAL antibody, an anti-human MMP-9 antibody (Ab16896) (Chemicon-Con, Temecula, CA), or a non-immune serum as a control. After incubating on ice for 30 min, the urine samples were mixed with 5 μl of RIPA-buffered Zymobin (ZyoMed Laboratories, South San Francisco, CA). Following an additional incubation on ice for 30 min, the antibody-antigen complexes were removed by centrifugation at 10,000 × g for 5 min. The supernatants were subjected to subcell gel electrophoresis to detect the remaining MMP activities.

**In Vitro Reconstitution of MMP-9-NGAL Complexes**—Recombinant human MMP-9 (OncoGene Sciences, Cambridge, MA) was diluted with gelatinase buffer (50 mM sodium acetate, pH 5.5, or 50 mM Tris-HCl, pH 7.0, 7.6, or 8.0, containing 5 mM CaCl₂, 1 μM ZnCl₂) to a final concentration of 0.1 μM. Recombinant human NGAL expressed and purified from Sf9 cells was diluted to 0.5 μM in the gelatinase buffer. To reconstitute MMP-9-NGAL complex, MMP-9 was mixed with NGAL in a molar ratio of 1:20 and was incubated at 37 °C for 1 h. The formation of MMP-9-NGAL complex was analyzed using substrate gel electrophoresis.

MMP-9 and NGAL were also individually diluted in normal control urine that contains no MMP activity. The possibility of MMP-9-NGAL complex formation in urine was investigated by mixing MMP-9 and individual proteins in molar ratios of 1:20. MMP-9-NGAL complex was detected using substrate gel electrophoresis.

The molecular identity of any complexes formed by mixing MMP-9 and NGAL was investigated with immunoprecipitation using the purified anti-NGAL antibody. After incubating the MMP-9 and NGAL mixture at 37 °C for 1 h, 1.0 μl of anti-NGAL antibody was added into MMP-9-NGAL mixtures. Immunoprecipitation was subsequently carried out as described above.

The MMP-9-NGAL complexes formed in the reconstitution experiments were cross-linked by incubating on ice for 1 h in the presence of 20-fold molar excess of dithiobis(sulfo)aminomethylpropionitrile (DTSSP) (Pierce). The resulting cross-linked complexes were analyzed using substrate electrophoresis.

For protection analysis, recombinant human NGAL expressed and purified from Sf9 insect cells was diluted to 0.4 μM in gelatinase buffer. MMP-9 was mixed with NGAL in different molar ratios ranging from 10:1 to 20:1 and was incubated at 37 °C for varying time periods. Aliquots of enzyme (10 ng) were collected at different time points and were subjected to gelatin zymography.

For protection analysis, recombinant human NGAL expressed and purified from Sf9 insect cells was diluted to 0.4 μM in gelatinase buffer. MMP-9 was mixed with NGAL in different molar ratios ranging from 10:1 to 20:1 and was incubated at 37 °C for varying time periods. Aliquots of enzyme (10 ng) were collected at different time points and were subjected to gelatin zymography.

The inhibition of MMP-9 degradation by NGAL was also studied using immunodepleted NGAL. Briefly, recombinant NGAL was incubated with anti-NGAL antibody and subsequently immunoprecipitated with protein A/G-agarose beads. MMP-9 was mixed with the immunodepleted NGAL for varying time periods, and enzyme degradation was determined using substrate electrophoresis.

The arbitrary activity of MMP-9 enzymatic bands was determined by scanning densitometry using Scion Image Analysis software (Scion Corporation, scioncorp.com). In some experiments, 1,10-phenanthroline (1,10 PA, Sigma) was used at a final concentration of 5.0 mM to inhibit MMP-dependent enzymatic activities.

