Increased Incidence of Matrix Metalloproteinases in Urine of Cancer Patients

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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 (zymography) to determine the frequency of detection of MMPs in urine of patients with a variety of cancers. Three molecular weight classes of urinary MMPs, M, 72,000, M, 92,000, and high molecular weight (M, >150,000), were detected reproducibly and correlated with disease status. The M, 72,000 and M, 92,000 species were identified as MMP-2 and MMP-9, respectively, and of these, MMP-9 is biologically active. The absence of these two MMP species correlated with organ-confined disease, and the high molecular weight species was an independent predictor of metastatic disease. 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 M, 92,000 collagenase (gelatinase B, MMP-9) were increased in rats bearing metastatic mammary carcinomas (3). In humans, serum levels of the M, 72,000 collagenase (gelatinase A, MMP-2) have been correlated with lung cancer metastasis and have been shown to be negative in response to combination chemotherapy (4). Serum levels of interstitial collagenase (MMP-1) have been reported to be increased in patients with metastatic prostate cancer (5), and gelatinase B (MMP-2) levels have been shown to be increased in serum of patients with colon and breast cancer (6). 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 collected from patients 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.

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References

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3. The abbreviations used are: MMP, matrix metalloproteinase; NED, no evidence of disease; CI, confidence interval; hMW, high molecular weight; TCC, transitional cell carcinoma.
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 discrim
inating 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

Results

One hundred-seventeen urine specimens obtained at random sam-
ping 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 adenocarci-
nomas 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 gel-
atin-containing SDS-polyacrylamide gels and evaluated for the pres-
ence 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 M, 150,000), a Mf,
92,000 form, and a M, 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

<table>
<thead>
<tr>
<th>Table 1 Patient population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer type</td>
</tr>
<tr>
<td>Prostate</td>
</tr>
<tr>
<td>---------------------------</td>
</tr>
<tr>
<td>No. of patients</td>
</tr>
<tr>
<td>Mean age (yr)*</td>
</tr>
<tr>
<td>Female/male</td>
</tr>
<tr>
<td>Detectable primary tumors</td>
</tr>
<tr>
<td>Detectable metastases</td>
</tr>
</tbody>
</table>

* Undetectable disease.
* Values are expressed as mean ± SD.
* NA, not applicable.

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two control groups ($P = 0.46$). 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 (all $P < 0.001$).

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 gelatinase are unknown. There was no correlation between the presence of the $M_r$ 20,000 species and the clinical status of the patients.

In our analysis of 117 urine specimens, we detected three predominant MMP species detectable by gelatin zymography (14). The $M_r$ 72,000 and $M_r$ 92,000 activities were recognized by anti-MMP antibodies in Western blots, confirming their identities as MMP-2 and MMP-9 (Fig. 1A, Lanes 2 and 3). The identities of the hMW and the $M_r$ 20,000 gelatinases are unknown. There was no correlation between the presence of the $M_r$ 20,000 species and the clinical status of the patients. Representation of the $M_r$ 100,000–116,000 species and the $M_r$ 45,000 species was too small for statistical analysis. Incubation of the substrate gels in 10 µM 1,10-phenanthroline, a metalloproteinase inhibitor, resulted in loss of proteolytic activity, verifying that the urine proteases are members of the metalloproteinase family (Fig. 1B, Lane 3; Ref. 9).

The hMW, $M_r$ 92,000, and $M_r$ 72,000 gelatinases were each significant multivariate (independent) predictors of disease status. Stepwise logistic regression indicated that the $M_r$ 92,000 ($P = 0.002$) and the $M_r$ 72,000 ($P < 0.001$) gelatinases were each independent predictors of cancer (Table 3). Each of these two enzymes was also an independent predictor of the subgroup of organ-confined cancers, indicating that the $M_r$ 92,000 and $M_r$ 72,000 species provided the most useful information for discriminating between patients with localized disease from those who are normal or show no evidence of cancer. The hMW gelatinase, but not the $M_r$ 92,000 or $M_r$ 72,000 species, was a multivariate predictor of metastatic cancer ($P < 0.001$). Remarkably, the estimated odds for metastatic cancer when the hMW species was present in urine was approximately 30 times greater than when the marker was absent (95% CI = 7.7–120.1). This model estimates that the probability of metastatic cancer is 74% when the hMW species is 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.

