The basic helix-loop-helix transcription factor DEC2 inhibits TGF-β-induced tumor progression in human pancreatic cancer BxPC-3 cells

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Received February 7, 2012; Accepted March 12, 2012

DOI: 10.3892/ijmm.2012.1037

Abstract. The basic helix loop helix (bHLH) transcription factor DEC2 is associated with the regulation of apoptosis, circadian rhythm and the response to hypoxia. However, the significance of DEC2 in pancreatic cancer remains unknown. Here, we showed for the first time that DEC2 inhibits the progression of human pancreatic cancer. Human pancreatic cancer BxPC-3 cells were treated with or without transforming growth factor-β (TGF-β), siRNA against DEC2, or a combination of TGF-β and DEC2 siRNA or DEC2 overexpression. The cells were analyzed by RT-PCR, real-time PCR, western blotting, immunofluorescent staining and ChIP assay. We also performed immunohistochemical analyses of DEC2 expression in surgically-resected pancreatic cancers. The expression of DEC2 was increased in TGF-β-treated BxPC-3 cells. In the presence of TGF-β, DEC2 overexpression decreased the migration and invasion of BxPC-3 cells. Knockdown of DEC2 by siRNA in the presence of TGF-β significantly increased the expression and nuclear concentration of slug. We also showed that DEC2 siRNA decreased the binding of DEC2 to the E-box of the slug promoter. Immunohistochemically, little DEC2 was detected in pancreatic cancer tissues, whereas significant amounts were detected in the adjacent non-cancerous pancreatic tissues. These results indicate that DEC2 has inhibitory effects against human pancreatic cancer that involve TGF-β and slug.

Introduction

Patients with pancreatic cancer have a poor prognosis with an overall 5-year survival rate of less than 5% (1). More than 80% of patients are diagnosed with pancreatic cancer at a locally advanced or metastatic stage (2). Understanding the molecular biology of pancreatic cancer, which can involve migration and invasion may provide insights into the development of novel tumor markers or new therapeutic strategies.

Transforming growth factor-β (TGF-β) inhibits cell proliferation and promotes tumor cell motility and invasion (3,4). In comparison with non-tumor pancreatic tissues, pancreatic tumors display increased TGF-β expression (5,6). In addition, TGF-β treatment has been demonstrated to induce epithelial-mesenchymal transition (EMT), which is a key step in the progression of various cancers. During EMT, cells undergo a developmental switch from a polarized, epithelial phenotype to a mesenchymal phenotype (7).

Transcription factors snail and slug are key factors for EMT. They downregulate the expression of various epithelial factors, including E-cadherin, claudins and β-catenin, and upregulate the expression of mesenchymal markers, including N-cadherin, vimentin and fibronectin (8,9).

Smads also play important roles in the progression of pancreatic cancer and Smad4 mutations are known to be present in more than 50% of pancreatic cancer cases (10,11). Smad2/3 are considered to be key regulators of pancreatic cancer. TGF-β activates the phosphorylation of Smad2 and Smad3 and the activated Smad complex binds to target gene promoters and regulates the transcriptional responses to TGF-β in the nucleus (12-14).

We have previously shown that tumor necrosis factor-α (TNF-α), hypoxia, paclitaxel, cisplatin, CLOCK/brain-muscle-arsnt-like-protein (BMAL) 1/2 and polyinosinic-polycytidylic
acid are upstream factors of the differentiated embryo chondrocyte 2 (DEC2) (BHLHE41/Sharp1), whereas its downstream factors include p53, Bax, Bim, vascular endothelial growth factor (VEGF) and interferon-β (IFN-β). We have also demonstrated that DEC2 is involved in the regulation of apoptosis, the response to hypoxia and the cell cycle in breast and oral cancer and sarcoma cells (15-21). However, the roles of DEC2 in pancreatic cancer remain unknown. In this study, we focused on the role of DEC2 in pancreatic cancer BxPC-3 cells subjected to TGF-β treatment and demonstrated that DEC2 has inhibitory effects on the tumor progression of BxPC-3 cells.

Materials and methods

Cell culture and treatment. Human pancreatic cancer BxPC-3 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in RPMI-1640 medium (Gibco-BRL, Breda, The Netherlands) supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 95% air and 5% CO₂. For the experiments, the cells were incubated with and without various concentrations of recombinant human TGF-β1 (R&D Systems, Minneapolis, MN, USA) for 24 h.

