Prognostic Significance of Type IV Collagen and Laminin Immunoreactivity in Urothelial Carcinomas of the Bladder

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Invasion of a carcinoma involves the degradation and penetration of the subepithelial basement membrane (BM). This phenomenon might be used for histopathologic evaluation of neoplasms of the bladder. The authors studied the clinicopathologic data and tissue specimens of 125 cases of urothelial carcinomas collected prospectively. Penetration of the BM was evaluated by immunohistochemical staining of the BM components laminin and type IV collagen. The use of this parameter as a prognostic indicator in bladder cancer was assessed. The 5-year survival rate of patients having tumors with an interrupted or absent BM was significantly lower than that of patients having tumors with an intact BM. The rate of progression was greater in tumors with an interrupted or absent BM than in tumors with an intact BM. No association was found between BM status and recurrence. However, a significant correlation between tumor stage and BM staining was found. A correlation was also found between ploidy and BM staining as well as between histologic grade and BM staining pattern. When evaluating histologic grade, stage, ploidy, age, and BM score as prognostic parameters, the stage of bladder carcinomas turned out to be the most important factor in predicting the survival rate and the progression-free survival. However, BM staining was found to be of value for early identification of microinvasion and is helpful for correct staging of urothelial carcinomas. Cancer 06:2583–2586, 1990.

The rapidly increasing knowledge in molecular biology and the new possibilities of biotechnology have opened new opportunities for the development of new approaches toward diagnosis and classification of neoplastic disease. Tumor invasion is an area that has benefited remarkably from these developments. Invasive growth of a carcinoma is defined as the penetration of malignant neoplastic cells through a basement membrane (BM). In this view the BM serves as an important structural barrier to progression of the neoplasm. To infiltrate and metastasize an epithelial neoplasm has to penetrate one or more BM layers. Basement membrane degrading proteases such as type IV collagenase play an important part in this process. Recent studies indicate that BM are not static structures destined only for destruction, but they can also be deposited in tumor tissue.

The study of BM morphologic features in invasive cancer is not new. Earlier attempts to outline BM in breast cancer by periodic acid-Schiff (PAS) staining were frustrated by the occurrence of PAS-reactive glycoproteins not only in BM but also in interstitial connective tissue. Immunohistochemical detection of BM components appears to be a more suitable approach toward the study of invasive growth. Visualization of the BM by immunohistochemical techniques with BM-specific antibodies is specific and provides very high resolution. This technique is reliable and reproducible and now forms one of the essential tools in the study of the role of the BM in neoplasia. Both laminin and type IV collagen can be used as markers of BM in tumors.

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There are only a few reports published concerning BM staining in bladder carcinomas. In one study a significant correlation was seen between BM continuity or discontinuity and progression (P = 0.036). In the other study bladder cancer patients with intact BM showed longer survival than patients with BM discontinuities (P < 0.001). We studied BM expression in bladder carcinomas to evaluate its use for the histopathologic identification of microinvasion. Furthermore, this study was performed to assess the usefulness of BM staining in comparison with grading, staging, and ploidy for the prediction of the biological behavior of bladder tumors.

Materials and Methods

The Department of Urology of the St. Maartens Gasthuis (Venlo, The Netherlands) provides a regional service for the Northern Limburg area. All patients diagnosed with primary bladder carcinoma between January 1979 and December 1988 were included in this study (n = 140).

Clinicopathologic information and transurethral resection specimens were collected prospectively. Staging was carried out according to the TNM system and grading into two groups, low and high grade, according to our criteria described elsewhere. Complete follow-up data up to December 1989 or until the time of death were obtained for all patients. The median follow-up was 21 months (minimum, 1 month; maximum, 109 months). Follow-up was conducted at least semiannually by cystoscopy.

Of the 125 evaluable cases 60 patients underwent only transurethral resection of the tumor, and 34 patients received adjuvant intravesical therapy. The other 31 patients did receive radiotherapy, cystectomy, or a combination of both.

As follow-up criteria we used survival, progression, and recurrence. Progression and recurrence were assessed by pathologists without knowledge of any previous clinical data.