**Modulation of MMP-9 Activity by NGAL in Cell Culture**—To investigate the biochemical consequences of MMP-9 and NGAL interaction in cells, MDA-MB-231, human breast carcinoma cells, were engineered to overexpress NGAL. cDNA encoding the full-length human NGAL open reading frame was subcloned into pcDNA3.1/GeneStorm vectors for expression in mammalian cells (Invitrogen, Carlsbad, CA). Endotoxin-free plasmid DNA was prepared using Universal Maxi DNA plasmid preparation kit (Boston BioProducts, Ashland, MA). Transfection of NGAL-expression constructs into human breast carcinoma cells was performed using Effectene according to the manufacturer’s manual (QIAGEN). Stably transfected cells were selected using Zeocin at 500 μg/ml (Invitrogen). Expression of NGAL by selected cell clones was confirmed by Western blot analysis using the anti-NGAL antibody. Serum-free medium conditioned by NGAL-expressing clones as well as control cells transfected with empty vectors or parental cells was prepared to determine the MMP-9 level elaborated by these cells (17). Briefly, 90% confluent cells were washed gently with phosphate-
buffered saline three times. Serum-free Dulbecco’s modified Eagle’s medium was added to cell culture and was conditioned for 20 h. Conditioned medium free of cell debris was then subjected to substrate electrophoresis to detect MMP-9 activity (18). Steady state mRNA levels of MMP-9, tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), and a house-keeping gene GAPDH in these transfected cells were determined by reverse transcription polymerase chain reactions (RT-PCR). Total RNA was extracted from phosphate-buffered saline-washed cells using Trizol according to the manufacturer’s instruction (Life Technologies, Inc.). 2.0 μg of total RNA was first treated with 0.8 units/ml DNase I (Roche Molecular Biochemicals), at 37 °C for 1 h. RT reactions were carried out in a 20-μl total volume consisting of 0.25 μg/ml random hexamers (Roche Molecular Biochemicals) using Omniscript RT kit (Qiagen, Santa Clarita, CA). PCR were performed in a total volume of 12.5 μl consisting of 10 pmol of each primer (Life Technologies, Inc.), 0.2 mm of each dNTP, and 0.25 unit of Advantage 2 DNA polymerase (CLONTECH, Palo Alto, CA). PCR reactions were performed using PTC-100 cycler (MJ Research, Waltham, MA) with thermal cycles of 30 s 95 °C denaturing, 30 s 60 °C annealing, and 60 s 68 °C elongation. Amounts of cDNA used in PCR reactions for each gene were individually determined to generate a linear amplification. Primers used for RT-PCR were: MMP-9 (accession number NM-004994) forward GGAGTACCTGGCGATTCTCAGGGG, reverse GCGTTTTCCTCCTGAAGACGG, CTGGGAAGACGG, inhibitor of matrix metalloproteinase-1 TIMP-1; accession number M12670) forward GGAATGCACAGTTGTTCCT, reverse GAAGCTCTTTCTGAGGCTT; and GAPDH (accession number J02642) forward ACGAGATCTGTCGTAGG, reverse TGATTITGGAAGATCCTGG. 

RESULTS

Biochemical Characterization of the ~125-kDa Urinary MMP Activity

Substrate Gel Electrophoresis of MMP Activities in Urine Samples—MMP activities contained in urine samples obtained from patients with breast cancer were assayed using substrate gel electrophoresis with gelatin as a substrate. 50 μl of freshly thawed urine samples were used in the analysis. At least three major MMP activities were readily detected in these urine samples, with apparent molecular masses of ~125,000, ~86,000, and ~65,000 (Fig. 1A, lanes 1, 2, and 3). Typically, no detectable MMP activity was found in the urine from normal controls (Fig. 1A, lane 4). The 86-kDa and the 68-kDa MMP activities have previously been determined to be MMP-9 and MMP-2, respectively (1). The identity of the 125-kDa urinary MMP remained unknown. Since the apparent size of the 125-kDa urinary MMP activity was similar to that reported for the MMP-9-NGAL complex purified from human neutrophils (16), the possibility that this 125 urinary MMP might be a complex of MMP-9 and NGAL was investigated. When analyzed together with purified human MMP-9-NGAL complex from neutrophils, the 125-kDa urinary MMP activity migrated at the same position as that of human neutrophil MMP-9-NGAL complex (Fig. 1B), suggesting that this 125-kDa urinary MMP could be a complex of MMP-9 and NGAL. These urinary gelatinolytic activities, excluding the 125-kDa activity, could be readily inhibited by 1,10-PA, a general metalloenzyme inhibitor, at a final concentration of 10 μM, verifying that they are indeed MMPs (data not shown).

