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Induction of p21waf1 Expression and Growth Inhibition by Transforming Growth Factor β Involve the Tumor Suppressor Gene DPC4 in Human Pancreatic Adenocarcinoma Cells

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Abstract

The tumor suppressor gene deleted in pancreatic cancer locus 4 (DPC4) is inactivated in about 50% of pancreatic adenocarcinomas. DPC4 was found to be homologous to Smad4 and may function as a transcription factor in the transforming growth factor β (TGF-β) receptor-mediated signal transduction pathway. We have investigated the role of DPC4 in the TGF-β receptor-mediated signal transduction cascade in five human pancreatic cancer cell lines (Panc-1, MDA-Panc-28, HS766T, Capan-1, and MiaPaCa-2). Our results demonstrate that the loss of responsiveness to TGF-β-induced growth inhibition correlates with the loss of expression of DPC4. We have shown that TGF-β induces p21waf1 expression in Panc-1 cells, whereas no induction of p21waf1 expression by TGF-β was detected in the other four cell lines lacking either DPC4 expression or the TGF-β type II receptor. No increase in p21waf1 mRNA stability was observed after treatment with TGF-β, which suggests that the induction of p21waf1 in Panc-1 cells is transcriptionally regulated by TGF-β. Our data also demonstrate that the expression of DPC4 is directly involved in TGF-β-mediated induction of the 3TP-lux reporter gene, which contains a known TGF-β-inducible plasminogen activator inhibitor promoter. These data suggest that: (a) TGF-β-mediated induction of p21waf1 and subsequent growth inhibition require the expression of DPC4; (b) p21waf1 is a downstream target gene of DPC4; and (c) transfection of the DPC4 gene restores the TGF-β-inducible gene expression. Inactivation of the tumor suppressor gene DPC4 and other components of the TGF-β signal cascades may abrogate one of the key negative controls of cell proliferation in pancreatic adenocarcinomas.

Introduction

Pancreatic adenocarcinoma is the fifth leading cause of adult cancer mortality in the United States (1). However, the epidemiology of pancreatic cancer provides few clues about its etiology and pathogenesis. Strategies for early detection of pancreatic adenocarcinoma have not yet been developed, and most pancreatic adenocarcinomas present with metastatic or locally advanced disease at the time of diagnosis (2). Therapeutic options for patients with advanced disease are few, because chemotherapy and irradiation are largely ineffective (2, 3). Metastatic disease often develops after potentially curative surgery (4). Nonetheless, recent findings have improved our understanding of the biology of pancreatic cancer and demonstrated that genetic and molecular alterations in adenocarcinomas of the pancreas involve the activation of specific oncopogenes and the inactivation of specific tumor suppressor genes (2, 5).

A recently identified candidate tumor suppressor gene, DPC4, has been found to be deleted or mutated in about 50% of pancreatic adenocarcinomas (5). A functional role for DPC4 was suggested by its peptide sequence, which is similar to those of the Drosophila melanogaster Mad protein and the Caenorhabditis elegans Mad homologues sma-2, sma-3, and sma-4 (5). Mad proteins have been linked to the TGF-β superfamily of cytokines that regulate cell differentiation and are potent inhibitors of cellular proliferation for most normal cells (6–8). Many cancer cells have been shown to lose responsiveness to TGF-β-induced growth inhibition (9, 10), suggesting that a defect in the TGF-β receptor-mediated signal transduction cascade may eliminate a critical negative control for cell proliferation. Recently, it has also been shown that the nuclear localization of DPC4 or Smad4 protein is regulated by the TGF-β receptor signal cascade (9). Another Smad family member, Smad1, is a direct substrate for TGF-β receptor, and it is activated and phosphorylated by the TGF-β receptor serine/threonine kinases (11). DPC4 also mediates transactivation of the plasminogen activator inhibitor promoter (3TP-lux reporter; Ref. 12). It has also been demonstrated that TGF-β-induced cell cycle arrest can be partially attributed to the regulatory effects of TGF-β on both the expression and activity of cdk inhibitors such as p21waf1, and the binding of these inhibitors to specific cdk complexes blocks their activity and causes cell cycle arrest (13, 14). The present study was designed to investigate the role of DPC4 in the TGF-β signal transduction cascade in human pancreatic adenocarcinoma cells.

