Urinary Type IV Collagenase: Elevated Levels Are Associated with Bladder Transitional Cell Carcinoma

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Abstract

Accumulating experimental evidence has linked the overproduction of extracellular matrix-degrading metalloproteinases with tumor cell invasion. In the present study one member of the metalloproteinase family, type IV collagenase (Mr 72,000 gelatinase), is shown to be elevated in the urine of patients with transitional cell carcinoma of the bladder. The form of the enzyme in the urine was studied by three independent methods: enzyme-linked immunosorbent assay, Western immunoblotting; and gelatin zymography. Immunoblotting revealed that the enzyme was present as a series of fragments, each retaining the amino terminus of the mature proenzyme. A prominent Mr 43,000 fragment was associated with the transitional cell carcinoma cases. Zymography demonstrated that multiple enzyme species with gelatinase activity were present in urine and that high-molecular-weight bands of substrate lysis corresponded to complexes between type IV collagenase and tissue inhibitor of metalloproteinases 2. The total amount of type IV collagenase antigen was significantly elevated in the urine of 37 transitional cell carcinoma patients (range, 0–1081 ng/ml; mean, 318.4 ± 147.3) compared to 19 normal controls (P ≤ 0.004) and 17 inflammatory disease controls (P ≤ 0.011). Immunohistochemical staining of bladder tumor biopsies verified that the transitional cell carcinoma cells were producing the Mr 72,000 enzyme. Thus, Mr 72,000 type IV collagenase, which is present in the urine in many forms including fragments and complexes with inhibitors, may be a useful marker for bladder cancer diagnosis or prognosis.

Introduction

Invasion is the most insidious aspect of bladder cancer. A high percentage of patients with noninvasive superficial TCCs 2 recur with invasive lesions. Progression to advanced stages and metastasis occurs in 20% of patients with superficial disease (1). Invasion of the bladder muscle is associated with a 60% incidence of ultimately lethal metastasis. The clinical problem indicates the need for accurate ways to detect TCC in its early stages. Because follow-up procedures may involve frequent examinations, development of less invasive diagnostic procedures is highly desirable. Current diagnostic methods to follow the clinical course of patients with bladder cancer include cytologic examination of bladder washings, urine flow cytometry, cystoscopic examination, computerized tomography, ultrasound imaging, and magnetic resonance imaging (2–5). To complement these procedures, previous investigators have attempted to identify urinary antigens which might serve as useful markers for TCC. A TCC-specific antigen has not been identified, although certain antigens in the urine have been correlated with TCC presence or TCC stage (6–10).

One approach to the identification of urine antigens associated with TCC is to choose known proteins which have been previously shown to play a role in cancer-associated mechanisms (10–13). Metalloproteinases which degrade the extracellular matrix fit this definition because they have recently been shown to be augmented in a wide variety of rodent and human tumors. One member of the metalloproteinase family, the Mr 72,000 type IV collagenase, was originally identified based on its ability to degrade type IV basement membrane collagen (14). This particular metalloproteinase was shown to be critical for tumor cell invasion by experiments in which a specific inhibitor (TIMP-2) to this enzyme prevented tumor cell invasion of the extracellular matrix in vitro (15). It has been hypothesized that Mr 72,000 type IV collagenase could facilitate the transition from in situ to invasive carcinoma. It could also play a role in the invasion of tissue structures which are encased in basement membranes including nerves, muscle, and blood vessels. Since these same basement membrane barriers are present in the bladder wall, TCC tumor cells which elaborate high levels of active Mr 72,000 type IV collagenase may have a selective advantage during tumor invasion. MMP-2 is one member of a family of at least eight matrix metalloproteinases which are secreted in a latent form requiring activation before they can degrade extracellular matrix substrate. Activation involves the removal of an amino terminal domain (16). In addition, endogenous metalloproteinase inhibitors, called TIMPs,

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2 The abbreviations used are: TCC, transitional cell carcinoma; TIMP, tissue inhibitor of metalloproteinases; ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulfate; MMP-2, Mr 72,000 type IV collagenase (Mr 72,000 gelatinase); NPD, no pathologic diagnosis.
constitute another level of enzyme regulation. Metalloproteinase activity is the result of a balance between activated enzyme and available TIMP (17). To date, two distinct members of the TIMP family have been identified: TIMP-1 and TIMP-2.

