Syndecan-2 Mediates Adhesion and Proliferation of Colon Carcinoma Cells*

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Syndecan-2 is a transmembrane heparan sulfate proteoglycan whose function at the cell surface is unclear. In this study, we examined the function of syndecan-2 in colon cancer cell lines. In several colon cancer cell lines, syndecan-2 was highly expressed compared with normal cell lines. In contrast, syndecan-1 and -4 were decreased. Cell biological studies using the extracellular domain of recombinant syndecan-2 (2E) or spreading assay with syndecan-2 antibody-coated plates showed that syndecan-2 mediated adhesion and cytoskeletal organization of colon cancer cells. This interaction was critical for the proliferation of colon carcinoma cells. Blocking with 2E or antisense syndecan-2 cDNA induced G1/G0 cell cycle arrest with concomitantly increased expression of p21, p27, and p53. Furthermore, blocking of syndecan-2 through antisense syndecan-2 cDNA significantly reduced tumorigenic activity in colon carcinoma cells. Therefore, increased syndecan-2 expression appears to be a critical for colon carcinoma cell behavior, and syndecan-2 regulates tumorigenic activity through regulation of adhesion and proliferation in colon carcinoma cells.

The syndecans are a family of cell-surface heparan sulfate proteoglycans that regulate cell behavior through the binding of extracellular matrix molecules and/or soluble ligands (1–3). This interaction regulates cell-ECM adhesion, migration, cytoskeleton organization, and gene expression through signal transduction pathways (2, 3). This interaction may be differentially regulated in cancer cells, since they are generally less adhesive and more migratory than normal counterparts. Therefore, it is probable that syndecans may influence adhesion to the ECM, cell morphology, and tumorigenic activity of cancer cells. Indeed, syndecan expression has been shown to suppress transformation and migration of several tumor cells (1, 4). Syndecan-1, in particular, has been associated with a tumor suppressor function. Transfection of syndecan-1 cDNA dramatically reverses the transformed phenotype of the S115 mammary epithelial-derived tumor cell line and inhibits soft agar colony formation (5). Expression of syndecan-1 is inhibited by malignant transformation of human keratinocytes (6). Moreover, syndecan-1 expression is decreased in a variety of cancer tissues (7–12). Similarly, syndecan-4, which is mainly involved in cytoskeletal and membrane reorganization to form stress fibers and focal adhesions at the later stage of primary fibroblast spreading (13), inhibits cell migration and tumor activity (14–16). RH-77 lymphoma cells, which readily invade type I collagen gels, fail to do so after expression of either syndecan-1 or -4 (16, 17). Consistently, mRNA expression of syndecan-1 and -4 are reduced significantly in several cancer cells including colon carcinoma cells (7, 10, 18, 19).

On the other hand, syndecan-2 shows somewhat different characteristics. Syndecan-2 is involved in regulation of cell adhesions in several cell lines including epithelial cells (20–22), neuronal cells (23, 24), and mesenchymal cells (25). Compared with syndecan-1 and -4, the role of syndecan-2 in cell migration has been less investigated. However, several reports indicate that syndecan-2 may positively regulate cell migration, since syndecan-2 is normally highly expressed in cells under migratory conditions (22, 24, 25). In particular, in Lewis lung carcinoma-derived P29 cells, syndecan-2 plays a major role in the interaction with fibronectin and regulates actin stress fiber formation in cooperation with integrin α5β1 (22). These reports indicate that syndecan-2 may function as a cell surface receptor in highly migratory tumor cells. Here, we present evidence that syndecan-2 plays a critical role in adhesion of colon carcinoma cells onto the ECM, and most importantly, this interaction is crucial for proliferation and tumorigenic activity in colon carcinoma cells.