Western Blot Analysis of Urine Samples—To further support the identification of the 125-kDa urinary MMP as being a complex of MMP-9 and NGAL, urine samples were subjected to Western blot analysis using a purified antibody against human NGAL (16), as well as a monospecific anti-human-MMP-9 antibody. Under non-reducing conditions, a protein band of ~125 kDa was consistently detected in urine samples that contain the 125-kDa MMP activity (Fig. 1C). The specificity of the NGAL antibody was confirmed using the purified human neutrophil MMP-9-NGAL complex. Under non-reducing conditions, the antibody recognized the 125-kDa MMP-9-NGAL complex in the concentrated urine sample, as well as the MMP-NGAL complex from patients with breast carcinoma (lanes 1, 2, and 3). Their identities are indicated by arrows on the right. In contrast, no MMP activities were detected in those samples from normal controls. The urine samples analyzed here are representative of ~50 urine samples from patients with breast carcinoma and normal controls. The molecular size markers are Perfect Protein Markers (Novagen). B, comigration of the ~125-kDa urinary MMP with purified human neutrophil MMP-9-NGAL complex. A representative urine sample containing the ~125-kDa MMP activity was analyzed together with purified human neutrophil MMP-9-NGAL, were separated on a 14% SDS-gel under non-reducing conditions and were subsequently subjected to Western blot analysis using a purified antibody against human NGAL. The positions of MMP-9-NGAL, MMP-9, and MMP-2 are denoted with arrows on the right. The molecular size markers are Perfect Protein Markers (Novagen). C, NGAL Western blot analysis. A representative urine sample containing the ~125-kDa MMP activity, together with purified human neutrophil MMP-9-NGAL, were separated on a 4–15% SDS-gel under non-reducing conditions and were subsequently subjected to Western blot analysis using monospecific antibody against human MMP-9. The positions of MMP-9-NGAL complex as well as free MMP-9 are marked (arrows on right).

Immunoprecipitation-Zymography—The identity of the ~125-kDa urinary MMP activity was further verified by immunoprecipitation. Urinary MMP activities that exist in the complex form with NGAL were immunoprecipitated using the purified anti-NGAL antibody and the monospecific anti-MMP-9 antibody. As shown in Fig. 2A, the anti-NGAL antibody specifically immunoprecipitated the 125-kDa urinary MMP activity, in a concentration-dependent manner. Increasing amounts of the 125-kDa urinary MMP activity was immunoprecipitated by elevating concentrations of the anti-NGAL antibody. When treated with the highest amount of anti-NGAL antibody, the 125-kDa MMP activity appeared to be completely removed from the urine. This anti-NGAL antibody had no apparent effect on any other MMP activities, i.e. those of the MMP-9-NGAL complex purified from human neutrophils (Fig. 1C). Similarly, the 125-kDa protein band was also detected in the same urine sample, as well as in the purified neutrophil MMP-9-NGAL complex using a monospecific anti-MMP-9 antibody (Fig. 1D). This antibody also detected the free MMP-9 of ~86 kDa with no cross-reaction to MMP-2 contained in the urine.