The purpose of the present study was to evaluate the levels of the M$_{72,000}$ type IV collagenase in the urine of patients with TCC compared to normal controls as well as patients with inflammatory bladder conditions. A monoclonal antibody specific for M$_{72,000}$ type IV collagenase was used in ELISA to measure the levels of enzyme in urine samples. Western blotting of urinary protein was used to evaluate whether the enzyme antigen was fragmented or complexed with other proteins. Gelatin zymography was used as an independent assay for the presence of enzyme fragments containing the active gelatinolytic fragments. In addition, we evaluated the urine for the presence of TIMP-2, the new member of the metalloproteinase inhibitor family previously shown to form a selective complex with the latent form of M$_{72,000}$ type IV collagenase (18). Finally, immunohistology was used to judge whether the M$_{72,000}$ type IV collagenase was being elaborated by the TCC cells or by the surrounding host tissue.

Materials and Methods
Patient Urine Collection. Thirty-one of 38 patient samples were obtained from patients entering Walter Reed Medical Center Urologic Clinic for routine diagnostic treatment. Twenty-four-h urine collections were obtained preoperatively from patients in a normovolemic state of hydration. The remaining 50 patient samples were spot urines obtained from patients with inflammatory conditions as well as patients with TCC. Normal volunteer urine samples were spot voids. The urine was stored frozen prior to assay. In general the urine metalloproteinase determination was conducted prior to receipt of the final pathologic diagnosis.

Gelatin Zymography. Eighteen-$\mu$l samples of unconcentrated urine were mixed with sample buffer consisting of 4% (w/v) SDS, 0.15 M Tris (pH 6.8), 20% (v/v) glycerol, and 0.5% (w/v) bromphenol blue. These were applied to a 10% SDS-acrylamide gel, containing 0.1% (w/v) gelatin as described, with modification (16, 19). One to ten ng of purified type IV collagenase from A2058 human melanoma were run concurrently as a positive control. After electrophoresis, the gels were submerged in 2.5% (v/v) Triton X-100 in order to remove the SDS prior to incubation in 0.05 M Tris, 0.2 M NaCl, and 5 mM CaCl$_2$ (pH 7.6), a buffer which supports enzyme activity. Gels were stained with 0.5% (w/v) Coomassie blue in 30% methanol/10% acetic acid and then destained in 30% methanol/10% acetic acid solution.

Western Blot Analysis. Urine samples were concentrated 10-fold by ultracentrifugation using Millipore Ultrafree-
Production and Characterization of Monoclonal Antibody. The monoclonal antibody against human M, 72,000 type IV collagenase was produced using standard protocols (21). Briefly, BALB/c mice were immunized with NH₂-terminal peptide (sequence APSPIIKFPGD-VAPKTDK) of procollagenase type IV coupled to keyhole limpet hemocyanin. The splenocytes of the immunized mouse were fused with mouse myeloma cells (P3X63Ag8,653). Hybrid cells were screened using ELISA and radioimmunoassays. The hybridomas were cloned by limiting dilution method, and the positive clone CA719E3C, which secretes CA-4001 monoclonal antibody of IgG1 subclass, was selected. The CA719E3C hybridoma was inoculated into pristane-pretreated mice to produce ascites fluids, and the monoclonal antibody in the ascites fluids was purified using high-performance liquid chromatography with a Gammabind Ultra column (Genex, Gaithersburg, MD).

ELISA Determination of Urine M, 72,000 Type IV Collagenase Concentrations. The ELISA characterization was performed by using the ELISA Kit from Kirkegaard and Perry Laboratories and Immulon Plates (DyneTech, Inc.). Serial dilutions of A2058-conditioned medium containing known quantities of M, 72,000 type IV collagenase were used to calculate a standard dilution curve. The concentrations of M, 72,000 type IV collagenase in urine samples were then estimated using this standard. The primary antibody was a monoclonal antibody CA-4001 against M, 72,000 type IV collagenase (Molecular Oncology, Inc.), which was diluted 1:1000, and the secondary antibody was goat anti-mouse IgG HRP-conjugate (BioRad), which was diluted 1:1000. The plates were read at 405 nm on a Titertek Multiskan plate reader.

