Matrix Metalloproteinase-9 Expression in Bladder Washes from Bladder Cancer Patients Predicts Pathological Stage and Grade

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ABSTRACT

The matrix metalloproteinases (MMPs), in particular the gelatinases (MMP-2 and MMP-9) have been associated with tumor cell invasion and metastasis in many human cancers. Here we examined the expression of proMMP-2 (gelatinase A) and proMMP-9 (gelatinase B) proteins in the cellular component of bladder washes obtained from 65 patients. Twenty-six patients had active bladder cancer, 24 had a history of bladder cancer but no evidence of active disease at the time of cystoscopy (recurrence-free), and 15 patients had lesions other than bladder cancer (controls). The results were correlated with the cytological findings of the bladder wash and the histopathological results of the tumor resection when performed. In patients with active transitional cell carcinoma of the bladder, 71% and 38% had expression and overexpression of the latent form of MMP-9 (proMMP-9), respectively. In contrast, neither latent nor active MMP-2 could be detected in any of the samples examined, regardless of tumor status. Overexpression of proMMP-9 correlated with higher grade (P = 0.003) and pathological stage (P = 0.04) of disease in the active bladder cancer group. No significant gelatinase expression was detected in the recurrence-free and control cases. Compared with urine cytology, proMMP-9 expression had an overall higher sensitivity for bladder cancer identification (71% versus 54%, P = 0.11). Detection of proMMP-9 in bladder washes may be a novel approach for the identification of patients with more aggressive forms of bladder cancer.

INTRODUCTION

An estimated 54,400 new cases of bladder cancer will be reported in the United States, with more than 12,500 deaths predicted for 1998 (1). It is the fourth most common cancer in men and the eighth most common in women. Seventy-five percent of bladder cancers are initially diagnosed as noninvasive (T0, TIS) or minimally invasive cancers (T1) (2). Bladder cancer has a 50% 5-year disease-free survival rate once the tumor invades the underlying stroma compared to 90% if the tumor is noninvasive. For patients with superficial cancers (T0, CIS, and T1) the standard treatment consists of transurethral tumor resection with or without intravesical therapy. However, 25% of superficial bladder cancers will eventually invade the bladder wall and require a radical cystectomy. Currently available prognostic factors are incapable of identifying which superficial tumors will become invasive. Thus, molecular markers to identify patients with bladder tumors at high risk for invasion are critical for the clinician to administer appropriate care.

Early stages of bladder cancer are characterized by the presence of a dysplastic and proliferative urothelium that initially grows into the lumen of the bladder. Cystoscopy and urine or bladder wash cytology is the traditional method of diagnosing bladder cancer. Although cystoscopy is highly effective at identifying papillary tumors, CIS is difficult to identify. Bladder wash cytology is relatively effective in diagnosing CIS but has a 20–40% false-negative rate in detecting papillary tumors, depending on the grade of the tumor (3). Novel methods to identify bladder cancer and determine the biological aggressiveness of the tumor are essential to better select the appropriate treatment for an individual patient.

Like all carcinomas, bladder cancer invasion into the neighboring stroma is the hallmark of metastasis formation (4). The proteolytic degradation of basement membranes has been considered an essential step for the invasion and metastatic spread of cancer cells (5). The MMPs are a family of zinc-dependent endopeptidases that have been associated with the ability of tumor cells to degrade extracellular matrix components during tumor cell invasion (5, 6). Considerable evidence has associated gelatinase A (MMP-2) and B (MMP-9), two members of the MMP family, with tumor metastasis because of their ability to degrade basement membrane collagen IV and their elevated expression in many malignant human tumors (7, 8), including bladder cancer (9–11). Previously, we have shown enhanced levels of both gelatinases in 42 cases of invasive bladder cancer by immunohistochemical studies (10). Davies et al. (9) demonstrated a positive correlation between higher levels of tumor expression and disease aggressiveness.
of proMMP-9 and invasive disease, using gelatin zymography. Overexpression of proMMP-2 in a rat bladder cancer cell line increased metastatic potential (12). In other studies, elevated levels of proMMP-2 (11, 13) and proMMP-9 (13) were detected in urine samples of patients with transitional cell carcinoma. In the present study, we analyzed the expression of gelatinases by gelatin zymography of the bladder washes from patients undergoing cystoscopy. Our goal was to evaluate the ability of this test to detect the presence of bladder cancer and to determine whether the presence of malignancy and degree of invasion correlate with the level of gelatinate expression. The data indicate that proMMP-9 expression in bladder washes may be a useful marker for bladder cancer detection and invasion.

