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High Levels of Tissue Inhibitor of Metalloproteinase-2 (TIMP-2) Expression Are Associated with Poor Outcome in Invasive Bladder Cancer[1]

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ABSTRACT

The matrix metalloproteinases (MMPs) and the tissue inhibitors of metalloproteinases (TIMPs) have been associated with tumor invasion and metastasis in many human cancers. Immunohistochemical studies were performed on frozen tumor samples from 42 patients with invasive bladder cancer treated by cystectomy with monoclonal antibodies against the M_r 72,000 gelatinase A (MMP-2), M_r 92,000 gelatinase B (MMP-9), and TIMP-2 to evaluate their significance in bladder cancer. Immunoreactivity for the gelatinases was predominantly tumor cell-associated, whereas strong TIMP-2 staining was mostly detected in the stroma. Tumor cells demonstrated moderate to strong reactivity for MMP-2 and MMP-9 in 71 and 71% of cases, respectively, which did not correlate with stage, grade, or outcome. Tumor cells were positive for TIMP-2 in 26 (62%) of 42 cases, and this correlated with a worse outcome (69 *versus* 25% died of disease; $P < 0.05$). In 31 (74%) of 42, there was moderate to strong stromal staining for TIMP-2; this also was associated with a poor outcome (65 *versus* 25% died of cancer; $P < 0.05$). Tumor basement membrane (BM) status was investigated using an antibody to type IV collagen. In 9 cases, the invasive tumor nests were surrounded by an intact BM; in 7 of these, stromal staining for TIMP-2 was absent. None of these 9 patients (0%) died of tumors compared with 7 (100%) of 7 with complete loss of BM staining ($P < 0.001$). These results suggest a potential role for TIMP-2 and BM staining as prognostic indicators in invasive bladder cancer.

INTRODUCTION

To metastasize, cancer cells must penetrate the ECM[3] barriers in a process involving, among other factors, the proteolytic degradation of ECM components. Many studies have shown a significant association between metastasis formation and expression of MMPs, a family of zinc-dependent endopeptidases known to degrade ECM components (1, 2). Of particular importance in tumor cell invasion are two members of the MMP family, the M_r 72,000 gelatinase A (MMP-2) and the M_r 92,000 gelatinase B (MMP-9), because these MMPs can cause the hydrolysis of BM type IV collagen (2, 3), and their expression is elevated in many types of human cancers, including breast (4–6), colon (6), prostate (7, 8), bladder (9), and ovarian (10) cancers.

Like all members of the MMP family, the activity of MMP-2 and MMP-9 is regulated at various levels, including transcription, secretion, activation, and inhibition by TIMPs (1–3). The TIMPs, which, as for today, include TIMP-1 (11), TIMP-2 (12–14), and TIMP-3 (15), are low-molecular-weight, secreted proteins that bind to the active form of the MMPs, inhibiting enzymatic activity. MMP-2 and MMP-9 are unique among other MMPs in that the latent forms of these proteinases can form complexes with TIMP-2 and TIMP-1, respectively. It has been suggested that disruption of the balance between MMPs and TIMPs in cancer may be a factor in the progression of tumors to more malignant phenotypes (3). For example, down-regulation of TIMP-1 was shown to induce a metastatic phenotype in NIH-3T3 cells (16). In other studies, decreased expression of TIMP-1 was correlated with increased collagenase activity and metastatic potential in several murine mammary carcinoma cell lines (17). TIMP-2 inhibited invasion of tumor cells *in vitro* (18, 19), and overexpression of TIMP-2 in melanoma cells markedly suppressed tumor growth and partially reduced metastasis formation (20, 21). Although these studies suggested a role for TIMPs in preventing tumor cell invasion, other studies showed a complex relationship between MMPs, TIMPs, and cancer. TIMP-1 protein (22) and mRNA (23) were both found to be elevated in human colon cancer. In addition, the level of TIMP-1 expression correlated with the extent of tumor cell invasion (23). TIMP-1 was found to be highly expressed in gliomas (24), and up-regulation of TIMP-2 mRNA expression was reported in the stroma of basal cell carcinomas and breast tumors (25). We have shown high levels of TIMP-2 protein in the stroma of breast, colon, and gastric tumors (26). In breast cancer, overexpression of stromal TIMP-2 significantly correlated with tumor recurrence (27).