Immunoperoxidase Staining on Paraffin Sections. Paraffin blocks of embedded human bladder tissue were obtained from the Surgical Pathology Department of the NIH and from Walter Reed Army Medical Center. Sections were cut 5 µm thick from formaldehyde-fixed, routinely prepared paraffin-embedded tissue.

After deparaffinizing and rehydration through xylene and graded alcohol serials, the slides were submerged for 30 min in 0.3% (v/v) hydrogen peroxide in absolute methanol to block endogenous peroxidase activity. The sections were blocked from nonspecific binding sites with 10% normal goat serum for 45 min and immediately incubated in 4 µg monoclonal M, 72,000 type IV collagenase (Molecular Oncology, Inc.), which was diluted 1:1000 and the secondary antibody goat anti-mouse IgG HRP-conjugate (BioRad), which was diluted 1:1000. The plates were read at 405 nm on a Titertek Multiskan plate reader.
dehydrated in graded alcohol serials and xylene, and mounted in permount.

Photographs were taken with an Olympus BH-2 microscope using Kodachrome 40 Type A, KPA film.

Results
Urinary samples were collected and analyzed prior to decoding the final pathologic diagnosis on the urologic clinic patients. The final patient cohort was classified into the following groups: 38 patients with transitional cell carcinoma; 17 patients with nonneoplastic inflammatory disease; and 19 patients with no pathologic diagnosis, which we considered as "normal controls." The types of inflammatory disease included cystitis, hematuria (NPD), renal stones, urethritis, hypospadia, and spermatocele. The TCCs ranged in grade and stage from inverted papilloma with mild atypia to grade III stage IV transitional cell carcinoma. Three patients of 38 were diagnosed as having a pathologic grade higher than level II. The rest of the patients fell into the category of grade I–II.

Zymography of Urine Samples. Urinary metalloprotei-nase functional activity was studied using gelatin zymography. At least 70% of the TCC urines demonstrated gelatinase activity by zymography. As shown in Fig. 1, the apparent molecular weights of gelatinolytic bands were 25,000, 45,000, 72,000, 92,000, and higher. The zymographic bands were abolished by pretreatment with EDTA (not shown), as would be predicted for metal atom (Zn)-dependent metalloproteinas.

Characterization of the Monoclonal Antibody CA-4001 against Human M, 72,000 Type IV Collagenase. The purified monoclonal antibody CA-4001 was specific against M, 72,000 type IV collagenase as determined by immunoprecipitation, ELISA, dot blot, and Western blot. As shown in Fig. 2 (Lane a), when conditioned medium from HT 1080 cells was separated by electrophoresis or gelatin-containing gels, the resulting zymogram revealed gelatinase bands consistent with HT 1080 cells producing both the proenzyme (M, 72,000) and the active (M, 62,000) forms of type IV collagenase as well as a M, 92,000 enzyme. When this same conditioned medium was separated on gels without gelatin for Western Blot analysis (Fig. 2, Lane b), the immunoblot revealed that monoclonal antibody CA-4001 reacts only with the proenzyme form of the M, 72,000 type IV collagenase. In immunoperoxidase staining of formalin-fixed, paraffin-embedded tissue the monoclonal antibody stained the tumor cell cytoplasm as previously reported for anti-peptide antibodies directed against the M, 72,000 amino terminus (16, 22, 23). When using 2.5 μg/ml of CA-4001 antibody the staining could be completely blocked with 5 μg/ml of the peptide used for immunizations, and about 50% reduction in the staining intensity was achieved with 0.5 μg/ml of the peptide (data not shown).

