Down-Regulation of E-Cadherin Gene Expression by Collagen Type I and Type III in Pancreatic Cancer Cell Lines

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ABSTRACT

E-cadherin-mediated cell-cell adhesion is reduced in epithelial tumors, which is thought to be a prerequisite to acquire invasive properties. We observed that several pancreatic carcinoma cell lines with high metastatic potential expressed normal levels of E-cadherin and possessed functional E-cadherin/catenin adhesion complexes. When the cell lines PANC-1, BxPC-3, and PaTu8988s were cultured either on type I or type III collagen, E-cadherin gene expression was repressed, and E-cadherin and catenin protein concentrations were reduced. In contrast, growth on fibronectin and collagen type IV had no influence. Collagen type I- or type III-dependent reduction of E-cadherin expression led to decreased cell-cell adhesion, increased proliferation, and migratory activity as well as morphological transformation. Overexpression of activated c-Src in PANC-1 cells mimicked collagen-induced E-cadherin down-regulation and changed the elevated cell proliferation and migration. Conversely, treatment of cells with the Src-inhibitors PP1 or herbimycin A resulted in complete suppression of collagen type I-induced E-cadherin decrease. Our data demonstrate that specific collagens are able to promote metastatic behavior by down-regulation of E-cadherin gene expression in a Src-kinase-dependent manner. This points toward a novel mechanism for substrate-dependent signaling and underlines the significance of extracellular matrix environment for tumor growth and invasiveness.

INTRODUCTION

Functional cell-cell contacts play a critical role in the organization of differentiated epithelial tissues. The transmembrane glycoprotein E-cadherin represents the predominant cadherin in epithelial cells and is responsible for cell-cell contacts via adherens junctions (1). The intracellular domain of E-cadherin attaches directly to β-catenin or γ-catenin, which associates with α-catenin, linking the protein complex to the actin cytoskeleton (2, 3).

The cadherin-catenin complex is highly dynamic and rearrangement of cell-cell adhesions can be observed in various cellular processes, such as epithelial cell scattering, proliferation, and migration (4–6). Pathological processes like cancer cell proliferation and metastatic events are correlated with defective E-cadherin-mediated cell-cell adhesion (7–10). Loss of E-cadherin/catenin-dependent cell-cell contact is often caused by mutations of the E-cadherin or the α-catenin gene (11–13). Another mechanism of altered cell-cell adhesion is the down-regulation of E-cadherin gene expression, for instance by modified methylation patterns of a 5' CpG island in the E-cadherin promoter (14, 15). Most recently, the transcription factor snail was described to contribute to down-regulation of E-cadherin gene expression by binding to an E-palindrome sequence in the E-cadherin promoter region. In different carcinoma cell lines, snail expression is responsible for dysfunctional E-cadherin cell-cell adhesion (16, 17). This strengthens the idea that the level of cadherin expression, rather than the level of catenins seems to be the rate-limiting step for E-cadherin complex formation and cell adhesion, thereby emphasizing the importance of accurate regulation of E-cadherin expression (18, 19).

In addition to defects in E-cadherin gene expression, posttranslational modifications like tyrosine phosphorylation, especially of β-catenin also contributes to loss of adhesion and dispersal of cancer cells (7, 20). Protein kinases of the Src-family are shown to affect cadherin-mediated cell-cell adhesion by phosphorylation of catenins and link signal transduction cascades to the regulation of cell adhesion (7, 21, 22). In fibroblasts, binding of integrins to their ligands leads to formation of focal adhesion plaques and to activation of focal adhesion kinase, which, in turn, recruits and activates the Src kinase (23). Furthermore, activation of Scr family kinases is required to disrupt cadherin-dependent cell-cell contacts (24).

As reported for other epithelial tumors, dramatic changes in E-cadherin-mediated adherens junctions are manifested in a substantial part of metastatic pancreatic tumors. In addition to mutations in the E-cadherin gene, defects in assembly and disassembly of the E-cadherin/catenin also contribute to metastatic behavior. This is underscored by clinical studies, which provide evidence that 30–60% of moderately to poorly differentiated pancreatic carcinomas show no changes of E-cadherin expression in immunohistological analyses (25, 26).

Pancreatic carcinomas differ from other solid tumors by enormous production and deposition of ECM around tumor cells (27). Beside mesenchymal cells, the epithelial tumor cells themselves contribute to production and assembly of ECM proteins (28).

