The Role of an 80 kDa Fragment of E-cadherin in the Metastatic Progression of Prostate Cancer

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ABSTRACT

Purpose: The purpose of this study was to evaluate an 80 kDa proteolytic fragment of E-cadherin as a potential biomarker for prostate cancer progression and to identify putative proteases that are responsible for the cleavage of E-cadherin.

Experimental Design: A wide spectrum of prostate cancer tissue and serum specimens representing different stages of prostate cancer was examined for the accumulation of the 80 kDa fragment of E-cadherin. Additionally, an expression array analysis was used to identify putative proteases that may have been involved in the cleavage of E-cadherin.

Results: A reproducible E-cadherin fragment was detected as a strong 80 kDa band in tissue samples. This fragment was detectable almost exclusively in metastatic sites. It was not visible in normal prostate tissue and was weak in 1 of 16 localized prostate cancers. The fragment is shed into the extracellular space and was detectable in patient serum in which the expression of the fragment showed a strong association with advanced prostate cancer. On the basis of cDNA expression analysis, several members of the metatllase and protease family could be identified as potentially responsible for the cleavage of the fragment from full-length E-cadherin.

Conclusions: In this study, we present the first report of serum levels of the 80 kDa fragment of E-cadherin in prostate cancer patients. This fragment is exclusively seen in neoplastic prostate tissue and may represent a useful biomarker of prostate cancer disease progression. This study also demonstrates an association of increased levels of several metalloproteases with metastatic prostate cancer and could provide a useful correlation between metalloproteinase expression/activity and E-cadherin cleavage and the metastatic progression of prostate cancer.

INTRODUCTION

The incidence of prostate cancer has increased over the past 10 years, in part because of prostate-specific antigen screening and public awareness. Men with clinically localized prostate cancer have excellent chances for long term cure. Treatment for advanced prostate cancer has not improved, however, since the introduction of antiandrogen therapy over 50 years ago. Therefore, novel approaches are needed to identify lethal prostate cancer at its earliest stages to impart aggressive clinical treatment.

E-cadherin is known to be important for proper cell-cell adhesion, and its cleavage has been linked to the malignant progression of adenocarcinomas including prostate cancer (1, 2). In the present study, we report the accumulation of an 80 kDa fragment of E-cadherin exclusively in hormone refractory metastatic prostate cancer tissue. This fragment has been previously described as a soluble peptide that was measurable in the serum of patients suffering from several types of adenocarcinoma (3–7). We investigated this fragment for its potential use as a serum marker for prostate cancer progression. The underlying pathomechanics for the cleavage of E-cadherin and the shedding of this 80 kDa fragment are not yet known. Metalloproteinases (MMPs) are believed to be one of the major players in tumor invasion and in metastasis via the degradation of extracellular matrix proteins (8). Thus far, several MMPs have been implicated in the extracellular cleavage and shedding of this unique E-cadherin fragment (9).

We looked for deregulation of MMPs during prostate cancer progression by expression array analysis (10, 11). The expression of several MMPs, i.e., MMP12 and MMP13, and disintegrin and proteases (ADAMs), i.e., ADAM 12 and ADAM 15, was found to be significantly altered at the mRNA level in metastatic prostate cancer.

This is the first report implicating members of the ADAM family of membrane disintegrins in the proteolytic cleavage of E-cadherin in metastatic prostate cancer. The resulting fragment is detected exclusively in the tissue and serum of metastatic prostate cancer cases, suggesting its potential use as a biomarker in advanced disease stage.

MATERIALS AND METHODS

Sample Collection. To examine the widest range of prostate cancer specimens and serum, we took clinical samples from the radical prostatectomy series and from the Rapid Autopsy Program at the University of Michigan. The specimens were processed within 20 min after surgical resection. Alternate...
sections of the prostate gland were submitted for histological review. The remaining sections were snap-frozen and stored. All of the samples used for cDNA expression array analysis and Western blot analysis were evaluated by the study pathologist (M. A. R.). All of the samples were trimmed to ensure >95% of the sample that was used represented the desired lesion. Areas of benign prostate tissue from prostates with prostate cancer were used as normal adjacent tissue in these experiments. In this setting, localized prostate cancer was defined according to the WHO classification (tumor-node-metastasis) 1997 as T1 to T3n. Patients with lymph node metastases were excluded. As a standard procedure, serum was collected from all of the individuals undergoing surgery. Clinical and pathology data for all patients were acquired with approval from the Institutional Review Board (IRB) at the University of Michigan and were maintained on a secure relational database (12). To study hormone-refractory prostate cancer, a rapid autopsy protocol was developed that allows men with advanced prostate cancer to consent to an autopsy immediately after death. To date, 23 complete autopsies have been performed with a median time of 2 h from death to autopsy. This procedure has been described in detail previously (13).