Knockdown of DEC2 by interference RNA. Short interference RNA (siRNA) against DEC2 was synthesized by Qiagen (Hilden, Germany). The sequences of the sense and anti-sense DEC2 siRNA and the negative control (scrambled) siRNA were described previously (17). For the siRNA transfection experiments, BxPC-3 cells were seeded at 5×10⁵ cells per 35-mm plate. After 24 h, the cells were transfected with the siRNA using the Lipofectamine RNA iMAX reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). After being transfected, the cells were incubated for 24 h and subjected to various analyses.

DEC overexpression. DEC1 or DEC2 overexpression was induced using the pcDNA vector as previously described (20). After being transfected, the cells were incubated for 24 h and subjected to the migration or invasion assay.

Western blotting. The cells were lysed using M-PER lysis buffer (Thermo Scientific, Rockford, IL, USA) and their protein concentrations (10 µg) were determined using the bicinchoninic acid (BCA) assay. Their lysates were subjected to SDS-PAGE and the proteins within them were transferred to PVDF membranes (Immobilion P, Millipore, Tokyo, Japan), which were then incubated with antibodies. The ECL, ECL-Plus or ECL-Advance western blotting detection systems (ECL, ECL-Plus or ECL-Advance, Minneapolis, MN, USA) for 24 h.

Real-time polymerase chain reaction (PCR) and reverse transcription-PCR. We prepared three independent RNA samples (n=3) for real-time PCR from the BxPC-3 cells. Total RNA was isolated and first-strand cDNA was synthesized as previously described (20). Real-time PCR was performed using SYBR-Green Master Mix (Invitrogen Life Technologies). The sequences of the primers for DEC1, DEC2 and 18S rRNA used in the real-time PCR and the sizes of their products were previously described (22). The sequences of the primers for slug used for the real-time PCR were as follows: slug-F, 5'-CCATTCCAGCCGACTA-3' and R, 5'-TCACTGCACCACAGTGAG-3'. The amplified products of slug were 69 bp. The sequences of the primers for DEC1, DEC2 and slug used for the RT-PCR were as follows: DEC1-F, 5'-GTCTGTTGAGTCACATCCCAG-3' and R, 5'-GAGTCGAGTCTCTTTGTTGAAAAG-3'; DEC2-F, 5'-CACCTCTATGGGAGATGCAC-3'; slug-F, 5'-GAGCAGTTTGCAGAGTGCA-3' and R, 5'-GAGTCTCTTTGTTGAGTA-3'. The amplified products of DEC1, DEC2 and slug were 534, 502 and 330 bp in length, respectively. The cDNA for DEC1, DEC2 and slug were amplified for up to 28 cycles. The PCR products were separated on 1.5% (w/v) agarose gels.

Chromatin immunoprecipitation (ChIP) assay. A ChIP assay was performed using a kit from Millipore, as described previously (16). Primers were designed to amplify a Smad binding element containing a DNA fragment from the DEC2 promoter and their sequences were as follows: human DEC2-F, 5'-GAGGAAATCGAGCCTTTCAA-3' and R, 5'-CGGCGGAATTCACAGTA-3'. Primers were also designed to amplify a DNA fragment containing the E-box in the slug promoter and their sequences were as follows: human slug-F, 5'-AGAGCAGACTTTGCAG-3' and R, 5'-GTGGGTTGCTAATTCAAAG-3'.

Immunofluorescent staining. The cells were seeded in a 4-chamber slide glass and incubated overnight. Then, they were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 30 min, before being permeabilized with 0.2% Triton X-100 in PBS for 10 min. The permeabilized cells were then washed in PBS twice and treated with 5% normal horse serum in PBS for 30 min (to minimize the non-specific adsorption of antibodies), before being incubated with anti-slug (1:300) antibody at 4°C overnight. Next, the cells were incubated for 1 h with goat anti-rabbit IgG antibody conjugated to Alexa 488 dye (Molecular Probes Inc., Tokyo, Japan), while nuclear staining was performed using 4',6-diamidino-2-phenylindole (DAPI). The cells were visualized using confocal laser scanning microscopy (Zeiss, LSM 710, Wetzlar, Germany).
DEC2 expression is induced in BxPC-3 cells by TGF-β. In this study, we used human pancreatic cancer BxPC-3 cells to functionally analyze DEC2 and investigate the protein expression of endogenous DEC2 and various EMT-related factors during TGF-β treatment. In these cells, TGF-β treatment induced Smad3 phosphorylation and upregulated the expression of DEC1, DEC2, slug and vimentin and downregulated the expression of claudin-1 (Fig. 1A). The highest DEC2 expression level was observed in the cells treated with 10 ng/ml of TGF-β for 24 h. TGF-β had little effect on the expression of Smad3, snail, E-cadherin, claudin-4 and N-cadherin. Next, we investigated the endogenous mRNA expression of DEC1 and DEC2 in the presence of TGF-β using real-time and RT-PCR. The mRNA expression levels of DEC1 and DEC2 were upregulated by TGF-β (Fig. 1B).