**MATERIALS AND METHODS**

**Study Population.** Bladder washes were collected from 65 patients undergoing cystoscopy from June 1996 to January 1997 at the Wayne State University/Karmanos Cancer Institute-affiliated Harper Hospital. Fifty-one men 44–89 years old (mean, 69.4 years) and 14 women 40–89 years old (mean, 63.6 years) represented the study cohort. Patient characteristics were collected by chart review. The pathological stages of those with active bladder cancer were classified according to the 1987 International Union Against Cancer TNM classification of urothelial cancer (14). Tumors were graded histologically, following the World Health Organization classification (15). The patients were stratified into three groups: group 1, transitional cell carcinoma present on cystoscopic bladder biopsy (active cancer); group 2, history of transitional cell carcinoma but no evidence of active disease (recurrence free); and group 3, no history of transitional cell carcinoma or active disease at the time of cystoscopy.

**Sample Collection.** After a patient underwent cystoscopy and the bladder was emptied and before intravesical manipulation, 100 ml of a sterile saline solution was pushed back and forth into the bladder 5 to 10 times. One-half of the sample was sent for cytological diagnosis, and the other half was processed for analysis of gelatinate expression.

**Cytology.** Samples of bladder washes were centrifuged at 2,000 rpm for 10 min, and two cytospin slides were prepared from the sediment of each sample and fixed in 95% ethanol. Fixed smears were stained by the modified Papanicolaou technique (16). Diagnostic interpretation of the cellular material was performed by a cytopathologist on the basis of criteria described previously (17). The cases were classified as positive or negative for malignant cells. All suspicious cases were included in the positive group. The bladder wash cytology results analyzed were from the original reports, carried out as a routine laboratory procedure, without subsequent revision.

**Sample Preparation for Gelatin Zymography.** Bladder washes (40–50 ml) were centrifuged (2,000 rpm, 10 min, 4°C) to obtain the cellular fraction. The cell pellets were washed twice with cold PBS (pH 7.2), centrifuged, and resuspended in 200–400 μl of lysis buffer [25 mM Tris buffer (pH 7.5), 100 mM NaCl, and 1% NP40] containing 1 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 2 mM benzamidine. The lysates were rocked at 4°C for 2 h, centrifuged (13,000 × g, 30 min, 4°C), and the supernatants were collected. The protein content in each supernatant was determined using the BCA Protein Kit (Pierce, IL). The samples were stored at −80°C until use.