Fig. 1. Biochemical characterization of the ~125-kDa urinary MMP activity. A, substrate gel electrophoresis of representative urine samples from breast cancer patients and normal controls. 50 μl of untreated urine samples were analyzed for MMP activities. Three major gelatinase activities were detected with apparent molecular weights of ~125 kDa, ~86 kDa, and ~68 kDa in urine samples from patients with breast carcinoma (lanes 1, 2, and 3). Their identities are indicated by arrows on the right. In contrast, no MMP activities were detected in those samples from normal controls. The urine samples analyzed here are representative of ~50 urine samples from patients with breast carcinoma and normal controls. The molecular size markers are Perfect Protein Markers (Novagen). B, comigration of the ~125-kDa urinary MMP with purified human neutrophil MMP-9-NGAL complex. A representative urine sample containing the ~125-kDa MMP activity was analyzed together with purified human neutrophil MMP-9-NGAL, were separated on a 4–15% SDS-gel under non-reducing conditions and were subsequently subjected to Western blot analysis using a purified antibody against human NGAL. The positions of MMP-9-NGAL, MMP-9, and MMP-2 are denoted with arrows on the right. The molecular size markers are Perfect Protein Markers (Novagen). C, NGAL Western blot analysis. A representative urine sample containing the ~125-kDa MMP activity, together with purified human neutrophil MMP-9-NGAL, were separated on a 4–15% SDS-gel under non-reducing conditions and were subsequently subjected to Western blot analysis using a purified antibody against human NGAL. The positions of MMP-9-NGAL, MMP-9, and MMP-2 are denoted with arrows on the right. D, MMP-9 Western blot analysis. A representative urine sample containing the ~125-kDa MMP activity, together with purified human neutrophil MMP-9-NGAL, were separated on a 4–15% SDS-gel under non-reducing conditions and were subsequently subjected to Western blot analysis using a purified antibody against human NGAL. The positions of MMP-9-NGAL, MMP-9, and MMP-2 are denoted with arrows on the right.
86-kDa MMP-9 or the 68-kDa MMP-2. The identity of the 125-kDa MMP activity was further explored by immunoprecipitation using the monospecific anti-MMP-9 antibody. Increasing amounts of both the 125-kDa MMP activity and the MMP-9 activity were specifically precipitated by the anti-MMP-9 antibody in a dose-dependent manner. Importantly, the MMP-2 activity was not affected by the anti-MMP-9 antibody (Fig. 2A). The specificity of immunoprecipitation was also confirmed using a non-immune control antibody that did not immunoprecipitate any of the MMP activities, even at the highest concentration (Fig. 2A). Taken together, these data support the finding that the ~125-kDa MMP activity in the urine of cancer patients is most probably a complex of MMP-9 and NGAL.

**In Vitro Reconstitution of the MMP-9-NGAL Complex**

After the identification of the 125-kDa gelatinase activity in the urine of cancer patients as being a complex of MMP-9 and NGAL, in vitro reconstitution experiments were performed to study the formation of the MMP-9-NGAL complex. Recombinant human MMP-9 and human NGAL were initially diluted in gelatinase buffers with different pH values (5.5, 7.0, 7.6, and 8.0) that resemble the pH range of normal urine. Diluted MMP-9 and NGAL were subsequently mixed in a molar ratio of 1:10 to final concentrations of 26 nM and 260 nM, respectively. After incubation at 37°C for 1 h, the formation of MMP-9-NGAL complexes was monitored using substrate gel electrophoresis. Two MMP activities of ~125 kDa and ~115 kDa formed in these reconstitution experiments.

The possibility that the MMP-9-NGAL complex could form in urine was directly tested by diluting MMP-9 and NGAL in normal control urine that contained no MMP activities. Diluted MMP-9 and NGAL were mixed in the molar ratio of 1:20 (MMP-9:NGAL) and were incubated at 37°C for 1 h. The formation of the 125-kDa gelatinase activity was detected, as was a 115-kDa MMP-9:NGAL complex (Fig. 3B). No MMP activity was detected in the control urine used as a diluent.

Immunoprecipitation was carried out to further verify that the 125-kDa and the 115-kDa gelatinase activities formed in vitro are indeed MMP-9-NGAL complexes. Purified anti-NGAL antibody was added to MMP-9 and NGAL mixtures at pH 7.0 and 8.0. Complexes containing NGAL were subsequently removed by immunoprecipitation. The anti-NGAL antibody specifically immunoprecipitated both the 125-kDa and the 115-kDa gelatinase complexes, demonstrating that both of these activities are indeed MMP-9-NGAL complexes (Fig. 3C).