Materials and Methods

Cell Culture. Human pancreatic cancer cell lines Panc-1 (CRL 1469, ATCC, Rockville, MD), MDA-Panc-28 (an established cell line from primary adenocarcinoma of the pancreas), HS766T (HTB 134; ATCC), Capan-1 (HTB 79; ATCC), and MiaPaCa-2 (CRL 1420; ATCC) were maintained in DMEM supplemented with 10% FBS (Life Technologies, Inc., Grand Island, NY). For TGF-β treatments, exponentially growing cells were treated with 5 ng/ml recombinant human TGF-β1 (R&D Systems, Inc., Minneapolis, MN) in DMEM with 0.5% FBS for 24, 48, and 72 h. Growth Inhibition Studies. The standard cell proliferation assay was performed as described previously (15). Briefly, the cell growth inhibition mediated by TGF-β was determined by thiazolyl blue (MTT) dye assay. A total of 2 × 104 cells/well were seeded in 96-well plates and treated with 5 ng/ml recombinant human TGF-β1 in DMEM with 0.5% FBS for 24, 48, and 72 h. Forty μl of 2 mg/ml thiazolyl blue (Sigma Chemical Co., St. Louis, MO) was added to each well and incubated for 4 h before the blue formazan crystals were dissolved in dimethyl sulfoxide. The absorbance was measured at 570 nm.

The abbreviations used are: TGF-β, transforming growth factor β; FBS, fetal bovine serum; ATCC, American Type Culture Collection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; cdk, cyclin-dependent kinase.
were added to each well at completion of the treatment, and plates were incubated at 37°C for 4 h. Wells were aspirated, and 100 μl of DMSO (Fisher Scientific Co., Pittsburgh, PA) per well were added. Plates were read at 570 nm in a spectrophotometer (E max precision microplate reader; Molecular Devices, Menlo Park, CA).

**Southern Blot and Northern Analysis.** DNA was isolated according to a modification of the Blin and Stafford procedure (16). Ten μg of HindIII-digested genomic DNA were analyzed by agarose gel electrophoresis and hybridization with a 32P-labeled 2.8-kb DPC4 cDNA probe. RNA was isolated as described by Chomczynski and Sacchi (17). Ten μg of total RNA were electrophoresed through a 1% agarose gel containing formaldehyde, transferred to a Magna Charge nylon transfer membrane (Micron Separations, Inc., Westboro, MA), UV cross-linked, and hybridized with a 32P-labeled 2.8-kb DPC4 cDNA probe. RNA isolated by the aid of LipofectAMINE reagent (Life Technologies, Inc., Gaithersburg, MD), and treated cells were exposed to 4 μg/ml actinomycin D (Sigma Chemical Co.) and harvested for RNA isolation for different amounts of time. Northern analysis for p21WAF1 expression was performed as described above.

**Western Blot Analysis.** Pancreatic tumor cell lines were incubated in the presence and absence of 5 ng/ml TGF-β1 for 4 and 12 h and harvested for protein extracts. Twenty-five μg of the protein lysates were resolved by SDS-PAGE, transferred to nylon membranes (Immobilon-P, Millipore, Bedford, MA), and detected with anti-p21WAF1 antibody as described previously (18). The subsequent Western blot analysis was carried out with an enhanced chemiluminescence Western blotting kit (Amersham) according to the manufacturer's recommendations.

**Transfections.** Liposome-mediated transient transfections of the 3TP-lux promoter-reporter construct were performed on Panc-1 and HS766T cells. Briefly, cells were grown to 70% confluence and transfected with 3 μg of 3TP-lux vector with or without 5 μg of DPC4 pCL-neo expression vector with the aid of LipofectAMINE reagent (Life Technologies, Inc., Gaithersburg, MD). pRL-CMV Renilla luciferase was cotransfected as a control reporter vector. Cells were treated with and without 5 ng/ml TGF-β1 in 10% serum media for 48 h. At the end of treatment, reporter activity was assayed in lysates with a dual-luciferase reporter assay system according to the manufacturer's instructions (Promega, Madison, WI).

