Dysadherin Expression Facilitates Cell Motility and Metastatic Potential of Human Pancreatic Cancer Cells

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ABSTRACT

Dysadherin is a membrane glycoprotein expressed strongly in several human cancers. Overexpression of dysadherin in tumor cells is closely associated with malignant phenotype (e.g., metastasis) and poor prognosis. In our analysis, six pancreatic cancer cell lines showed a positive correlation between dysadherin expression and cell motility. Introduction of small interfering RNA (siRNA) against dysadherin into the Panc-1 cell line caused reduction of dysadherin expression and suppression of cell motility. In contrast, stable transfection of a dysadherin expression vector into the Capan-1 cell line increased cell motility. In vivo, the metastatic potential of orthotopically transplanted Capan-1 tumor cells in severe combined immunodeficient mice was increased by dysadherin overexpression. Cell morphology and actin organization were also influenced by modulation of dysadherin expression. Cells transfected with dysadherin siRNA tended to have a relatively larger, more spread shape and increased transverse actin stress fibers compared with parent cells and cells transfected with control siRNA. Our study suggests that dysadherin is able to modulate actin structures, stimulate cell motility, and contribute directly to the metastatic potential of human pancreatic cancer cells.

INTRODUCTION

Pancreatic cancer is a lethal disease; because of its aggressive growth and rapid metastasis to the lymph nodes and liver, only 20% of patients admitted with ductal adenocarcinoma of the pancreas undergo surgical resection, and, at best, 25% of those survive for 5 years (1, 2). The molecular mechanism responsible for the aggressiveness of pancreatic cancer is still elusive, although one can speculate that a molecule whose expression is specifically associated with invasion and metastasis might provide a clue.

Recently, we reported the cloning and characterization of dysadherin, a cancer-associated antigen that is a cell membrane glycoprotein with an FYXD motif (3, 4). Previous studies have indicated that dysadherin is expressed to various extents in different types of tumors, such as stomach, colon, pancreatic, and breast tumors (3–6). Only a limited number of normal cell types, including lymphocytes, endothelial cells, and basal cells of stratified squamous epithelium, show dysadherin expression. Stable transfection of a dysadherin expression vector into PLC/PRF/5 liver cancer cells resulted in reduced cell–cell adhesiveness and a markedly higher number of metastatic nodules in mouse xenografts (3).

The pattern of dysadherin expression in cancer tissue implies that this protein plays an important role in cancer cell invasion and metastasis (5). The expression of dysadherin is significantly correlated with clinicopathological variables such as distant metastasis and poor prognosis in pancreatic ductal adenocarcinoma, thyroid carcinoma, and colorectal carcinoma (6–8). In addition, the number of dysadherin-overexpressing tumor cells is correlated with the histopathological grading of tumor nests: almost all cancer cells of infiltrative and poorly differentiated tumor nests show overexpression of dysadherin, whereas a much smaller proportion of cells in well-differentiated tumor nests do so (6). These findings indicate that overexpression of dysadherin is strongly associated with the invasiveness of pancreatic cancers.

Several phenotypes of cultured cells observed in vitro are considered to reflect the metastatic potential of the cells in vivo. One such in vitro phenotype is cell motility. A previous study showed that a human pancreatic cancer cell line selected in vivo for metastatic ability had increased cell motility in vitro (9). Our previous observations also indicate that, among human liver cancer cell lines, the level of cell motility in vitro is correlated with the ability to form metastases in vivo and that overexpression of dysadherin in a human liver cancer cell line causes increased metastasis (3, 10–12).

The aim of this study was to clarify whether dysadherin overexpression contributes to cancer metastasis through the ability of dysadherin to stimulate cell motility. We also attempted to demonstrate the universal significance of dysadherin overexpression in human cancers, regardless of the organs from which they are derived. Accordingly, in addition to our previous work using a liver cancer cell line (3), we analyzed six human pancreatic cancer cell lines in the present study to demonstrate the relationship between dysadherin expression level and cell motility in these cell lines. Furthermore, we modulated dysadherin expression in these pancreatic cancer cell lines using small interfering RNA (siRNA) technology and cDNA transfection, and we analyzed the motility, actin organization, focal contact formation, and metastasis potential of the cells.

MATERIALS AND METHODS

Cell Culture. All cell lines were obtained from American Type Culture Collection (Manassas, VA). Panc-1, Mpanc-96, Miapaca-2, HPAF-IL, BxPC-3, and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma, St. Louis, MO) containing 10% heat-inactivated fetal calf serum, and Capan-1 was cultured in RPMI 1640 (Sigma) containing 10% heat-inactivated fetal calf serum. All cell culture was performed at 37°C under 5% CO2.

