N-Cadherin Expression and Epithelial-Mesenchymal Transition in Pancreatic Carcinoma

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ABSTRACT

Purpose: Loss of intercellular adhesion and increased cell motility promote tumor cell invasion. In the present study, E- and N-cadherin, members of the classical cadherin family, are investigated as inducers of epithelial-to-mesenchymal transition (EMT) that is thought to play a fundamental role during the early steps of invasion and metastasis of carcinomas. Cell growth factors are known to regulate cell adhesion molecules. The purpose of the study presented here was to investigate whether a gain in N-cadherin in pancreatic cancer is involved in the process of metastasis via EMT and whether its expression is affected by growth factors.

Experimental Design: We immunohistochemically examined the expression of N- and E-cadherins and vimentin, a mesenchymal marker, in pancreatic primary and metastatic tumors. Correlations among the expressions of N-cadherin, transforming growth factor (TGF)β, and fibroblast growth factor 2 were evaluated in both tumors, and the induction of cadherin and vimentin by growth factors was studied in cultured cell lines.

Results: N-cadherin expression was observed in 13 of 30 primary tumors and in 8 of 15 metastatic tumors. N-cadherin expression correlated with neural invasion (P = 0.008), histological type (P = 0.043), fibroblast growth factor expression in primary tumors (P = 0.007), and TGF expression (P = 0.004) and vimentin (P = 0.01) in metastatic tumors. Vimentin, a mesenchymal marker, was observed in a few cancer cells of primary tumor but was substantially expressed in liver metastasis. TGF stimulated N-cadherin and vimentin protein expression and decreased E-cadherin expression of Panc-1 cells with morphological change.

Conclusion: This study provided the morphological evidence of EMT in pancreatic carcinoma and revealed that overexpression of N-cadherin is involved in EMT and is affected by growth factors.

INTRODUCTION

Cadherins, calcium-dependent cell adhesion molecules, are involved in maintaining the epithelial structure of a variety of tissues and play important roles in embryonic development and maintenance of normal tissue architecture (1). It has been well established that E-cadherin plays a role in tumor progression and metastasis, because loss of E-cadherin expression has been found to correlate with an invasive and undifferentiated phenotype in many carcinomas including pancreatic carcinoma (2–7). N-cadherin (neural cadherin), another adhesion molecule, is associated with a heightened invasive potential in cancer. A recent study demonstrated that overexpression of N-cadherin in breast carcinoma correlates with invasiveness as a result of N-cadherin-mediated interactions between cancer and stromal cells (8). The phenotype of breast cancer cell lines was found to undergo dedifferentiation from epithelial to mesenchymal as a result of N-cadherin transfection without a loss of E-cadherin expression (9). In squamous epithelial cells, expression of N-cadherin produced a scattered phenotype with an epithelial-to-mesenchymal transition (EMT) in association with a reduction in E- and P-cadherins (10). In N-cadherin transfected breast cancer cells, N-cadherin promotes motility and invasion, but the reduction in the expression of E-cadherin does not necessarily correlate with either of these two (11). These findings indicate that N-cadherin, functioning as adhesion molecules, may be more important than E-cadherin for metastasis and invasion.

Changes in cell adhesion, regulated by environmental signals such as growth factors, appear to be necessary for dynamic cellular movement and maintenance of tissue patterning. Growth factors and cytokines can modulate expression of E-cadherin; for example, transforming growth factor (TGF)β induces dedifferentiation of the phenotype of normal mammary epithelial cells from epithelial to fibroblastic, which correlates with a reduction in the expression of E-cadherin (12). Fibroblast growth factor (FGF)-1 and FGF-2 enhance E-cadherin-mediated cell-cell adhesion and reduce in vitro invasion in cancer cells (13, 14). Furthermore, N-cadherin-dependent motility may be mediated by FGF receptor signaling, but the mechanism of regulating cadherin expression is not known (8, 11).

Pancreatic cancer has a very poor prognosis, and the 5-year survival rate for patients who underwent surgical resection is reported to be only 8.1–24.0% (15–18). The reasons for such poor prognosis are a high incidence of local recurrence, lymph node metastasis, hepatic metastasis, and peritoneal dissemination. As pancreatic cancer progresses, a high rate of neural
invasion, which is associated with poor prognosis, is observed and increases even more as the cancer becomes undifferentiated (19–21). One of the reasons that pancreatic cancer extends along the neural bands is probably due to the abundance of nerves inside and around the pancreas. Another possibility is that the adhesion molecules, which define the affinity of cancer cells to neural band, subsequently affect the motility of cancer cells. One study of the relationship between neural cell adhesion molecule expression and neural invasion found no correlation (22). Because N-cadherin is highly prevalent in neuronal tissues and is also found in fibroblasts, muscles, vascular endothelium, and peritoneal mesothelial cells (23–26), it is important to investigate the association between the expression of N-cadherin in pancreatic cancer and its invasiveness including neural invasion.

