Cadherin Switching in Human Prostate Cancer Progression

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ABSTRACT

The progression of carcinomas is associated with the loss of epithelial morphology and a concomitant acquisition of a more mesenchymal phenotype, which in turn is thought to contribute to the invasive and/or metastatic behavior of the malignant process. Changes in the expression of cadherins, “cadherin switching,” plays a critical role during embryogenesis, particularly in morphogenetic processes. Loss of E-cadherin is reported to be associated with a poor prognosis; however, thus far, evidence (R. Umbas, et al., Cancer Res. 54: 3929–3933, 1994) for up-regulation of other cadherins has only been reported in vitro, i.e., we have found evidence (M. J. G. Bussemakers et al., Int. J. Cancer, 85: 446–450, 2000) for cadherin switching in prostate cancer cell lines (up-regulation of N-cadherin and cadherin-11, two mesenchymal cadherins, in cell lines that lack a functional E-cadherin-catenin adhesion complex). Here, we report on the immunohistochemical analysis of the expression of N-cadherin and cadherin-11 in human prostate cancer specimens. N-cadherin was not expressed in normal prostate tissue; however, in prostate cancer, N-cadherin was found to be expressed in the poorly differentiated areas, which showed mainly aberrant or negative E-cadherin staining. Cadherin-11 is expressed in the stroma of all prostatic tumors, in the area where stromal and epithelial cells are found. In addition, cadherin-11 is also expressed in a dotted pattern or at the membrane of the epithelial cells of high-grade cancers. In a number of metastatic lesions, N-cadherin and cadherin-11 are expressed homogeneously. These data raise the possibility that cadherin switching plays an important role in prostate cancer metastasis.

INTRODUCTION

For patients with prostate cancer, it is now well documented that decreased expression of E-cadherin is associated with a poor prognosis (1, 2). The biological functional relation between loss of E-cadherin expression and acquisition of invasive behavior has been described by several groups (3–6), and, moreover, the restoration of an epithelial phenotype and a concomitant reduction in invasiveness after DNA-mediated transfection of E-cadherin has been reported by several independent investigators (6, 7). The clinical data that show in several epithelial-derived malignancies that the loss of E-cadherin expression is not only associated with invasiveness but also with metastatic behavior and poor clinical outcome (1, 2, 8–11) suggest that this event is pivotal in the complex metastatic cascade (12). Clearly, the mere loss of cell–cell contact and communication cannot be the sole explanation for the observed relation between loss of E-cadherin-mediated adhesion and poor clinical outcome.

Recently, a number of papers have been published that describe the inappropriate expression of nonepithelial cadherins by epithelial cells as a putative novel mechanism for promoting the interaction with the stroma, thereby facilitating invasion and metastasis (13–16). Indeed, we were able to show that human prostate cancer cell lines, which lack expression of either E-cadherin or the catenins and, therefore, lack an E-cadherin-mediated cell–cell adhesion, show expression of several other cadherins (17). Most prominent is the expression of the mesenchymal N-cadherin and cadherin-11 in these cell lines. Moreover, in other epithelial-tissue derived cancer cell lines, e.g., human bladder, melanoma, and breast cancer cell lines (15, 16, 18–20), which lack functional E-cadherin-mediated adhesion, expression of other cadherins is observed. Considering that the de novo expression of N-cadherin and cadherin-11 may distinguish changes in cell-adhesion processes that are important for the invasive and metastatic capacity of the prostate cancer cells, we decided to investigate the expression of N-cadherin and cadherin-11 in human prostate cancer specimens by single- and two-parameter immunofluorescence. By analyzing the protein expression patterns of these cadherins and correlating them to the expression patterns of E-cadherin or α-catenin, we aimed at establishing the pathological role of induced expression of N-cadherin and cadherin-11 in prostate cancer progression.

