Isolation and Characterization of a Complementary DNA (PD-1) Differentially Expressed by Human Pancreatic Ductal Cell Tumors

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Abstract
A complementary DNA (cDNA) library from a cloned subline (CD-11) of a well differentiated human pancreatic tumor cell line, HPAF, was subjected to differential screening using single stranded cDNA probes synthesized from mRNA of the well differentiated cell clone CD-11 and a poorly differentiated pancreatic tumor cell line, Panc 1. A cDNA clone (PD-1) was identified which had an insert of 626 base pairs (bp). PD-1 cDNA hybridized to a transcript of about 650 bp on Northern blot analysis, suggesting that the cDNA was close to full length. Densitometric analysis of Northern blots showed that a well differentiated pancreatic tumor line had a 5-fold higher PD-1 expression as compared to the poorly differentiated line, Panc 1. Nucleotide sequence analysis of the PD-1 cDNA and its deduced amino acid sequence showed an open reading frame of 399 bp. In addition to the open reading frame, the sequence had a 5' untranslated region of 61 bp and a 3' untranslated tail of 147 bp. The nucleotide sequence did not show any significant homology to any other sequence in the GenBank or EMBL databases; however, the translated protein showed 35% homology to bacterial ribosomal proteins over 112 amino acids. Sequence analysis of the PD-1 cDNA and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of its in vitro transcription/translation product suggest that this gene encoded a protein of 16,000 daltons. Northern blot analysis with a panel of 11 pancreatic cell lines, a fibroblast line, a melanoma line, and normal and tumor pancreatic tissue showed that PD-1 expression was relatively higher in the more differentiated pancreatic tumor lines as compared to normal and poorly differentiated lines with few exceptions.

Introduction
The differentiation pathway for pancreatic ductal epithelial cells has not been elucidated. This contributes to a poor understanding of the biology of the pancreatic tumors, which are among the more lethal tumors in man. Investigation of the molecular basis of normal and tumor cell differentiation may facilitate new approaches to the diagnosis and therapy of this disease.

Differential hybridization methods have been successfully used to isolate unknown genes whose levels of expression change under different cellular conditions (1-4). We have previously constructed a cDNA1 library (5) from a clone of a well differentiated human pancreatic tumor cell line, HPAF/CD-11 (6). This library was subjected to differential screening with single stranded cDNA probes generated from mRNA prepared from the CD-11 clone and a poorly differentiated human pancreatic tumor cell line, Panc 1 (7). One cDNA clone (PD-1) detected an mRNA that was expressed at 5-fold higher levels in CD-11. The nucleotide sequence and an analysis of the expression of this newly described gene in several pancreatic tumor cell lines are reported herein.

Results and Discussion
A cDNA library from a cloned subline of a well differentiated human pancreatic tumor cell line, HPAF/CD-11 (240,000 plaques), was screened using single stranded cDNA probes synthesized from mRNA from the poorly differentiated cell line Panc 1 and the well differentiated cell line CD-11. One of the clones selected after several rounds of screening showed significantly higher levels of expression in CD-11 when compared to Panc 1. Restriction enzyme analysis of purified phage DNA showed an insert size of approximately 625 bp; this cDNA was named PD-1. Northern blot analysis showed that PD-1 cDNA hybridized to a transcript of approximately 650 bp, as shown in Fig. 1B, suggesting that the cDNA was close to full length. Densitometric analysis of this Northern blot showed that CD-11 cells demonstrated a 5-fold higher expression of PD-1 as compared to Panc 1. The same filter was probed with a β-actin cDNA probe (8) as a control for the quality and quantity of mRNA, as shown in Fig. 1A. Ethidium bromide staining of ribosomal RNA (data not shown) confirmed these results.

The overexpression of several mRNA species in tumor cell lines has been shown to be a consequence of gene amplification (3). To investigate this possibility, PD-1 cDNA was hybridized to a Southern blot of genomic DNA from CD-11 and Panc 1, as shown in Fig. 2. Purified genomic DNA from these lines was digested with EcoRI, BamHI, and HindIII. PD-1 cDNA hybridized to several bands, suggesting that this gene could be part of a multigene family or that it was a very large gene which contained multiple restriction sites. None of the bands detected in Southern blots showed amplification in CD-11 or deletion in Panc 1.

