Increased Incidence of Matrix Metalloproteinases in Urine of Cancer Patients

Marsha A. Moses, Dmitri Wiederschain, Kevin R. Loughlin, David Zurakowski, Carolyn C. Lamb, and Michael R. Freeman

Abstract

Matrix metalloproteinases (MMPs) have been implicated in mechanisms of metastasis in experimental cancer models and in human malignancies. In this study, we used substrate gel electrophoresis to determine the frequency of detection of MMPs in urine of patients with a variety of cancers. Three molecular weight classes of urinary MMPs, Mr 72,000, Mr 92,000, and high molecular weight (Mr ≥150,000), were detected reproducibly and correlated with disease status. The Mr 72,000 and Mr 92,000 species were identified as MMP-2 and MMP-9, respectively, by Western blot analysis. The presence of biologically active MMP-2 (P < 0.001) or MMP-9 (P = 0.002) was an independent predictor of organ-confined cancer, and the high molecular weight species (P < 0.001) was an independent predictor of metastatic cancer. This is the first study to demonstrate that analysis of urinary MMPs may be useful in determining disease status in a variety of human cancers, both within and outside of the urinary tract.

Introduction

Tumor cells metastasize to distant sites by disassembling the complex extracellular matrices that delimit tissue spaces and surround blood vessels. A specialized class of matrix-degrading enzymes, the MMPs, has been implicated in metastasis in experimental models and in human tumors. Elevated production of MMP enzymes by cancer cells and tumor stroma correlates with the malignant or metastatic phenotype. Overproduction of MMPs by a tumor communicating with the vascular and lymphatic systems might result in increased levels of MMP activity in other body fluids, such as blood or urine. This possibility is consistent with previous demonstrations that levels of other regulatory molecules overproduced by tumors and measured in body fluids of cancer patients, such as the angiogenic peptide basic fibroblast growth factor, have been shown to be independent predictors of disease status (1, 2).

Elevated levels of MMPs have been found in serum and plasma of animals bearing experimental tumors and in human patients. Serum and plasma levels of the Mr 92,000 collagenase (gelatinase B, MMP-9) were increased in rats bearing metastatic mammary carcinomas (3). In humans, serum levels of the Mr 92,000 collagenase (gelatinase A, MMP-2) have been correlated with lung cancer metastasis and have been seen to decline in response to combination chemotherapy (4). Serum levels of interstitial collagenase (MMP-1) have been and gelatinase B (MMP-2) levels have been shown to be increased in serum of patients with colon and breast cancer (5). Recently, Gohji et al. (7, 8) reported that the ratio of serum MMP-2 to tissue inhibitor of metalloproteinase 2 and serum levels of MMP-2 and MMP-3 (stromelysin-1) correlated positively with recurrence of urothelial cancer following complete tumor resection. These data are consistent with the conclusion that MMPs are important mediators of cancer progression and that MMP levels are frequently elevated in predictable ways in body fluids of cancer patients.

There has been relatively little study of the possibility that MMP analysis of human urine might provide important clinical information. In light of the potential of clinical urinary analysis for cancer diagnosis and prognosis, we addressed three questions in this study. (a) Can MMPs be detected in human urine in their intact, biologically active forms? (b) Does the detection of urinary MMPs correlate with disease status in cancer patients? (c) If such a correlation can be demonstrated, does it apply to cancers outside of the urinary tract?

Materials and Methods

Patient Urine Collection. Patients were chosen for the study, their urine was collected, and their clinical status was verified and monitored by a urologist (K. R. L.) and a radiation oncologist (C. C. L.). Urine was collected according to the institutional biotechnical guidelines pertaining to discarded clinical material. Urine samples were collected in one of two ways. Patients seen in an ambulatory care setting provided a voided specimen; those who were undergoing cystoscopic or surgical procedures were catheterized prior to the planned procedure. All patients in the NED group were treated by surgical resection. Prior to analysis, urine samples were tested for the presence of blood using Ames Multistix 7 reagent strips (Miles, Elkhart, IN), and specimens containing blood were not analyzed.