MATERIALS AND METHODS

Surgical Specimens. On radical prostatectomy or transurethral resection, prostatic adenocarcinoma specimens were snap-frozen in liquid nitrogen. Non-malignant prostate specimens were obtained from cystoprostatectomy. Metastases were obtained from operation (pelvic lymph nodes dissection). Four µm serial sections were used for immunohistochemical analysis. One of the serial sections was stained with H&E to determine the histopathological grading using the Gleason score (21).

Immunofluorescence. Sections were fixed using parafomaldehyde (3%) for E-cadherin, N-cadherin, cadherin-11, and α-catenin staining. On preincubation with normal sheep serum (diluted 1:20 in PBS) for 30 min., the sections were incubated for 1 h with the primary antibody. Next, the sections were incubated with biotin-labeled secondary antibody, followed by a streptavidin-fluorescein detection step (Amersham). Antibodies against E-cadherin (HECD-1 mouse monoclonal antibody, Takara) and N-cadherin [GC4 (mouse monoclonal antibody, Sigma)], an N-cadherin rabbit polyclonal antibody (Takara), cadherin-11 mouse monoclonal antibody (Ref. 17, clone No.16a), and a rabbit polyclonal antibody against α-catenin, C2081 (Sigma), were used at a dilution of 1:100, 1:30, 1:30, 1:100, and 1:1000, respectively.

Specificity of the antibodies against N-cadherin and Cadherin-11 was determined by the comparison of steady-state mRNA levels assessed by Northern analysis with immunofluorescence, using a panel of cell lines (17). There was a full correlation between mRNA and immunofluorescence results. Moreover, Western analysis using these antibodies identified proteins of the expected MW (M,) 135,000 for N-cadherin (17) and M, 120,000 for Cadherin-11.

N-cadherin, cadherin-11, and E-cadherin staining using the mouse monoclonal antibodies was performed for all of the samples and scored by three independent observers (K. T., A. v. B., and E. T. G. R.). E-cadherin was scored as either normal (membrane staining) or aberrant (negative or heterogeneous staining) as previously described by Schipper et al. (22) and Umbas et al. (23). N-cadherin staining was judged negative when <5% of the prostate cells were positive for N-cadherin, or when no significant N-cadherin staining was observed. N-cadherin staining was considered positive when 61% of the prostate cancer cells showed expression. Furthermore, the staining pattern was scored, i.e., membranous, or dotted. Cadherin-11 staining was scored negative when weak diffuse staining was observed, particularly in the stroma surrounding cancer cells, or when no significant staining was observed. Cadherin-11 staining was considered positive when 61% of the prostate cancer cells showed expression, typically in a dotted pattern.
Double staining, using FITC- and TRITC-labeled secondary antibodies were performed to investigate relative localization of E-cadherin and N-cadherin (rabbit polyclonal) and $\alpha$-catenin and cadherin-11. The samples were visualized by either standard epiluminescence fluorescence microscopy (Zeiss, axioskop) using the appropriate filter combinations or CSLM (Leitz DMIRBE, Leica).

**Statistical Analyses.** The $\chi^2$ test was used to determine the correlation between staining and Gleason score. The odds ratio was used to determine the correlation of N-cadherin or cadherin-11 with E-cadherin.

**RESULTS**

**Expression Pattern of N-Cadherin and Cadherin-11 in Human Prostate Cancer Specimens.** Using degenerate PCR cloning strategies, we have recently identified several cadherins to be induced in prostate cancer cell lines that lack membranous E-cadherin/$\alpha$-catenin complexes (17). In particular, N-cadherin and cadherin-11, the expression of which was confirmed by Western blot analysis, seemed to be of potential relevance for prostate cancer progression. To gain insight into the role of N-cadherin and cadherin-11 in human prostate cancer progression, we used immunofluorescence analysis to study the expression patterns of these cadherins in human primary and metastatic prostate cancer.

In nonmalignant preexisting acini, we never found expression of N-cadherin, i.e., none of the 12 samples analyzed showed positive staining. For cadherin-11, we occasionally found a very weak diffuse staining in the stromal cells, which is in agreement with the putative mesenchymal nature of cadherin-11 (13).