The exceptionally high ratio of ECM:tumor cells in pancreatic carcinomas and the presence of E-cadherin in cultured pancreatic carcinoma cell lines prompted us to address the influence of different ECM components on E-cadherin-mediated cell-cell adhesion. The pancreatic cancer cell lines BxPC-3, PANC-1, and PaTu8988s contain functional E-cadherin-based adherens junctions. However, a mesenchymal transformation occurred when these cell lines were cultured on collagen type I or type III, which possibly contributed to enhanced invasive properties. This is apparent by significantly reduced E-cadherin and catenin protein concentrations and decreased cell-cell aggregation. Cells on collagen type I or III show enhanced proliferation and cell migration compared with TCP, collagen type IV, or fibronectin. Transfection of PANC-1 cells with an activated c-Src tyrosine kinase revealed Src-dependent, cell-matrix-induced down-regulation of E-cadherin gene expression.

MATERIALS AND METHODS

Antibodies. Monoclonal anti-E-cadherin and anti-γ-catenin were purchased from Transduction Laboratories (Lexington, KY). Monoclonal anti-actin and polyclonal anti-β-catenin and anti-α-catenin were from Sigma Chemical (St. Louis, MO). Polyclonal anti-integrin α2 and anti-integrin β1, were from Chemicon International (Temecula, CA). Anti-integrin αv monoclonal antibody was purchased from Serotec (Oxford, United Kingdom). A

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The abbreviations used are: ECM, extracellular matrix; TCP, tissue culture plastic; ILK, integrin-linked kinase; ERK, extracellular signal-regulated kinase.
monoclonal anti-src antibody was supplied by Upstate Biotechnology (Raleigh, NC). Cy3-conjugated antiamouse IgG was from Dianova (Hamburg, Germany) and Alexa 488-conjugated antirabbit antisem from Molecular Probes (Eugene, OR).

**Cell Lines and Culture Conditions.** The cell lines PANC-1 and BxPC-3 were obtained from American Type Culture Collection (Rockville, MD). PaTu8988s was kindly provided by H. Kern and P. Elsässer (University of Marburg, Germany). Epithelial cells were grown in Dulbecco’s modified Eagle medium complemented with 10% FCS, 1% nonessential amino acids, and 1% l-glutamine (all from Life Technologies, Karlsruhe, Germany). For analyses of substrate influence, the cells were seeded in 100-mm dishes coated with collagen type I, type III, or type IV or fibronectin, or on normal TCP. For collagen type III, which was of human origin. Mouse collagen type IV was concentrated at 10 µg/cm2 in coated culture dishes, and human fibronectin had an amount of 2 µg/cm2. All of the coated dishes were purchased from Becton Dickinson (Heidelberg, Germany). For immunofluorescence examination, the cells were seeded on collagen type I- or fibronectin-covered coverslips (Becton Dickinson). Phase-contrast microscopy was done using an inverse photomicroscope (Carl Zeiss, Oberkochen, Germany).

**Generation of Stable Transfected PANC-1 Cells and Src Kinase Assay.** Activated c-Src mutant Y527F was kindly provided by S. Courtneidge (Sugen Inc., South San Francisco, CA) and subcloned in the expression vector pcDNA3.1 (Invitrogen, Groningen, the Netherlands). PANC-1 cells, 70% confluent, were transfected with 15 µg pcDNA3.1-Src(Y527F) or pcDNA3.1 plasmid, both linearized by EcoRI digestion, using 40 µl of liposomal transfection reagent DMRIE-C (Life Technologies) according to the manufacturer’s instructions. For generation of stable transfected cells, G418-resistant (1 mg/ml) cell clones were subcloned, and Src expression was controlled by Northern blot and Western blot analyses. Two cell lines, exhibiting a strong (p19) or a moderate (p5d) overexpression of Src(Y527F) were chosen for these experiments.

The activity of the Src kinase was determined by an in vitro phosphorylation assay performed following the procedure described by Bolen et al. (29). One h after seeding on different substrates, the cells were lysed and the Src kinase immunoprecipitated. The kinase activity was estimated by the phosphorylation of the Src substrate enolase. The reaction was initiated by the addition of [γ-32P]ATP to the reaction and of rabbit muscle enolase to the precipitated src. Labeled proteins were detected after SDS polyacrylamid gel electrophoresis by autoradiography.