Sample Preparation and Substrate Gel Electrophoresis. Samples were frozen immediately after collection and stored frozen (−20°C) until assay. The samples were thawed, and creatinine concentrations were determined using a commercial kit (Sigma Chemical Co., St. Louis, MO) according to manufacturer’s instructions. Aliquots (1 ml) of each sample were dialyzed against double-distilled water in 18-mm dialysis tubing (Spectra/Por membrane, molecular weight cut-off (MWCO); Mr 3500, Spectrum, Houston, TX) with two exchanges of 4 liters each (per 20 urine samples). Following dialysis, urine samples were centrifuged at 4000 rpm for 5 min at 4°C, and the supernatants were collected. Urine samples (30 μl) were mixed with buffer consisting of 4% SDS, 0.15 M Tris (pH 6.8), 20% (w/v) glycerol, and 0.5% (w/v) bromphenol blue. Samples were applied, without boiling, into wells of a 4% acrylamide Laemmli stacking gel/10% SDS-acrylamide separating gel containing 0.1% (w/v) gelatin (Life Technologies, Inc., Detroit, MI) on a mini-gel apparatus as described previously (9, 10). Gels were run at 15 mA/gel during stacking and at 20 mA/gel during the resolving phase at room temperature. After electrophoresis, the gels were soaked in 2.5% Triton X-100 with gentle shaking for 15-30 min in 0.5% Coomassie Blue R-250 in acetic acid, isopropl alcohol, and water (1:3:6); destained in acetic acid, ethanol, and water (1:3:6); photographed; and dried for permanent record. Both proenzymes...
and activated proteinases appeared as zones of substrate clearing. Different MMPs were distinguished from each other on the basis of their molecular weights. The identity of these MMPs was confirmed by Western blot analysis using anti-MMP antibodies (Oncogene Science, Cambridge, MA). Antibodies purchased from Biogenesis Ltd. (Sandown, NH) and Chemicon International, Inc. (Temecula, CA) were also tested. In all cases, anti-MMP-9 antibodies cross-reacted with MMP-2. As positive controls, purified human MMP-2 and MMP-9 (Biogenesis Ltd.) were subjected to gel electrophoresis as described above. To verify that the proteolytic activities detected were metal-dependent proteinases, samples were subjected to incubation in substrate buffer in the presence of 1,10-phenanthroline, a MMP inhibitor.

Data Collection and Analysis. Zymograms were processed and evaluated independently by two investigators who had no knowledge of the clinical status of the individuals from whom the urine specimens were obtained.

Statistical Analysis. MMPs detected in the urine of patients with organ-confined disease and with metastatic cancer were compared to the normal/NED control groups. Sensitivity and specificity were calculated using standard formulas and were expressed as percentages. Ninety-five % CIs were derived using Pratt’s method (11). The likelihood ratio for a positive test result was determined as the fraction of true-positives divided by the fraction of false-positives [sensitivity/(1 – specificity)] to provide an indicator of the discriminating power for each MMP (12). Stepwise logistic regression was used to establish the independent predictors of cancer and to estimate the probability for combinations of the MMP markers in the final multivariate model (13). One-way ANOVA was performed to assess differences in creatinine levels among the groups, with a Bonferroni correction for multiple comparisons. Fisher’s exact test was used for comparison of proportions. All statistical tests were conducted using a two-sided α level of 0.05. Data analysis was performed using SAS for Windows (Version 6.11; SAS Institute Inc., Cary, NC).

Results

One hundred-seventeen urine specimens obtained at random sampling times were analyzed. Sixty-eight specimens were obtained from individuals with prostate, renal, bladder, breast, and a variety of other types of cancer (Table 1). Specimens taken from patients with organ-confined cancers were obtained prior to surgical or other therapeutic intervention. Gleason sum scores of the prostate adenocarcinomas ranged from 5 to 9, with 26 of 28 (93%) of the prostate adenocarcinomas scored as Gleason grades 5–7. Prostate cancer patients had an average serum prostate-specific antigen value of 12.4 ± 12.5 ng/ml (mean ± SD) taken near the time of sampling. The bladder cancer group included grades I–III. The renal cancer group included grades II–III. Twenty-one patients had metastatic cancer at the time of sampling. All of the breast cancer patients had metastatic disease. Thirty urine specimens were obtained from healthy male and female volunteers (normals), and 19 samples were obtained from former cancer patients (male and female) showing NED at the time of sampling.