In cancer specimens, particularly high-grade (Gleason score: 7; see next section on “Increased Expression...”), we found expression of N-cadherin in the carcinoma cells (Fig. 1, A, D, and G). In the primary cancer specimens, the expression pattern is heterogeneous, with a predominant expression at the membrane (Fig. 1, A and D). Interestingly, in the high-grade tumors, N-cadherin was expressed more homogeneously, and, moreover, the pattern of staining changed from a membranous honeycomb-like staining to a dotted pattern (Fig. 1, D). Because we performed double-label immunofluorescence (FITC for N-cadherin and TRITC for E-cadherin), we were able to study the expression of both cadherins simultaneously. On comparison of the individual fluorescent dyes, it became evident that in the heterogeneously staining primary tumors, the pattern of staining was complementary, i.e., cells showed either a predominant expression of E-cadherin or N-cadherin at the membrane (Fig. 1A versus 1B, and Fig. 1D versus 1E). In the undifferentiated tumors (Gleason score: 9, 10) and metastases, E-cadherin was mostly negative; and all of the cancer cells were positive for N-cadherin albeit in two patterns, a membranous and a dotted one (Fig. 1, G, H, and I). The complementary staining pattern is best illustrated in the double exposures of N-cadherin and E-cadherin-associated immunofluorescence (Fig. 1, C and F).
These results are in agreement with a switch from E-cadherin expression to N-cadherin expression in the progression of the malignant process, and one might, therefore, expect intermediate patterns in which this putative transition is evident. The standard epiluminescence fluorescence microscopical evaluation occasionally revealed patterns that were suggestive for such intermediate processes, but we considered the judgement of these subtle changes equivocal. Hence, we performed CSLM analysis of the double-label immunofluorescence. Now we could clearly see that in some areas a transition from E-cadherin to N-cadherin was evident. In Fig. 2A, we see—besides E-cadherin- and N-cadherin-positive cell clusters—cell-cell contacts where E-cadherin staining and N-cadherin staining are juxtaposed (arrowheads), i.e., not colocalized. In this case, the pattern of N-cadherin staining is similar to that for E-cadherin. However, occasionally we found processes in which E-cadherin and N-cadherin were expressed in the same cell, albeit in different structures (Fig. 2B). This figure also clearly illustrates that the cells that do express N-cadherin have a marked decrease in E-cadherin immunoreactivity (in the periphery of the process).

The cadherin-11 immunoreactivity was completely different from that of N-cadherin. In nonmalignant areas, only weak staining was observed in the stroma (Fig. 3A). Interestingly, the expression of cadherin-11 was strongly induced in cancer areas with a typical dotted pattern (Fig. 3B), which was very similar to that observed for N-cadherin. In high-grade cancers and metastases, the expression was no longer confined to the stromal cells but was clearly observed in the cancer cells (Fig. 3, C and D). Sometimes the expression seemed to be at the stromal-epithelial interface, but coexpression of E-cadherin/α-catenin and cadherin-11 was also found in the typical dotted pattern. In the cells coexpressing E-cadherin/α-catenin and cadherin-11, the de novo expression of cadherin-11 was associated with a decreased expression of E-cadherin.

**Increased Expression of N-Cadherin and Cadherin-11 in High-Grade Prostate Cancer and Prostate Cancer Metastases.** On the basis of the detailed analysis of expression patterns of N-cadherin and cadherin-11, we decided to score the samples as follows. N-cadherin was either negative or positive, whereby we discriminated between membranous expression and the dotted pattern in the carcinoma cells. For cadherin-11, we scored the dotted pattern differentially between stroma and epithelium, i.e., a dotted stromal pattern or a dotted stromal + epithelial pattern. When we confronted the immunofluorescence analysis with the histopathological data, it became clear that there was a clear correlation with the Gleason score (Table 1). The membranous expression of N-cadherin increased from 5% of low-grade cases (Gleason score: 4–6) to 50% in intermediate- and high-grade cases (Gleason score: 7 and 8–10, respectively). This correlation was statistically significant, as was the correlation with decreased expression of E-cadherin (Table 2). In other words, there was a significant correlation between decreased expression of E-cadherin and increased expression of N-cadherin. Moreover, there was a trend toward a change of localization of the cadherin complexes from a typical pattern associated with the adhesion belt to a dotted pattern. We found the highest percentage of tumor cells expressing N-cadherin in the dotted pattern in the metastases (Table 1).