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The abbreviations used are: cDNA, complementary DNA; PD-1, pancreatic differentiation 1; SDS, sodium dodecyl sulfate; bp, base pair(s); kb, kilobase(s); poly(A)+, polyadenylated.
Expression of PD-1 mRNA was further evaluated in a panel of pancreatic tumor cell lines representing various morphological stages of differentiation, as shown in Fig. 3A. The differentiation grade for the various tumor cell lines was determined from published morphological and ultrastructural descriptions of the cell lines and corresponding tumors and was based on the grading system of Kern et al. (9). The PD-1 probe hybridized to an mRNA of approximately 650 bp in all cell lines tested. The relative expression of PD-1 was higher in all well differentiated and moderately differentiated cell lines: well differentiated cell lines HPAF (Lane 7), Colo 357 (Lane 8), and Capan-1 (Lane 10) and moderately differentiated cell lines T3M4 (Lane 1), AsPc-1 (Lane 2), and BxPc-3 (Lane 3). Poorly differentiated cell lines Panc 1 (Lane 6) and HGC 25 (Lane 5) showed lower expression. One poorly differentiated line, Mia Paca (Lane 4), showed higher expression. Two other pancreatic cell lines of undefined differentiation status, Panc 89 (Lane 11) and SW 979 (Lane 9), also showed higher levels of PD-1 expression.

Expression of PD-1 mRNA was also studied in nonpancreatic cell lines, normal pancreatic tissues, and several other normal tissues. The data presented in Fig. 4A show that PD-1 expression was very low in the human fibroblast line HUFF (Lane 7), the melanoma cell line SK-MEL-28 (Lane 5), and normal human B-lymphocytes transformed with Epstein-Barr virus (Lane 6). Normal pancreatic tissue (Lane 3) expressed PD-1 in lower amounts than that seen in well differentiated pancreatic tumor tissue (Lane 4). This may be attributable to the fact that ductal epithelial cells, the probable cell of origin of most pancreatic tumors, represent only about 10% of the cell constituency of the normal adult pancreas. If PD-1 expression was predominantly associated with ductal cells, this would explain the lower levels seen in RNA preparations from total pancreatic tissue. PD-1 expression at levels similar to that seen in pancreas was also seen in Northern blots of lung, colon, cystic duct, gallbladder, kidney, and liver (data not shown).

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The complete nucleotide sequence and the deduced amino acid sequence of the cloned cDNA are presented in Fig. 5. There was a single open reading frame of significant length that coded for a putative protein of approximately 16 kilodaltons. The entire sequence showed an open reading frame of 399 bp, a 5′ untranslated region of 61 bp, and a 3′ untranslated region of 147 bp. The noncoding 3′ region also contained the polyadenylation signal AATAAA, as overlined in Fig. 5.

The production of PD-1 protein was confirmed by in vitro translational analysis using a rabbit reticulocyte lysate. PD-1 mRNA transcripts were expressed from the pBS vector using T-7 RNA polymerase and placed into a rabbit reticulocyte lysate assay. The translated protein is shown in Fig. 6. A protein band of approximately 17 kilodaltons was seen (Lane 2) when PD-1 cDNA was placed in the correct orientation, which was not produced in lysates which contained no exogenous transcripts (Lane 4) or an antisense construct (Lane 1). The translated protein size was nearly identical to that predicted from the sequence analysis.