**EXPERIMENTAL PROCEDURES**

**Materials and Antibodies—** Monoclonal cyclin D1 and polyclonal p21, p27, cyclin D2, cyclin E, CDK2, and CDK4 antibodies were purchased from Santa Cruz, Inc. (Santa Cruz, CA). Monoclonal p53 antibody was obtained from Calbiochem. Texas Red-conjugated affinity-purified anti-mouse IgG, was obtained from Rockland, Inc. (Gilbertsville, PA). Fluorescein-conjugated AffiniPure F(ab')2 fragment donkey anti-chicken IgY was purchased from Jackson ImmunoResearch laboratories, Inc. (West Grove, PA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Amresco Inc. (Solon, OH), and Effectene was purchased from Qiagen (Hilden, Germany). Isopropyl-b-D-thiogalactopyranoside, glutathione-Sepharose beads, and other chemicals were purchased from Sigma.

**Cell Culture, Morphology, and Treatment—** One normal colorectal cell line (CCD-18Co), three colorectal adenocarcinoma cell lines (SW403, LoVo, COLO205), and three colorectal carcinoma cell lines (HCT116, KM12SM, KM1214) were purchased from the Korean cell line bank. HCT116 were grown in McCoy's 5a, KM1214 were grown in Dulbecco's modified Eagle's medium, and KM12SM were grown in minimum essential medium (Invitrogen) supplemented with 10% fetal bovine serum and with penicillin (100 units/ml) and streptomycin (10 μg/ml, Invitrogen) at 37 °C in 5% CO2 in a humidified atmosphere. For treatment with epidermal growth factor (EGF), KM1214 and...
Syndecan-2 Mediates Adhesion and Proliferation of Cancer Cells

KM12SM cells were starved for 24 h in serum-free media with or without 2E (0.5 μg/ml), and then 10 μl EGFP was added for 5–30 min. RNA Extraction and Reverse Transcription-PCR—Total RNA extracted from cultured cells was used as the template for reverse transcriptase reaction. Aliquots of cDNA were amplified using the following primers syndecan-1 (forward 5'-GTCTGGGGGATGACTCTGAC-3' and (backward) 5'-GTAT-TCTCCCCGGAGGTTTCT-3'; human syndecan-2 (forward) 5'-ACATCTCCCTTGGT-TGAAAGG-3' and (backward) 5'-TAATCTCATCTCCTCCCAGG-3'; human syndecan-3 (forward) 5'-GTCTGGTCCTGAGATTGCTG-3' and (backward) 5'-TGGGCGCTTTGTTAGATG-3'; human glyceraldehyde-3-phosphate dehydrogenase (forward) 5'-GGATGACTGACCTAGCTCAACG-3' and (backward) 5'-TCTACGCCCAGTCAGTCCACC-3'; integrin β1 (forward) 5'-GCCGATATCTGGAAATTTGG-3' and (backward) 5'-TCTCAGCAAACCCAC-3'. After an initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s (except glyceraldehyde-3-phosphate dehydrogenase and integrin β1 at 60 °C), and extension at 72 °C for 30 s were carried out. The reaction products were analyzed in 1.5% agarose gels. The amplified DNA fragments (syndecan-1, 552 bp; syndecan-2, 539 bp; syndecan-3, 397 bp; glyceraldehyde-3-phosphate dehydrogenase, 600 bp) were cloned and sequenced to confirm the PCR products.

Immunoblotting—After cultures were washed twice with PBS (500 μl/10-cm diameter plate), the cells were lysed in radiolabeled precipitation medium containing 1% Triton, pH 7.4, 10 mM NaF, 2 mM Na3VO4) containing a protease inhibitor mixture (1 μg/ml aprotinin, 1 μg/ml antipain, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 μg/ml peptatin A, 20 μg/ml phenylmethylsulfonyl fluoride). Cell lysates were clarified by centrifugation at 13,000 rpm for 15 min at 4 °C, denatured with SDS sample buffer, boiled, and analyzed by SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride membranes (Amersham Biosciences) and probed with the appropriate antibodies followed by species-specific horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences). Signals were detected by enhanced chemiluminescence (ECL; Amersham Biosciences).

Transfectant Transfections of Antisense Syndecan-2 cDNA—For syndecan-2 antisense cDNA, a 5' fragment of 150 bp was excised using the polynuker HindIII site and an internal HindIII site and retested into pcDNA3.1 cut with EcoRI. KM1214 cells (2 x 105) were plated on 6-cm diameter culture dishes, incubated at 37 °C for 24 h, and then transfected with 4 μg of mock- or antisense syndecan-2 in pcDNA3.1 using Effectene reagent (Qiagen).