The purpose of this study presented here was to investigate whether a gain in N-cadherin in pancreatic cancer is involved in the process of metastasis via EMT and whether its expression is affected by growth factors. To this end, the expression of N- and E-cadherins and vimentin, a mesenchymal marker, was immunohistochemically examined in pancreatic primary and metastatic tumors. In addition, clinicopathological parameters including patient prognosis were assessed in relation to N-cadherin expression. Correlations among the expressions of N-cadherin, TGFβ, and FGF were evaluated in both primary and metastatic tumors. Finally, the induction of cadherin and vimentin by growth factors was examined in cultured cell lines.

MATERIALS AND METHODS

Antibodies and Growth Factors. Monoclonal mouse immunoglobulin (IgG) antibodies to N-cadherin were purchased from Zymed Laboratories Inc. (San Francisco, CA), E-cadherin from Takara Bio Inc. (Shiga, Japan), and vimentin from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies to E-cadherin immunoglobulin (IgG) antibodies to N-cadherin were purchased separately. A total of 45 samples were used for immunohistochemical examination 20% of the cancer cells were stained and a high N-cadherin in invasion, which is associated with poor prognosis, is observed and increases even more as the cancer becomes undifferentiated (19–21). One of the reasons that pancreatic cancer extends along the neural bands is probably due to the abundance of nerves inside and around the pancreas. Another possibility is that the adhesion molecules, which define the affinity of cancer cells to neural band, subsequently affect the motility of cancer cells. One study of the relationship between neural cell adhesion molecule expression and neural invasion found no correlation (22). Because N-cadherin is highly prevalent in neuronal tissues and is also found in fibroblasts, muscles, vascular endothelium, and peritoneal mesothelial cells (23–26), it is important to investigate the association between the expression of N-cadherin in pancreatic cancer and its invasiveness including neural invasion.

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staining were also divided into two groups in the same way as that of N-cadherin staining.

**Cells.** Five human pancreatic cancer cell lines, AsPC-1, BxPC-3, Capan-2, MiaPaCa-2, and Panc-1, were purchased from American Type Culture Collection (Rockville, MD). Cells were grown in monolayer culture in RPMI 1640 (Life Technologies Inc., Gaithersburg, MD) containing 10% fetal bovine serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37°C in a humidified atmosphere composed of 95% air and 5% CO₂.

After the cells had been incubated for 24 h at 37°C, fresh serum-free medium was added alone or supplemented with 5 or 10 ng/ml of FGF-2 or TGFβ. The cells were then kept for an additional 24–48 h at 37°C. For the activation of the FGF receptor, 1 μg/ml of heparin was added to the FGF-2. Protein expression of N-, E-cadherin, and vimentin with or without FGF and TGFβ treatment was evaluated by using Western blot and immunocytochemical analysis.

**Protein Extraction and Western Blotting.** Cells were harvested and lysed with radioimmunoprecipitation assay buffer [10 mM PBS (pH 7.4), 0.1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS containing 1 mM of phenylmethylsulfonyl fluoride and gantrexate mesilate]. Total extracts were cleared by centrifugation at 14,000 for 10 min at 4°C, and the extracted protein was then subjected to Western blotting as described previously (3). Fifty-μg aliquots of protein were loaded onto 7.5% SDS-polyacrylamide gels and transblotted to a 0.45-μm immobolin-P transfer membrane (Millpore, Bedford, MA). The blots were blocked at 4°C overnight with 5% nonfat milk in TBST [50 mM Tris-HCL (pH 7.4), 150 m M NaCl, and 0.2% Tween-20] and reacted with appropriately diluted primary antibody solutions (1:100) for 1 h at room temperature. The enhanced chemiluminescence system (Amersham Life Sciences, Amersham, United Kingdom) was used for the detection of bound antibodies. Primary antibody-bound membranes were incubated for 1 h at room temperature with horseradish peroxide-conjugated, antimouse IgG diluted with TBST and Tris-buffered saline. The membranes were treated with enhanced chemiluminescence reagents according to the manufacturer’s protocol. The membranes were exposed to X-ray film for 1–15 min. Protein expression was measured with the ATTO spot analyzer system AE-6920M (ATTO Corporation, Tokyo, Japan). The quantity of target protein was calibrated by that of β-actin, and relative intensities were obtained.