Thirty μl of each urine sample were electrophoresed through gelatin-containing SDS-polyacrylamide gels and evaluated for the presence of zones of proteolytic activity after incubation of the gels in renaturing conditions. Representative results are shown in Fig. 1A (Lane 1). There was an association between the patients’ disease status (i.e., presence of cancer) and the presence of three gelatinase activities, a hMW form (greater than or equal to Mr 150,000), a Mr 92,000 form, and a Mr 72,000 species (Table 2). The frequencies of detection of one or more of these three urinary MMP species in each experimental group were: normal plus NED, 8 of 49 (16%); NED, 2 of 19 (11%); normal, 6 of 30 (20%); cancer, 48 of 68 (71%); and metastatic cancer, 19 of 21 (90%). The frequency of detection of at least one MMP species was significantly higher for both the cancer group and the metastatic cancer group, compared to each control group (P < 0.001). There were no significant differences between the

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Table 1 Patient population

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>Prostate</th>
<th>Renal</th>
<th>Bladder</th>
<th>Breast</th>
<th>Other</th>
<th>NED*</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>28</td>
<td>10</td>
<td>10</td>
<td>9</td>
<td>11</td>
<td>19</td>
<td>30</td>
</tr>
<tr>
<td>Mean age (yr)*</td>
<td>63 ± 6.4</td>
<td>61 ± 11</td>
<td>64 ± 16</td>
<td>49 ± 11</td>
<td>49 ± 21</td>
<td>68 ± 6.9</td>
<td>32 ± 6.0</td>
</tr>
<tr>
<td>Female/male</td>
<td>0/28</td>
<td>4/6</td>
<td>2/8</td>
<td>9/0</td>
<td>6/5</td>
<td>6/13</td>
<td>8/22</td>
</tr>
<tr>
<td>Detectable primary tumors</td>
<td>28</td>
<td>10</td>
<td>10</td>
<td>9</td>
<td>11</td>
<td>NA*</td>
<td>NA</td>
</tr>
<tr>
<td>Detectable metastases</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>9</td>
<td>7</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

* Undetectable disease.

* Values are expressed as mean ± SD.

* NA, not applicable.
Zymographic profiles were identical in repeat assays of the same urine cancer patients (66%) but not in any of the other urine specimens. The urine of five of the specimens obtained from metastatic breast cancers, although this did not reach statistical significance ($P = 0.46$). The detection frequencies of one or more of these three MMPs were significantly higher for prostate (21 of 28, 75%), bladder (8 of 10, 80%), and breast (9 of 9, 100%) cancers as compared to the control groups ($P < 0.001$).

In all specimens examined, four molecular weight classes of gelatin-degrading activity were frequently observed: the hMW class was seen in 48 (41%) of the samples; the $M_r$ 92,000 form was seen in 52 (44%); the $M_r$ 72,000 form was seen in 23 (20%); and a $M_r$ 20,000 form was seen in 34 (29%). Gelatinases with $M_r$s of 100,000-116,000 and 45,000 were detected in 4 (3.4%) and 5 (4.3%) of the specimens, respectively. A gelatinase of approximately 125,000 was detected in 52 (44%); the $M_r$ 72,000 form was seen in 23 (20%); and a $M_r$ 20,000 form was evident after storage for over 24 h at 4°C.

The hMW gelatinase, but not the $M_r$ 92,000 or $M_r$ 72,000 species, was confined cancers, although this did not reach statistical significance ($P = 0.08$). The difference in detection of at least one MMP was higher for metastatic cancers than it was for organ-confined cancers, although this did not reach statistical significance ($P = 0.08$). The detection frequencies of one or more of these three MMPs were significantly higher for prostate (21 of 28, 75%), bladder (8 of 10, 80%), and breast (9 of 9, 100%) cancers as compared to the control groups ($P < 0.001$).