Recombinant Syndecan-2 and -4E—The extracellular domains of syndecan-2 and syndecan-4 were cloned into pGEX-5X-1. These constructs were used to transform E. coli DH5α, and recombinant fusion protein and the glutathione S-transferase ectodomain of syndecan-2 (2E) and -4 (4E) were induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 7 h. The fusion proteins were purified with glutathione-Sepharose beads. Purified 2E and 4E were used after dialysis in 50 mM Tris-HCl, pH 8.0.

Plating Experiment—35-mm bacteria culture plates were coated with either 20 μg/ml syndecan-2 or syndecan-4 antibody in PBS overnight at 4 °C. The coated plates were washed with phosphate-buffered saline (PBS), blocked with 0.2% heat-inactivated bovine serum albumin for 1 h at room temperature, and then washed again with PBS (3 x 5 min). KM1214 and LoVo cells were detached with 0.05% trypsin-0.53 mM EDTA, suspended in serum-free media containing 0.25 mg/ml soybean trypsin inhibitor, and centrifuged. Cells were resuspended in serum-free media, plated on the coated plates, and incubated for various periods of time at 37 °C. To study the morphological changes, cells were incubated with or without 2E for 24 h in 5% CO2 in a humidified atmosphere. Cells were photographed at 20 x magnification with a digital camera (Olympus).

Cell Proliferation Assay—Cell proliferation was measured by a colorimetric assay using MTT. In brief, KM1214 and KM125M cells were harvested with 0.05% trypsin/EDTA and seeded into 35-mm dishes at 1 x 104 cells/dish. After allowing cells to attach to the plate for 24 h, fresh medium containing 2E or 4E (0.25 μg/ml) was added. After incubation, the medium containing 0.5 mg/ml MTT (Sigma) was added to each plate in a volume of 100 μl, and cells were incubated for 1 h. The medium was then removed, and 200 μl of dimethyl sulfoxide was added to each well at room temperature. The mean concentration of absorbance at 570 nm in each set of samples was measured using a 96-well microtiter plate reader (Dynatech, Chantilly, VA). Also, the growth activity of syndecan-2 antisense transfected cells was performed as described above.

Fluorescence-activated Cell Sorting—Colon cancer cells were cultured in 10-cm diameter dishes then washed with PBS and released trypsin (w/v), 1 mM EDTA followed by the addition of PBS. After pellets formed, cells were resuspended in PBS and counted. Cells (1 x 105/ml) were incubated with anti-syndecan-2 or anti-syndecan-4 in 10% fetal bovine serum in PBS for 1 h on 4 °C. Then PBS containing 0.05% Tween 20 was added, and the cells were washed three times and incubated for 1 h in 1% fluorescein-conjugated mouse anti-mouse or anti-chicken in 10% fetal bovine serum in PBS for 30 min. Syndecan-2 or syndecan-4 expression was analyzed by flow cytometry. For the cell cycle distribution, KM1214 cells were cultured in 10-cm diameter dishes containing 7 ml of Dulbecco's modified Eagle's medium supplemented with or without 0.75 μg/ml 2E or 4E. After 24–36 h, cells were collected by centrifugation (1,000 rpm, 10 min) and washed with PBS. Cells were treated with RNase (250 μg/ml in PBS) and then stained with propidium iodide (50 μg/ml in PBS) at 37 °C for 3 h. The cells were analyzed for DNA content by flow cytometry, and cell cycle distribution was analyzed by MULTI CYCLE software.

Anchorage-independent Growth in Soft Agarose—Each well of a 6-well culture plate was coated with 3 ml of bottom agar mixture (Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 0.6% agar). After the bottom layer had solidified, 2 ml of top agar mixture (Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 0.3% agar) containing either 1 x 105 cells with 2E or antisense transfected cells was added to each well, and the cultures were incubated at 37 °C in a humidified 5% CO2 and 1% O2 atmosphere for 5 days, normal growth medium was changed every 5 days, and then 100 μl of medium was added, and the cultures were washed three times and incubated for 4,000 rpm, 10 min) and washed with PBS. Cells were treated with RNase (250 μg/ml in PBS) and then stained with propidium iodide (50 μg/ml in PBS) at 37 °C for 3 h. The cells were analyzed for DNA content by flow cytometry, and cell cycle phase distribution was analyzed by MULTI CYCLE software.

