Overexpression of pRB in Human Pancreatic Carcinoma Cells: Function in Chemotherapy-Induced Apoptosis

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Background: Human pancreatic adenocarcinomas are highly resistant to chemotherapy. The p16 tumor-suppressor protein is inactivated in more than 90% of human pancreatic cancers. The p16 protein transcriptionally inhibits expression of retinoblastoma tumor-suppressor gene pRB. The pRB protein transcriptionally inhibits expression of the p16 gene. Because pRB normally inhibits chemically induced apoptosis in nonpancreatic tissues, we investigated whether pRB is involved in resistance to chemotherapy-induced apoptosis in pancreatic cancer cells.

Methods: pRB expression was examined by immunohistochemistry in 106 human pancreatic tissue specimens. The human pancreatic tumor cell line Capan-1 (pRB+/p16−) was stably transfected with p16 to functionally inactivate pRB. pRB gene expression was examined by western and northern blot analyses, and pRB function was assessed by electrophoretic mobility shift assays and promoter transactivation studies for the transcription factor E2F. Changes in cell sensitivity to chemotherapy were measured by assays for cytotoxicity and apoptosis. Results: pRB was overexpressed in pancreatic ductal adenocarcinomas but was hardly detectable in other pancreatic malignancies, chronic pancreatitis, or nontransformed human pancreatic tissue. Expression of p16 in Capan-1 cells resulted in the loss of pRB gene and protein expression concomitant with increased activity of the transcription factor E2F, which was not detected in wild-type or control-transfected Capan-1 cells. Wild-type and control-transfected Capan-1 cells were resistant to chemotherapy-induced apoptosis, but pRB-depleted (i.e., p16-transfected) Capan-1 cells were highly sensitive. The effect was specific to pRB depletion because two other human pancreatic cancer cell lines that retained high pRB expression after p16 transfection were resistant to chemotherapy-induced apoptosis. Conclusions: Overexpression of pRB is associated with human pancreatic duct-cell cancer and may allow pancreatic cancer cells to evade chemotherapy-induced apoptosis. [J Natl Cancer Inst 2002;94:129–42]
a repressor of progression through G1. [reviewed in (16–18)]. CDK activity in turn is governed by the accumulation and binding of cyclins and is modulated by two distinct families of inhibitory proteins (CDK inhibitors [CKIs]). Members of the inhibitor of CDK4 (INK4) family (p15, p16, p18, and p19) specifically inhibit the activity of CDK4 and CDK6 and subsequent pRB phosphorylation (17,18).

The functional interdependence of pRB and p16 expression in human pancreatic cancer is of particular interest for two reasons. First, human pancreatic cancer carries the highest frequency of p16 alterations of all human malignancies, with more than 90% of all pancreatic cancers having functionally inactive p16 (19). Second, several studies in a variety of tissues revealed a bidirectional transcriptional regulation between pRB and p16; pRB can transcriptionally inhibit p16 expression (20,21), and restoration of p16 expression can transcriptionally inhibit pRB expression (22–24). This functional interplay has been observed in a variety of tumors and is documented often by the mutually exclusive expression patterns for pRB and p16 (25).

In addition to pRB’s key role in cell-cycle regulation, recent experimental evidence (26–30) suggested that pRB might also exert a protective function against apoptosis. For example, pRB knockout mice die during gestation with excessive induction of apoptosis in some tissues (26,27). Furthermore, expression of pRB in pRB-deficient SAOS-2 osteosarcoma and bladder carcinoma cells inhibited apoptosis initiated by ionizing radiation or interferon gamma independent of the cellular p53 status (28–30). On the basis of these observations, we hypothesized that altered expression of pRB might be involved in the regulation of apoptosis in human pancreatic adenocarcinoma and might contribute to the chemotherapy-resistant phenotype. We tested this hypothesis by analyzing the expression of pRB in pancreatic cancer cells undergoing malignant transformation and by assessing the function of pRB expression in chemotherapy-induced apoptosis in pancreatic cancer cell lines.

**MATERIALS AND METHODS**

**Materials**

Dulbecco’s modified Eagle medium (DMEM), RPMI-1640 medium, and Geneticin (G418) were obtained from Life Technologies (Karlsruhe, Germany). Fetal calf serum (FCS) was purchased from Biochrom (Berlin, Germany). Human pancreatic cell lines Capan-1, Panc-1, Capan-2, BxPc3, MiaPaca, and Hs766T were obtained from the American Type Culture Collection (Manassas, VA). DanG cells were obtained from Deutsches Krebsforschungszentrum (Heidelberg, Germany). The following antibodies were used: mouse anti-human anti-pRB antibody (clone G3–245; BD Biosciences, Heidelberg, Germany); mouse anti-human anti-Ki67 antibody (MIB-1; dianova, Hamburg, Germany); mouse monoclonal anti-human-p16 (clone DCS-50.1; NeoMarkers, Union City, CA); mouse anti-human anti-polyadenosine-diphosphate-ribose-polymerase (PARP) antibody (Calbiochem, Bad Soden, Germany); mouse anti-human anti-p107 (Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-human anti-RB (BD Biosciences); mouse monoclonal anti-human anti-cyclin A (BD Biosciences); and the rabbit polyclonal anti-mouse secondary antibody, hematoxylin, and alkaline phosphatase/anti-alkaline phosphatase (APAAP) complex (DAKO, Hamburg, Germany). The following vectors were purchased: pRC/CMV and pcDNA3 (Invitrogen, Carlsbad, CA), pE2F-Luc (Clontech Laboratories, Palo Alto, CA), and pRL-Tk-Luc (Promega Corp., Madison, WI). Full-length human pRB and p16 complementary DNA (cDNA), which was kindly provided by Dr. K. Brand, Max Delbrück Center for Molecular Medicine, Berlin-Buch, Germany, was subcloned into HindIII and XhoI sites of pRC/CMV. Chemotherapeutic agents used were mitomycin C, 5-fluorouracil (5-FU), doxorubicin (Sigma, Taufkirchen, Germany), and gemcitabine (Lilly, Gießen, Germany).

