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EXPRESSION OF MARKERS FOR TRANSITIONAL CELL CARCINOMA IN NORMAL BLADDER MUCOSA OF PATIENTS WITH BLADDER CANCER

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ABSTRACT

Purpose: Because we have found that random mucosal biopsies have no additional prognostic value to conventional histopathology, we studied biopsies of histologically normal bladder mucosa with several molecular markers believed to be associated with the development of transitional cell carcinoma.

Materials and Methods: Six groups of patients with an increasing stage of bladder tumor were selected: (1) benign disease (for example, benign prostatic hyperplasia, n = 8); (2-4) low (n = 10), intermediate (n = 9) and high risk (n = 7) superficial tumors; (5) progressive superficial tumors resistant to optimal conservative therapy (n = 6); (6) invasive or disseminated tumors at presentation (n = 5). We studied the expression of cytokeratin (used as an epithelial marker), fibronectin, E-cadherin (HECD-1), I-CAM, human leukocyte antigen (HLA)-I, HLA-II and epidermal growth factor receptor (EGF-R) in cold-cup biopsies of normal mucosa.

Results: Fibronectin, HECD-1, I-CAM and HLA-II expression showed no significant changes in the different groups. There was a significant increase in the expression of HLA-I (p = .003) and EGF-R (p = .0001) with a higher stage of the tumor.

Conclusion: An increasing EGF-R expression in normal looking mucosa of patients with increasing stages of bladder tumors could be a prognostic factor or might indicate that this increase in expression is not tumor specific but is seen in the whole bladder.

Key Words: bladder; carcinoma, transitional cell; biopsy; tumor markers, biological

Of the patients presenting with transitional cell carcinoma (TCC) of the urinary bladder, approximately 65% have superficial tumors (<pT1). The remaining patients present with invasive (≥pT2) or metastatic (N+, M+) disease.† Superficial bladder cancer is characterized by inconsistent biological behavior. The risk of tumor recurrence and progression can vary. Of all superficial bladder cancer patients a small group will only have 1 episode of superficial tumor. After transurethral resection (TUR) they will be without recurrences with or without adjuvant treatment ("low risk tumors"). Another small group of patients will present with "high risk" tumors from the start. These patients are at risk for progression to invasive disease, despite maximal adjuvant treatment. The majority of patients, however, will have regularly recurrent bladder tumors without obvious signs of progression ("intermediate risk"). To differentiate between these risk groups prognostic factors are used, such as tumor grade, stage, size and multiplicity, recurrence rate, and possibly the result of random mucosal biopsies. As we have shown, these selected mucosal biopsies are of no additional value to conventional histopathology in a multivariate analysis. However, the high recurrence rate and multiplicity in superficial bladder cancer suggest that the entire mucosa is primed to become malignant (field defect theory). Therefore, we looked at molecular changes in mucosal biopsies, using several markers associated with development of TCC. We analyzed normal-appearing mucosa in 6 groups of patients with an increasing stage and grade of bladder tumors. The expression of these markers in normal mucosa is believed to be a potential indicator of a mucosal field defect, and might be an additional prognostic factor in the work-up of patients with bladder cancer.

MATERIALS AND METHODS

In all, 6 groups of patients were identified. For each group 10 patients were selected (table 1). In every patient urethroscopy was done, and cold-cup mucosal biopsies were taken from normal-appearing mucosa. At least 2 biopsies were taken from the left and right lateral bladder wall. In case of a tumor on one of the lateral walls, the biopsy was taken 2 cm away from the tumor. Biopsies were stored in liquid nitrogen. Serial frozen sections were obtained from the biopsy material. One section from each series for the immunohistochemical analysis was stained with hematoxylin and eosin to determine the histopathological diagnosis. In a pilot study we tested the feasibility of this technique, and the results showed that biopsies stored in this manner were adequate for these investigations. There were no artifacts due to storage in liquid nitrogen and there was little mechanical damage.