Immunohistochemical Technique

Routinely formalin fixed (10% formaldehyde in phosphate-buffered saline [PBS]) and paraffin-embedded tissue blocks of the transurethral resections were sectioned at 4 μm. Sections were deparaffinized, rehydrated, and pretreated with pepsin (Sigma Chemical Co., St. Louis, MO) (0.1% in 0.1 normal [N] hydrochloric acid [HCl] for 30 minutes at room temperature) to enhance immunoreactivity. Endogenous peroxidase was blocked in 0.3% hydrogen peroxide (H2O2) in methanol for 30 minutes. After washing in PBS (3 × 5 minutes) the sections were incubated with polyclonal anti type IV collagen antiserum (diluted 1:3000 in PBS with 1% bovine serum albumin [BSA]) and polyclonal anti-laminin antiserum (diluted 1:100 in PBS with 1% BSA) overnight at 4°C in a moist chamber. Both primary antisera were raised in rabbits. Their immunospecificity was documented previously.

After washing in PBS the sections were incubated with peroxidase-labeled swine anti-rabbit Ig antibodies (Dako, Copenhagen, Denmark) (diluted 1:40 in PBS with 1% BSA) for 30 minutes at room temperature. After final washing with PBS a diaminobenzidin H2O2 substrate was used to visualize the immunoreactivity. In 125 cases satisfactory immunohistochemical evaluation was accomplished, which was assessed using blood vessels as an internal control. For statistical evaluation the immunoreactivity of BM at the tumor/stromal interface was scored semiquantitatively into two patterns as follows: (1) tumors with intact BM; and (2) tumors with patchy or absent BM.

Chromosome Analysis

For microscopic analysis of chromosomes, tumor samples were collected in 0.5% sodium citrate with 0.5 μg/ml colcemid. After incubation for 1 hour at room temperature, the tissue was mechanically disaggregated in 5 ml Hanks' balanced sodium solution (Hanks' BSS). Afterward a solution of 19 ml Hanks' BSS and 6 ml colcemid was added. After incubation for 30 minutes at 37°C, hypotonic treatment in a solution of 6 ml PBS and 24 ml 0.052 mol/l potassium chloride was followed by fixation in methanol-acetic acid 7:3. Chromosomes were routinely stained with Giemsa. This direct method has been described elsewhere in detail.

Analyzable metaphases, at least five (average, 28) per case were photographed and underwent karyotyping according to the Paris nomenclature. The tumors were classified according to their modal chromosome number and chromosome range. The latter classification distinguishes the tumors with diploid or hypodiploid (46 chromosomes or less) cells from those with hyperdiploid (more than 46 chromosomes) cells.

Statistical Analysis

Prognostic factors that were included in the analyses, besides BM pattern, were as follows: age, sex, T category (stage), histologic grade, ploidy, and therapy.

Kaplan-Meier survival curves for time to death, progression, and recurrence were analyzed by the log-rank test. The association between the various prognostic parameters was analyzed using a chi-square test. When a significant difference between the groups with a different pattern of BM deposition occurred, a multivariate analysis using Cox proportional hazards model was executed, to determine the prognostic value of BM continuity after correction for the mentioned extraneous prognostic factors. All statistical analysis were conducted with the

Results

Immunohistochemical Findings

Paraffin sections of 140 transitional cell carcinomas of the bladder were stained. Both antisera against type IV collagen and laminin showed strong staining of blood vessels and around muscle fascicles. Only when these internal controls stained properly the immunostaining was considered to be appropriate. In most cases laminin staining was somewhat weaker than staining with type IV collagen. In 125 cases reliable immunohistochemical evaluation could be performed. Cases with unreliable immunostaining were found to be evenly distributed over low-grade and high-grade malignant bladder carcinoma categories.

Clinicopathologic Data

Of the 125 lesions that could be analyzed, 52 showed almost intact BM and 73 lesions showed patchy or absent BM (Figs. 1, 2, and 3). In these tumors the tumor stage could not be established with certainty in the hematoxylin and eosin (H & E)-stained sections in eight of 125 cases (6%). Of 46 tumors with intact BM staining 38 were non-infiltrating in the H & E-stained sections (82.6%). Of 71 tumors with patchy or absent BM staining 58 were infiltrating in H & E-stained sections (81.7%). A strong cor-

Fig. 1. Transitional cell carcinoma with continuous BM staining.

Fig. 2. Transitional cell carcinoma with interrupted BM staining and microinvasive growth in the lamina propria.