In the present study, we wished to examine the expression of MMP-2, MMP-9, and TIMP-2 in 43 cases of invasive bladder cancer. The survival of patients with invasive bladder tumors is very poor, and presently there are no good prognostic indicators of tumor invasion. A recent study showed that levels of MMP-2 and MMP-9 as determined by zymography correlated with tumor grade (9). However, TIMP-2 expression was not examined. In the present study, we examined the expression of TIMP-2 in conjunction with that of MMP-2 and MMP-9 in bladder carcinoma, and we present evidence suggesting a strong correlation between high levels of TIMP-2 immunostaining and poor outcome in patients with invasive bladder cancer.

MATERIALS AND METHODS

Study Population. Forty-three patients undergoing radical cystectomy (42 cases) or partial cystectomy (one case) for invasive (stage T1 or higher) bladder carcinoma between 1983 and 1992 at Harper Hospital (Detroit, MI) were selected for this study. The primary criteria for selection were the availability of fresh frozen tissue within the frozen tumor bank and sufficient clinical follow-up for tumor-specific survival analysis. Patient characteristics, including age at the time of cystectomy, sex, race, and follow-up information, were all obtained by chart review and physician contact when necessary. The study group comprised 31 men and 12 women. The mean age was 66 (median, 67; range, 34–84) years. There were 35 white patients and 8 African-American patients. Treatment was radical cystectomy in 42 cases and partial cystectomy in 1 case (an invasive carcinoma arising in a diverticulum). Cystectomy was performed in 2 patients with stage T_1 cancer, both with extensive transitional cell carcinoma *in situ*, which had failed at least two courses of intravesical *Bacillus Calmette* therapy. A third patient with stage T_1 cancer involving a diverticulum underwent partial cystectomy. In 6 cases, adjuvant radiation therapy was included, and in an additional 12 patients, adjuvant chemotherapy was given. Follow-up for the entire study population ranged from 6 to 84 (median, 18) months. Overall, 22 (51%) of 43 of the patients in this study group died of bladder cancer. Follow-up in the 21 patients not dying of cancer

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[3] The abbreviations used are: ECM, extracellular matrix; MMP, matrix metalloproteinase; BM, basement membrane; TIMP, tissue inhibitor of metalloproteinase; mAb, monoclonal antibody; TCC, transitional cell carcinoma.

(alive or dead of some other cause) ranged from 8 to 84 (median, 23) months. Of this group, 12 patients were alive and clinically free of disease, 3 were alive with local recurrence but no distant metastasis, and 6 had died of other causes (4 with no clinical evidence of carcinoma at the time of death and 2 with local recurrence only).

Pathological Materials. All specimens used in this study had been obtained at the time of radical cystectomy or partial cystectomy. Samples of tumors were snap frozen and stored at −70°C. H&E-stained sections cut at the beginning and end of sectioning were evaluated to confirm the presence of tumors within the frozen tissue samples. A total of 42 cases fulfilled the minimal criteria for inclusion. All histological sections from the radical cystectomy specimens were reviewed. Tumors were classified according to their histological types, and histological grades were assigned according to the criteria of Mostofi *et al.* (28). Pathological stages were assigned according to the 1987 International Union Against Cancer (29) tumor-staging system following histological review of the cystectomy specimens. In addition, the presence or absence of angiolymphatic invasion was recorded.

Immunohistochemical Procedures. Sections (5 μm) cut from the frozen tissue blocks were immersed in cold acetone for 10 min and then rehydrated. Slides were then incubated with the primary antibodies for 20 min at room temperature. Positive and negative controls were used for all antibodies. After washing in PBS (pH 7.2) without calcium and magnesium, the sections were incubated with the secondary antibody (biotinylated rabbit antimouse) at a 1:200 dilution. The slides were washed again in PBS and then incubated for 10 min with an avidin and biotinylated peroxidase complex (Vectastain; Vector Laboratories). Finally, the sections were washed in double-distilled water, counterstained, and mounted in Aquamount (Lerner Laboratories) under coverslips.