We studied the expression of cytokeratin (CK18-2), fibronectin, E-cadherin (HECD-1), intercellular adhesion molecule (I-CAM), human leukocyte antigen (HLA) class I, HLA class II and the epidermal growth factor receptor (EGF-R). The antigen G250, which is renal cell carcinoma specific with only minor expression on the major bile ducts, was used as a negative control. The monoclonal antibodies (MAbs) used for this testing are listed in table 2. Expression of the MAbs was tested by means of direct immunohistochemical staining for MAbs for HLA class I and II† and indirect methods for the other MAbs. The indirect immunoperoxidase technique used in this study was performed at room temperature. The frozen sections were fixed with acetone for 10 minutes and air dried. Preincubation was done with 10% normal rabbit serum in
phosphate-buffered saline (PBS) for 30 minutes and subsequently with primary antibody for 1 hour. The primary antibody concentration was typically determined by using serial dilutions on a positive control. The optimal concentration was chosen as the lowest with which a clear positive signal was obtained at a minimal background. The optimal dilution is also indicated in Table 2. After rinsing with PBS samples were incubated with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dakopatts A/S, Glostrup, Denmark) diluted 1:100 in PBS containing 1% bovine serum albumin (BSA) for 30 minutes, and rinsed again with PBS before detection of the peroxidase activity with 3,3'-diaminobenzidine 0.6 mg/ml (Sigma Chemical Co., St. Louis, Missouri) in 0.65% imidazol/PBS containing 25 μl of 30% H2O2. Following brief rinsing with water, the sections were incubated with 0.5% CuSO4 (in 0.9% NaCl) for 5 minutes to intensify the reactions. After rinsing with water, the sections were counterstained with hematoxylin, dehydrated and mounted. Control experiments were done with 1% BSA in PBS instead of primary antibody. Staining was scored semiquantitatively by 3 different investigators. For each antigen a total of 10 high-power fields was scored. In each high-power field the percentage of clearly positive staining cells was estimated, after determination of the optimal primary antibody concentration. No variations in intensity of staining were considered.

For statistical analysis of the 6 different groups of patients the Kendall test against rank-correlation was used.

**RESULTS**

First, conventional histology of the biopsies was studied. Only patients in whom this histology was normal, that is, without mucosal denudation, dysplasia or tumor, were evaluated. The remaining number of patients in each group was 8 in group 1 (benign disease), 10 in group 2 (low risk), 9 in group 3 (intermediate risk), 7 in group 4 (high risk), 6 in group 5 (progressive superficial tumors in spite of optimal conservative therapy) and 5 in group 6 (invasive or disseminated tumors at presentation).

The epithelial structures in the biopsy specimens were analyzed with the various antibodies. The results of the antibody expression, together with the results of the statistical analysis are listed in Table 3. G250 expression, used as a control for nonspecific staining, was negative in all cases.

The epithelial phenotype was confirmed by using cytokeratin 18 (CK18-2), an epithelial-specific intermediate filament protein that is known to be expressed in all cell layers of the mucosa. CK18-2 was chosen because the epitope recognized by this antibody is, unlike the one recognized by RGE53, not partially masked in normal urothelium. Although cytokeratin 18 expression was not used as a tumor marker, aberrant expression patterns have to be kept in mind in relation to stage and grade of TCC. In our study no significant changes in CK18-2 expression were noted (p = .04) although expression decreased in the higher groups.

**DISCUSSION**

Because superficial bladder cancer is characterized by an unpredictable clinical course, prognostic factors are of particular interest. The high recurrence rate and multiplicity led some investigators to formulate a “field defect theory”, presuming that carcinogenic factors in urine can affect the whole mucosa. Therefore, selected mucosal biopsies were believed to carry prognostic value. However, the value of histologic examination of mucosal biopsies of normal-appearing mucosa is contradictory and probably very limited. We investigated such biopsies with several markers, commonly used to study the development of TCC, in 6 groups of patients with tumors of increasing clinically malignant behavior. Of some markers, like cytokeratin 18 and fibronectin, little is known about the relation of the expression of these markers to TCC behavior. The relation of I-CAM expression and cancer has only been described recently. In kidney cancer an increased I-
CAM expression is found compared with normal kidney tissue. Such expression is also found in bladder cancer cell lines, and a relation was found between I-CAM expression and susceptibility for killer cells. Moreover, I-CAM expression was stimulated with interferon-gamma (IFN-γ), indicating a possible mode of action of BCG, which increases urinary IFN-γ. However, the relation between I-CAM expression and the prognosis of bladder cancer is unclear. Loss of E-cadherin expression or inhibition of E-cadherin by addition of antibodies is associated with invasive phenotype of several tumors. We found low E-cadherin expression to be associated with invasive bladder cancer. Reduced expression of HLA-I has been described in various tumors and has been associated with the ability of tumor cells to escape immunologic surveillance and with malignant potential (invasiveness, metastases, dedifferentiation) of the tumor.