Western Blot for Analysis of Type IV Collagenase Fragments. The presence of the urinary antigen was verified by immunoblotting of urine samples concentrated 10-fold by ultrafiltration. Using either a monoclonal or a polyclonal antibody directed against the amino terminal peptide as primary antibody, the Western blot demonstrated that the major form of the enzyme in the urine was an amino terminal fragment with a molecular weight of 72,000. The monoclonal antibody CA-4001 (Fig. 3) recognized this major fragment species in all of the TCC cases but not in any of the control cases. The polyclonal affinity-purified antibody exhibited a higher sensitivity and thereby revealed a collection of type IV collagenase fragments. As shown in Fig. 4, the polyclonal antipeptide antibody blots demonstrated that the M, 45,000 fragment was more prominent in the TCC patients compared to the controls.

Western Blot for Analysis of Enzyme-Inhibitor Complexes. It has been previously established that latent M, 72,000 type IV collagenase forms a specific complex with TIMP-2, a new member of the tissue inhibitor metalloproteinase family. Western bloting using a specific antiTIMP-2 antibody (which fails to recognize TIMP-1) was used to investigate potential complex formation. Without reduction of disulfide bonds, significant levels of TIMP-2 enzyme complex were detected with apparent molecular weights of M, 93,000 and ≥ M, 200,000 as shown in Fig.
This is consistent with studies of specific high-molecular-weight complex formation as previously described (24). Because the specific nature of the complex is known to require intact disulfide bonds, we treated samples with 2-mercaptoethanol. As shown in Fig. 5B, all of the high-molecular-weight complexes were eliminated following this reduction.

**ELISA Determination of Type IV Collagenase Concentrations.** The direct ELISA assay in the present study used the mouse monoclonal antibody CA-4001, which recognizes the amino terminal peptide of the latent form of the Mr 72,000 type IV collagenase. Specificity was verified by competition with a synthetic peptide corresponding to this antigen (16). The urine was not concentrated prior to ELISA. Final metalloproteinase concentration levels in the urine were calculated by running a full dilution curve on each sample compared with a parallel dilution curve using known amounts of human Mr 72,000 type IV collagenase (Fig. 6).

As shown in Fig. 7, the enzyme concentration was significantly elevated in the urine of patients with transitional cell carcinoma of all grades compared to normal controls or inflammatory controls. The mean collagenase level for the TCC group as a whole was 318.5 ng/ml (Table 1). Analysis of the subgroup diagnosed as having stage TIII TCC (Table 1) demonstrates significantly elevated (P ≤ 0.001) levels of Type IV collagenase compared to normals. When the ELISA results were compared with those from immunoblots, a general concordance was observed.

**Immunoperoxidase Staining of TCC Sections.** The potentially relevant source of urinary metalloproteinase in pa-
patients with transitional cell carcinoma was thought to be the tumor cells themselves. In order to test this assumption, immunoperoxidase staining using the same monoclonal antibody CA-4001 used in the ELISA and Western blot studies was conducted on histopathological sections of human transitional cell carcinoma. As previously reported for anti-peptide antibodies directed against the Mr 72,000 amino terminus (16, 22, 23), immunoreactivity is preserved in formalin-fixed, paraffin-embedded tissue. Positive-staining controls could be competed with the antigen peptide to verify specificity. Sections of normal urinary bladder epithelium (Fig. 8A) were negative for immunoreactivity. In contrast, sections of papillary (Fig. 8B) and transitional cell carcinoma were positive. The staining was localized predominantly to the cytoplasm of the neoplastic epithelium. These results support the assumption that the source of the urinary Mr 72,000 type IV collagenase as well as enzyme inhibitor complexes and enzyme fragments were at least partially derived from the carcinoma itself. Thus, urinary Mr 72,000 type IV collagenase levels may serve as a useful marker of TCC.