The nucleotide sequence of PD-1 did not show any significant homology to any sequence in the GENBANK or EMBL databases. However, a protein homology search through the GENBANK and EMBL databases, using the Fast A program of the GCG sequence analysis software package (10) (Genetics Computer Group, Madison, WI), revealed that the deduced PD-1 protein sequence showed 35% homology in 111 amino acids to ribosomal protein L23 from Haloarcula sp. (11) and 35% over 112 amino acids to ribosomal protein L22 from Halobacterium halobium (12). This homology may be serendipitous and unrelated to the function of PD-1, or it is possible that PD-1 represents a human ribosomal protein or ribosomal associated protein not previously sequenced. We have previously investigated the expression of mRNA for three mammalian ribosomal proteins, rpS16, rpL30, and rpL32, in the same pancreatic cell lines as were investigated with the PD-1 probe (3). The mRNA levels for these three proteins were not significantly different among the different pancreatic tumor cell lines, with the single exception of a concomitant amplification of the gene and mRNA for rpS16 in the Panc 1 cell line. Therefore, the amplification of PD-1 expression is not a consequence of a general amplification of ribosomal proteins expressed in well differentiated pancreatic tumor cell lines. It is interesting to note, however, that one of the striking morphological differences between well differentiated and poorly differentiated pancreatic tumor cell
lines is the ratio of free to endoplasmic reticulum-bound ribosomes. The generation of antibodies against recombinant PD-1 protein will enable us to determine whether its expression is correlated with this or other distinctions in subcellular morphology between differentiated and undifferentiated cell types.

The deduced PD-1 protein sequence was also searched for homology with protein motifs known to be associated with a biological activity, using the motif component of the search program and database available from Intelligenetics. This search revealed that several potential phosphorylation sites exist within the protein including: putative cyclic AMP and cyclic GMP protein kinase sites at residues 32 and 116; putative casein kinase II sites at residues 15 and 87; and putative protein kinase C sites at residues 12, 15, and 116. The preponderance of hydrophobic amino acids at residues 3–13 suggests that this region may be a signal sequence, raising the possibility that PD-1 is secreted, since no obvious transmembrane region was found. Obviously, one would not expect to find a signal sequence on a ribosomal protein. If the PD-1 protein is secreted, it will be important to determine whether it has biological activity as a cytokine or differentiation factor. Further studies will evaluate the hypothesized phosphorylation and secretory function of PD-1.

In summary, this report presents the nucleotide and translated protein sequence for the PD-1 cDNA. Higher expression of this gene was observed in differentiated pancreatic tumor lines as compared to undifferentiated cell lines, and expression was lower or undetectable in melanoma, normal fibroblast cell lines, and normal pancreatic tissues.

Materials and Methods

Cell Lines. The HPAF cell line was isolated from the ascitic fluid of a patient with pancreatic adenocarcinoma (6), and a very well differentiated cloned subline, CD-11, has been previously described (6). The Panc 1 pancreatic tumor cell line was obtained from the American Type Culture Collection, and the line and its xenograft are classified as a poorly differentiated cell line (7). The sources of other pancreatic lines of various states of differentiation are: Colo 357, obtained from Dr. George Moore (Denver, CO); SW 979, Panc 89, and QGP-1, obtained from Dr. H. Kalthoff (Hamburg, Federal Republic of Germany); AsPc-1, BxPc-3, Mia Paca, Capan-1, HGC 25, the human B-lymphocyte cell line NALM-6, and the SK-MEL-28 melanoma were obtained from the American Type Culture Collection. Human foreskin fibroblast cells (HUFF) were obtained from primary cultures established by Dr. Kay Singer, Duke University Medical Center. Cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Normal pancreata and other organs were obtained from the Duke University Comprehensive Cancer Center tumor and tissue bank.

Total RNA and DNA Purification. Total cellular RNA from various tumor cell lines was isolated by the guanidine isothiocyanate-cesium chloride cushion ultracentrifugation method (13). Cells were washed twice with ice-cold phosphate-buffered saline and lysed with a solution containing 4 M guanidine isothiocyanate-0.05 M sodium acetate-250 mM 2-mercaptoethanol. Total RNA was recovered via sedimentation through a 5.7 M CsCl-0.025 M sodium acetate cushion in a Beckman SW 40 Ti rotor centrifuged at 32,000 rpm for 18 h. RNA pellets were resuspended in 0.3 M sodium acetate and precipitated with ethanol. Poly(A)* mRNA was further purified on two cycles of oligo(dt) cellulose affinity chromatography (14). Genomic DNA from HPAF/CD-11 and Panc 1 cell lines was purified by the SDS-Proteinase K digestion method and then extracted with phenol-chloroform (15).