The hMW gelatinase, but not the $M_r$ 92,000 or $M_r$ 72,000 species, was confined cancers, although this did not reach statistical significance ($P = 0.08$). The difference in detection of at least one of these gelatinases was detected and 8% when it is not detected. For organ-confined cancers, the probability of cancer is 96% when both the $M_r$ 92,000 and $M_r$ 72,000 gelatinases are detected and 29% when neither is detected. In fact, the probability of organ-confined cancer was calculated to be over 50% when at least one of these gelatinases was detected.

The sensitivity, specificity, and likelihood ratios for the hMW, $M_r$ 92,000, and $M_r$ 72,000 enzymes for detection of metastatic cancer, organ-confined cancer, and all cancers (metastatic plus organ-confined) are shown in Table 4. Specificity values were highest for the $M_r$ 72,000 gelatinase, which correctly classified all but one of the 49 normal/NED patients as being cancer-free. Given that the $M_r$ 72,000 and the $M_r$ 92,000 species alone were multivariate predictors of disease status for organ-confined cancers and for outcomes of all cancers, we analyzed the statistical performance characteristics of these two markers in combination (Table 4). This analysis resulted in an increase in sensitivity in comparison to the evaluation of each marker alone. Calculation of likelihood ratios indicated that each of the hMW, $M_r$ 92,000, and $M_r$ 72,000 gelatinases had significant discriminatory power when samples were grouped into metastatic cancer, organ-confined cancer, or all cancer categories.

There was no association between MMP detection in the zymograms and urinary creatinine concentration (Table 2), indicating that the presence of gelatinase activity in urine is unlikely to be a result of variations in solute concentration or functional status of the kidney.
cancers (n = 47), and all cancers (n = 68). The results of the analysis of the combination activity detectable by zymography increases when cancer is present in cases. These data suggest that the incidence of urinary gelatinase difference in the prevalence of one or more of these MMPs in the clinical categories shown in Table 1 and between individuals with the clinical status of cancer patients was conducted by Margulies et al.

To evaluate potential differences in prevalence of MMPs between the clinical categories shown in Table 1 and between individuals with cancer and the control groups (normal plus NED), specimens were scored as positive or negative in the three major molecular weight classes in a double-blind manner. There was a statistically significant finding that intact MMPs are also present in urine and that evaluation of these enzymes provides useful information on the disease status of patients is a unique observation with potentially important clinical ramifications. It is also significant that this information was derived using small volumes (30 μl) of urine, obtained directly from patients with no sample concentration, few processing steps, and no expensive equipment. The sensitivity and specificity values obtained using the urinary hMW, M₉, 92,000, and M₉, 72,000 enzymes as clinical indicators are comparable to, or better than, those observed for a number of serum tumor markers currently in use to monitor disease recurrence (17-19). These data support the feasibility of using MMP enzyme assays as a first look clinical assay or as a means to provide correlative and supportive information in assessing the clinical course of disease in cancer patients.

Interestingly, the M₉, 72,000 enzyme demonstrated the highest specificity (98%) and highest positive likelihood ratios for organ-confined cancers (16.0) and all cancers (22.0). The likelihood ratio, which is the ratio of true-positives to false-positives, is generally considered to be the most important statistical parameter in the consideration of the usefulness of a clinical test (20). This suggests the possibility that analysis of urinary MMP-2 might provide important information in a clinical setting in which malignancy is suspected but is, as yet, undetected by other diagnostic methods.

MMP enzyme analysis might also be used in combination with other standard methods to increase the specificity and predictive value of these measurements. Our results also suggest that analysis of urinary MMPs by zymography in subsequent studies may provide additional, novel information. For example, zymography detects a series of enzyme species in a single evaluation, allowing for the interesting possibility that certain cancers may display characteristic zymographic patterns. Consistent with this possibility is our observation of a M₉, 125,000 MMP species in the breast cancer urine specimens that was not detected in any of the other pathological or control specimens.

In conclusion, this study suggests that the evaluation of urinary MMPs by zymography reflects disease status in patients with organ-confined and metastatic tumors, both within and outside the urinary tract.

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References


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