Discussion

In the present study, urinary levels of the extracellular matrix-degrading metalloproteinase MMP-2 were compared in patients falling into three classes: (a) normal; (b) with a diagnosis of transitional cell carcinoma of the bladder; and (c) with nonneoplastic inflammatory conditions. Three different methods were used to analyze the urinary antigen: zymography; Western blotting; and ELISA. Zymography demonstrated direct enzyme activity, whereas ELISA and Western blotting detected immunoreactive enzyme. Immunohistochemistry verified direct production of the antigen by carcinoma cells. Both polyclonal and monoclonal antipeptide antibodies, directed against a peptide comprising the amino terminal end of the latent enzyme, were used in the immunoassays. The advantage of these antibodies is that the peptide is unique for this enzyme (14), and the peptide itself can be used to compete for antigen recognition in the immunoassay. The results, derived from all of the methods, demonstrated an increase in levels of the MMP-2 enzyme in cancer patients compared with patients with inflammatory conditions or with normal controls (Fig. 7). Although there is an overlap in individual values of the urine enzyme concentration in patients with transitional cell carcinoma compared to normals and inflammatory controls, there is a highly statistically significant difference between the groups. Moreover, all the tumors in this limited series which were classified as stage III or higher were associated with urine enzyme values above the median. This finding is in keeping with the concept that the invasive phenotype is associated with the increased elaboration of matrix-degrading metalloproteinases.

Direct ELISA assays were conducted on whole urine, without prior concentration. Twelve- or 24-h collections were used to control for excretion rates. A full dilution curve with each patient’s unknown sample was compared with a dilution curve for known antigen to calculate the final urinary concentration. The concentrations ranged from 0 ng/ml to more than 1000 ng/ml. It is likely that a significant proportion of the enzyme was derived directly from the bladder tumor mass. As shown by immunohistochemistry, the carcinoma cells clearly produce the enzyme. In vitro studies with cultured tumor cells have shown this enzyme to be secreted into the conditioned media (20).

The Western blotting studies (Fig. 4) corroborated the ELISA assays in every sample examined. In addition, the Western blotting revealed that a large percentage of the Mr 72,000 antigen had been fragmented into smaller pieces. The predominant species migrated with an apparent size of Mr 44,000. Zymography demonstrated that this fragment, when present, retained gelatinolytic activity in some but not all patients. This suggests that this Mr 44,000 fragment may retain a portion of the enzyme-active site. However, the proteolytic activity of this truncated active site may be less stable than the native

**Table 1** Comparison of type IV collagenase concentrations in the urine of the cohort subgroups

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Collagenase* (ng/ml (mean ± SEM))</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (19)</td>
<td>73.1 ± 23.4</td>
<td></td>
</tr>
<tr>
<td>Inflammatory (17)</td>
<td>86.8 ± 24.2</td>
<td>0.64</td>
</tr>
<tr>
<td>Benign prostatic hyper trophy (9)</td>
<td>87.3 ± 36.0</td>
<td>0.74</td>
</tr>
<tr>
<td>Renal/prostate (5)</td>
<td>76.3 ± 61.7</td>
<td>0.95</td>
</tr>
<tr>
<td>TCC (37)</td>
<td>318.4 ± 147.3</td>
<td>0.004</td>
</tr>
<tr>
<td>TCC, TII, and TIV (3)</td>
<td>837.4 ± 147.3</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Collagenase concentrations were measured by ELISA using a parallel assay with purified type IV collagenase of known concentration as a standard curve.
* P values were calculated by t test, comparing each cohort subgroup with the normal controls.
results clearly indicated that a significant proportion of the enzyme (more than half) was complexed with TIMP-2. Furthermore, the high-molecular-weight gelatinase activity, which was seen on zymography, appeared to be related to these complexes. The basement membrane surrounding the bladder muscle and underlying the transitional cell epithelium is breached during invasion and metastatic progression. Several different studies have reported a correlation between the increased loss of type IV basement membrane collagen in bladder tumors and invasive stage, clinical aggressiveness, and metastasis (11, 13, 25). Type IV collagen is a substrate for the Mr 72,000 enzyme. Therefore, the increased production or activation of this enzyme might be causally related to certain aspects of invasion. It remains to be determined exactly what percentage of the urine enzyme is in the latent or in the conformationally activated form. It is also uncertain whether the increased proteolysis may be due to reductions in TIMP-2 rather than increased molar production of enzyme. However, the results of the present study suggest that measurement of urinary levels of the Mr 72,000 type IV collagenase enzyme, in all its various forms and fragments, may provide a noninvasive method of monitoring the development and progression of transitional cell tumors in patients.

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References


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