Immunohistochemical stains were evaluated by two independent observers without knowledge of the clinical outcomes. For MMP-2, MMP-9, and TIMP-2 antibodies, reactivity was assessed in both tumor cells and the stroma around the tumor. Reactivity was scored using a combined quantitative (percentage of tumor cells or stroma with immunoreactivity) and qualitative (intensity of staining: none, weak, or strong). These parameters were then combined as: 0, absent (no) staining; 1, focal (<10% of tumor cells or stroma) and weak; 2, diffuse (>10% of tumor cells or stroma) and weak or focal (<10% of tumor cells or stroma) and strong; and 3, diffuse and strong. Type IV collagen (BM) staining was assessed around the invasive tumor cell nests. The pattern observed was scored as: 0, intact, complete BM around tumor nests; 1, focal loss; 2, extensive loss (>50% of tumor surface); and 3, no BM staining.

mAbs. The characterization of the mouse mAbs against human MMP-2 (CA-406 and CA-801) and TIMP-2 (T2–101) has been reported previously (26, 27). The mAb against human MMP-9 (CA-209) recognizes both the native and denatured enzyme as well as the precursor (M_r 92,000) and active (M_r 85,000) forms and a pro-MMP-9/TIMP-1 complex, suggesting that the epitope of the antibody does not involve the TIMP-1-binding region. In addition, CA-209 recognizes pro-MMP-9 produced by human fibrosarcoma HT1080 cells and does not cross-react with MMP-2 or other proteins in the conditioned media of HT1080 cells (30). The specificity of the mAbs used was determined by immunoblot analysis and immunoprecipitation of the antigens expressed by a variety of nonmalignant and malignant human cell lines and with tissue extracts from a bladder tumor, and no cross-reactivity was detected.

Statistical Analysis. Frequency tables for quantitative demographic and clinical data as well as distribution characteristics for quantitative variables such as age were generated. Significant bivariate associations were tested for in all study variables by χ^2 test (two-sided test) and Fisher's exact test for small samples (one-sided test). Each variable was tested for prognostic significance for bladder cancer (disease) specific survival using Kaplan-Meier survival curves and the log-rank test. Patients dying of bladder cancer are considered failures; patients alive or dying of other causes are considered censored. The number of cases available ($n = 42$) was insufficient for meaningful assessment by Cox multivariate regression analysis. To look for possible significant associations between variables and survival, bivariate analysis was performed. By applying the χ^2 test and Fisher's exact test, all variables were cross-paired to assess for significant univariate associations. Kaplan-Meier survival curves and log-rank tests were subsequently applied. All statistical analysis were performed using the SAS statistical software program (SAS Institute, Inc., Cary, NC). Results were considered significant at $P = 0.05$.

RESULTS

Pathological Findings. The study group comprised 41 TCCs, 1 squamous cell carcinoma, and 1 small cell carcinoma. There were 14 cases of TCC in which the tumors showed areas of squamous differentiation, 3 cases with evidence of glandular differentiation, and 2 cases with areas of small cell differentiation. The histological grade was 2 of 3 in 5 cases and 3 of 3 in the remaining 38 cases. Grade was not a significant predictor of outcome ($P = 0.066$, log-rank test). The pathological stages, based on the radical cystectomy specimen, were T_1 in 3 cases, T_2 in 3 cases, T_{3a} in 7 cases, T_{3b} in 15 cases, and T_4 in 15 cases. Tumor stage was a highly significant predictor of outcome for the entire group ($P = 0.0002$, log-rank test). Lymph nodes were evaluated in all of the patients. Lymph node metastasis were present in 16 cases (37%), and lymph node status was a significant predictor of outcome in the study group ($P < 0.02$, log-rank test).

Immunohistochemical Distribution of MMP-2 and MMP-9. Immunohistochemical results were available in 42 cases; in one case, insufficient tumor was present in the frozen tumor block. Immunostaining with MMP-2 and MMP-9 mAbs was predominately localized in the tumor cells with cytoplasmic immunoreactivity (Fig. 1, *A*, *B*, and *H*). Tumors showed moderate or strong immunoreactivity (score, 2 or 3) for MMP-2 and MMP-9 in 30 (71%) of 42 cases each; in 27 (64%) of 42 cases, both enzymes were moderate or strongly coexpressed. There was no significant association between expression of either MMP-2 ($P = 0.253$, log-rank test; Fig. 2*A*) or MMP-9 ($P = 0.819$, log-rank test; Fig. 2*B*) and survival from bladder cancer. No correlation was found between MMP-2 or MMP-9 expression and histological grade or histological type (all $P > 0.05$, χ^2 test). Also, combined expression of MMP-2 and MMP-9 did not correlate with survival ($P > 0.10$, log-rank test).