**Immunocytochemical Analysis.** After the cells had been grown on glass coverslips to 50% confluence, they were washed with PBS and fixed with 100% ethanol and 100% acetic acid (9:1) for 10 min on ice. Only for vimentin processing, the cells were incubated with 2% Triton X in PBS. For all of the other processes, they were incubated with the N-, E-cadherin, and vimentin monoclonal antibody for 1 h at room temperature. Nonspecific protein was blocked with 2% normal goat serum in PBS for 30 min. After washing, Cy3-conjugated secondary antismouse IgG was applied in the dark followed by incubation for 1 h at room temperature. Finally, the cells were observed under a fluorescence microscope.

**Statistical Analysis.** Relationships between the clinicopathologic characteristics of the 30 patients with high and low N- and E-cadherins were examined with the χ² test or Fisher’s exact probability test. Survival rates were calculated with the Kaplan-Meier method, and the differences between high and low N-cadherin expression groups were evaluated with the log-rank test. The results in in vitro experiments are expressed as the mean value ± SD. Statistical differences among each time point were assessed by ANOVA. The Turkey-Kramer test for post-hoc multiple comparisons was used when ANOVA was significant. P values < 0.05 were considered statistically significant.

**RESULTS**

**Overexpression of N-Cadherin and Reduced Expression of E-Cadherin in Pancreatic Cancer Tissue.** The staining of N-cadherin in primary pancreatic cancer tissue was mainly identified in the cytoplasm of cancer cells, infiltrating cells, and neural bands (Fig. 1A). In noncancerous tissues, acinar, ductal, and islet cells were not stained with N-cadherin. Thirteen of the 30 pancreatic cancers (43%) were positive for N-cadherin expression. In metastatic liver tumors, N-cadherin immunoreactivity was strongly identified in noncancerous hepatic cells as well as in the cytoplasm of metastatic cancer cells (Fig. 1, B and C). Eight of 15 metastatic liver tumors (53%) were positive for N-cadherin expression.

In primary cancer tissues, E-cadherin expression in cancer cells was heterogeneous or negative compared with that in normal epithelial tissues and was characterized by patterns with variable degrees of membrane and cytoplasmic staining (Fig. 1D).
 Twenty pancreatic cancers (66%) were found to have reduced expression of E-cadherin. This expression was preserved in the noncancerous hepatic cells but reduced in the metastatic cancer cells of the metastatic liver tumors (Fig. 1E). Reduced expression of E-cadherin was also detected in 11 (73%) metastatic liver tumors.

Correlation between N- and E-Cadherin Expression and Clinicopathological Features Including Survival Analysis. Table 1 summarizes the relationship between N- and E-cadherin expression and the clinicopathological features of the pancreatic cancers. N-cadherin expression in primary tumors significantly correlated with the extent of intrapancreatic nerve invasion and histological grade: tumors with positive nerve invasion and poorly differentiation had higher expression of N-cadherin. The survival rates for the 13 patients with N-cadherin-positive tumors and 17 with N-cadherin-negative tumors were not significantly different (Fig. 2). Moreover, there was no significant correlation between reduced E-cadherin expression and any of the clinicopathological factors.

Correlations among N-cadherin, E-cadherin, FGF-2, TGFβ, and Vimentin Expression in Pancreatic Cancer Tissue. TGFβ and FGF expressions were observed in fibroblasts, islet cells, and acinar cells in noncancerous tissue, but those in cancer cells were heterogeneous (Fig. 3, A, B, D, and E). Vimentin, a mesenchymal marker, was mainly observed in fibroblasts that surrounded the cancer cells and in a few cancer cells in primary tumors (Fig. 3C). However, vimentin expression was substantially in cancer cells of hepatic metastasis (Fig. 3, F and G). The relationship between the expression of N-cadherin staining and those of E-cadherin, FGF2, TGFβ, and vimentin was analyzed on the basis of expressions only in cancer cells. In primary tumors, there was a significant correlation between N-cadherin expression and FGF-2: tumors with a higher expression of FGF-2 also showed higher expression of N-cadherin (Table 2). Metastatic liver tumors demonstrated significant correlations between N-cadherin and TGFβ and vimentin: tumors with a higher expression of N-cadherin and vimentin also showed a higher expression of TGFβ (Table 3). When expression of these factors in primary tumors and hepatic metastases...
were compared, the expression of N-cadherin and vimentin was higher in the latter than in the former, but the difference did not reach statistical significance. No correlation could be established between overexpression of N-cadherin and reduced expression of E-cadherin.