**Results**

To determine the effects of TGF-β on human pancreatic adenocarcinoma cell lines, the standard cell proliferation assays were performed using five well-characterized cell lines (Panc-1, MDA Panc-28, HS766T, Capan-1, and MiaPaCa-2) in the presence and absence of TGF-β (Fig. 1; Ref. 15). The results show statistically significant growth inhibition by TGF-β in Panc-1 cells after 48 and 72 h of treatment. TGF-β induced a 20% growth inhibition at 24 h (P = 0.07 by Student’s t test), a 26% growth inhibition at 48 h (P = 0.01 by Student’s t test), and a 33% growth inhibition at 72 h of treatment (P < 0.001 by Student’s t test). The other four cell lines did not demonstrate any TGF-β-induced growth inhibition in the assays (Fig. 1). Both the control and treated MDA Panc-28 and MiaPaCa-2 cells experienced a decrease in viable cells, whereas the control and treated Capan-1 and HS766T cells had a little increase in viable cells. Our data indicate that the TGF-β receptor-mediated signal cascade for growth inhibition is functional in Panc-1 cells but is defective in the other four cell lines. This finding is consistent with earlier reports that many malignancies have lost responsiveness to TGF-β-induced growth inhibition, suggesting that inactivation of the components of the TGF-β signal cascades may abolish one of the key negative controls of cell proliferation. These TGF-β-unresponsive and TGF-β-responsive pancreatic tumor cell lines serve as useful tools to further study the alterations in TGF-β receptor-mediated growth inhibition in pancreatic tumors.

To determine whether the loss of TGF-β-mediated growth inhibition in human pancreatic tumor cell lines correlates with the inactivation of tumor suppressor gene DPC4, we characterized it in the five human pancreatic cancer cell lines using Northern and Southern blot analysis. Our results show that a full-length DPC4 mRNA was expressed in a TGF-β-responsive cell line, Panc-1, but was absent from the TGF-β-unresponsive cells lines HS766T and Capan-1 (Fig. 2A). A truncated form of DPC4 mRNA was detected in TGF-β-unresponsive MDA Panc-28 cells (Fig. 2A). The growth of MiaPaCa-2 cells, which express a full-length DPC4 mRNA, was not inhibited by TGF-β. The unresponsiveness to TGF-β-induced growth inhibition in MiaPaCa-2 cells can be explained by the lack of TGF-β type II receptors in this cell line (19). Southern blot analyses demonstrated that the loss of expression of DPC4 in HS766T cells is caused by the homozygous deletion of the DPC4 gene, whereas no deletion in the gene was detected in the other four cell lines studied (Fig. 2B). These results suggest that the inactivation or loss of expression of DPC4 or TGF-β type II receptor in the TGF-β signal transduction pathway abolishes one of the critical negative controls for cell proliferation in HS766T, Capan-1, MDA Panc-28, and MiaPaCa-2 cells.

It has been demonstrated that TGF-β induces the expression of cdk inhibitors such as p21WAF1, and TGF-β-induced growth inhibition is partially attributed to the regulatory effects of TGF-β on both the level and activity of the G1 cyclins and their cdk partners (14). We performed Northern blotting analyses to determine whether the expression of p21WAF1 is inducible by TGF-β. The results showed that p21WAF1 expression was induced by TGF-β in Panc-1 cells but not in HS766T, Capan-1, MDA Panc-28, and MiaPaCa-2 cells (Fig. 3). In the Panc-1 cells, a dose- and time-dependent induction of p21WAF1 was...
The role of tumor suppressor gene DPC4 in pancreatic cancer.

We have characterized the expression of DPC4 in Panc-1 cells. DPC4 has been linked to the TGF-β signal transduction pathway, which is known to mediate inhibition of cell proliferation. In our studies with five human pancreatic cancer cell lines, we found that one cell line, Panc-1, demonstrated TGF-β-mediated growth inhibition. We have demonstrated that the loss of TGF-β-induced growth inhibition and TGF-β-induced p21 expression correlate with the loss of DPC4 gene expression. DPC4 is directly involved in TGF-β signal transduction. Our data suggest that TGF-β-mediated induction of p21 and subsequent growth inhibition require the expression of DPC4 and that p21 is a candidate downstream target gene in human pancreatic cancer cells.