Cadherin-11, is a so-called mesenchymal-type cadherin, and, indeed, we found very weak expression in stroma surrounding nonmalignant secretory acini. In all cancers, there was a markedly increased expression of cadherin-11, and of particular interest was the change in the cellular origin of the expression. In the low-grade tumors, expression was confined to the stromal cells, whereas in the intermediate- and high-grade tumors, the carcinoma cells also expressed cadherin-11. The induced expression of cadherin-11 in the cancer cells was significantly correlated with an increasing Gleason score (Table 3). Moreover, in 9 (60%) of 15 of the metastatic lesions, (homogeneous) expression of cadherin-11 in the typical dotted pattern was also found. As is illustrated by Table 4, the most commonly observed pattern is that of mutual exclusiveness: when E-cadherin is expressed, there is no expression of cadherin-11 in the carcinoma cells, and when the E-cadherin staining pattern is aberrant, cadherin-11 is expressed in a dotted pattern in the cancer cells.

**DISCUSSION**

More than 90% of all malignant tumors are carcinomas and, thus, of epithelial origin. Interestingly, late in tumor progression, carcinoma cells lose the epithelial differentiation and acquire a more spindle-shaped/mesenchymal morphology, a phenomenon that has been suggested to
correlate with metastatic potential (14). In carcinomas, loss or down-regulation of E-cadherin expression is often observed, which correlates with the degree of malignancy of the tumor (12). Moreover, mechanisms that interfere with E-cadherin-mediated cell adhesion (e.g., changes affecting the catenins) have been suggested to be involved in epithelial-mesenchymal transition and in increased cellular motility.

Recently, the first reports appeared that described inappropriate expression of nonepithelial cadherins by epithelial cells as functionally related to disrupted cell-cell adhesion. A human squamous carcinoma cell line that displayed a scattered fibroblastic phenotype was found to express N-cadherin along with a decreased expression of E- and P-cadherin (24). In addition, in several breast cancer cell lines that showed a reduced expression of E-cadherin, high levels of N-cadherin expression were found (15). Temporary, up-regulation of expression of N-cadherin has also been suggested to play an important role in the migration of melanoma cells through the endothelium (16, 25). For prostate cancer, we previously found that cell lines that lack functional E-cadherin complexes do express N-cadherin and cadherin-11 (17). In this report, for the first time, we describe the de novo expression of N-cadherin and cadherin-11 using two parameter immunofluorescence. N-cadherin shows aberrant expression.

Table 1 N-cadherin expression in prostate cancer

<table>
<thead>
<tr>
<th>N-cadherin expression</th>
<th>n</th>
<th>Negative</th>
<th>Dotted</th>
<th>Membrane + dotted</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients</td>
<td>83</td>
<td>33</td>
<td>22 (26.5%)</td>
<td>28 (33.7%)</td>
</tr>
<tr>
<td>Gleason score</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4–6</td>
<td>21</td>
<td>17</td>
<td>3 (14.3%)</td>
<td>1 (4.76%)</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>2</td>
<td>1 (16.7%)</td>
<td>3 (50.0%)</td>
</tr>
<tr>
<td>8–10</td>
<td>41</td>
<td>12</td>
<td>12 (29.3%)</td>
<td>17 (41.5%)</td>
</tr>
<tr>
<td>LN^a metastasis</td>
<td>15</td>
<td>2</td>
<td>6 (40.0%)</td>
<td>7 (46.7%)</td>
</tr>
</tbody>
</table>

^a LN, lymph node.