Since DEC2 has five CAGAC Smad3 binding elements (SBE) in its promoter, we investigated whether Smad3 binds to the SBE in the DEC2 gene. We selected a Smad3 binding target sequence containing a single SBE, which was located near to the transcription starting point of the DEC2 gene (Fig. 1C). The binding of Smad3 to the SBE in the DEC2 promoter was significantly increased in the TGF-β treated cells compared with the non-TGF-β treated cells.

DEC2 negatively regulates slug expression. To clarify the biological functions of DEC2 in TGF-β-induced tumor progression, we examined whether the transfection of DEC2 siRNA altered the expression of EMT-related factors. In the presence of TGF-β, DEC2 siRNA upregulated the expression of DEC1 and slug, whereas it had little effect on the Smad3 phosphorylation and the expression of Smad3, snail, E-cadherin, claudin-4 and N-cadherin. Next, we investigated the endogenous mRNA expression of DEC1 and DEC2 in the presence of TGF-β using real-time and RT-PCR. The mRNA expression levels of DEC1 and DEC2 were upregulated by TGF-β (Fig. 1B).

Using immunofluorescence analysis, we examined whether DEC2 siRNA affects the nuclear/cytoplasmic amounts of slug. As shown in Fig. 2D, DEC2 siRNA transfection had little effect on the expression of Slurk in the absence or presence of TGF-β. In the presence of TGF-β, the addition of control siRNA significantly increased the nuclear concentration of Slug in the presence of TGF-β. Since the slug gene has one CAGCTG E-box element in its promoter, we investigated whether DEC2 binds to this E-box. In the presence of TGF-β, the addition of control siRNA significantly increased the binding of DEC2 to the slug promoter CAGCTG.
E-box compared with that observed after DEC2 siRNA transfection, whereas in the absence of TGF-β, control siRNA only slightly increased the binding of DEC2 to the slug promoter CACGTG E-box compared with DEC2 siRNA (Fig. 2E).

DEC2 overexpression inhibits the invasion and migration induced by TGF-β. Migration and invasion are important phenomena in tumor progression. Thus we examined whether DEC2 is involved in invasion and migration. We performed an invasion assay in which we transiently transfected the cells with a DEC1- or DEC2-expressing plasmid. As a result, we found that in the presence of TGF-β, the number of pcDNA-transfected invasive BxPC-3 cells was increased ~32-fold compared with the number of pcDNA-transfected cells observed in the absence of TGF-β (Fig. 3A). In the presence of TGF-β, the number of invasive DEC1 pcDNA-transfected cells was increased about 4-fold compared with the number of pcDNA-transfected cells and the number of invasive DEC2 pcDNA-transfected cells was decreased about 4-fold compared with the number of invasive pcDNA-transfected control cells. We also found that in the presence of TGF-β, DEC2 pcDNA delayed cell migration for 24 h compared with that observed in the cells transfected with pcDNA (Fig. 3B). On the other hand, in the presence of TGF-β, DEC2 siRNA increased the amount of migration detected at 24 h compared with that observed in the cells transfected with control siRNA.

DEC2 protein expression in human pancreatic cancer tissues and the adjacent non-cancerous pancreatic tissues. We examined the immunohistochemical expression of DEC2 in human pancreatic cancer tissues. Photographs of the DEC2 expression in representative cases are shown in Fig. 4. Significant DEC2 immunoreactivity was detected in the non-cancerous pancreatic tissues (94%; 16/17 cases) compared with the adjacent cancer tissues and it was predominantly localized within the cytoplasm of the non-cancerous pancreatic cells, although very weak DEC2 immunoreactivity was found in the adjacent cancer cells in all cases.