RNA Interference. The basic strategy for design of siRNAs specific for dysadherin was based on previous studies (13–15). The siRNA sequences chosen to target dysadherin were positions 141–162 (Dys141) and 498–520 (Dys498) in the nucleotide sequence of dysadherin (GenBank accession number AB072911). The lamin A/C siRNA used as a control has been described previously (16). As the other negative control, a siRNA (Negacon) was designed with sequence 5’- GUUUCGAGGACUACUACAUUU-3’ (sense) and 5’- AUGUAGUAGUGCCUCGAAAACUU-3’ (antisense). The sequence of this negative control siRNA was subjected to BLAST search (17), and no highly homologous sequences were detected in the GenBank database. Although all experiments were performed using these two negative controls, there was no difference between them in the results obtained. All siRNAs were purchased from Dharmacon Research (Lafayette, CO). Twenty-four hours after inoculation of cells into 24-well flat-bottomed plates at 1 x 104 cells per...

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well, siRNA transfection was performed using Oligofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

Establishment of Capan-1 Cells Stably Expressing Dysadherin. The expression vector for dysadherin, pcDNA3-L3HSV, has been described previously (3). Transfection was performed with LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions. Stable transfectants were selected by incubation with Geneticin (500 μg/mL; G418; Invitrogen), and dysadherin expression was determined by immunofluorescence and immunoblotting analyses.

Reverse Transcription-Polymerase Chain Reaction. Total RNA was extracted from each cell line with TRIZOL reagent (Invitrogen) followed by treatment with DNase I (Promega, Madison, WI). The total RNA was reverse transcribed with Omniscript Reverse Transcriptase (Qiagen, Hilden, Germany) using an oligo(dT) primer (Invitrogen). The template cDNA was amplified by the use of Hotstart Taq polymerase (Qiagen). For standardization of the amount of template cDNA, expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each sample was quantified. The primer sets for amplification of dysadherin and GAPDH cDNA were as follows: dysadherin, 5'-TCCACTGTGACACCACGA-3' (forward primer) and 5'-AAACACCA-GATGGCGTGAAGGT-3' (reverse primer); and GAPDH, 5'-AGGAGA-GAGAGACACCTCACTGC-3' (forward primer) and 5'-ATGACAAGGTC-GCGGCTCC-3' (reverse primer). Polymerase chain reaction products were subjected to agarose gel electrophoresis and visualized by ethidium bromide staining.

Immunoblot Analysis. The cell lysates were subjected to 10% SDS-PAGE, and then separated proteins in the gel were electroblotted to polyvinylidene difluoride membranes (Immobilon; Millipore, Canton, MA). Anti-dysadherin monoclonal antibody (mAb) NCC-M53 and anti-Na⁺/K⁺-ATPase mAb (Affinity BioReagents, Inc., Neshanic Station, NJ) were used as described previously (6, 18). The membrane was incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and visualized by using an enhanced chemiluminescence kit (Amersham Biosciences, Buckinghamshire, United Kingdom).

Immunofluorescence and Cell Morphology. A total of 2 × 10⁵ cells per well were grown on glass coverslips in 12-well flat-bottomed plates for 24

![Fig. 1. Correlation between dysadherin expression and cell motility in human pancreatic cancer cell lines. A shows an immunoblot analysis of dysadherin expression (top panel) and Na⁺/K⁺-ATPase as a loading control (bottom panel) in human pancreatic cancer cell lines. The cell motility index calculated by the Array Scan software (see Materials and Methods) is indicated in B. Cell lines and the number of cells analyzed are indicated at the bottom of the graph. Error bar, SD. A representative image of a trace of movement of each cell is shown in C (×40). The closed red lines indicate the margins of cell areas visualized by rhodamine-phalloidin-stained F-actin. The blue lines indicate the margins of track areas.](http://www.cancerres.aacrjournals.org)
hours, and siRNA transfection was performed as described above. The cells were fixed with 4% formaldehyde followed by 100% ethanol at −20°C. Permeabilization was performed with 0.1% Triton X-100, and nonspecific binding was blocked with 2% normal swine serum.