**DNA Microarrays.** The construction of the cDNA microarrays was described recently (10, 11). In brief, plasmid templates for a maximum of 10,000 transcripts were isolated from bacterial clones and inserts that were amplified by polymerase chain reaction (PCR). Purified PCR fragments were printed onto glass slides and cross-linked with the DNA targets. The cDNAs that were generated from prostate cancer samples (prostate cancer) and benign prostate tissue (reference) samples were labeled with distinguishable fluorescent dye and hybridized to the cDNA microarray. The cDNA microarray was analyzed using a scanner, and fluorescence ratios were determined for each gene. Color intensities were converted into ratios of gene expression. These ratios were imported into a database for further analysis.

**Immunoblot Analysis.** Representative prostate tissue specimens used for Western blot analyses were homogenized in NP40 lysis buffer containing 50 mmol/liter Tris-HCl (pH 7.4), 1% NP40 (Sigma, St. Louis, MO) and the complete proteinase inhibitor cocktail (Roche, Indianapolis, IN). All of the experimental samples were quantitated using the Bradford assay, and E-cadherin levels were measured on a per-total-protein basis. Thirty μg of protein extracts were electrophoresed through a 6% SDS-polyacrylamide gel under reducing conditions. The resolved proteins were transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ). After blocking in 10% nonfat dry milk, the primary antibody was applied to 1:1000 dilution overnight at 4°C. To detect the full-length of E-cadherin (120 kDa) and the 80 kDa fragment, the human E-cadherin-1 monoclonal antibody (HECD-1, Zymed Laboratories, San Francisco, CA), raised against the extracellular domain, was used. The E-cadherin cytoplasmic antibody was C20820 (BD Transduction Laboratories). After washing, the membrane was incubated with specific horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech) for 1 h at room temperature. The signals were visualized using the enhanced chemiluminescence and autoradiography.**

**RESULTS**

As reported in one of our earlier studies, Western blot analysis revealed considerable accumulation of a 97 kDa E-cadherin fragment in localized prostate cancer (14). This fragment was significantly increased in localized tumor samples as compared with matched histologically normal prostate tissue. Interestingly, we noticed the appearance of an 80 kDa fragment in one of the tumor samples (Fig. 1, arrow), and to a much lesser extent in 3 other tumor samples. This 80 kDa fragment was not detected in any of the matched normal controls. Examination of prostate cancer metastases demonstrated that the 80 kDa fragment was present in all of the metastatic samples, including a subset of seven metastatic liver samples derived from seven different patients (Fig. 2A). The 80 kDa fragment was not detectable in matched normal liver controls that were harvested from the same patient. The 80 kDa E-cadherin fragment was not only present in the liver metastases but was also present at high levels in a variety of metastatic organ sites of the same patient (Fig. 2B). These findings indicate that the generation of the 80 kDa fragment is strongly associated with metastatic progression, because it is not detected in any normal tissue examined and is detected very rarely in localized prostate cancer.

We attempted to map the truncation of E-cadherin that generated the 80 kDa in metastatic prostate cancer. To determine its origin from full-length protein, we used domain-specific antibodies. Fig. 3C provides a schematic map of the HECD-1 (Zymed Laboratories; Catalog no. 13-1700) and C20820 (BD Transduction Laboratories; Catalog no. 610181) antibody epitopes of E-cadherin. Western blots of protein lysates, derived from metastatic liver samples, demonstrated that the 80 kDa fragment is strongly recognized by the NH₂-terminal antibody human E-cadherin-1, which suggests that the 80 kDa fragment originates from the extracellular domain of full-length E-cadherin. This was confirmed by using a second, cytosolic domain-specific antibody C20820, which, although able to de-
Detection of E-cadherin and its cleaved fragments in human prostate cancer tissue and matched normal controls. E-cadherin Western Blot analysis of four patient groups (A–D) with four patients each. Tumor (T) and matched normal (N) tissue samples were dissected and proteins extracted. One strong 80 kDa band (arrow) is seen in patient sample 3, group A. Lysates of nonstarved LNCaP cells are included for comparison. kDa, Mr in thousands.

Because of its extracellular localization and the fact that the 80 kDa fragment has been found in the serum of other cancer diseases of the stomach (4, 5), colon (7) and bladder (3, 6). The 80 kDa fragment can be produced and detected in the supernatant of LNCaP or MCF-7 cells after serum deprivation (15). As demonstrated in Fig. 3B, the soluble 80 kDa fragment from the media of serum-starved LNCaP cells migrates at the same molecular weight as the fragment observed in metastatic tissue.