**Gelatin Zymography.** Gelatin zymography was performed using 10% SDS-polyacrylamide gels containing 0.1% gelatin, as described previously (18). Equal amounts of protein (2 μg) from each bladder wash were mixed with Laemmli sample buffer without reducing agents and subsequent boiling. Recombinant human proMMP-9 and proMMP-2 produced in a vaccinia expression system (19) were used as controls. At the end of the electrophoretic run, the gels were incubated (20 min, 22°C) in a solution of 2.5% Triton-X100 in distilled H2O, and then washed in distilled H2O for another 20 min. The gels were then incubated (16 h, 37°C) in 50 mM Tris-HCl, 5 mM CaCl2 (pH 8), and then stained with 0.25% Coomassie Blue in a solution of 10% methanol and 5% acetic acid. Bands with gelatinolytic activity, detected as clear bands against the blue-stained gelatin background, were visualized after the gels were destained with 10% methanol and 5% acetic acid. To analyze the expression of gelatinases in the cellular precipitates of the bladder washes, samples were subjected to gelatin zymography. It is known that some members of the MMP family, and in particular the gelatinases, can be detected in complex mixtures using SDS-polyacrylamide gels impregnated with gelatin because of their ability to degrade denatured collagen. After electrophoresis under nonreducing conditions, the proteins in the sample are allowed to renature by removal of the SDS, which results in the in situ degradation of substrate, indicated by a gelatinolytic band at the corresponding size of the proteinase. The intensity of the bands was analyzed by densitometry using an Ambis Radioanalytic Imaging System. This system software allowed the quantification of the gelatinases expression in the samples, measuring the intensity rate of the bands against the baseline control. An intensity rate of 25–89% of the control was defined as expression, and 90% or more was defined as overexpression.

**Statistical Analysis.** The Fisher exact test was used to determine the significance between gelatinase expression and overexpression and stage and grade of disease. For statistical comparison between levels of gelatinases, we used the Student t test.

**RESULTS**

Of the 65 patients examined, 26 had active cancer, 24 were recurrence-free, and 15 were control patients who underwent cystoscopy for a variety of noncancerous reasons, including

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**Table 1** Cytology and proMMP-9 in study cohort

<table>
<thead>
<tr>
<th>Samples (n = 65)</th>
<th>Cytology</th>
<th>proMMP-9</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
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<tr>
<td>Control patients</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>No active bladder cancer</td>
<td>1</td>
<td>23</td>
</tr>
<tr>
<td>Active bladder cancer</td>
<td>13</td>
<td>13</td>
</tr>
</tbody>
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* Tissue diagnosis was reported as folicular cystitis; patient had two subsequent visits with voiding cytology specimens reported as negative for malignancy.

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of the samples derived from patients with bladder cancer showed significantly higher levels of proMMP-9 expression when compared with the control patients. In contrast, expression of proMMP-2 or MMP-2 could not be detected in any of the samples examined regardless of pathological stage and grade. When an arbitrary known amount of recombinant proMMP-9 was used as a standard, samples were classified as expressing and overexpressing proMMP-9 as described in “Materials and Methods.” Table 4 describes the expression and overexpression of proMMP-9 according to pathological stage in patients with active bladder cancer. Although there are only a few cases, patients with TIS were more likely to have a positive cytology (67%) or proMMP-9 expression (100%) compared with patients with T, disease (22% had positive cytology and 44% had proMMP-9 expression). In patients with invasive bladder cancer (T,–T,4), cytology studies were positive in 9 of 14 cases, whereas 12 of 14 cases had proMMP-9 expression. In this study, proMMP-9 expression had 71% sensitivity, 95% specificity, and 91% positive predictive value in detecting bladder cancer. Conversely, cytology had 50% sensitivity, 95% specificity, and 81% positive predictive value. However, because of small sample size these numbers were not statistically significantly (P = 0.11). A positive cytology and proMMP-9 expression were generally more common in patients with grade III bladder cancer. Interestingly, 30 and 40% of the patients with grade I or II bladder cancer had a positive cytology or proMMP-9 expression, compared with 62 and 94% of patients (P = 0.02) with grade III disease, respectively.

Overexpression of proMMP-9 was common in invasive or grade III tumors (Tables 4 and 5). Eight of the 14 patients with T,–T,4 tumors overexpressed proMMP-9 compared to 2 of 12 patients with CIS or T, disease (P = 0.04). Similarly, 63% (10 of 16) of the patients with grade III tumors and 0% (0 of 10) of the patients with grade I or II tumors overexpressed proMMP-9, respectively (P = 0.003).