Table 3. Staining of antigens in mucosal biopsies: percentage (with range) of positive cells in high-power field.

<table>
<thead>
<tr>
<th>Group</th>
<th>HLA I</th>
<th>HLA II</th>
<th>HECD-1</th>
<th>CK18-2</th>
<th>FIBRO</th>
<th>I-CAM</th>
<th>EGFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60</td>
<td>25</td>
<td>100</td>
<td>100</td>
<td>30</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>(WK-100)</td>
<td>(0-100)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>7</td>
<td>100</td>
<td>100</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>(10-30)</td>
<td>(5-10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>35</td>
<td>7</td>
<td>100</td>
<td>100</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>(5-80)</td>
<td>(5-10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>&lt;50</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(WK-60)</td>
<td>(0-5)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>5</td>
<td>90</td>
<td>25</td>
<td>95</td>
<td>90</td>
<td>90</td>
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<td>90</td>
</tr>
<tr>
<td>(70-100)</td>
<td>(5-90)</td>
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<td></td>
</tr>
<tr>
<td>6</td>
<td>100</td>
<td>15</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(6-60)</td>
<td>(5-60)</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

| p = 0.003 | p = 0.031 | p = 0.083 | p = 0.040 | p = 0.07 | p = 0.001 |

In case antigen expression was seen, staining scored as positive (yes versus no). Differences in staining intensity were not considered. Statistical analysis according to Kendall exact rank correlation (not possible for fibronectin).
pared different groups of patients with an increasing stage of primary tumor. Looking at the markers we used, one might expect a rise in EGF-R expression and a fall in HLA class I and E-cadherin expression with increasing malignant potential of the mucosa. The relation between HLA class II, cytokeratin, fibronectin and I-CAM expression and TCC behavior is inconsistent. The fact that most markers show no changes in our study indicates that normal mucosa in patients with bladder tumor reacts similarly to normal mucosa, and this does not support the “field defect” theory. The increasing HLA-I expression is the opposite of what was expected and is difficult to understand. Like others, we have found an abnormal distribution of EGF-R expression (not only in basal cells) in normal-appearing mucosa, even in patients without histological evidence of bladder carcinoma. However, the increasing expression of EGF-R might indicate a malignant potential of the normal mucosa and might be of prognostic value. On the other hand, since this increased EGF-R expression especially is found in patients with invasive lesions, it might also indicate that increased EGF-R expression, which is believed to be associated with TCC progression, is a general phenotypic change in the urothelium.

In conclusion, normal mucosa, adjacent to bladder tumors, harbors potential prognostic information. It is unclear whether or how this is related to the progression of transitional cell carcinoma. Immunohistochemical examination of normal-appearing mucosa in bladder cancer patients with the markers presented here was not conclusive. Our results indicate that the expression of EGF-R in normal mucosa might be of value, but that unambiguous interpretation is not possible.

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REFERENCES

    III, Gonzalez-Zulueta, M., Nichols, P. W., Skinner, D. G. and
    Jones, P. A.: Role of chromosome 9 in human bladder cancer.

    cytometric deoxyribonucleic acid studies on exophytic tumor
    and random mucosal biopsies in untreated carcinoma of the

32. Rao, J. Y., Hemstreet, R. E., III, Bonner, R. B., Jones, P. L., Min,
    K. W. and Fradet, Y.: Alterations in phenotypic biochemical
    markers in bladder epithelium during tumorigenesis. Proc.

33. Waterfield, M. D., Mayes, E. L., Stroobant, P., Bemmet, P. L.,
    Young, S., Goodfellow, P. N., Banting, G. S. and Ozanne, B.: A
    monoclonal antibody to the human epidermal growth factor