**Immunohistochemistry.** For protein detection, cells were rinsed with ice-cold PBS and lysed by scraping into 500 µl of RIPA buffer (50 mM Tris/HCl (pH 7.2), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 400 µM aprotinin, 50 µM leupeptin, 1 µM soybean trypsin inhibitor, and 0.5 mM Pefabloc) (all from Roche Diagnostics, Mannheim, Germany) and homogenization in a Dounce homogenizer. Cell debris was removed by centrifugation and protein concentration was determined by the bicinchoninic acid method. For coimmunoprecipitation assays, cells were lysed in precipitation buffer (50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% NP40, 1 mM magnesium chloride, 1 mM calcium chloride, 400 µM aprotinin, 50 µM leupeptin, 1 mM soybean trypsin inhibitor, and 0.5 mM Pefabloc) and homogenization was performed in a Dounce homogenizer. One mg of lysate was preclared by incubation with protein G-agarose. After centrifugation, the supernatant was incubated for 2 h at 4°C with 5 µg of anti-E-cadherin antibody preincubated protein G-agarose. The immunoprecipitates were washed three times with ice-cold precipitation buffer and resuspended in 30 µl g of loading buffer. SDS-PAGE was performed according to standard procedures. Forty µg of lysate or the resuspended immunoprecipitates was separated. Proteins were blotted by semidry technique onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) and immunoreactive proteins were visualized by using the enhanced chemiluminescence (ECL) Western blotting detection system (Fierce, Rockford, IL) or by using alkaline phosphatase-conjugated antibodies (Dianova, Hamburg, Germany) and nitroblue tetrazolium/bromo-chloro-indolyl phosphate staining.

**Aggregation Assay.** DNA synthesis was determined by [methyl-3H]thymidine incorporation as described previously (31). Briefly, cells were plated in 24-well plates (5 × 104 cells/well) and allowed to attach for 24 h. Serum starvation was performed by incubation with DME + 0.1% BSA for 36 h, followed by stimulation with growth medium (DME + 10% FCS) for an additional 36 h. [3H]thymidine (1 µCi/well) was added for the last 24 h. Incorporated, acid-insoluble radioactivity was determined by liquid scintillation counting after solubilization in 0.1% NaOH-1% SDS. For determination of cell number, 2 × 105 cells were seeded in 35-mm tissue culture dishes with 2-mm2 grids on the bottom (Nunc, Wiesbaden, Germany) and incubated in growth medium. Cell numbers from five individual grids were determined every 24 h for at least 4 days or until the cells reached confluency. Cultures were refed every 2d day.

**Cell Migration Assay.** One × 105 cells per well were seeded in DME on top of uncoated, collagen type I- or fibronectin-coated polyethylene terephthalate (PET) membranes of transwell inserts (12-well inserts, pore 8 µm; Becton Dickinson, Heidelberg, Germany). After 3 h, mitomycin C (10 µg/ml) was added to the cells to inhibit cell proliferation. Afterward, medium in the bottom chamber was replaced by 1.2 ml of assay medium. Cells in the upper chamber were treated with DME without any supplements. After 48 h of incubation, cells were fixed with 4% (w/v) paraformaldehyde and stained with hematoxylin, and nonmigrated cells on the upper side of the porous membrane were wiped away. Migrated cells were visualized under the microscope, and five visual fields were counted for quantification.

**Aggregation Assay.** To determine the Ca2+-dependent cell-cell-adhesion, aggregation assays were performed as described previously (32) with some modifications: cells were washed with PBS and treated with 0.01% trypsin in HEPES-buffered saline (37 mM NaCl, 5.4 mM KCl, 0.34 mM NaH2PO4, 5.6 mM glucose, and 10 mM HEPES) containing 2 mM CaCl2. Trypsinized cells were centrifuged, washed twice with HEPES with 2 mM CaCl2 and resuspended in the same buffer. The cells were singled using an pipette until no aggregates were monitored. To examine the calcium dependency, EDTA and EGTA to a final concentration of 5 mM were added. To specify E-cadherin-dependency, 5 µM of neutralizing antibody against E-cadherin (DECM1; Sigma Chemicals, St. Louis, MO) was added to the experiment. Cells were allowed to aggregate for 30 min at 37°C with a constant rotation of 70 rpm. The aggregates were documented by photography, and the extent of cell aggregation was calculated by measurement of the aggregation diameter. Three independent assays were performed in quadruplicate.

**RNA Studies.** RNA was extracted using the RNaseasy midi kit from Qiagen (Hilden, Germany) according to manufacturer’s instructions. For Northern blot detection, 20–30 µg of total RNA were separated by electrophoresis and transferred on Hybond-N membranes (Amersham Pharmacia Biotech, Uppsala, Sweden) as described previously (33). Ethidium bromide staining of the agarose gels and hybridization with an 18S rRNA probe were used to verify equal loading and blotting of RNA. A cDNA probe for human E-cadherin and 18S RNA were kindly provided by Dr. Thomas Gress (University of Ulm, Ulm, Germany). Blots were hybridized with [32P]dCTP-labeled cDNA probes as described earlier (33).