**DISCUSSION**

Bladder carcinoma is an important public health problem. It is the fifth most common human cancer, with more than 54,000 new cases estimated in 1998 accounting for more than 12,000 deaths (1). Transitional cell carcinoma, the most common histopathological type (94%), usually presents as a superficial lesion in 70–80% of the cases, recurs in 50–70% of patients, and progresses to muscle invasive disease or presents...
proMMP-9 in Bladder Washes

**Fig. 1** Zymography of gelatinase expression in bladder washes from patients with no history of bladder cancer. The cellular fraction (2 μg/lane) of bladder washes was subjected to gelatin zymography as described in “Materials and Methods.” Lane 1, squamous metaplasia; Lane 2, cystitis glandularis; Lane 3, no bladder lesion identified by cystoscopy; Lane 4, nephrogenic adenoma; Lanes 5 and 6, dysplasia; Lane 7, cystitis follicularis; Lanes 8 and 9, cystitis cystica; and Lane 10, no bladder lesion identified by cystoscopy. Purified human recombinant proMMP-9 (10 ng/lane) and proMMP-2 (containing a fraction of active MMP-2, 10 ng/lane) were used as standards.

**Fig. 2** Zymography of gelatinase expression in bladder washes from patients with history of bladder cancer but no active disease at time of cystoscopy. Samples (2 μg/lane) of bladder washes prepared as described in Fig. 1 were subjected to gelatin zymography. Lanes 1, 2, 4, and 8, cystitis follicularis; Lane 3, cystitis glandularis; Lane 5, nephrogenic adenoma; Lane 6, squamous metaplasia; and Lane 7, dysplasia. Recombinant proMMP-9 and proMMP-2 were used as standards as described in Fig. 1.

de novo at an invasive stage in 15–30% of cases. Stage and grade represent the most reliable prognostic indicators of this disease. Fewer than 2% of patients with grade I tumors, approximately 11% of patients with grade II tumors, and 45% of patients with grade III:ptT3 tumors are likely to progress within 2 years of diagnosis. Patients with bladder cancer that infiltrates the muscle wall have a worse outcome, with 50% developing metastasis within 2 years and a 5-year survival rate between 20 and 40%. (21) However, recent data in pT2–pT3a, N0, M0 patients have revealed increased 5-year survival rates (60–70%) after radical treatment (2). This highlights the importance of distinguishing aggressive superficial tumors that will eventually invade and muscle-invasive tumors that will metastasize.

Here we have examined the expression of gelatinases in the cellular component of bladder washes from three patient categories: those with active histologically proven transitional cell carcinoma; those with a history of bladder cancer but are currently recurrence-free; and those undergoing cystoscopy for benign conditions that have no history of bladder cancer. We wished to test whether expression of these enzymes in gelatin zymography correlates with the presence of disease and/or the invasiveness of the tumor. Bladder washes are known to contain cellular elements, derived from the bladder wall, that after isolation aid in the cytological diagnosis of bladder tumors. Trott and Edwards (22) reported that bladder wash cytologies were more accurate in detecting the presence of bladder cancer in comparison with voided urine cytology. It has been shown that the barbotage action enhances cell shedding and provides better preserved cells for diagnostic examination (22). Our data indicate that the cellular pellets of bladder washes can be used to detect gelatinases in patients with bladder cancer, using a simple zymographic assay. Analysis of 65 cases revealed that proMMP-9 is the major gelatinase expressed in the bladder washes, whereas proMMP-2 could not be detected. The expression of proMMP-9 correlated strongly with the presence of bladder cancer because 73% of patients with bladder cancer had proMMP-9 expression in their bladder wash, whereas only 5% of patients with benign bladder conditions had proMMP-9 expression (P = 0.001). Expression of proMMP-9 correlated with higher tumor grade (P < 0.02), but interestingly, overexpression correlated with tumor grade (P = 0.003) and pathological stage (P < 0.04), suggesting a potential prognostic value for proMMP-9 in patients with active bladder cancer. In contrast, our data indicate that cytological analyses of the same samples failed to correlate with either tumor grade (P = 0.12) or pathological stage (P = 0.11).