RESULTS

Syndecan-2 Is Highly Expressed in Tumor Cells—We investigated mRNA expression of each syndecan family member in several colon cancer cell lines including normal (CDD-18co), weekly metastatic (COLO205, SW403, and LOVO), and highly metastatic cell lines (KM1214, KM125M, and HCT116, Fig. 1A). mRNA expression of syndecan-1 was decreased in most colon cancer cell lines. On the other hand, syndecan-2 mRNA levels were increased by 2–5-fold in all cancer cell lines tested compared with normal colon cell line. Syndecan-4 expression levels were decreased in highly metastatic cell lines, whereas integrin β1 expression levels were not significantly changed. Cell surface expression of syndecan-2 was correspondingly increased in colon carcinoma cell lines, whereas syndecan-4 was not (Fig. 1B). These data suggest that syndecan-2 may be related to tumorigenic activity in colon carcinoma cells.

Syndecan-2 Mediates Adhesion of Colon Carcinoma Cells on the ECM—Because it is known that syndecans regulate cell-ECM interactions (1, 2), we investigated whether increased expression of syndecan-2 regulates the adhesion of colon cancer cells to the ECM. The function of syndecan-2 core proteins as a cell surface receptor was directly analyzed by purified recombinant syndecan-2 (2E), corresponding to the extracellular domain of syndecan-2, and extracellular domain of recombinant syndecan-4 (4E) as a control (Fig. 2A). The addition of 2E completely blocked the adhesion of colon cancer cells on the ECM in two different experimental conditions. First, cells were detached and replated onto tissue culture plates in the presence of 0.75 μg/ml either 2E or 4E (Fig. 2B). In the absence of 2E (Control), both KM1214 and KM125M normally attached and spread onto tissue culture plates at 24 h after plating. In the presence of 2E, however, these cells were not attached at all, even after 48 h. In contrast, their attachment and spread normally occurred in the presence of the same amount of 4E. Similarly, either 2E or 4E was attached on the non-replicating cells, and the morphological changes were monitored (Fig. 2C). Unexpectedly, at 24 h after the addition of 2E but not 4E, cells started rounding and floating off from the tissue culture plate. We presumed that this was due to interruption of cell interaction with the ECM through syndecan-2.

To more directly access the involvement of syndecan-2 in...
adhesion on the ECM, highly metastatic KM1214 and weakly metastatic LoVo cells were detached and replated onto antibody-coated plates (Fig. 3). Compared with cells on either bovine serum albumin- or syndecan-4 antibody-coated plates that remained unattached, both colon carcinoma cells on syndecan-2 antibody-coated plates were normally attached (90 ± 8%, 89 ± 6%) and spread (46 ± 9%, 40 ± 2%) at 12 h after plating. It was even more efficient than normal culture conditions on

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**Fig. 1.** Syndecan-2 expression is increased in colon cancer cell lines. A, total RNA was extracted from human colon cancer cell lines, and mRNA expression was analyzed by reverse transcription-PCR using each primer as indicated. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. The reaction products were analyzed in 1.5% agarose gels (top panel). Representative results from three independent experiments are shown. Quantified syndecan-2 mRNA levels compared with normal cells are shown (bottom panel). B, colon cancer cells were incubated with anti-syndecan-2 or anti-syndecan-4 antibodies, and each protein expression level was analyzed by flow cytometry. IgG was used as a control.