Discussion

We have demonstrated that the loss of TGF-β-induced growth inhibition and TGF-β-induced p21 expression correlate with the loss of DPC4 gene expression. DPC4 is directly involved in TGF-β-mediated induction of the 3TP-lux reporter gene, which contains a known TGF-β-inducible promoter. Our data suggest that TGF-β-mediated induction of p21 mRNA and protein correlate with the increased proliferation in cells kept in the media containing 0.5% serum, whereas the cell lines that express low basal levels of p21 mRNA and protein show no change or decrease in growth in the media containing 0.5% serum.

The candidate tumor suppressor gene DPC4 was recently identified and cloned by Hahn et al. (5). DPC4 was found to be deleted or mutated in 50% of the pancreatic carcinoma xenografts examined (5). DPC4 has been linked to the TGF-β signal transduction pathway, which is known to mediate inhibition of cell proliferation. In our studies with five human pancreatic cancer cell lines, we found that one cell line, Panc-1, demonstrated TGF-β-mediated growth inhibition. We have characterized the expression of DPC4 in Panc-1 cells. Furthermore, treatment of Panc-1 cells with TGF-β resulted in a significant induction of the G1 cdk inhibitor p21 mRNA. This induction of p21 gene expression lasted for at least 48 h after treatment with TGF-β, the same amount of time it took for growth inhibition to become statistically significant (Figs. 1 and 3). TGF-β has been previously reported to induce p21 in human squamous cell carcinoma cell lines (14). Induction of p21 and subsequent G1 cell cycle arrest could account for at least one of the mechanisms of growth inhibition mediated by TGF-β. Because Panc-1 cells are known to possess a mutated p53 tumor suppressor gene (21), induc-
Fig. 3. p21<sup>−/−</sup> mRNA induction by TGF-β in Panc-1 cells. A, Northern blot analysis of 10 μg of total RNA from Panc-1 cells after 4 h of treatment with various doses of TGF-β-1. Hybridization with p21<sup>−/−</sup> and GAPDH cDNA probes is shown. B, Northern blot analysis of Panc-1 total RNA after treatment with 5 ng/ml TGF-β for various amounts of time. C, quantitation from six, two, and two independent experiments on Panc-1 cells after 4, 8, and 12 h of treatment, respectively, with 5 ng/ml TGF-β. Quantitation of both p21<sup>−/−</sup> and GAPDH was performed to correct for differences in loading. Values are expressed relative to the control, which was given an arbitrary value of 1. Data in the graph represent the means ± SE. Induction of p21<sup>−/−</sup> at 4 h of treatment was statistically significant (P < 0.05 by t test). D, lack of p21<sup>−/−</sup> mRNA induction by TGF-β in MDAPanc-28, MiaPaCa-2, HS766T, and Capan-1 cell lines. Representative Northern blot analysis of 10 μg of total RNA from five human pancreatic cancer cell lines after treatment with 5 ng/ml TGF-β-1 for 0, 4, and 12 h. Hybridization with p21<sup>−/−</sup> and GAPDH cDNA probes is shown. Densitometry quantitation and correction for loading differences were performed as described. E, lack of TGF-β-inducible p21<sup>−/−</sup> expression in Western blot analysis. Twenty-five μg of cell extracts from the indicated human pancreatic cancer cell lines that were treated with 5 ng/ml of TGF-β-1 for 0, 4, and 12 h, were used in the analysis and probed with anti-p21<sup>−/−</sup> and anti-β-actin antibodies as described previously (18). F, effect of TGF-β on p21<sup>−/−</sup> mRNA stability in Panc-1 cells. Cells were pretreated with and without 5 ng/ml TGF-β for 8 h. Both untreated and treated cells were then exposed to 4 μg/ml actinomycin D for various amounts of time and harvested for RNA isolation. A semilogarithmic plot of the decay of p21<sup>−/−</sup> mRNA is shown. mRNA levels at time 0 in either the absence or presence of TGF-β were arbitrarily defined as 1. G, lack of activation of JTP-lux after TGF-β1 treatment in HS766T cells is restored by cotransfection with a DPC4 expression vector.
tion of p21\textsuperscript{wafl} expression by TGF-\(\beta\) must occur through a p53-independent pathway. Interestingly, none of the other four pancreatic cancer cell lines exhibited TGF-\(\beta\)-mediated growth inhibition or p21\textsuperscript{wafl} induction. The lack of responsiveness to TGF-\(\beta\) in MiaPaCa-2 cells could be explained by the fact that although DPC4 transcripts are expressed in these cells, they are known to lack the TGF-\(\beta\) type II receptor necessary for activation of the signal transduction cascade leading to growth inhibition and possibly to p21\textsuperscript{wafl} induction (19). MDAPanc-28, a cell line derived from a primary adenocarcinoma, showed a truncated form of the DPC4 transcript and different restriction enzyme fragments when compared with those of the other cell lines (data not shown). HS766T, a pancreatic cancer cell line derived from a metastatic lymph node, showed a homozygous deletion of DPC4 and no expression of the DPC4 transcript. Capan-1, a cell line derived from a pancreatic cancer metastasis in the liver, demonstrated a loss of expression of DPC4 as determined by Northern blot analysis. Thus, our data show that the loss of TGF-\(\beta\)-inducible p21\textsuperscript{wafl} expression in all these pancreatic tumor cell lines can be explained by the absence of functional DPC4 protein. However, what led to the high basal expression levels of the p21\textsuperscript{wafl} gene in MiaPaCa-2 cells, which seem to be independent of p53 and DPC4 proteins, remains unknown. Our data, summarized in Table 1, suggest that a high basal level of p21\textsuperscript{wafl} expression not only fails to inhibit cell proliferation but also increases growth in HS766T and Capan-1 cells under low-serum conditions. This observation may not be explained by the presence of mutated p21\textsuperscript{wafl}\ protein in these cells, because p21\textsuperscript{wafl} mutation is a rare event in tumors (22). It is possible that in these cells, p21\textsuperscript{wafl} protein functions as an adapter required to efficiently assemble active cyclin/cdk complexes as in the reported cases (23, 24). This may explain in part the increase in growth of these two cell lines in low-serum conditions. Another explanation is suggested by a recent report that p21\textsuperscript{wafl} protein inhibited p300-associated cyclin E-Cdk2 activity and stimulated \(\kappa\)B enhancer-dependent gene expression (25). The interaction of RelA NF\(\kappa\)B and cyclin/CDK/p21\textsuperscript{wafl} complex through the p300/CBP coactivators may affect cell cycle progression.