We noted that in the in vitro reconstitution experiments, the 115-kDa enzyme complex appeared to be the major complex formed by mixing recombinant MMP-9 and NGAL. Given the fact that the monomeric form NGAL represents the major protein (>95%) in the recombinant NGAL used in the study as revealed by protein silver staining (Fig. 4A), one would expect that the 115-kDa form is the major complex generated in the in vitro reconstitution experiments. Since both the 115- and the

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**Fig. 2. Immunoprecipitation of the urinary 125-kDa MMP activity using anti-NGAL antibody and anti-MMP-9 antibody.** A, 50 μl of urine samples (1:1 v/v diluted with RIPA) containing the 125-kDa MMP activity were mixed with increasing amounts of anti-NGAL antibody, anti-MMP-9 antibody, or a non-immune control antibody. After incubating on ice for 30 min, the antibody-antigen complexes were removed by immunoprecipitation. The supernatants were subjected to substrate gel electrophoresis to detect the remaining MMP activities. The 125-kDa MMP antigen complexes were removed by anti-MMP-9 antibody, or a non-immune control antibody. After incubating on ice for 30 min, the antibody of urine samples (1:1 v/v diluted with RIPA) containing the 125-kDa MMP activity were mixed with increasing amounts of anti-NGAL antibody, NGAL complexes occurred in the supernatants. Formation of these MMP-9/NGAL complexes was monitored using substrate gel electrophoresis. Mixing of MMP-9 and NGAL generated two MMP activities with molecular sizes of ~125 kDa and ~115 kDa. These species and MMP-9 are denoted with the arrows. Purified human neutrophil MMP-9:NGAL was included as a control. B, recombinant human MMP-9 and NGAL were diluted in normal urine containing no MMP activities and were subsequently mixed in molar ratio of 1:20 (MMP-9 to NGAL). After 1 h of incubation at 37°C, MMP-9:NGAL complex formation was analyzed using substrate gel electrophoresis. Two MMP activities of ~125 kDa and ~115 kDa formed in these reconstitution experiments. C, immunoprecipitation of MMP-9-NGAL complexes formed in reconstitution experiments. One μl of the purified anti-NGAL antibody was added into MMP-9:NGAL (1:10) mixtures and incubated at 37°C for 1 h. Immunoprecipitation was carried out as described in Fig. 2. The 125-kDa as well as the 115-kDa complexes were readily immunoprecipitated using the purified anti-NGAL antibody under both pH conditions. The positions of the 125-kDa and the 115-kDa MMP-9:NGAL and MMP-9 are denoted with the arrows. The molecular size markers are Perfect Protein Markers (Novagen).

**Fig. 3. In vitro reconstitution of MMP-9-NGAL complexes.** A, recombinant human MMP-9 and NGAL were diluted in gelatinase buffers with different pH values and were then mixed in a molar ratio of 1:10 (MMP-9 to NGAL). In vitro reconstitution was carried out at 37°C for 1 h. These reconstitution experiments resulted in the generation of the ~125-kDa MMP activity as well as a MMP activity migrating at ~115 kDa. These species and MMP-9 are denoted with the arrows. Purified human neutrophil MMP-9:NGAL was included as a control. B, recombinant human MMP-9 and NGAL were diluted in normal urine containing no MMP activities and were subsequently mixed in molar ratio of 1:20 (MMP-9 to NGAL). After 1 h of incubation at 37°C, MMP-9:NGAL complex formation was analyzed using substrate gel electrophoresis. Two MMP activities of ~125 kDa and ~115 kDa formed in these reconstitution experiments. C, immunoprecipitation of MMP-9-NGAL complexes formed in reconstitution experiments. One μl of the purified anti-NGAL antibody was added into MMP-9:NGAL (1:10) mixtures and incubated at 37°C for 1 h. Immunoprecipitation was carried out as described in Fig. 2. The 125-kDa as well as the 115-kDa complexes were readily immunoprecipitated using the purified anti-NGAL antibody under both pH conditions. The positions of the 125-kDa and the 115-kDa MMP-9:NGAL and MMP-9 are denoted with the arrows. The molecular size markers are Perfect Protein Markers (Novagen).
The potential biochemical consequences of the interaction between MMP-9 and NGAL was investigated using recombinant MMP-9 and NGAL. We first analyzed these two recombinant proteins by protein silver staining to determine the purity of proteins being studied. In each case, only a single protein band was observed in the MMP-9 or the NGAL protein preparation. Each sample was separated on a 4–15% gradient gel that were subsequently silver stained. In each case, a single protein band was detected in the MMP-9 or the NGAL protein preparation. The identities of proteins being studied. In each case, only a single protein band was observed in the MMP-9 or the NGAL protein preparation. The identities of the proteins were confirmed by immuno precipitation studies using the anti-NGAL antibody (Fig. 2B). Taken together, these results showed that MMP-9 and NGAL can be co-purified from cell lysates and may form complexes in vitro, with a molecular weight of approximately 150 kDa.