Discussion

DEC2 regulates apoptosis, the response to hypoxia, differentiation and circadian rhythms (16-18,20,23). However, the role of DEC2 in tumor progression is poorly understood. Epithelial mesenchymal transition (EMT) is characterized by the loss of epithelial factors and upregulated mesenchymal marker expression and results in a spindle cell morphology and invasive phenotype (3,8). We showed that TGF-β increased the migration and invasion of BxPC-3 cells and upregulated their DEC2 expression. In addition, TGF-β affected the levels of some EMT-related molecules, such as p-Smad3, slug, claudin-1 and vimentin, although it had little effect on the expression of snail, claudin-4, E-cadherin and N-cadherin and the cell morphology. Nishioka et al (24) also found that TGF-β had little effect on the expression of E-cadherin and the cell morphology in BxPC-3 cells. These results suggest that TGF-β upregulates the malignancy of BxPC-3 cells by increasing their migration and invasion and upregulating their slug expression, but does not affect the EMT.
Figure 2. DEC2 negatively regulates slug. (A) BxPC-3 cells were transfected with control siRNA or siRNA against DEC2. At 24 h post-transfection, the cells were treated with TGF-β (10 ng/ml) and incubated for 24 h and their lysates were subjected to western blot analyses of DEC1, pSmad3, Smad3, slug, claudin-1, vimentin and actin. One representative of at least three independent experiments with similar results is shown. (B) BxPC-3 cells were treated as above and total-RNA was prepared and subjected to real-time and RT-PCR for DEC2, slug and claudin-1. Each value represents the mean ± SE (bars) of three independent experiments. *p<0.05, according to the t-test. (C) BxPC-3 cells were transfected with pcDNA or DEC2 pcDNA. At 24 h post-transfection, the cells were treated with or without TGF-β (10 ng/ml) for 24 h and their lysates were subjected to western blot analyses of DEC2, slug and actin. (D) BxPC-3 cells were treated as above and the cells were fixed, incubated with anti-slug antibody and visualized using Alexa488-conjugated secondary antibody (green). The cells were also counterstained with DAPI (blue) in order to detect their nuclei. A merged image that is representative of at least two independent experiments with similar results is shown. The arrows indicate strong colocalized amounts. (E) BxPC-3 cells were transfected with control siRNA or siRNA against DEC2. At 24 h post-transfection, a ChIP assay was performed using BxPC-3 cells that had been treated with or without TGF-β (10 ng/ml) for 24 h. PCR was performed as above.
We found that Smad3 binds to the SBE in the DEC2 promoter. These findings suggest that TGF-β and Smad3 are upstream factors of DEC2. It was reported that TGF-β increases the expression and promoter activities of snail and slug in various cancer cells (25-27). Transcription factors snail and slug play important roles in TGF-β-induced tumor progression and have E-boxes in their promoters (28,29). However, snail was not upregulated by TGF-β in the BxPC-3 cells. Thus, we focused on the effects of DEC2 on slug. Slug enhances invasion in various cancer cells, involving upregulation of metalloproteinase-9 and downregulation of E-cadherin (30,31). Slug also enhances proliferation and migration in prostate and ovarian cancer cells (32,33). We showed that DEC2 knockdown or overexpression in the presence of TGF-β affected both the expression and the nuclear concentration of slug. In addition, DEC2 bound to the E-box in the slug promoter. These findings suggest that DEC2 negatively regulates slug through its E-box. We also showed that DEC2 inhibits the expression of DEC1, as previously described (34), as well as the migration and invasion induced by TGF-β. DEC1 is highly expressed in various tumors (21,35-37), whereas DEC2 is highly expressed in non-cancerous pancreatic ducts compared with cancer tissues. DEC2 also negatively regulates DEC1 through its E-box (34). Based on the above findings, DEC2 may inhibit tumor progression by suppressing slug or DEC1. Further studies are needed to clarify the details of the mechanism by which DEC1 regulates slug.

In the present study, we demonstrated that DEC2 inhibits TGF-β-induced tumor progression through slug in BxPC-3 cells and DEC2 is expressed at relatively low levels in cancer tissues compared with its levels in non-cancerous tissues. Further studies are needed to clarify the role of DEC2 in the effects induced by TGF-β and to elucidate the detailed molecular mechanisms by which DEC2 inhibits tumor progression.

Acknowledgements

This study was supported by Grants-in-Aid for Science from the Ministry of Education, Culture, Sports, Science, and Technology.
of Japan; a Grant for Hiroasaki University Institutional Research; and the Fund for the Promotion of International Scientific Research.

References