In summary, the four pancreatic cancer cell lines that do not exhibit TGF-\(\beta\)-mediated growth inhibition and p21\textsuperscript{wafl} induction have either a deletion, loss of expression, or truncation of DPC4 or a lack of the TGF-\(\beta\) type II receptor. Previous published work shows that Panc-1 and HS766T share a subtype of the TGF-\(\beta\) type I receptor SKR1 as well as a similarly low expression level of ALK5, the major type I receptor; the work also shows that HS766T cells express relatively high levels of the TGF-\(\beta\) type II and III receptors, whereas Panc-1 cells express low levels of these receptors (26). Therefore, we believe that the differential response to TGF-\(\beta\) observed between these two cell lines cannot be explained on the basis of their TGF-\(\beta\) receptor status but is most probably caused by the lack of the DPC4 gene in HS766T cells. DPC4 plays an important role in the TGF-\(\beta\) signal transduction cascade, as demonstrated by its ability to restore TGF-\(\beta\) activation of 3TP-lux when transfected into cells that lack the DPC4 gene. p21\textsuperscript{wafl} may serve as a downstream target gene of DPC4 in this pathway. Additional studies are in progress to determine the DPC4-responsive elements in the p21\textsuperscript{wafl} promoter. Our results suggest that in human pancreatic cancer cell lines, a functional DPC4 gene is required for the TGF-\(\beta\)-mediated induction of p21\textsuperscript{wafl} and subsequent growth inhibition to occur. These results support the role of DPC4 as a tumor suppressor gene in human pancreatic adenocarcinoma and may provide a basis for gene therapy strategies that can restore the signal transduction pathways of TGF-\(\beta\)-mediated growth suppression.

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References


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