In the case of MMP-9-NGAL complexes in the urine of cancer patients, the 125-kDa gelatinase activity was the major form detected. In addition, we did in fact detect the presence of both the 125-kDa and the 115-kDa MMP activities in some urine samples from breast cancer patients (Fig. 2B). Their identities as being MMP-9-NGLA complexes were confirmed by immunoprecipitation studies using the anti-NGAL antibody (Fig. 2B). Taken together, our data showed that MMP-9 and NGAL are capable of forming MMP-9-NGAL complexes with preserved MMP activities that are consistent with those activities detected in the urine of cancer patients.

Protection of MMP-9 from Degradation by NGAL in Vitro

The effect of NGAL on MMP-9 degradation in vitro was then studied by mixing MMP-9 (0.1 µM) and NGAL (1.0 µM) prior to the incubation. In the presence of NGAL, MMP-9 degradation was readily inhibited resulting in a decrease in the enzymatic degradation rate as evidenced by an increase in the remaining amounts of enzyme at each time point compared with MMP-9 incubated by itself (Fig. 4C). The protection of MMP-9 from degradation by NGAL was further investigated using an immunodepleted NGAL preparation. In contrast to the inhibitory effect of intact NGAL on MMP-9 degradation, immunodepleted NGAL had no apparent protection of MMP-9. MMP-9 degradation occurred at a similar rate (Fig. 4D) as when MMP-9 was incubated alone (Fig. 4B).

We noted that the activity of the MMP-9-NGAL complex also decreased during the incubation, and at the same time the free MMP-9 seemed to be protected in the presence of NGAL. We speculate that this may be a function of a potentially dynamic and transient nature of the interaction between MMP-9 and NGAL in vitro. This is supported by the observation that in the presence of DTSSP, a cross-linking reagent, the complexes were readily detected after incubating MMP-9 and NGAL for 2 h (Fig. 4E). However, in the absence of the cross-linking reagent, very little MMP-9-NGAL complex was detected, suggesting that NGAL may form transient complexes with MMP-9. However, since this interaction is dynamic in vitro, the dissociation of MMP-9 from NGAL would release free MMP-9 that is prone to degradation, thereby resulting in a decrease in the free MMP-9 pool. This loss of free MMP-9 during the incubation would be subsequently reflected as decreases in the formation of MMP-9-NGAL complexes.

The protective effect of NGAL on MMP-9 degradation in vitro was further characterized by mixing MMP-9 with increasing amounts of NGAL, resulting in molar ratios of MMP-9:NGAL of 10:1, 5:1, 1:1, 1:5, 1:10, and 1:20. The protective effect of NGAL was determined by monitoring enzymatic activity of the remaining MMP-9 using substrate electrophoresis. These results clearly demonstrated a dose-dependent protection of MMP-9 from degradation by NGAL (Fig. 5A). In the presence of increasing amounts of NGAL, degradation of MMP-9 decreased and resulted in an increase in the remaining MMP-9 activity. The modulation of MMP-9 activity by NGAL was also investigated in buffers with different pH values. Protection of MMP-9 from degradation by NGAL was effective at all of the pH conditions tested (Fig. 5B). As quantified by scanning densitometry analysis, it was evident that NGAL was capable of protecting MMP-9 since more MMP-9 activity remained in the presence of NGAL as compared with MMP-9 incubated alone. Taken together, NGAL appears to be capable of protecting MMP-9 from degradation in a dose-dependent manner, resulting in the preservation of MMP-9 activity.