Cells were incubated with anti-dysadherin mAb and anti-paxillin mAb (BD Bioscience and Clontech) followed by fluorescein isothiocyanate-labeled secondary antibody. Alexa fluoro 594-conjugated phalloidin (Molecular Probes, Eugene, OR) was used to visualize F-actin. The samples were then mounted with Vectashield (Vector Laboratories, Burlingame, CA) and examined by multi-photon fluorescence microscopy (Bio-Rad Laboratories, Hercules, CA).

Six visual fields (×100) per coverslip were randomly selected, avoiding any overlapping, for the morphologic analysis. Cells were classified as spindle type or spread type by visual observation. We defined spindle type as a cell morphology with few processes of the cell membrane (one or two) with few actin stress fibers and spread type as a polygonal shape with many actin stress fibers. The experiments were performed in triplicate.

**Cell Motility Assay.** Cell motility was determined by use of the Array Scan system with HitKit HSC reagent kits (Cellomics, Pittsburgh, PA; ref. 19). In experiments involving siRNA transfection, the transfected cells were trypsinized 48 hours after transfection. A total of $7.5 \times 10^5$ cells per well were inoculated into collagen I-coated 96-well plates covered with blue fluorescent beads. The plates were then incubated for 18 hours at 37°C under 5% CO$_2$. As cells moved across the layer of fluorescent beads, they phagocytosed and pushed aside the beads, clearing phagokinetic tracks behind them. The track area, recognized as a negative image in a background of blue fluorescent beads, was proportional to the distance of cell movement. The cells were fixed with 4% paraformaldehyde and stained with rhodamine-conjugated phalloidin. The rhodamine signal corresponding to the submembranous cortical actin layer of a cell was defined as the margin of respective cell area. The Array Scan computer software captured nine visual fields from each well of a 96-well plate. The motility index was calculated as the total track area divided by the total cell area in all visual fields in a well. The motility index of a cell line was calculated as the average of the motility index in three independent trials (8 wells per trial).

**Orthotopic Implantation.** Severe combined immunodeficient mice [SCID (6 weeks of age)] were obtained from CLEA Japan (Tokyo, Japan) and maintained in a specific pathogen-free environment. Experimental orthotopic implantation was performed as described previously (20). Each mouse received injection in the pancreas with $1 \times 10^6$ cells under appropriate anesthetic procedures. Local or metastatic tumor formation was evaluated macroscopically 35 days later.

**Statistical Analysis.** The differences in cell number, cell areas, and cell motility between cell lines were examined using Student’s t test.

**RESULTS**

**Dysadherin Expression Is Positively Correlated with Cell Motility.** The expression level of dysadherin was low in Capan-1 and HPAF-II cells, moderate in Panc-1 and Miapaca-2 cells, and high in...
M panc-96 and BxPC-3 cells (Fig. 1A). BxPc-3 and M panc-96 cells, which strongly expressed dysadherin, showed a higher cell motility index than did Capan-1 and HPAF-II cells, which had the lowest expression of dysadherin (Fig. 1B and C). Cell motility was clearly correlated with the level of dysadherin expression among the six human pancreatic cancer cell lines, although discordance was observed between the cell lines with moderate dysadherin expression (Miapaca-2 and Panc-1).

Establishment of Small Interfering RNA-Mediated Reduction of Dysadherin Expression. We used siRNA technology not only in Panc-1 cells but also in HeLa cells, which are frequently used in siRNA experiments and strongly express dysadherin.

The expression of dysadherin was suppressed in Panc-1 cells and HeLa cells transfected with Dys141 siRNA, whereas the parent cells and cells transfected with control siRNA (lamin A/C) preserved dysadherin expression (Fig. 2A). This reduction of dysadherin protein seemed to be caused by the RNA interference reaction because a reverse transcription-polymerase chain reaction experiment confirmed the reduction of dysadherin mRNA (Fig. 2B). The other siRNA, Dys498, was less efficient than Dys141 in reducing dysadherin expression (data not shown). Thus, we used Dys141 siRNA for further study. All siRNA-transfected Panc-1 and HeLa cells showed growth curves similar to those of host cells and control cells (data not shown). Unlike the situation in Panc-1 and HeLa cells, dysadherin expression was not suppressed in BxPc-3, M panc-96, and Miapaca-2 cells by transfection with dysadherin siRNA (data not shown). Besides, lamin A/C expression was not suppressed in these cells by transfection with lamin A/C siRNA (data not shown). Therefore, the differences in levels of dysadherin expression among pancreatic cancer cell lines are attributable to the efficiency of siRNA transfection.