**Up-Regulation of N-Cadherin by Growth Factors in Cancer Cells.** N- and E-cadherins and vimentin protein expression levels in pancreatic cancer cell lines were evaluated by Western blot analysis (Fig. 4). N-cadherin and E-cadherin were detected as a single band corresponding to the respective molecular sizes of 136 kDa and 123 kDa, which is consistent with their known molecular weight. Expression levels of N- and E-cadherin varied among five pancreatic cancer cell lines. N-cadherin was expressed in BxPC-3, Panc-1, and more strongly in Capan-2, whereas E-cadherin expression was observed in the four cell lines except MIAPaCa-2. Vimentin was detected as a single band corresponding to the molecular size of 56 kDa and was expressed in the four cell lines except BxPC-3.

Changes in the expression of N-cadherin, E-cadherin, and vimentin as a result of TGFβ or FGF-2 treatment was examined by Western blot analysis and immunocytochemistry. TGFβ treatment (5 ng/ml) significantly increased N-cadherin and vimentin protein expression and decreased E-cadherin expression in Panc-1 cells (Fig. 5, A and C). FGF-2 treatment (10 ng/ml) also increased N-cadherin expression in BxPC-3 cells, but E-cadherin expression was not markedly changed (Fig. 5B). Immunocytochemistry confirmed changes in N- and E-cadherin and vimentin in Panc-1 cells in response to changes in TGFβ and N-cadherin and in BxPC-3 cells in response to changes in FGF-2 (Fig. 6). Immunoreactivity for N- and E-cadherin was mainly observed in cell membrane and for vimentin in cytoplasm (Fig. 6). It was noted that TGFβ treatment caused Panc-1 cells to form scattered appearance of cell clusters. Other cell lines were refractory to the treatment with TGFβ and FGF-2.

**Table 2.** N-cadherin expression in primary pancreatic cancer

<table>
<thead>
<tr>
<th>N-cadherin</th>
<th>Negative (n = 17)</th>
<th>Positive (n = 13)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-cadherin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>6</td>
<td>4</td>
<td>0.794</td>
</tr>
<tr>
<td>Reduced</td>
<td>11</td>
<td>9</td>
<td>0.176</td>
</tr>
<tr>
<td>TGFβα*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>8</td>
<td>3</td>
<td>0.007</td>
</tr>
<tr>
<td>High</td>
<td>9</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>FGF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>11</td>
<td>2</td>
<td>0.712</td>
</tr>
<tr>
<td>High</td>
<td>6</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Vimentin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>15</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

*TGF, transforming growth factor; FGF, fibroblast growth factor.
expression means that the tumor cells have been converted to a metastatic phenotype, for example, EMT. In the study presented here, we could not find any correlation between N- and E-cadherin expression in primary pancreatic or in metastatic tumors. An N-cadherin transfection study of breast cancer cells demonstrated recently that N-cadherin promotes motility and invasion and that a reduced expression of E-cadherin does not necessarily correlate with motility or invasion (11). N-cadherin itself might have the potential to promote tumor progression and metastasis, because in our study overexpression of N-cadherin and reduced expression of E-cadherin was much more evident in metastatic than in primary tumors. In addition, vimentin, a mesenchymal marker, was strongly expressed in cancer cells of hepatic metastasis, which in turn was significantly associated with the expression of N-cadherin. Although it is very difficult to provide firm evidence of EMT in cancer tissue, these results suggest that during the metastatic process, EMT may occur, and pancreatic cancer cells may convert to a metastatic phenotype so that the process is related to the changes in cadherin expression.

A number of studies have shown that epithelial cells can be induced to scatter in response to environmental signals such as growth factors (35, 41, 42). It was shown that TGFβ induces a mesenchymal transdifferentiation and modulates E-cadherin expression in epithelial cells (34, 43, 44). Transfection of N-cadherin into breast cancer cells resulted in increased cell migration and invasion, which was greatly enhanced by the

### DISCUSSION

Important steps in the development of metastasis and local recurrence in vivo have been linked to enhanced cell-cell adhesion or cell-matrix adhesion in the tumor itself or to enhanced cancer cell extrication at different sites (4). Analysis of adhesion molecules in human cancer cell lines suggested that those molecules might influence the migration of tumor cells (33). To infiltrate host tissues, cancer cells of epithelial origin have to separate from the tumor mass by breaking their cell-cell contacts, also known as adhesions functions (34, 35). Various studies of clinical tumor tissue samples and tumor cell lines demonstrated that reduced expression of E-cadherin is associated with tumor progression and enhanced cell invasiveness (36–38).