Expression of both gelatinases was less often detected within the stroma and usually was weak and diffuse. Stromal staining was poorly defined and could not be localized to the cytoplasm of any cellular component (Fig. 1*A*). The impression was that it was present predominantly in the extracellular matrix. Scattered macrophages stained for MMP-9 (not shown). For MMP-2, moderate to strong (score, 2 and 3) stromal staining was present in 9 (21%) of 42 cases, weak staining (score, 1) was present in 6 (14%) of 42, and no detectable reactivity was present in 27 (64%) of 42 tumors. There was no correlation between MMP-2 stromal reactivity and cancer survival ($P = 0.747$, log-rank test). Stromal expression of MMP-9 was moderate (score, 2) in 3 (7%) of 42 cases, weak (score 1) in 4 (9%) of 42, and absent in 35 (83%) of 42; no cases showed strong stromal reactivity. However, there was a weak but not statistically significant association between stromal staining for MMP-9 and reduced cancer-specific survival ($P = 0.073$, log-rank test; data not shown).

Immunohistochemical Distribution of TIMP-2. TIMP-2 immunoreactivity was detected in both the tumor and the stroma (Fig. 1, *C–F* and *I*). Moderate to strong cytoplasmic immunoreactivity for TIMP-2 was present in the tumor cells in 16 (38%) of 42 cases, weak reactivity was present in 10 (24%) of 42, and no staining was detected in 16 (38%) of 42 carcinomas. There was no correlation between TIMP-2 expression and grade or histological type ($P > 0.05$, χ^2 test). As shown in Fig. 2*C*, the presence of any tumor cell staining for TIMP-2 was associated with decreased survival ($P = 0.012$, log-rank test). The presence of TIMP-2 within the stroma could be detected in 31 (76%) of 41 cases, with the majority (28 of 31) of the cases having moderate to strong staining and 3 cases having weak immunoreactivity. Moderate to strong stromal TIMP-2 staining correlated strongly with poor survival ($P < 0.001$, log-rank test; Fig. 2*D*). Bivariate analysis of TIMP-2 stromal staining with lymph node status showed TIMP-2 expression to be significantly associated with reduced sur-