**Immunofluorescence Analysis.** For immunohistochemical examination, substrate-coated coverslips purchased from Becton Dickinson (Heidelberg, Germany) with confluent grown cells were fixed in –20°C cold methanol/acetone (1:1) for 20 min. Unspecific binding was blocked by treatment with PBS containing 3% BSA. Cells were incubated for 1 h at 37°C with anti-E-cadherin, anti-β-catenin, or anti-α-catenin as indicated under “Antibodies”, diluted in PBS + 0.3% BSA. Protein localization was visualized by incubation with a secondary Cy3- or Alexa 488-conjugated antibody. Cells were examined by confocal laser-scanning microscopy (ECS 4D Leica, Wetzlar, Germany).
RESULTS

Collagen Disrupts Functional E-Cadherin Adhesion Complexes. To prove the idea that ECM proteins are able to modify cell-cell adhesion, we studied in detail the E-cadherin/catenin complex in the cell lines BxPC-3, PANC-1, and PaTu8988s, which were all derived from human pancreatic carcinomas.

E-cadherin was found to be colocalized with α- and β-catenin in cell-cell contacts as shown by immunohistological staining in BxPC-3 and PANC-1 cells (Fig. 1A). Comparable data were obtained for PaTu8988s (data not shown). When cell lysates were separated into triton-insoluble and triton-soluble fractions, most of E-cadherin and β- and α-catenin was detected in the triton-insoluble fraction, which indicated that this protein complex was associated with the cytoskeleton (Fig. 1B). Because only small amounts of γ-catenin were observed mainly in the triton-soluble fraction, we assume that this catenin is of minor function in cell-cell adhesion in these cell lines.

To confirm that functional E-cadherin/catenin complexes were formed, aggregation assays were carried out (Fig. 1C). For quantification, the size of aggregates was measured after 30 min, and average values were used to compare the outcome of different experiments. During reaggregation, the number of single cells decreased and large cell aggregates were formed in the three cell lines. The presence of either EGTA/EDTA (5 mM each) or an antibody against E-cadherin (DECMA1) blocked cell aggregation, demonstrating that the observed cell-cell adhesion was Ca²⁺ dependent and was mediated by E-cadherin.

To test the influence of ECM on E-cadherin-mediated cell-cell adhesion, we cultured BxPC-3, PANC-1, and PaTu8988s cells on collagen type I, type III, or type IV or on fibronectin in comparison to TCP (uncoated TCP) for 2 days. Thereafter, E-cadherin and catenin expression, as well as protein localization, was examined by immunofluorescence analysis. As shown in Fig. 2A, BxPC-3 cells grown on fibronectin revealed typical membrane localization for E-cadherin, β-catenin, and α-catenin. In contrast, cells grown on collagen type I showed a strong reduction in E-cadherin and β-catenin staining. Obviously no α-catenin was visible in areas of cell-cell contacts. The cell lines PANC-1 and PaTu8988s showed comparable cellular effects when cultured on collagen...
Collagen type I as demonstrated by E-cadherin staining (Fig. 2B). Cells grown on collagen type III exhibited similar changes as examined for those on collagen type I (data not shown). To confirm and quantify the described E-cadherin/catenin reduction, we performed immunoblot analyses. Cells cultured on collagen type I or type III exhibited a marked reduction in the protein content of E-cadherin and $\beta$- and $\alpha$-catenin in total cell lysates as compared with controls grown on TCP (Fig. 3A). In BxPC-3 cells, the signals for E-cadherin and $\beta$-catenin were nearly completely lost (91% reduction), whereas the amount of $\alpha$-catenin was less dramatically affected (64% reduction). Similar results were obtained for PANC-1 and PaTu8988s cells (Fig. 3A). The expression level of $\gamma$-catenin, which is hardly detectable in the three cell lines, was not influenced (data not shown). The concentration of constitutively expressed proteins, such as $\beta$-actin, was not altered in these samples. The decrease in E-cadherin and $\beta$- and $\alpha$-catenin content was collagen type I- and III-specific because none of the analyzed cell lines exhibited altered concentrations on growth on fibronectin (Fig. 3A) or collagen type IV (exemplary documented for E-cadherin in Fig. 3B).