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**Fig. 2.** Exogenous recombinant syndecan-2 extracellular domain inhibits adhesion and spreading in colon cancer cells. A, purified glutathione S-transferase (GST) ectodomain of syndecan-2 (2E) and -4 (4E) were separated on 10% SDS-PAGE and stained with Coomassie Blue. B, both KM1214 and KM12SM cells were detached with trypsin and replated onto tissue culture plates in the presence of 0.75 μg/ml either recombinant 2E or 4E. C, control. Either recombinant 2E or 4E (0.75 μg/ml) was added into exponentially growing cells and incubated at 37 °C. After 24 h, morphological changes were monitored, and photographs were taken under a phase-contrast microscope attached to a digital camera. Representative results from five independent experiments are shown.
tissue culture plates. The number of either attached or spread KM1214 cells on syndecan-2 antibody-coated plates was 1.5 and 2.2 times higher than cells on normal tissue culture plates (T/C plate), respectively (Fig. 3A). These results strongly suggest that syndecan-2 mediates the adhesion of colon cancer cells to the ECM.

Syndecan-2 Regulates Proliferation of Colon Carcinoma Cells—Engagement of cells on the ECM is important for cell growth (35, 36). Because syndecan-2 is expressed highly in colon cancer cells, syndecan-2 may play a critical role in the tumorigenic activity in colon cancer cells. We investigated whether syndecan-2 regulated proliferation of cancer cells. Both KM12SM and KM1214 cells were cultured in the presence of low amounts (0.25 μg/ml) of either 2E or 4E, and cell numbers were quantified using a colorimetric assay (Fig. 4A). In the presence of 2E, but not 4E, both cell lines showed no net increase in cell number, implying that blocking of syndecan-2 function with 2E caused severe growth arrest. Consistent with these data, transfection of 4 μg of antisense syndecan-2 cDNA reduced cell surface expression of syndecan-2 (Fig. 4B, left panel).
panel) and induced cell cycle arrest in KM1214 cells (Fig. 4B, right panel). Both 2E-treated and antisense syndecan-2-transfected cells showed increased expression of cyclin-dependent kinase inhibitors p53, p21, and p27 (28) and decreased expression of cyclin E and cyclin D2 (Fig. 5, A and B). Furthermore, fluorescence-activated cell sorting analysis using propidium iodide staining confirmed that 2E induced cell cycle arrest at G0/G1 phase (Fig. 5C). Exposure of cells for 36 h with 2E caused inhibition of progression from the G1/G0 to S and G2/M phase in KM1214, which resulted in an increase of 1.35 in the number of cells in the G0/G1 phase compared with control cells. In contrast to 2E, 4E did not significantly affect cell growth. All these data strongly suggest that syndecan-2 is important for proliferation in colon carcinoma cells.

Several studies show that EGF receptors are expressed at high levels in a variety of epithelial cancers including colon cancer, and activation of EGF receptors appears to be critical for the growth of many tumors (37–40). Thus, we investigated EGF-mediated mitogen-activated protein kinase activation in colon cancer cells. Compared with control cells, 2E-pretreated cells showed decreased mitogen-activated protein kinase activation in response to 10 nM EGF (Fig. 6). Therefore, increased cell growth was observed in 2E-pretreated cells, which is consistent with the data of other studies (37–40). These results support the notion that syndecan-2 is important for proliferation in colon carcinoma cells.
expression of syndecan-2 is closely correlated with increased proliferative activity in colon cancer cells.

Increased Expression of Syndecan-2 Is Important for Tumorigenic Activity of Colon Cancer Cells—To investigate the effect of syndecan-2 on tumorigenic activity, we performed anchorage-independent growth assay in soft agar. The colony-forming ability of KM1214 cells was reduced ~70% in the presence of syndecan-2E (0.75 μg/ml) compared with normal cells (Fig. 7A). Similarly, transfection of antisense syndecan-2 cDNA into KM1214 cells significantly reduced colony formation in soft agar in a dose-dependent manner (Fig. 7B). Therefore, expression of syndecan-2 was crucial for anchorage-independent growth in colon cancer cells.

DISCUSSION

Cell adhesion to the ECM is mediated by specific cell surface receptors, and progression of colon and other cancers has been associated with changes in their level of expression and/or activity. Cancer cells change adhesive properties, and this is associated with changes in their level of expression and/or receptors, and progression of colon and other cancers has been known to be further investigated in detail.

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