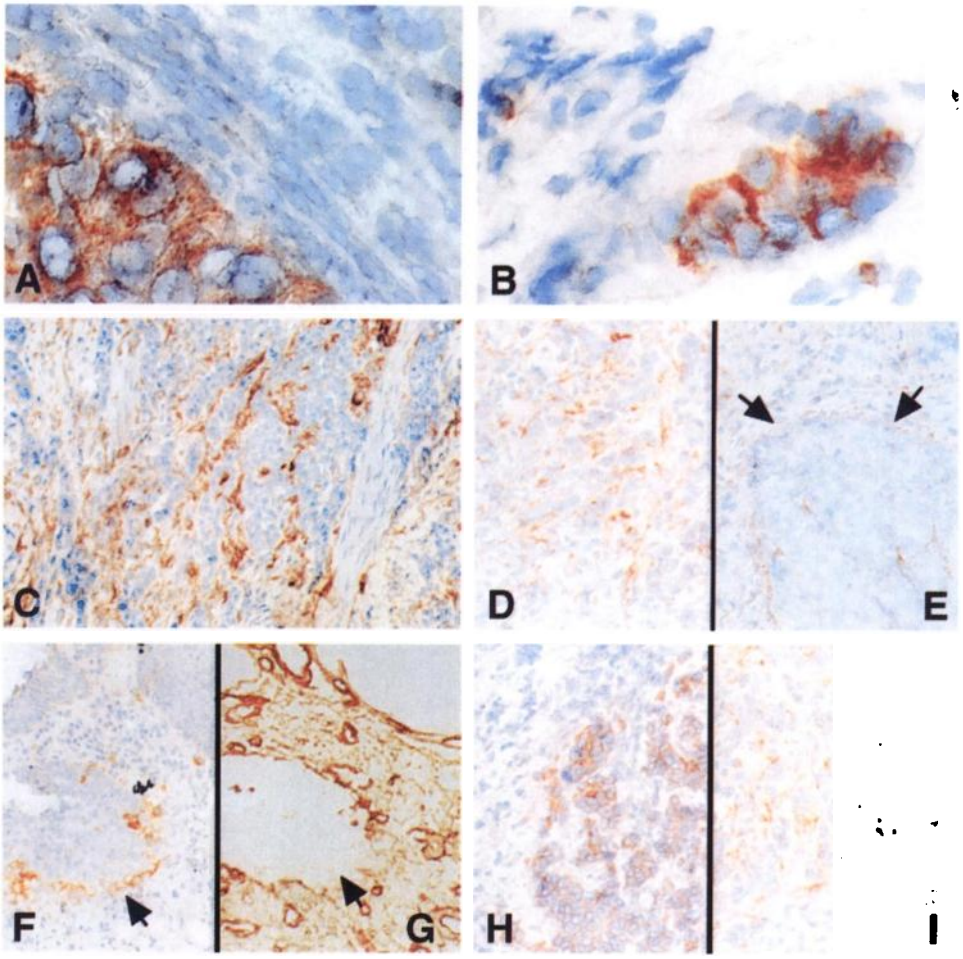

Fig. 1. *A* and *B*, immunostaining of a TCC with mAbs to either MMP-2 (*A*) or MMP-9 (*B*) in the same tumor specimen. Note the strong staining in the cytoplasm of the tumor cells and weak immunoreactivity in the adjacent stroma. ×400. *C–E* and *I*, invasive transitional carcinoma stained with a mAb against TIMP-2. Note the strong TIMP-2 immunoreactivity within the stroma between the infiltrative tumor nests (*C, D,* and *I*). Note the presence of weak stromal TIMP-2 in the deep invasive margin of the pushing tumor (*E, arrows*; contrast with *D*). ×100. *F*, TIMP-2 immunostaining in a TCC *in situ* adjacent to an invasive bladder cancer. Note the strong localized presence of TIMP-2 at a site of early microinvasion along the tumor-stroma interface (*arrow*) and the absence of staining in the uninvolved stroma (*right bottom corner*). ×100. *G*, same field as *F*, but stained with an antibody to type IV collagen, showing the loss of type IV collagen immunoreactivity at the site of microinvasion (*arrow*) and coincident with TIMP-2 immunoreactivity (compare with *F, arrow*). ×100. *H*, invasive TCC with strong cytoplasmic MMP-2 immunoreactivity. ×100. *I*, same field as *H*, but showing strong stromal TIMP-2 immunoreactivity. ×100. *D, E, H,* and *I*, from the same tumor specimen.

vival in the lymph node-negative patients ($P < 0.05$, log-rank test) but not in the lymph node-positive patients ($P > 0.10$, log-rank test).

TIMP-2 stromal staining was apparently localized in the extracellular matrix and could not be appreciated in fibroblasts or myofibroblasts. In all cases, the stromal expression of TIMP-2 was seen immediately adjacent to the invasive tumor nests. The strongest reactivity was noted around infiltrative tumor nests (Fig. 1, *C* and *D*), and weak staining was present when tumor nests were larger and with more of a pushing border (Fig. 1*E*, *arrows*). In one of the slides, intense reactivity for TIMP-2 was noted in the stroma-tumor interface immediately adjacent to a focus of early invasion associated with carcinoma *in situ* (Fig. 1*F*, *arrow*). In areas without carcinoma, the stroma was negative (Fig. 1*F*, *right bottom corner*). Likewise, the connective tissue surrounding a large number of infiltrating tumor nests was also negative compared with the stroma between invasive tumor cells (Fig. 1*C*). Comparison of the staining with anti-MMP-2 and anti-TIMP-2 mAbs in the same field revealed the differential localization of enzyme (tumor cells) and inhibitor (stroma; Fig. 1, *H* and *I*) in areas of invasive bladder cancer.

Type IV Collagen Immunostaining. A complete BM, as indicated by a continuous layer of type IV collagen immunoreactivity, was present around the invasive tumor nests in 9 (21%) of 42 cases. Focal loss of this layer was detected in 7 of 42 tumors, extensive loss was detected in 17 of 42, and in 7 of 42 tumors, there was the absence of type IV collagen immunoreactivity around tumor cells. As shown in Fig. 3, the pattern of type IV collagen staining was strongly associated with poor bladder cancer outcome ($P = 0.0016$, log-rank test). No significant correlation was detected between type IV collagen staining and either MMP-2 or MMP-9 in tumor cells. However, there were weak associations between the presence of stromal MMP-2 and stromal MMP-9 with loss of type IV collagen reactivity ($P = 0.029$ and 0.074, respectively, Fisher's exact test).