As expected by the data shown before, $\beta$-catenin as well as $\alpha$-catenin coimmunoprecipitated with E-cadherin in lysates from BxPC-3 cells grown on TCP or fibronectin (Fig. 3C). In contrast, in cells cultured on collagen type I or type III, only trace amounts of coimmunoprecipitated $\beta$-catenin and no $\alpha$-catenin were detectable (Fig. 3C). This argues that the residual E-cadherin found in these cells is partially complexed with $\beta$-catenin but is no longer associated to $\alpha$-catenin. Again, similar results were obtained for PANC-1 and PaTu8988s (data not shown).

**Collagen Affects E-Cadherin Gene Expression.** We sought to investigate whether the observed reduction of E-cadherin protein content was attributable to an altered E-cadherin gene expression. For this purpose, we carried out Northern blot analysis of total RNA from cells

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![Fig. 2. Collagen type I reduced the concentration of the E-cadherin/catenin complex. Immunostaining of E-cadherin as well as $\beta$- and $\alpha$-catenin of BxPC-3 cells cultured on fibronectin (FN) or collagen type I, (A). B, PANC-1 and PaTu8988s cells grown on fibronectin or collagen type I stained for E-cadherin. Bar, 43 $\mu$m.](image-url)
cultured on different substrates. E-cadherin mRNA concentrations were significantly reduced in cells grown on collagen type I or type III compared with those in cells cultured on TCP and fibronectin (Fig. 4A). To verify this result, we performed reporter assays using the -178/+17 fragment of the E-cadherin promotor (34; Fig. 4B). E-cadherin promotor activity was reduced in BxPC-3 and PANC-1 cells cultured on collagen type I or type III as seen by the decrease in luciferase activity of the transfected reporter construct. No changes were observed in cells grown on fibronectin compared with TCP (Fig. 4C).

**Collagen Type I and III Enhances Proliferation and Migration.** To analyze whether collagen as substrate induces mesenchymal changes in pancreatic epithelial cancer cells, we tested several functional parameters. BxPC-3 cells exhibited a typical epithelial-like shape and grew in clusters on TCP or fibronectin even in subconfluent cell cultures, as analyzed by phase-contrast microscopy. This morphology changed markedly in cells cultured on collagen type I or type III to a more spindle shaped, mesenchymal phenotype. In addition, BxPC-3 cells changed their behavior and preferred to grow as single cells (Fig. 5A). Furthermore, BxPC-3 cells showed accelerated cell proliferation on collagen type I and type III, as determined by counting the cells over a period of 4 days (Fig. 5B). This was verified by [3H]thymidine incorporation assays performed with BxPC-3 and PANC-1 cells (Fig. 5C). Growth on collagen type I or type III resulted in an 87 and 103% up-regulation of DNA-synthesis for BxPC-3 or in an 83 and 79% up-regulation for PANC-1 cells, respectively, compared with cells grown on TCP. Both cell lines showed no significantly altered proliferation rate when cultured on fibronectin relative to TCP. Moreover, migration of BxPC-3 cells and PANC-1 through collagen type I was much higher than through fibronectin. Fig. 5D shows migration analysis, using a transwell migration system, where cells were confluenitly seeded on substrate-coated membranes with 8-μm pores. After 48 h, a 2.7-fold increase of BxPC-3 cells were counted on the bottom side of the membrane in case of collagen type I-coated membranes compared with fibronectin-coated ones. Using PANC-1 cells, we observed a 5-fold increase.

Collagen Type I and III Up-Regulates α2 Integrin Subunit. We next asked whether collagen receptors of the integrin superfamily were also affected by culturing cells on different substrates. Immunoblot analyses revealed that, in contrast to reduction of cell-cell adhesion molecules, the expression of specific integrin subunits was up-regulated under these conditions. As shown in Fig. 6, incubation of BxPC-3, PANC-1, and PaTu8988s cells on collagen type I or type III resulted in increased amounts of integrin subunit α2 compared with cells grown on TPC or fibronectin. The integrin α1 subunit, also

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**Fig. 3. Reduction of E-cadherin/catenin concentrations in cells grown on collagen type I or type III. A, total cell lysates from BxPC-3, PANC-1, and PaTu8988s grown on TCP, collagen type I, collagen type III, or fibronectin were analyzed by Western blotting. A 30 μg sample was separated per lane in 10% SDS-PAGE and stained with E-cadherin and β- and α-catenin antibodies. Restaining of blots for β-actin is shown to demonstrate equal loading. B, E-cadherin content of cells grown on collagen type IV compared with cells on TCP. C, immunoprecipitation of E-cadherin from lysates of cells grown on TCP, collagen type I, and fibronectin was followed by Western blotting. E-cadherin and catenin concentrations were analyzed by staining with antibodies against E-cadherin, β-catenin, and α-catenin. On the left, positions of the Mr markers in thousands.