Establishment of Dysadherin-Overexpressing Capan-1 Human Pancreatic Cancer Cells. After stable transfection with a dysadherin expression vector, three Capan-1 clones with the highest level of stable dysadherin expression were selected (Fig. 2C). These three dysadherin-overexpressing clones and the mock-transfected clones showed growth curves similar to that of parental Capan-1 cells (data not shown).

Change in Dysadherin Expression Affects the Motility of Human Pancreatic Cancer Cells. The three dysadherin-overexpressing Capan-1 clones showed significantly increased cell motility compared with parental Capan-1 and mock-transfected cells (Fig. 3A and B). On the contrary, Panc-1 cells transfected with dysadherin siRNA showed a significant reduction of cell motility compared with parental Panc-1 cells and Panc-1 cells transfected with lamin A/C siRNA (Fig. 3C and D). Reduction of cell motility by dysadherin siRNA transfection was also observed in HeLa cells (data not shown).

Reduction of Dysadherin Expression Induces Change in Cell Morphology and Actin Organization. Panc-1 cells transfected with dysadherin siRNA tended to show relatively larger, more spread shapes, and the margin of cells became unclear compared with control siRNA-transfected cells or parental cells (Fig. 4A). The Array Scan system revealed that Panc-1 cells transfected with dysadherin siRNA had significantly larger cell areas than did control cells (Fig. 4B). We counted the number of spindle-type cells (less adherent) and spread-type cells (more adherent) by visual observation, and we found that Panc-1 cells transfected with dysadherin siRNA showed significantly more spread-type cells (i.e., more adherent) than did control cells (Fig. 4C). These morphologic changes were more apparent in nearly all HeLa cells transfected with dysadherin siRNA.

According to observation by confocal microscopy, dysadherin siRNA transfection markedly reduced dysadherin expression in almost all Panc-1 and HeLa cells. In control cells, dysadherin was expressed on the cell membrane in juxtaposition to the submembranous cortical actin layer (Fig. 5A), as described previously (3). The cortical actin layer was decreased in dysadherin siRNA-transfected Panc-1 cells (Fig. 5B). More filamentous transverse actin stress fibers were found in dysadherin-reduced cells than in control cells (Fig. 5A and B). We also found similar changes in actin filaments in HeLa cells transfected with dysadherin or control siRNAs (Fig. 6A). To examine whether the increase in stress fibers was related to the formation of focal adhesions, we also analyzed paxillin expression in Panc-1 and HeLa cells transfected with dysadherin or control siRNAs. Paxillin was clustered more conspicuously at focal adhesions in dysadherin...
siRNA-transfected HeLa cells than in control siRNA-treated HeLa cells (Fig. 6B and C). We also found a similar tendency in dysadherin siRNA-treated Panc-1 cells and Capan-1 cells stably transfected with an expression vector (Clone 2) or mock-transfected (Mock 2) were stained for dysadherin (green) and F-actin (red). Original magnification, ×600.

**Fig. 5.** Immunofluorescence analysis of dysadherin and F-actin in Panc-1 (A and B) and Capan-1 (C and D) cell lines. Dysadherin siRNA or control siRNA-treated Panc-1 cells and Capan-1 cells stably transfected with an expression vector (Clone 2) or mock-transfected (Mock 2) were stained for dysadherin (green) and F-actin (red). Original magnification, ×600.

The layer of actin filaments at the cortical regions was more conspicuous in dysadherin-overexpressing Capan-1 cells than in mock-transfected cells (Fig. 5C and D). We failed to observe transverse actin stress fibers in any of these cells. In addition, dysadherin-overexpressing clones tended to show membrane filopodia on the surface of their cell membrane, and dysadherin was localized there (Fig. 5D).

**Dysadherin Overexpression in Human Pancreatic Cancer Cells Promotes Metastasis in Mice.** At 35 days after transplantation, dysadherin-transfected Capan-1 cells showed multiple metastases to the liver, whereas parental Capan-1 cells and mock transfecants did not (Table 1). All of the transplants (dysadherin-transfected, mock-transfected, and parental Capan-1 cells) showed almost equally efficient growth at the primary injection site. This is consistent with our previous observation that the expression level of dysadherin does not strongly affect tumor cell growth (3).

**DISCUSSION**

In this study, we investigated the role of dysadherin expression, which significantly correlates with clinicopathological factors in pancreatic cancer and with cell morphology, cell motility, and metastatic potential. The siRNA-mediated reduction of dysadherin expression induced formation of actin stress fibers, reduction of submembranous F-actin, and suppression of cell motility in Panc-1 human pancreatic cancer cells. On the contrary, dysadherin overexpression in the human pancreatic cancer cell line Capan-1 caused an increase in the amount of submembranous F-actin, high motility, and frequent metastasis in SCID mice. Our results clearly indicate that dysadherin plays an important role in actin reorganization, cell morphology, cell migration, and metastasis.