No correlation was found between the intensity of staining of type IV collagen and expression of TIMP-2 in the tumor cells. There was, however, a highly significant association between the presence of TIMP-2 in the stroma and decreased type IV collagen staining ($P < 0.0001$, Fisher's exact test). In one case, an area with *in situ* carcinoma lacking a continuous layer of type IV collagen (Fig. 1*G*) showed strong TIMP-2 staining in the same location (Fig. 1*F*). Bivariate analysis was performed to look for potential significant associations with regard to bladder cancer survival. These indicated significant bivariate associations between BM staining pattern and stromal TIMP-2 expression ($P = 0.0001$) and between BM status and stromal MMP-2 ($P = 0.05$), MMP-9 ($P = 0.05$), and combined MMP-2 and MMP-9 expression ($P < 0.05$). Another strong bivariate association was noted between stromal TIMP-2 and stromal MMP-2 ($P = 0.003$) and MMP-9 ($P = 0.0025$). Survival analyses were also performed, evaluating combinations of TIMP-2, MMP-2, and MMP-9 expression. These all revealed insignificant results; however, the validity of these analyses is limited by the small numbers in each subgroup. Larger numbers would be required to allow for more definitive testing of these associations with the Cox regression model.

DISCUSSION

An estimated 50,000 new cases of bladder cancer are reported annually in the United States, with more than 8,500 deaths predicted

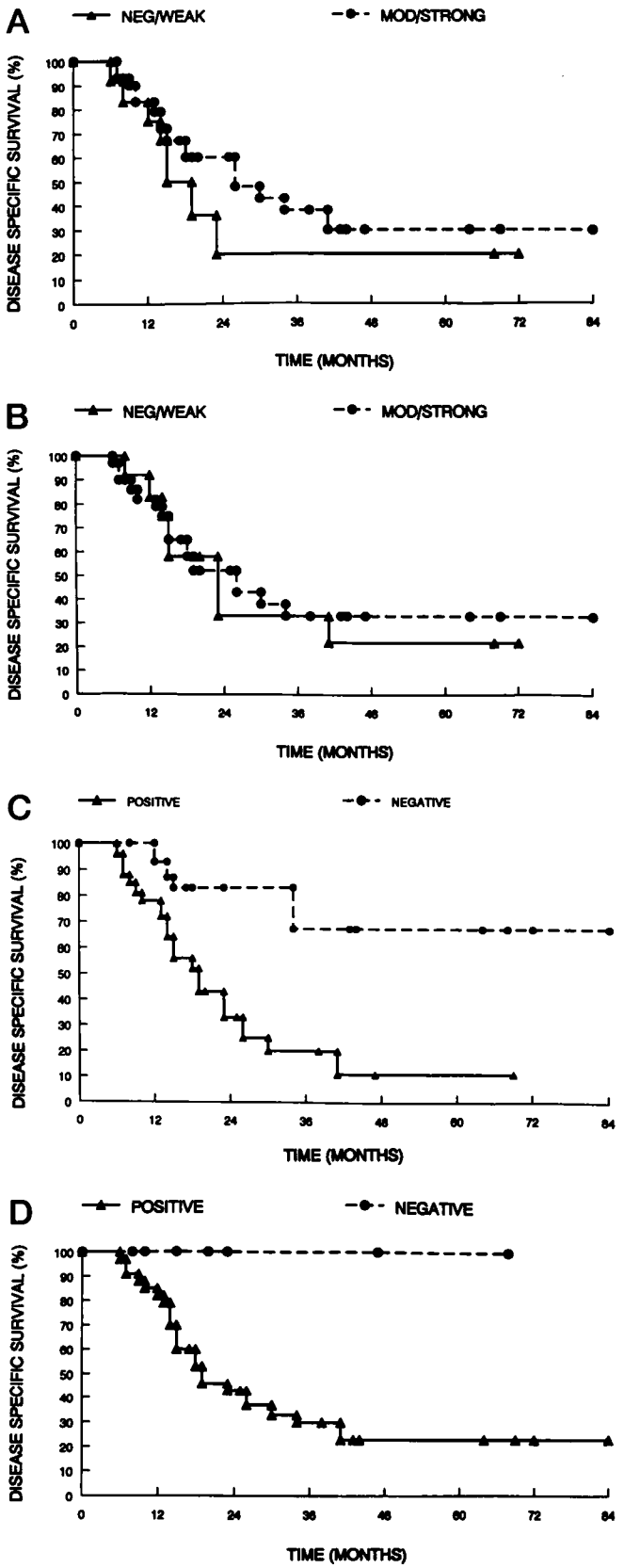

Fig. 2. Kaplan-Meier survival curves correlating disease-specific survival with MMP-2 (*A*) and MMP-9 (*B*) tumor immunostaining and tumor (*C*) or stromal (*D*) TIMP-2 immunostaining.