Table 2 Correlation of N-cadherin expression with E-cadherin expression

<table>
<thead>
<tr>
<th>E-cadherin expression</th>
<th>n</th>
<th>Negative</th>
<th>Dotted</th>
<th>Membrane + dotted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>45</td>
<td>26</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Aberrant</td>
<td>38</td>
<td>7</td>
<td>12</td>
<td>19</td>
</tr>
</tbody>
</table>

χ^2 = 14.20

P = 0.0008

Fig. 3. CSLM analysis of the double-label immunofluorescence for cadherin-11 (FITC) and α-catenin (TRITC). Original magnification, ×630. Normal prostate specimen (A), primary prostate cancer (B), and lymph node metastasis (C, D). In nonmalignant areas, only weak staining is observed in the stroma (A). The expression of cadherin-11 is strongly induced in stromal areas with a typical dotted pattern (arrowhead, B). In high-grade cancers and metastases, the expression was no longer confined to the stromal cells but clearly observed in the cancer cells (C and D, arrowheads). α-catenin shows aberrant expression.

The typical honeycomb pattern associated with localization in a belt-like structure disappeared, and a dotted pattern emerged. This pattern is remarkably similar to that observed for cadherin-11 in the stromal cells, and it is tempting to speculate that this dotted pattern represents an as yet poorly characterized junctional complex mediating interaction between mesenchymal cells.

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Moreover, cadherin-11 has been suggested to be up-regulated during tumor progression: it may play a role in stromal-epithelial interactions and, thus, may be involved in migration. This would be in agreement with the interaction of stromal and cancer cells in a subtype of gastric cancer that is characterized by overexpression of cadherin-11 (26). A role for cadherin-11 in tumor cell invasion and metastasis is, furthermore, suggested for breast cancer: in most invasive breast cancer cell lines, expression of cadherin-11 is observed, whereas no expression can be detected in any noninvasive cell line (27). In prostate cancer cell lines, expression of cadherin-11 is found in those lines that have lost functional expression of E-cadherin, attributable to loss of expression of α-catenin (17).

Double-label immunofluorescence analysis of cadherin-11 expression revealed no expression of cadherin-11 in nonmalignant prostate tissue. However, all of the prostate cancer specimens did show a dotted staining pattern in the stromal cells surrounding the tumor cells and at the interface of stroma and tumor cells. Further detailed analysis of the expression of cadherin-11 provided unequivocal evidence that in high-grade cancers, the tumor cells also express cadherin-11. Even more interesting is the homogeneous expression of cadherin-11 in the prostate metastatic lesions. The striking resemblance of the pattern of N-cadherin and cadherin-11 staining, i.e., in a dotted pattern, indicates that the switch in cadherin expression and concomitant change of pattern is, in fact, related to the functional interaction between cancer and stromal cells. This, in turn, could be an essential step in the metastatic cascade. Clearly, we can at this point only speculate about the biological functional relation of the cadherin switch and increased metastatic propensity. However, the significant correlation with histological grade together with the fact that we find enrichment of this effect in the metastases (i.e., the metastases show a rather homogeneous pattern for both cadherin-11 and N-cadherin expression) corroborates the hypothesis that N-cadherin/cadherin-11-positive cells have a selective advantage to metastasize. It is noteworthy, however, that up-regulation of E-cadherin in metastases has also been reported (1, 28), which in fact could mean that cadherin switching is a dynamic process. The functional role of reexpression of E-cadherin is yet poorly understood. Cadherin-11, also termed osteoblast(OB)-cadherin is expressed at high levels in osteoblasts, which makes it, furthermore, tempting to speculate that there is a potential role of cadherin-11 in homing toward secondary sites, particularly bone.

We, therefore, conclude that we for the first time were able to provide evidence of cadherin switching in advanced human prostate cancer. The change in pattern—resembling the pattern of cadherin expression in stromal cells—suggests a functional role of homotypic cadherin-mediated interactions, in the later stages of cancer progression.

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