### Discussion

In this study, we report that three MMPs are independent predictors of disease status for patients with a variety of cancers. This is the first report demonstrating that: (a) MMPs in urine correlate with the presence of malignant disease; (b) MMP-2 and MMP-9 are present in urine in their intact and functional forms; (c) MMP-2/MMP-9 and a hMW MMP species can serve as independent predictors of organ-confined cancer and metastatic cancer, respectively; and (d) these correlations are not confined to tumors within the genitourinary tract.

In our analysis of 117 urine specimens, we detected three predominant molecular weight classes of gelatinases ($M_r$ 150,000, $M_r$ 92,000, and $M_r$ 72,000) in urine by substrate gel electrophoresis. The $M_r$ 72,000 and $M_r$ 92,000 species were identified as MMP-2 (gelatinase A) and MMP-9 (gelatinase B) by Western blot analysis. The identities of the hMW class, the $M_r$ 125,000 MMP species detected only in the

### Table 2： Gelatinase profile

<table>
<thead>
<tr>
<th>Specimens positive for any MMP (%)</th>
<th>Prostate</th>
<th>Renal</th>
<th>Bladder</th>
<th>Breast</th>
<th>Other</th>
<th>Metastatic</th>
<th>NED</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>All specimens</td>
<td>75%</td>
<td>40%</td>
<td>80%</td>
<td>100%</td>
<td>64%</td>
<td>90%</td>
<td>11%</td>
<td>20%</td>
</tr>
<tr>
<td>Detectable primary tumors</td>
<td>75%</td>
<td>40%</td>
<td>80%</td>
<td>100%</td>
<td>64%</td>
<td>90%</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Detectable metastases</td>
<td>50%</td>
<td>67%</td>
<td>NA</td>
<td>100%</td>
<td>86%</td>
<td>90%</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Specimens positive for a specific MMP (%)</th>
<th>hMW</th>
<th>$M_r$ 92,000</th>
<th>$M_r$ 72,000</th>
<th>Creatinine (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMW</td>
<td>57%</td>
<td>30%</td>
<td>30%</td>
<td>131 ± 70</td>
</tr>
<tr>
<td>$M_r$ 92,000</td>
<td>64%</td>
<td>30%</td>
<td>30%</td>
<td>134 ± 78</td>
</tr>
<tr>
<td>$M_r$ 72,000</td>
<td>39%</td>
<td>10%</td>
<td>36%</td>
<td>91 ± 52</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td></td>
<td></td>
<td></td>
<td>85 ± 42</td>
</tr>
</tbody>
</table>

**Table 3： Multivariate predictors of disease status**

<table>
<thead>
<tr>
<th>Marker</th>
<th>$\beta$ coefficient</th>
<th>SE</th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metastatic cancers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hMW</td>
<td>3.4</td>
<td>0.71</td>
<td>30.5</td>
<td>7.7–120.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Organ-confined cancers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$M_r$ 92,000</td>
<td>1.57</td>
<td>0.52</td>
<td>4.8</td>
<td>1.7–13.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>$M_r$ 72,000</td>
<td>2.58</td>
<td>1.00</td>
<td>13.1</td>
<td>3.6–93.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>All cancers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$M_r$ 92,000</td>
<td>1.89</td>
<td>0.48</td>
<td>6.6</td>
<td>2.6–17.0</td>
<td>0.002</td>
</tr>
<tr>
<td>$M_r$ 72,000</td>
<td>2.5</td>
<td>1.07</td>
<td>12.3</td>
<td>1.7–87.3</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>


\[a\] NA, not applicable.

\[b\] Values are expressed in mean ± SD.
TCC specimens we examined (10 bladders and 1 kidney). It is interesting to note that the pattern of the low molecular weight species of gelatinases in urine appears random, whereas the pattern seen with the higher molecular weight species, i.e., the hMW, M, 125,000 (in breast cancer), M, 92,000, and M, 72,000 gelatinases appears to be nonrandom. There is ample evidence in the literature to suggest that the lower molecular weight forms are most likely degradative products of the higher forms that arise by autocatalysis or some other proteolytic mechanism (16), which may occur during handling, storage, or preparation of the specimens after collection.

Our results are consistent with previous reports indicating that MMPs may be elevated in the serum of cancer patients. However, the 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, M, 92,000, and M, 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 corroborative and supportive information in assessing the clinical course of disease in cancer patients.

Interestingly, the M, 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 M, 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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