**Fig. 4. E-cadherin expression was reduced in cells grown on collagen type I or type III. A, Northern blot analysis of BxPC-3 and PANC-1 cells cultured on different substrates. Thirty μg of total RNA were separated in a 1% agarose gel and blotted onto nylon membrane. E-cadherin mRNA is detected by hybridization with a 32P-human cDNA probe. Rehybridization with an 18S rRNA probe shows equal loading of the gel. B, both cell lines, cultured on different substrates, revealed reduced E-cadherin gene expression after transfection with a reporter vector containing the E-cadherin promotor. A 200-bp fragment (−178/+17) of the E-cadherin promotor (34) was cloned in a luciferase reporter construct. C, measurement of E-cadherin promotor activity. Cotransfection with a renilla luciferase construct was used to normalize the obtained data. Luciferase activity in cells on TCP was set as 1, means are shown of three independent assays performed in duplicates; bars, ± SD. •, statistically significant reductions (P < 0.002; Student’s t test).
known as a receptor for collagen, was likewise up-regulated, although to a lesser extent (data not shown). In contrast, the concentration of the most abundant β integrin subunit, namely β1, was not altered by different substrates, as shown for all of these cell lines.

**Src Inhibitors Abolish Reduction of E-Cadherin, and Active Src Mimics the Collagen-induced Effects.** It is well documented that c-Src kinase is activated by integrins occupied by their ligands and that activated c-Src reduces cadherin-mediated cell-cell adhesion (24, 35, 36), selecting this kinase as a suitable candidate for mediating collagen-induced decrease of E-cadherin gene expression in pancreatic carcinoma cell lines.

To examine whether Src tyrosine kinase is involved in substrate-mediated regulation of cell-cell adhesion, the src kinase activity was estimated in cells grown on different substrates. As demonstrated in Fig. 7A Src autophosphorylation as well as phosphorylation of the Src substrate enolase was clearly enhanced in PANC-1 cells cultured on collagen type I or type III compared with cells grown on TCP or fibronectin. To verify the involvement of Src, we treated the cell lines with Src kinase inhibitors PP1 or herbimycin A in the presence of Src substrate enolase was clearly enhanced in PANC-1 cells cultured on collagen type I or type III compared with cells grown on TCP or fibronectin.

**Fig. 6.** The amount of integrin subunit α2 was elevated in cells cultured on collagen type I or type III. Total protein lysates were prepared from BxPC-3, PANc-1, and PaTu8988s. Samples of 60 μg of protein were separated in each line and blotted. The blots were probed with anti-integrin α2 and anti-integrin β1. Equal loading of the blots is shown by detection of β-actin.

**Fig. 5.** Determination of functional parameters of pancreatic carcinoma cells cultured on collagen type I or type III. A, morphological changes were analyzed by phase-contrast microscopy of BxPC-3 cells grown on different substrates. Bar, 200 μm. B, to determine the proliferation rate, 2 × 10^5 BxPC-3 cells were seeded in 35-mm cell culture dishes, which were uncoated (TCP) or coated with collagen type I, type III, or fibronectin. The cell number was determined by counting five individual grids every 24 h. Means of the calculated cell numbers of a representative experiment of three are shown; bars, ± SD. C, differences in cellular proliferation rates were confirmed by comparison of [3H]thymidine incorporation in BxPC-3 and PANC-1 cells. Means of four independent assays performed in triplicate are given in percentage of incorporation relative to those contained in mock-transfected cells; bars, ± SE. D, for cell migration assays, 1 × 10^5 BxPC-3 or PANC-1 cells were seeded in transwell inserts in DME. The membranes of the inserts contained 8-μm pores and were coated either with collagen type I or with fibronectin. After 24 h, the migrated cells per visual field were counted. The number of cells migrated through collagen type I was given in relation to those migrated through fibronectin, which were set as 1. Means are shown of four independent assays performed in quadruplicate; bars, ± SD. *, statistically significant reductions (P < 0.002, Student t test).
decrease in transcript and protein concentration, we observed a reduced E-cadherin promoter reporter activity in Src-transfected PANC-1 (Fig. 9B), which strongly indicated that c-Src activity indirectly controls E-cadherin gene expression.

As a functional consequence, PANC-1 cells, overexpressing activated c-Src(Y527F), showed enhanced migration in transwell migration assays either in the absence of a chemoattractant (3.7-fold for clone p19 and 3-fold for psd) or in the presence of LPA (1.7-fold for both clones), compared with mock transfected controls (Fig. 9C). Most strikingly, our results for Src-transfected PANC-1 cells resemble the data obtained by pancreatic cancer cell lines cultured on collagen type I or III (Figs. 2–5).