MMP activity has recently been associated with the cleavage of E-cadherin (9, 16). Statistical analysis of cDNA expression data for normal prostate, localized prostate cancer, and metastatic tissue samples using a 10k expression array (10, 11) revealed that neither matrilysin nor stromelysin were significantly overexpressed in the metastatic tissue samples, as anticipated from previous reports (9, 16). In fact, Matrilysin showed a significant underexpression in the metastases compared with localized prostate cancer (Mann-Whitney test, P = 0.007; Table 1). Sixteen different members of the MMP family and their inhibitors were represented on the cDNA chip of the University of Michigan and suitable for analysis. Ten of these 16 targets encoding different proteins were significantly up- or down-regulated on the RNA level in metastatic tissue compared with localized prostate cancer. Detailed results of the statistical analysis are given in Table 1. On the basis of this analysis, increased highly significant difference between the serum levels of 61 localized prostate cancer cases and serum levels of 16 patients with metastatic disease (P < 0.001). The concentrations of the 80 kDa fragment in the serum of the three categories is graphically shown in a box plot (Fig. 4). Within the group of patients with localized prostate cancer, a possible association between 80 kDa serum levels and clinical parameters was tested. Four categories of 80 kDa serum concentration were created according to the calculated quartiles of the 61 patients. There was no association (Cramer’s V; value <0.24) between expression of the E-cadherin fragment in the serum and any of the following clinical parameters: age, preoperative prostate-specific antigen, tumor stage, Gleason score (categorized Gleason sum: <7; 7; >7), multifocal tumor, seminal vesicle involvement, surgical margin, tumor size, weight of prostate gland, and race.

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activity of MMP12, MMP13, ADAM1, ADAM12, and ADAM15 could be associated with increased disruption of E-cadherin in the metastatic sites of prostate cancer, as reflected by the increased presence of the 80 kDa fragment. Interestingly, two tissue inhibitors of MMPs (TIMPs), were significantly decreased (TIMP2, \( P < 0.001 \); TIMP3, \( P < 0.001 \)), at the RNA transcript level in metastatic samples, which may result in an increased activity of several MMPs. We have independently validated the changes in gene expression for ADAM15 and TIMP2 and TIMP3 by Western blot and in a human prostate cancer tissue array (data not shown).

**DISCUSSION**

The first report demonstrating an association between altered cell adhesion and tumor progression was published nearly 60 years ago (17). Early reports of E-cadherin proteolysis and cleavage emerged 40 years later when Wheelock and Damsky presented the discovery of a novel 80 kDa species of E-cadherin in the media of mouse and human mammary tumor cells (15). This proteolytic fragment, which was derived from the NH2-terminal extracellular domain of E-cadherin, could be generated by a number of stress stimuli, such as serum deprivation and high calcium loading and was particularly intriguing because of its ability to disrupt the lateral adhesion of mammary epithelium. It was demonstrated that MMP activity could recapitulate the generation of this fragment in cell culture systems (9, 18). In recent studies, decreased E-cadherin expression, either alone or in combination with other biomarkers, has been associated with prostate cancer progression (19, 20).

We hypothesized that specific proteolytic cleavage events target and inactivate E-cadherin and result in the disruption of interepithelial adhesion and the promotion of the malignant progression of prostate epithelial cells. In our previous studies, we have observed that increased cleavage of E-cadherin, generating 97 kDa/100 kDa fragments, was significantly associated with localized prostate cancer as compared with adjacent normal tissue (2, 14). In this study, we present the first report of the existence of the 80 kDa E-cadherin fragment in the extracellular compartment of human tumor tissue. Interestingly, this fragment seemed to be almost exclusively present in the metastatic sites of prostate cancer and was independent of the organ site to which the cancer had spread. A time course experiment and comparison with adjacent normal tissue showed that the appearance of the 80 kDa fragment was associated with increased disruption of E-cadherin in the metastatic sites of prostate cancer, as reflected by the increased presence of the 80 kDa fragment. Interestingly, two tissue inhibitors of MMPs (TIMPs), were significantly decreased (TIMP2, \( P < 0.001 \); TIMP3, \( P < 0.001 \)), at the RNA transcript level in metastatic samples, which may result in an increased activity of several MMPs. We have independently validated the changes in gene expression for ADAM15 and TIMP2 and TIMP3 by Western blot and in a human prostate cancer tissue array (data not shown).