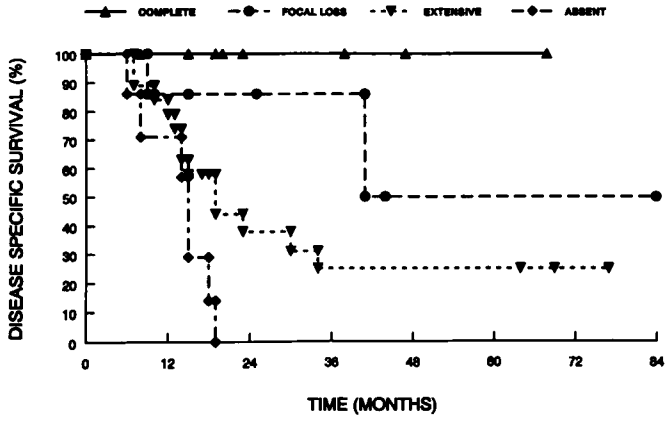

Fig. 3. Kaplan-Meier survival curves correlating disease-specific survival with BM status as determined by staining with an antibody to human type IV collagen. *Complete* (▲), continuous type IV collagen staining of the BM; *extensive* (▼), extensive loss of type IV collagen staining.

annually. More than 70% of patients present with superficial disease, which is traditionally managed by local therapy. Approximately 20% of patients, however, present with muscle-invasive disease requiring more radical therapy (31). With the advent of effective adjuvant and neoadjuvant chemotherapy, it has become increasingly important to define those patients with poor prognoses who would most likely benefit from such additional therapy. At the present time, the single most important prognostic indicator in patients with TCC is the tumor stage (32–34). Although histological grade has been shown to be a highly significant predictor of recurrence and progression in noninvasive TCC (33, 35), it has proven to be much less significant in muscle-invasive tumors (36). This is because the vast majority of such tumors fall within the high-grade group. Other pathological features, which have been evaluated in the past, have been the presence or absence of angiolymphatic invasion (37, 38) and the presence of squamous or glandular differentiation (39, 40). More recently, studies of the *p53* suppressor gene have suggested that this feature may be an important prognostic indicator in this subgroup of bladder cancer patients (41).

In the present study, we evaluated the relationship between gelatinases (MMP-2 and MMP-9), TIMP-2 expression, and basal lamina status with outcome in 43 cases of invasive bladder cancer. We used a series of mAbs to human MMP-2, MMP-9, and TIMP-2 with a high degree of specificity for their respective antigens. Our data show a strong immunoreactivity for both proteinases in the tumor cells, with both cytoplasmic and cell surface distribution in most of the cases. A small number of cases, however, showed stromal staining for MMP-2 or MMP-9, but the intensity of the staining was always weaker than that of the tumor cells. Stromal staining seemed localized to the ECM, and it was not possible to be certain of the cellular source of the proteases in this location. Areas of benign tissue within the neoplasm showed no immunoreactivity with the MMP mAbs, suggesting a specific expression of gelatinases in malignant tissue. The expression of these proteinases, however, failed to significantly correlate with survival in this set of invasive bladder cancer specimens. It may be of significance that our mAbs do not distinguish between active and latent enzymes (26, 27, 30). Therefore, a correlation with poor prognosis and enzyme activation could not be established. Davies *et al.* (9) correlated MMP-2 activation with tumor grade using zymography of bladder tumor extracts, which detected the presence of active MMP-2 forms in the more malignant tumors compared with the low-grade tumors. In this study, we have only examined cases of invasive bladder cancer with uniformly high grades, which consistently

showed high levels of gelatinase staining. Thus, a differential expression of gelatinases in low- and high-grade bladder tumors cannot be ruled out. In areas with *in situ* carcinoma, however, we could not detect immunostaining for the gelatinases in the tumor cells, suggesting that the presence of these proteinases is associated with invasive cancer. In agreement with our results, immunohistochemical studies in prostate cancer showed a lack of correlation between expression of MMP-2 and tumor grade or stage. Nevertheless, the exclusive immunolocalization of MMP-2 in the dysplastic epithelium and prostatic adenocarcinoma suggested a role for MMP-2 in prostate tumor invasion (8).