DISCUSSION

We have demonstrated that E-cadherin gene expression in epithelial pancreatic carcinoma cell lines is down-regulated by collagen type I and type III. E-cadherin and catenins form functional adhesion complexes on fibronectin or TCP in the analyzed cells. However, the cells dramatically reduce E-cadherin protein and its gene expression when cultured either on collagen type I or III. Loss of E-cadherin correlated with increased proliferation and migration behavior. This effect was abolished by treatment with the Src kinase inhibitors PP1 and herbimycin A but could be mimicked by overexpression of activated c-Src. These results shed new light on the potential of ECM to promote tumor growth and metastatic potential, even in cancer cells that are able to form functional cadherin/catenin adhesion complexes.

A mutual interaction between substrate- and cell-cell adhesion in tumors has already been postulated; however, a cross-talk on the level of gene transcription has not been reported before. A coordinated regulation between cadherin- and integrin-mediated substrate adhesion is documented for different cell types (38–47). With one exception (42), in which a coordinated regulation of motile activity by integrins and N-cadherin in myoblasts is reported, all of these reports describe an inverse relationship between cadherins and activated integrins. Inhibition of E-cadherin function by using an anti-E-cadherin antibody prevented loss of integrin expression in differentiating keratinocytes (38). The introduction of E-cadherin into Xenopus-derived fibroblasts caused down-regulation of integrins and reduced adhesion to fibronectin and laminin (39). Only two of these cross-talks...
Fig. 9. Activated Src-induced transformation in Panc-1 cells. A, E-cadherin mRNA concentration was reduced in Src-transfected cells. Twenty μg of total RNA per lane was analyzed by Northern blot hybridization with a human E-cadherin-specific probe. Ethidium bromide staining of the 18S rRNA is shown to verify equal loading of the gel. B, to quantify the activity of the E-cadherin promoter, c-Src(Y527)-transfected cells were additionally transfected with the E-cadherin reporter vector. The luciferase activity was measured and normalized to a cotransfected renilla luciferase expression vector. Promoter activity in Src-containing cells was expressed in relation to mock-transfected Panc-1, which was set as 1. Means of three independent assays are shown; bars, ± SD. C, migration of Src transfected Panc-1 cells in response to LPA, as attractant, compared with BSA. The cells were seeded in DME in transwell inserts containing 8-μm pores. After 24 h, the cell number at the lower side of the inserts was estimated. The quantity of migrated cells is expressed in relation to mock-transfected cells in the presence of BSA. Means are shown of three independent assays performed in quadruplicate; bars, ± SD. *, statistically significant reductions (P < 0.01, Student t test).

were analyzed in more detail in terms of signaling cascades (43, 46). Both revealed that the canonical wnt/wingless (wg) signaling pathway mediated the coupling of cell-cell and cell-substrate adhesion. This is attributable to an enhanced stabilization of cytoplasmic β-catenin, which can interact with Lymphoid-enhancing factor/T-cell receptor transcription factors leading to nuclear localization of both proteins. Nuclear complexes of Lef/β-catenin may represent a potential mechanism to down-regulate the gene encoding for E-cadherin. In the study of Wu et al. (45), the activation or overexpression of ILK in epithelial cells was associated with loss of epithelial characteristcs, which includes enhanced fibronectin matrix assembly, loss of E-cadherin concentrations, loss of expression of keratins 14 and 18, and increased concentrations of vimentin. ILK, comparable with wnt/wg signals, reduced the Gsk-3β activity, stabilized the cytoplasmic β-catenin pool, and resulted in nuclear translocation of Lef/β-catenin. We speculated that this transcriptional active complex induced the observed down-regulation of E-cadherin.

In this study on pancreatic carcinoma cells, we can exclude the possibility that collagen-induced E-cadherin down-regulation is based on activation of the wnt/wg signaling pathway, because β-catenin concentration was found to be decreased. This is probably attributable to an active GSK-3β phosphorylation of β-catenin and an intact ubiquitin proteasome system, which effectively degrades β-catenin. Furthermore, we did not find β-catenin in the nucleus nor an activation of Lef/Tcf responsive promoter constructs.4 This clearly argues against an influence of the canonical wnt/wg pathway or an influence of the ILK.