Moses et al. (13) found MMP expression in the urine of cancer patients that correlated with disease status. In addition, they demonstrated that expression of proMMP-9 and proMMP-2 in urine were comparable to or better than other tumor markers in predicting metastatic cancer of any tumor type (13). These investigators measured proMMP-9 expression in urine and hypothesized that metastatic tumors secrete proMMP-9 in the urine. We, however, measured proMMP-9 expression in exfoliated bladder cells. Therefore, our data are specifically indicative of the role of proMMP-9 in bladder cancer. In addition, our data are in agreement with previous studies indicating the importance of proMMP-9 in cancer progression (23–25). In our previous immunohistochemical study (10), we could not find a positive correlation between gelatinases expression and pathological stage, tumor grade, and/or outcome in 42 cases of invasive bladder cancer. However, we found that the expression of TIMP-2 in the tumor stroma strongly correlated with poor survival. Attempts to measure TIMP-1 and TIMP-2 by immunoblot analysis in the bladder washes were unsuccessful and probably require a more sensitive detection assay. Thus, the levels of TIMPs in the bladder washes remain to be determined. However, a previous study of 69 patients with bladder cancer demonstrated high serum levels of TIMP-1 and a positive correlation between TIMP-1 levels and invasion (26). These results and our results in the present and previous studies further demonstrate a complex relationship between levels of proteinases and their inhibitors in cancer progression (10, 27).
Expression of gelatinases has been correlated with tumor invasion and metastasis in many human tumors, including bladder cancer. Davies et al. (9) correlated proMMP-2 activation with tumor grade, using zymography of bladder tumor extracts that detected the presence of active MMP-2 forms in the more malignant tumors compared to the low-grade tumors. In the case of proMMP-9, the studies by Davies et al. (9) showed that levels of the latent form of the enzyme are enhanced in invasive bladder cancer, in agreement with our results. Interestingly, the expression of gelatinase mRNAs by in situ hybridization is mostly in stroma cells, with occasional expression in the tumor cells (9). Although we have not determined the precise nature of the cells in the bladder washes, they are most likely to contain epithelial and some inflammatory cells as reported previously (22). However, it is unlikely that the major source of proMMP-9 in the bladder washes are inflammatory cells because bladder washes obtained from patients with inflammatory conditions showed no presence of proMMP-9, suggesting that proMMP-9 expression is associated with the epithelial cells. Indeed, immunohistochemical studies indicated that both proMMP-9 and proMMP-2 are present in bladder cancer cells (10). In other studies, human bladder cancer cell lines cultured in vitro were shown to express both gelatinases after exposure to basic fibroblast growth factor (28). Thus, bladder cancer cells under certain conditions can potentially express both proMMP-9 and proMMP-2. Alternatively, expression of gelatinases in bladder cancer cells may be the result of paracrine secretion of enzymes by stromal cells and subsequent binding of the enzymes to the tumor cells (18).

It was interesting to observe the lack of proMMP-2 detection in the bladder washes in spite of previous reports of proMMP-2 presence in bladder tumors (10) and in the urine of patients with transitional carcinoma (11). The reason for this discrepancy is unclear but may be due to a preferential entrapment of proMMP-9 in the bladder wall, whereas proMMP-2 is readily secreted and detectable in the urine. Alternatively, the samples of the bladder washes are too small to allow for the detection of low levels of proMMP-2. Moses et al. (13) detected proMMP-2 in only 30% of the urine specimens from patients with bladder cancer, whereas proMMP-9 was detected in 70% of the cases in the urine samples. Although the precise role of these enzymes in bladder cancer progression remains unclear, our data suggest that expression of proMMP-9 in bladder washes can be useful for identifying patients with bladder can-
cer and differentiating high-grade cancers from superficial disease.

REFERENCES


Matrix metalloproteinase-9 expression in bladder washes from bladder cancer patients predicts pathological stage and grade.


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