Protection of MMP-9 from Degradation by NGAL in Cell Culture

The protective effect of NGAL on MMP-9 degradation was further investigated in cell culture using MDA-MB-231 human breast carcinoma cells. Elevated levels of NGAL in medium...
conditioned by cells stably transfected with NGAL expression constructs (N-2 and N-5) were confirmed by Western analysis using the anti-NGAL antibody (Fig. 6A). In contrast, neither the parental cells (231) nor empty vector-transfected cells (C-3) expressed any appreciable amounts of NGAL (Fig. 6A). MMP-9 activity elaborated by these cells was then determined using substrate zymography analysis of serum-free medium conditioned by these cells. Only trace amounts of MMP-9 were detected either in the parental cells (231) or in control vector-transfected cells (C-3). However, MMP-9 activity was detected in cells overexpressing NGAL (N-2 and N-5) (Fig. 6B). Therefore, it appeared that elevated NGAL expression resulted in an increase in MMP-9 activity.

The modulation of MMP-9 activity by NGAL was further investigated to determine whether this increase in MMP-9 activity in NGAL-overexpressing cells was directly caused by changes in MMP-9 transcription or indirectly on the posttranslational level. Steady state MMP-9 mRNA levels were determined using RT-PCR analysis. No apparent differences in MMP-9 mRNA were detected among these cells (Fig. 6C). At the same time, expression levels of the endogenous MMP-9 inhibitor, TIMP-1, and a house-keeping gene, GAPDH, were also determined. Overexpression of NGAL had no apparent influence on mRNA levels of TIMP-1 or GAPDH (Fig. 6C). Combined with our in vitro results, it is likely that the increases in MMP-9 activity in cells overexpressing NGAL resulted from the protection of MMP-9 by NGAL.

**DISCUSSION**

In this study, we report the first identification of a high molecular weight MMP in the urine of cancer patients that was previously shown to be predictive of disease status (1). This newly identified urinary MMP species is a complex of MMP-9 and NGAL. Several independent lines of evidence support this finding: a) the ~125-kDa urinary MMP activity comigrates with the purified human neutrophil MMP-9-NGAL complex; b) a purified anti-NGAL antibody as well as a monospecific anti-MMP-9 antibody consistently detected the 125-kDa protein band in urine samples that has the ~125-kDa MMP activity; c) these same antibodies specifically immunoprecipitated the ~125-kDa MMP activity in urine in a concentration-dependent manner; and d) the ~125-kDa MMP activity can be reconstituted in vitro by mixing MMP-9 and NGAL.
As we previously reported, intact MMP activities could be detected from a small volume (30 μl) of urine sample directly collected from cancer patients without further preparation (1). This detection of MMP activities was shown to correlate with disease status. MMP-2, MMP-9, or both, and the high molecular weight MMPs serve as independent predictors of organ-confined or metastatic cancers, respectively. Identification of these high molecular weight MMPs in urine of cancer patients may be useful in the development of non-invasive markers for selecting cancer therapeutics and for monitoring of clinical cancer therapies.

Data presented in this study demonstrated that the ~125-kDa MMP activity in the urine of cancer patients is a complex of MMP-9 and NGAL. NGAL was first identified as a 25-kDa protein copurified with human neutrophil gelatinase B (MMP-9) (16). Binding of NGAL and MMP-9 has been shown to result in a gelatinase activity of ~135 kDa detected in specific granules of human neutrophils stimulated with phorbol myristate acetate (16). In neutrophils, NGAL and MMP-9 are stored in specific granules, while MMP-9 is also present independently in gelatinase granules (21, 22). The MMP-9-NGAL complex detected in the urine of cancer patients is probably not directly derived from leucocytes in the urine samples since we specifically excluded any urine samples that contained leucocytes. However, it remains possible that the urinary MMP-9-NGAL complex may be composed of MMP-9 and NGAL secreted by neutrophils that have infiltrated into the tumor sites.