Recently, repression of E-cadherin in carcinoma cell lines could be assigned to binding of the transcription factor snail to E-palindrome sequences in the E-cadherin promoter (16, 17). Although Panc-1 and PaTu8988s were found to be positive for snail mRNA in reverse transcription-PCR studies, we were not able to demonstrate a correlation between changes of snail expression with different substrates the cells were cultured on.4 Furthermore, the use of a promoter variant with a mutated E-palindrome sequence (34) revealed a down-regulation of E-cadherin by collagen type I or III (data not shown) that was comparable with the wild type promoter. These findings make the involvement of the transcription factor snail in substrate-induced E-cadherin regulation in BxPC-3, Panc-1, and PaTu8988s cells most unlikely.

We obtained strong evidence that the tyrosine kinase c-Src plays a crucial role as mediator of the collagen/E-cadherin coupling observed in pancreatic tumor cells: (a) blocking of Src kinase activity by incubation with specific inhibitors reverted the substrate-induced effects; (b) overexpression of activated c-Src reduced E-cadherin gene expression in Panc-1 cells; and (c) Src overexpression induced a mesenchymal transition, which mimics the cellular effects induced by collagen type I and type III in nontransfected cells.

In many cell types, the ECM-induced stimulation of integrins results in activation of focal adhesion kinase (FAK), of Src and thereby the Ras/ERK pathway (48–50). Src kinase family members are well known to transduce ECM-initiated signals via focal adhesion complexes (48–51). We observed a dramatic up-regulation of collagen receptors in BxPC-3, Panc-1, and PaTu8988s cells cultured on collagen, which could result in a substrate-induced integrin stimulation after Src activation.

Until now there is limited information about the mechanism of how Src regulates gene expression. Src is able to enhance binding of the Sp1 transcription factor to the promoter of the urokinase receptor (52). In addition, Src induces binding of transcription factors to the CCAAT box, such as nuclear factor-Y (NF-Y), which was found to be necessary to promote expression of the osteopontin- and the bone sialoprotein-promoter (53, 54). Furthermore, Src-regulated gene expression is described in the literature via the Ras/ERK pathway (55). Lu et al. showed increased cadherin and β-catenin synthesis on stable expression of an inhibitory ERK mutant (44). With respect to these data, the Ras/ERK pathway might be attractive to be analyzed in more details. The E-cadherin promoter construct used in our assays contains regulatory elements associated with the Ras/ERK pathway (AP-2, CAAT box, and SP-1 sites). Whether one of these is involved in the response to collagen type I or III remains to be clarified.

Concentrations of β- and α-catenin were also reduced when cells were seeded on collagen type I or III. Although, we cannot exclude the possibility that Src signaling might also affect promoter elements of the catenin genes, it seems more likely that catenin decrease is an

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4 A. Menke and B. Seidel, unpublished results.
indirect effect induced by a changed E-cadherin concentration. A coordinated expression of the different members of the E-cadherin/catenin complex may account for this effect. Different groups reported that in response to altered E-cadherin gene transcription, the expression of β- and α-catenin was affected similarly (39, 56, 57). The mechanism underlying the coordinated expression of cadherins and catenins is still unclear, and the possibility remains that cadherin-complex formation increases the half-life of catenins (58).

Up to now, dysfunction of the E-cadherin/catenin complex in tumor cell lines was correlated with phosphorylation-induced complex disassembly (7, 35). Genda et al. (36) analyzed the significance of fibronectin and collagen substrate for the hepatocellular carcinoma cells. They observed that disassembly of the E-cadherin-catenin complex was induced by β1 and β2 integrin subunits. Our results do not exclude the possibility that besides a reduction of E-cadherin gene expression, there is a disassembly of the E-cadherin adhesion complex as an additional mechanism. Cells grown on collagen showed residual E-cadherin and β-catenin, which accumulated in the Triton-soluble fraction (data not shown). This indicates that β-catenin, even when it is bound to E-cadherin, is not necessarily linked to the cytoskeleton. This was confirmed by the lack of α-catenin in E-cadherin communoprecipitation studies of lysates from pancreatic carcinoma cells grown on collagen type I (Fig. 2C). However, the most prominent effect of collagen type I and type III was the down-regulation of E-cadherin gene expression. This might be specific to pancreatic carcinoma cells reflecting the high level of ECM in most pancreatic tumors (27).

In conclusion, we have shown that collagen type I and type III can induce an epithelial-mesenchymal transition in epithelial pancreatic carcinoma cells. The tyrosine kinase c-Src was identified as a central mediator in signal transduction leading to reduced E-cadherin gene expression. This represents a novel regulatory mechanism of mutual interactions and their implications for cadherin function. J. Cell. Biochem., 61: 514–523, 1996.

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Down-Regulation of *E-Cadherin* Gene Expression by Collagen Type I and Type III in Pancreatic Cancer Cell Lines


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