Fig. 3. Transitional cell carcinoma without normal BM staining.

relation was observed between T category and BM staining (chi-square = 48.6, \( P < 0.0001 \)) (Table 1), as well as between grade and the BM staining pattern (chi-square
= 28.8, P < 0.0001). Of 37 low-grade lesions 29 showed intact BM (78.4%), whereas of 87 high-grade lesions only 24 showed intact BM (27.5%) (Table 2). A correlation was also found between ploidy and BM staining (chi-square = 27.9, P < 0.0001). In 100 of 125 cases chromosomal analysis could be successfully performed. Of the 24 diploid tumors 20 showed intact BM (83.3%), and of the 76 hyperdiploid tumors 19 showed intact BM (25.0%) (Table 3).

The eight cases in which the stage could not be established with certainty were all of high-grade malignancy. In seven of them chromosomal analysis was performed. Of these cases six were hyperdiploid and one was diploid. With BM staining it was possible to establish the correct stage in all eight cases. Microinvasive growth was seen in four tumors while the other four were non-invasive. Of the four patients with infiltrating tumor, one showed progression and eventually died of metastatic disease after 14 months. Another of these patients died of unrelated disease after 2 months. One of the patients with a non-infiltrating tumor died after 30 months without any sign of bladder tumor. All other five patients are alive without disease. The median follow-up for these eight cases was 23 months (minimum, 2 months; maximum, 49 months).

The association between BM staining and the subsequent clinical course was further assessed. The BM staining pattern proved to be of no value for the prediction of recurrence-free survival (Fig. 4). There was, however, a borderline significant correlation between BM staining pattern and progression-free survival (log-rank chi-square = 3.906, P = 0.048). As is shown on actuarial survival curves (Fig. 5) patients with tumors with intact BM showed less progression than patients with tumors with patchy or absent BM.

We also found a significant correlation between survival and BM staining patterns (log-rank chi-square = 8.45, P = 0.004). Patients with tumors with intact BM showed a highly significant longer survival than those with tumors with interrupted or absent BM (Fig. 6).

In view of the effects on survival and progression-free survival, we studied the interrelation between different variables using Cox proportional hazards model. We first calculated the univariate prognostic value for survival and progression-free survival regarding age, grade, stage, ploidy, and therapy. Age did not influence the correlation between BM staining pattern and survival parameters. Stage, however, largely eliminated the value of BM expression as a prognostic indicator. Also, the other variables, such as grade, ploidy, and therapy did not show a significant independent correlation with survival and progression-free survival in addition to tumor stage.

In the group of high-grade tumors (n = 87) 26% showed intact and 74% showed patchy or absent BM staining. A slight difference in survival was observed, which appeared not to be statistically significant (chi-square = 2.234, P = 0.135). The same was observed for progression-free survival (chi-square = 1.533, P = 0.2157).

When we evaluated the hyperdiploid group (tumors with more than 46 chromosomes per cell), 19 of 76 tumors (25.0%) showed intact BM, whereas 57 tumors showed patchy or absent BM (75.0%). Between these groups we found no differences in survival or progression-free survival. A slight, but not significant difference in survival was seen between the two BM patterns in Stage Ta (chi-square = 1.754, P = 0.1854). In Stage T1 tumors BM pattern did not correlate with survival. Likewise, the BM pattern did not correlate with progression-free survival either in Ta or in T1 tumors.

**Discussion**

In carcinomas, a dynamic interaction occurs at the interface between tumor cells and the surrounding mesenchymal stroma. Collagenases, including type IV-specific collagenase, and other proteases, such as plasminogen activators, cathepsins, and heparinase, form a cascade system of enzymes facilitating extracellular matrix break-

**Table 1. BM Expression Versus Stage of Infiltration (T)**

<table>
<thead>
<tr>
<th>BM</th>
<th>Ta</th>
<th>T1</th>
<th>T2/T3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>38</td>
<td>8</td>
<td>0</td>
<td>46</td>
</tr>
<tr>
<td>II</td>
<td>13</td>
<td>39</td>
<td>19</td>
<td>71</td>
</tr>
<tr>
<td>Total</td>
<td>51</td>
<td>47</td>
<td>19</td>
<td>117</td>
</tr>
</tbody>
</table>

BM I: intact basement membrane; BM II: interrupted or absent basement membrane. $x^2 = 48.6, P < 0.0001$.