The immunohistochemical localization of MMP-2 and MMP-9 in bladder cancer cells does not completely agree with the reported mRNA expression of gelatinases in bladder tumors, which was found mostly in the stromal cells (9). A similar differential localization of gelatinase protein and mRNA was also observed in other tumor systems (6, 10, 25, 26, 27) and with other MMPs, including the membrane-type MMP-1, a physiological activator of MMP-2 (42, 43). Also, the mRNA of collagenase-3, an MMP overexpressed in breast tumors, is found exclusively in the tumor stroma, whereas the protein is localized in the cancer cells.[4] The reason for these discrepancies is presently unknown. Tumor cell-associated MMPs may be the result of translocation and binding of secreted proenzyme forms or of activated enzymes from producing neighboring stromal cells to cell surface receptors on the cancer cells. A recent immunoelectron microscopy study of gastric and skin cancers demonstrated MMP-2 localized in the lumen of the endoplasmic reticulum of the tumor fibroblasts, but it was also found in the cytosol of the cancer cells (44), suggesting a possible mechanism of enzyme uptake by the tumor cells. At present, it is unknown how tumor-stroma interactions can modulate the localization of MMPs.

We have shown that TIMP-2 was mostly (76% of the cases) localized in the tumor stroma around nests of invasive tumor cells positive for gelatinase expression. However, 38% of the cases showed moderate to strong staining of TIMP-2 in the tumor cells. The highest levels of TIMP-2 staining were noted around nests of tumor cells invading in an infiltrative fashion, a pattern associated with extensive or complete loss of BM staining. This was reflected in the significant association between BM loss and stromal TIMP-2 expression. In contrast, islands of invasive tumor with well-circumscribed borders and intact basal lamina (pushing tumor) showed weak TIMP-2 immunoreactivity that was localized in the invasive edge of the tumor. It was interesting to observe a clearly defined localization of TIMP-2 in the tumor-stroma interface in an area of carcinoma *in situ* adjacent to the invasive tumor. A similar pattern of TIMP-2 localization was observed in *in situ* carcinoma of the breast (27), which is known to progress into invasive carcinomas. Thus, this pattern of TIMP-2 expression may represent an early stromal response to tumor microinvasion. The molecular mechanisms responsible for the up-regulation of TIMP-2 in the stroma adjacent to invasive tumor cells remain unknown but may require a close association between tumor and stromal cells, possibly involving tumor-specific signals to the stromal cells. Indeed, we observed strong TIMP-2 immunoreactivity only in the stroma between clusters of infiltrative bladder tumor cells, whereas the connective tissue surrounding the tumor mass was negative (Fig. 2*C*).

A striking observation of this study was the strong correlation ($P < 0.001$) between stromal and tumor TIMP-2 immunostaining, loss of BM, and poor survival in patients with invasive bladder cancer. This supports the importance of BM breakdown for tumor invasion and metastasis. The majority of tumor-related deaths of bladder cancer patients are due to metastatic disease. If the current results can be confirmed in independent data sets, determination of these related markers could be valuable in identifying patients at high risk for metastases and most likely to benefit from adjuvant therapy. Elevated levels of TIMPs were also described in breast cancer (26, 27), gliomas (24), and colorectal tumors (22, 23). In breast cancer, enhanced expression of stromal TIMP-2 was a strong indicator of tumor recurrence (27), and in colorectal cancer, high levels of stromal TIMP-1 mRNA correlated with the presence of lymph nodes and distant metastases (23). This positive relationship between TIMP expression and cancer is paradoxical, because both TIMP-1 and TIMP-2 are known inhibitors of MMP activity. However, recent studies have shown that TIMP-2 plays a role in the activation of pro-MMP-2 by membrane-type MMP (45). Thus, TIMP-2 may regulate both the inhibition and activation of MMP-2. In other studies, TIMP-1 and TIMP-2 were shown to posses *in vitro* mitogenic activity for certain cells (46, 47); however, their role in tumor cell proliferation *in vivo* remains elusive.

[4] C. Lopez-Otin, personal communication.

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