Interestingly, human NGAL contains sequence similarities to mouse 24p3 and rat neu/HER2/c-erbB-2-related lipocalin, both overexpressed in oncogene-mediated cell transformation (10, 14, 15). Under normal conditions, expression of human NGAL is restricted to breast, lung, trachea, and bone marrow (10, 15). However, elevated levels of NGAL expression have been reported in human breast tumors as well as in adenocarcinomas of lung, colon, and pancreas (11–13). This suggests that an increased production of NGAL by tumor cells may be associated with the tumorigenic phenotype and might contribute to the elevated levels of MMP-9-NGAL complex in the urine. The presence of this complex can be detected with substrate gel electrophoresis as well as by antibody-based assays.

The source of the ~125-kDa MMP-9-NGAL activity in the urine of cancer patients is currently under investigation. Given that the glomerular filtration limit is ~45 kDa (23), it is unlikely that this large protein complex could be directly filtered from serum into urine. The possibility that the MMP-9-NGAL complex forms in urine was investigated using an in vitro reconstitution assay. Our results demonstrate the feasibility of the formation of the 125-kDa MMP activity in gelatinase buffers with pH values in the range of urinary pH as well as in normal urine. In addition to the 125-kDa species, a 115-kDa activity was also observed in our in vitro reconstitution experiment. At least three possibilities exist to explain the formation of these two forms. NGAL has been shown to form dimers in complex with the monomeric NGAL. Finally, the possibility exists that the formation of MMP-9 and NGAL complex results in protein conformational changes that interfere with protein migration under non-reducing conditions. Immunoprecipitation experiments using the purified anti-NGAL antibody demonstrated that both the 125-kDa and 115-kDa gelatinase activity formed in vitro by mixing MMP-9 and NGAL are indeed complexes of MMP-9 and NGAL. The 125-kDa proteolytic activity formed in vitro has the same apparent molecular size as the gelatinase activity detected in urine samples from cancer patients. We did in fact, detect the presence of the 125-kDa as well as the 115-kDa activities in some urine samples from breast cancer patients. The identity of these activities being the complexes of MMP-9-NGAL was also confirmed by immunoprecipitation experiments using the anti-NGAL antibody (Fig. 2B). Therefore, the possibility exists that MMP-9 and NGAL are separately excreted into urine where they subsequently form MMP-9-NGAL complexes.

The effect of NGAL on MMP-9 was studied using an in vitro system employing recombinant human MMP-9 and NGAL, as well as in a cell culture system in which NGAL was overexpressed in MDA-MB-231 human breast carcinoma cells. In both of these systems, we observed the protection of MMP-9 from degradation by NGAL. This dose-dependent protective effect inhibited MMP-9 from degradation and resulted in a significant increase in MMP-9 activity. The protection of MMP-9 from degradation likely resulted from the interaction between MMP-9 and NGAL since this effect occurs posttranslationally and is independent of changes in MMP-9 or TIMP-1 expression. If this protection also occurs in vivo, the elevated expression of NGAL either derived from tumor cells or from infiltrating inflammatory cells might very likely result in an increase in the local concentration of MMP-9 enzyme, which can in turn influence various aspects of tumor progression including tumor angiogenesis and metastasis (25–27). The biological significance of the modulation of MMP-9 activity by NGAL is currently under investigation.

In conclusion, we have determined the identity of a high molecular weight gelatinase activity that we have previously detected in urine samples of cancer patients (1). This proteolytic activity is a complex of MMP-9 and NGAL. NGAL appears to exert a protective effect on MMP-9 and prevents the latter from degradation both in vitro and in cells. Given the correlation between the presence of this MMP activity in urine and disease status, this newly identified MMP-9-NGAL complex may play an active role in tumor progression. Together with other MMP species in urine, they may represent a family of diagnostic targets that could be useful in both choosing the most appropriate clinical therapeutic regimen and in monitoring treatment responses.

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REFERENCES
Significance of MMP-9-NGAL Complex

The High Molecular Weight Urinary Matrix Metalloproteinase (MMP) Activity Is a Complex of Gelatinase B/MMP-9 and Neutrophil Gelatinase-associated Lipocalin (NGAL): MODULATION OF MMP-9 ACTIVITY BY NGAL

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