Acquisition of metastatic phenotype of cancer cells consists of multiple steps including EMT. Changes in cadherin expression patterns may play a role in the process of EMT and cellular motility (39). Nonepithelial cadherin, including N-cadherin, was found to induce a mesenchymal-scattered phenotype associated with reduced E- and P-cadherin in squamous epithelial cells (10). In prostate cancer, especially undifferentiated tumors and metastases, E-cadherin was mostly negative, and all of the cancer cells were positive for N-cadherin in what is called “the cadherin switch” (40). The purpose of the current study was to investigate whether a gain in N-cadherin in pancreatic cancer is involved in the process of metastasis via EMT and whether its expression is affected by growth factors. In epithelial cells the resultant loss of E-cadherin and the increase in N-cadherin

### Table 3 N-cadherin expression in hepatic metastasis.

<table>
<thead>
<tr>
<th>N-cadherin</th>
<th>Negative (n = 7)</th>
<th>Positive (n = 8)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-cadherin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Reduced</td>
<td>4</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>TGFβ⁺</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (n = 7)</td>
<td>6</td>
<td>1</td>
<td>0.004</td>
</tr>
<tr>
<td>High (n = 8)</td>
<td>1</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>FGF2</td>
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<td></td>
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</tr>
<tr>
<td>Low (n = 6)</td>
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<td>4</td>
<td>0.398</td>
</tr>
<tr>
<td>High (n = 9)</td>
<td>5</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Vimentin</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Low (n = 10)</td>
<td>7</td>
<td>3</td>
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</tr>
<tr>
<td>High (n = 5)</td>
<td>0</td>
<td>5</td>
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</table>

* TGF, transforming growth factor; FGF, fibroblast growth factor.

![Fig. 4](image) Western blot analysis of E-cadherin, N-cadherin, and vimentin in five pancreatic cancer cell lines. Thirty μg of total proteins extracted from cancer cells were loaded onto each lane.

![Fig. 5](image) Western blot analysis of changes in N-cadherin, E-cadherin, and vimentin expression by in transforming growth factor (TGF)β and fibroblast growth factor (FGF)2 treatment in pancreatic cancer cells. A, Panc-1 cells were incubated with 5 ng/ml TGFβ for 0, 24, and 48 h. B, BxPC-3 cells were incubated with 10 ng/ml FGF2 for 0, 24, and 48 h. Fifty μg of total proteins were loaded. C, quantitative analysis with image intensifier. n = 3; * significant changes against control (ANOVA). N-cadherin and vimentin expressions were significantly induced, and E-cadherin expression was reduced by 48 h of TGFβ in Panc-1 cells. N-cadherin expression was induced, but E-cadherin and vimentin expressions were not changed by 24 h of FGF2 in BxPC-3 cells; bars, ±SD.
The presence of FGF-2 and accompanied by up-regulation in matrix metalloproteinase-9 activity (8, 11). In our study, we investigated the correlation between the expression of growth factors and cadherin in pancreatic cancer cells in connection with EMT. In primary tumors, there was a significant correlation between N-cadherin expression and FGF-2. In metastatic liver tumors, there were also significant correlations between N-cadherin and TGFβ/H9252 and vimentin: metastatic tumors with a higher expression of TGFβ/H9252 also had a higher expression of N-cadherin and vimentin was found in Panc-1 cells, and high expression of N-cadherin was observed in BxPC-3 cells. Note that TGFβ treatment resulted in scattered appearance of cell clusters.

In conclusion, the study reported here provided morphological evidence of the occurrence of EMT in pancreatic cancer cells.
carcinoma and found that overexpression of N-cadherin is involved in EMT and is affected by growth factors. Because EMT is an important process in the invasion and metastasis of malignant tumor cells (31, 47, 48), it is possible that N-cadherin is the adhesion molecule not only to acquire the fibroblastic morphology of EMT but also to obtain invasive and metastatic potential. To confirm this, it will be necessary to perform an N-cadherin transfection study with an invasion and motility assay.

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