Differential Screening. The HPAF/CD-11 cDNA library was subjected to differential hybridization using single stranded cDNA probes made from mRNA of Panc 1 and CD-11 cells. The probes were synthesized using mouse mammary leukemia virus reverse transcriptase (BRL, Gaithersburg, MD) and random hexamer primers (Phar-
macya, Piscataway, NJ). The reaction was carried out in 50 μl of buffer containing 50 μM Tris (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 3 mM MgCl₂, 3 μg random hexamer primers, 200 μM dATP, 200 μM unlabeled dCTP, 200 μM dGTP, 200 μM dTTP, 50 μCi [α-32P]dCTP, 5 μg actinomycin D, 45 units of RNasin (Promega Biotech, Madison, WI) and 300 units of mouse mammary leukemia virus reverse transcriptase. Following incubation at 37°C for 1 h, the reaction was stopped by adding EDTA to a 20 mM concentration, and cDNA-RNA fragments larger than 100 bp were separated by Sephadex G-100 chromatography. RNA in the cDNA-RNA hybrid was hydrolyzed at 65°C for 30 min in an equal volume of 0.6 N NaOH and 30 mM EDTA. The specific activity of cDNA obtained was 0.5–1.5 x 10⁶ cpm/μg of RNA. For screening, triplicate nitrocellulose membranes were lifted and subjected to alkaline hydrolysis and neutralization (15). Prehybridization, hybridization, and washing were as previously described (4). Plaques which hybridized strongly with the Panc 1 cDNA probe, but not with the CD-11 cDNA probe, were selected. The differential reactivity was confirmed through at least two additional screening cycles.

**Sequencing.** Single phage plaques selected after differential screening were grown to large quantities using either plate lysates or liquid culture followed by glycerol gradient purification (15). Inserts cut with EcoRI from purified DNA were subcloned into pBS +/- vectors (Stratagene, La Jolla, CA). Both single stranded and double stranded sequencing were used. Single stranded cDNA was prepared using standard techniques with the + and – strands pBS phagemids that are hybrid for 1 phage and pBS (Stratagene). Sequencing was performed using Sequenase T-4 DNA polymerase under conditions recommended by the supplier (United States Biochemicals, Cleveland, OH).

**In Vitro Transcription and Translation.** PD-1 cDNA was subcloned into pBS (Stratagene) in both orientations at the EcoRI site. The recombinant plasmid was linearized with HindIII, extracted with phenol-chloroform, and ethanol precipitated. Linear plasmids containing inserts in both orientations were transcribed using T-7 polymerase following the instructions of the supplier (Promega). The transcripts were translated in a rabbit reticulocyte lysate (Promega) using the manufacturer’s procedure with 50 μCi of [35S]methionine (Amersham). The products were analyzed by electrophoresis on a 10% SDS-polyacrylamide gel. Immediately after electrophoresis, gels were enhanced for radioactive signal using EN3'HANCE (DuPont-NEN), dried at 60°C for 1 h, and autoradiographed.

**Northern and Southern Blot Analysis.** Total RNA (20 μg) and/or purified poly(A)+ RNA were fractionated by electrophoresis on 1.2% agarose gels containing 0.66 M formaldehyde and transferred to nitrocellulose via capillary blotting. Genomic DNA was digested with the indicated restriction enzymes and separated by electrophoresis on 0.8% agarose gels. Southern blotting was performed using standard procedures (15). cDNA probes were labeled with [32P]dCTP using a random-primed labeling kit (Boehringer Mannheim, Indianapolis, IN) and were separated from free label by Sephadex G-50 column chromatography (Pharmacia). Prehybridization and hybridization for both Northern and Southern blots were carried out in a solution of 5X saline-sodium phosphate-EDTA, 50% formamide, 5X Denhardt’s reagent, 200 μg/ml of sheared salmon sperm DNA, and a minimum of 10⁶ cpm/ml of probe at 42°C for 18 h. Blots were washed twice with 2X standard saline citrate containing 0.1% SDS at room temperature for 15 min followed by four washes with 0.2X standard saline citrate-0.1% SDS at 60°C for 1 h.

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**References**

1. Cochran, B. H., Reifel, A. C., and Stiles, C. D. Molecular cloning of