**Table 1** Comparison of expression data derived from localized prostate cancer (Loc PCA) and metastatic prostate cancer (PCA mets) tissue samples

<table>
<thead>
<tr>
<th>Expression Up-regulated</th>
<th>Loc PCA vs.</th>
<th>PCA mets</th>
<th>( P )</th>
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<tr>
<td>MMP1</td>
<td>0.112</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP7</td>
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<tr>
<td>MMP9</td>
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<tr>
<td>MMP12</td>
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<td>MMP13</td>
<td>0.046</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP15</td>
<td>0.000</td>
<td></td>
<td></td>
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<tr>
<td>MMP16</td>
<td>0.905</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP17</td>
<td>0.865</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADAM1(^a)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ADAM10</td>
<td>0.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADAM11</td>
<td>0.063</td>
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<tr>
<td>ADAM12</td>
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<td></td>
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</tr>
<tr>
<td>ADAM15</td>
<td>0.003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIMP1</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>TIMP2</td>
<td>0.001</td>
<td></td>
<td></td>
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<tr>
<td>TIMP3</td>
<td>0.000</td>
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\(^a\) ADAM, a disintegrin and MMP; TIMP, tissue inhibitor of MMP.
ance of the 80 kDa fragment was independent of ischemic processes, which is in accordance with the literature (data not shown; Ref. 21).

The 80 kDa fragment of E-cadherin is of interest not only because its appearance has been associated with increased invasiveness (15, 22) but also because it is detectable in the serum. In this study, we demonstrated a very strong association between the soluble 80 kDa fragment and the presence of prostate cancer metastasis in both tissue and serum. This is in agreement with several studies reporting the existence of the fragment in the serum of patients suffering from other types of cancer (3–7). The potential use of the 80 kDa fragment as a diagnostic marker has been the subject of controversy for other tumor types such as bladder (6, 23) or colon (4, 5, 7, 24). In this setting, there was increased expression of the 80 kDa fragment in the serum of patients compared with that in the controls, but this difference did not reach statistical significance. Whether the variance in serum expression is associated with predictive value in patients with localized prostate cancer remains a matter of speculation. We could not demonstrate a strong association between the clinical and pathological parameters and the expression of the 80 kDa fragment in localized prostate cancer cases. Future analyses, including more cases and a long follow-up time, may demonstrate an association with tumor burden and may possibly reveal predictive information between the presence of the E-cadherin fragment and the failure during follow-up after cancer-specific therapy.

Although a number of membrane proteins are cleaved within their ectodomains by MMP activity, specific enzymes have not been identified in most cases. The same is true for the cleavage of the E-cadherin ectodomain. Two secreted MMPs, matrilysin and stromelysin, have been implicated in the proteolysis of E-cadherin and the generation of the soluble 80 kDa fragment (9, 16, 18). However, this is in contradiction to a study that postulated an unidentified membrane-associated MMP that is responsible for E-cadherin cleavage (25). Additionally, MCF-7 mammary carcinoma cells, which are known to generate large amounts of the 80 kDa fragment, do not exhibit any measurable amounts of stromelysin-1 (26). The identification of the protease that is responsible for generating the 80 kDa fragment of E-cadherin in metastatic prostate cancer has not been accomplished. Additional support for this comes from the presented analysis of cDNA microarray expression data of normal prostate, localized prostate cancer, and metastatic prostate cancer (10, 11). Analysis of a 10k gene chip did not indicate increased expression of matrilysin or stromelysin in metastatic prostate cancer. In fact, on the transcript level there was a significant decrease in matrilysin expression in prostate cancer tissue compared with normal tissue. Of the MMPs represented on the array, only five were significantly up-regulated, including three ADAMs. The adamalysin family of transmembrane glycoproteins is involved in cell adhesion and proteolytic ectodomain processing of cytokines and adhesion molecules (27). They are modular MMPs that contain an RGD integrin-binding domain (disintegrin), and cysteine-rich, epidermal growth factor-like domains, followed by a transmembrane region and cytoplasmic domain. One of the best characterized members is the tumor necrosis factor-convertase (TACE, ADAM17), which processes pro-tumor necrosis factor, tumor necrosis factor recep-

tors, interleukin-6 receptor, and l-selectin (28). As described above, examination on the mRNA level demonstrated increased expression of ADAM-15 in metastatic prostate cancer. It is also of interest to note that the chromosomal location for ADAM-15 on 1q21, is a region of specific high-level amplification in prostate cancer metastasis (29–32).

In this study, we present the first report of the existence of an 80 kDa fragment of E-cadherin in the extracellular compartment of tumor tissue. This fragment is exclusively seen in neoplastic prostate tissue. The potential role of this 80 kDa fragment in tumorigenesis and its suitability as a tissue or serum biomarker with predictive value will be the subject of additional studies. This study also demonstrates an association of increased levels of several MMPs and metastatic prostate cancer. These results provide evidence that the MMP activity could enhance tumor invasion and metastasis by cleavage and inactivation of E-cadherin.

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