Our previous study showed that aggregation of dysadherin by anti-dysadherin antibody-coated beads triggered recruitment of actin filaments on the cell membrane in human umbilical vein endothelial cells (3). Actin organization is one of the major mechanisms for regulation of cell migration (21–25). Based on the previous and present studies, dysadherin may recruit and/or maintain actin filaments at the leading edge, where actin fiber utilization is believed to actively occur during cell movement (26). Our data therefore suggest that the recruitment of filamentous actin toward the cell membrane is a possible mechanism of facilitation of cell motility by dysadherin overexpression. Alternatively, dysadherin might suppress or disrupt formation of focal contacts and facilitate cell movement. On the other hand, several signaling molecules, such as Rho G-proteins and phos-
phatidylinositol 3'-kinase, are known to play important roles in the regulation of cell motility (27, 28). It is a very interesting possibility that dysadherin overexpression might stimulate such signal-mediating molecules. Further investigation is still necessary to address the molecular mechanisms by which dysadherin overexpression affects cell motility.

A previous study (3) suggested the involvement of dysadherin in E-cadherin stability in liver cancer cells, and dysadherin expression showed significant negative correlation with E-cadherin expression in thyroid carcinoma by immunohistochemical analysis (7). However, in human pancreatic ductal adenocarcinoma and colon cancer, immunohistochemical analyses showed that mutually exclusive expression of E-cadherin and dysadherin in cancer cells occurred in some cases but, overall, was not statistically significant (6, 8). In addition, in the present study, alteration of dysadherin expression in pancreatic cancer cells did not cause changes in the expression of cadherins (data not shown). The reason for the retention of E-cadherin expression in pancreatic cancers with dysadherin overexpression is unclear. It is possible that a strong signal for maintaining E-cadherin expression exists in pancreatic cancer tissue and that dysadherin could (only partially in most cases) antagonize the E-cadherin–maintaining signals.

The siRNA we designed for dysadherin was a very efficient suppressor of the expression of the target protein. Because dysadherin appears to promote the invasiveness of pancreatic cancer, dysadherin siRNA may represent an approach to the prevention or therapy of tumor metastasis in vivo. Alternatively, small molecule antagonists against dysadherin would be of interest as antimetastasis agents. The use of anti-dysadherin agents, including siRNA, in the management of metastasis in pancreatic cancer depends on whether dysadherin plays any important roles in normal tissue, and it will be important to address this point in the near future.

The present study demonstrates the biological significance of dysadherin overexpression in human pancreatic carcinomas. Our results suggest that dysadherin modulates actin organization and cell motility in pancreatic ductal adenocarcinoma cells. The relationship between dysadherin expression and metastatic potential in SCID mice indicates that dysadherin functions as an inducer of metastasis of pancreatic cancer by facilitating cell motility.

Table 1 Dysadherin overexpression in human pancreatic cancer Capan-1 cells facilitates metastasis in mice

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Liver metastasis</th>
<th>Lymph node metastasis</th>
<th>Peritoneal dissemination</th>
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<tbody>
<tr>
<td>Capan-1</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mock 1</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Clone 2</td>
<td>6</td>
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<td>2</td>
</tr>
<tr>
<td>Clone 3</td>
<td>7</td>
<td>5</td>
<td>1</td>
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NOTE: After stable transfection with a dysadherin expression vector, three Capan-1 clones (Clones 1–3) with the highest level of dysadherin expression were selected. Parental Capan-1 cells and two mock transfectants (Mock 1 and Mock 2) were also injected into SCID mice as described in Materials and Methods.

Fig. 6. Reduction of dysadherin expression modulates actin organization and clustered focal contact in HeLa cells. A shows dysadherin siRNA-treated HeLa cells stained for dysadherin (green) and F-actin (red). Most dysadherin siRNA-treated HeLa cells show reduced dysadherin expression. Arrowheads indicate HeLa cells without reduced dysadherin expression after transfection with dysadherin siRNA. These nonresponding cells, which were probably nonresponsive because of inefficient introduction of siRNA, are considered “internal controls.” Dysadherin siRNA-treated (C) or control siRNA-treated (B) HeLa cells were stained for paxillin (green) and F-actin (red). A–C, ×600.

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