**Table 2. BM Expression Versus Tumor Grade**

<table>
<thead>
<tr>
<th>BM</th>
<th>Low grade</th>
<th>High grade</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>29</td>
<td>24</td>
<td>53</td>
</tr>
<tr>
<td>II</td>
<td>8</td>
<td>64</td>
<td>72</td>
</tr>
<tr>
<td>Total</td>
<td>37</td>
<td>88</td>
<td>125</td>
</tr>
</tbody>
</table>

BM I: intact basement membrane; BM II: interrupted or absent basement membrane. $x^2 = 28.8, P < 0.0001$.

**Table 3. BM Expression Versus Chromosomal Numbers**

<table>
<thead>
<tr>
<th>Chromosomal number</th>
<th>Diploid</th>
<th>Hyperdiploid</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>20</td>
<td>19</td>
<td>39</td>
</tr>
<tr>
<td>II</td>
<td>4</td>
<td>57</td>
<td>61</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>76</td>
<td>100</td>
</tr>
</tbody>
</table>

BM I: intact basement membrane; BM II: interrupted or absent basement membrane. $x^2 = 27.9, P < 0.0001$. 


Conversely, as a host reaction to the invading neoplasm, extracellular matrix components, including BM material and interstitial collagen, may also be deposited around tumor cells. A desmoplastic stromal reaction around tumor cells, involving myofibroblasts, might play a role in BM deposition at the tumor stromal interface. Numerous immunohistochemical studies have documented loss of continuity of BM in many different malignant neoplasms.

The initial working hypothesis of these studies was that in benign (noninvasive) neoplasms an intact BM would be found, whereas in invasive malignant neoplasms the BM would be interrupted or absent. This has proven to be an oversimplification, as also reflected in the results we obtained. On one hand some noninvasive bladder tumors showed discontinuities in the BM, whereas on the other hand some invasive bladder carcinomas showed intact BM. We then argued that a positive balance between deposition and breakdown of BM components, resulting in intact BM, could be a sign of competent host response to the neoplasm. This might correlate with a better prognosis.

In the current study a direct correlation between T category and BM loss was observed. A proportion of the Ta lesions (intraepithelial tumors) showed incomplete BM, suggesting that although invasion had not yet occurred, there was already significant discontinuity of the BM barrier. Conversely some of the T1 lesions (tumors with invasion into the lamina propria) showed intact BM, reflecting the production of BM components even in invasive carcinomas. Conn et al. reported similar observations in their study on bladder carcinoma. Daher et al. reported intact as well as interrupted BM in T1 tumors. In their study no information was presented concerning Ta tumors.

Long-term follow-up studies are necessary to evaluate the biological significance of these observations. Statistical evaluation of clinical follow-up data in our study resulted in a highly significant correlation between BM deposition pattern and survival. Furthermore, a borderline significant correlation between progression-free survival and BM de-
position pattern was found. According to both survival parameters patients with tumors with intact BM had a better prognosis than patients with tumors with patchy or absent BM. No correlation was found with the recurrence-free survival, the mean number of recurrences per year, and the recurrence rate.

These results are in concordance with the literature in regard of recurrence-free survival and progression-free survival. As to survival, the only comparable data are found in the study of Daher et al. They documented a significant difference in short-term survival between their two groups of BM staining pattern, which did not include Ta tumors.

Our material was also analyzed for a number of additional parameters. For each parameter the prognostic value was calculated. All parameters with a significant correlation with survival or progression-free survival were further tested by a multivariate analysis. In the Cox proportional hazard model the additional value of BM deposition pattern was calculated after correction for other parameters. The following variables were found to have prognostic value: age, stage of the tumor, histologic grade, and ploidy. We found that tumor stage was the most important independent prognostic parameter for survival (log-rank test chi-square = 21.6, P < 0.0001) and progression-free survival (log-rank test chi-square = 9.88, P = 0.0017). The other variables did not add essential additional information.

In contrast to earlier studies, our results document that as a prognostic indicator BM staining is of limited additional value in comparison with grade and stage. Nevertheless, BM staining is of practical value for correct staging of bladder carcinomas because in eight of 125 cases (6%), the tumor stage could not be established with certainty in the H & E-stained sections. Basement membrane staining, which can be performed on routinely fixed and embedded material, facilitates the assessment of microinvasive growth of bladder cancer, which may be overlooked or only suspected in the H & E-stained sections. As such this rather simple technique is indirectly important for prediction of survival and progression-free survival because it helps to establish the correct stage of the neoplasm.

REFERENCES