**Patient Samples**

A total of 106 pancreatic specimens that had been surgically resected and routinely processed at the Charité University Hospital, Berlin, Germany, were analyzed in this study. Institutional review board approval was obtained, and the patients provided written informed consent. None of the patients received chemotherapy or radiation therapy before surgery. Of the tissue samples from these patients, 66 were diagnosed as ductal adenocarcinoma, 25 were diagnosed as chronic pancreatitis, four were diagnosed as acinar cell carcinomas, six were diagnosed as neuroendocrine tumors, and five were diagnosed as normal pancreas.

**Cell Culture**

Human pancreatic tumor cell lines were grown in subconfluent monolayer cultures in RPMI-1640 medium containing 15% FCS (Capan-1), 20% FCS (AsPC-1), or 10% FCS (DanG) or in DMEM containing 10% FCS (Panc-1, Capan-2, BxPc3, MiaPaca, and Hs766T). Culture media were supplemented with 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were cultured in a humidified atmosphere of 95% air and 5% CO2 at 37°C.

**Immunohistochemistry**

Immunohistochemical staining for pRB and Ki67 proteins was performed on formalin-fixed, paraffin-embedded tissue specimens with the use of the APAAP method according to the instructions of the manufacturer (DAKO). Sections (5 μm thick) were mounted on slides, deparaffinized in xylene, and rehydrated in a series of graded ethanol solutions. The tissue sections were then submerged in 0.01 M citrate buffer (pH 6.0), and antigen retrieval was performed by heating the slides in a water bath at 96°C for 45 minutes. The slides were then incubated with a mouse monoclonal anti-pRB antibody (1:200 in buffer D, which consisted of RPMI-1640 medium supplemented with 10% heat-inactivated FCS and 1 mg/mL sodium azide, which was adjusted to pH 7.5 by the addition of 1 M Tris base) overnight at 4°C. After the slides were washed in Tris-buffered saline (TBS), they were incubated with a rabbit anti-mouse secondary antibody (diluted 1:50 in buffer D), rinsed in TBS, and then incubated with the APAAP complex (diluted 1:50 in buffer D) for 30 minutes at room temperature. After a brief rinse in TBS, the slides were incubated again with the APAAP complex and then rinsed in TBS before they were counterstained with hematoxylin. The same protocol of antigen retrieval and immunostaining was followed for Ki67, with the exception that the cells were incubated with a mouse monoclonal anti-Ki67 antibody (diluted 1:20 in buffer D). Staining of normal gut tissue adjacent to pancreatic specimen served as internal control to monitor the quality of immunostaining for pRB and Ki67 reactivity. All tissue specimens were evaluated for both pRB and Ki67 immunoreactivity. Sections treated with buffer D without primary antibody were used as negative controls. After a representative
area of the tissue section was selected, digital images of each section were captured with an AxioCam Digital Camera (Zeiss, Jena, Germany) and processed with Adobe Photoshop software (San Jose, CA). The presence or absence of nuclear staining was evaluated independently by two investigators blinded to the type of specimen and stain. For each sample, 500 cells were counted and the percentage of positively stained tumor nuclei was calculated.

For immunocytochemical staining of cultured tumor cell lines, 4 × 10^6 Capan-1 cells per chamber were seeded into one four-chamber slide (Nalge Nunc, Naperville, IL) and allowed to attach overnight. Adherent cells were fixed with acetone/methanol for 15 minutes at −20 °C and incubated with mouse monoclonal anti-p16 (1:50 diluted in buffer D) or anti-pRB (1:333 diluted in buffer D) antibodies overnight at 4 °C. Cells then were washed with TBS and incubated with secondary antibodies and the APAAP complex as described above.

**Western Blot Analysis**

Whole-cell lysates were prepared from 5 × 10^7 cells grown on 10-cm dishes. Cells were washed twice with phosphate-buffered saline (PBS) and lysed directly in a buffer containing 20 mM Tris (pH 7.8), 150 mM sodium chloride, 1% glycerol, 0.5% Nonidet P-40 (NP-40), 50 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 2 μM EDTA, 2 μM leupeptin, and 3 μg/mL aprotinin. Lysates were centrifuged for 10 minutes at 10,000g at 4 °C. The protein concentrations of the supernatants were determined with the use of a protein assay kit (Bio-Rad, München, Germany), and then the supernatants were adjusted to 1 mg/mL in Laemmli buffer. Samples were heat denatured, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and electroblotted to polyvinylidene difluoride membranes (NEN, Boston, MA). Membranes were blocked for 1 hour at room temperature in 5% nonfat dry milk dissolved in PBS containing 0.1% Tween 20 (PBST) and then incubated with monoclonal antibodies (anti-pRB, 1:1000; anti-p16, 1:1000; anti-p107, 1:100; anti-RB2, 1:1000; and anti-cyclin A, 1:1000) diluted in PBST overnight at 4 °C. Membranes were washed three times in PBST and incubated with goat anti-mouse or goat anti-rabbit secondary horseradish peroxidase-conjugated antibodies for 2 hours at room temperature. After a final wash in PBST, the protein bands were visualized with the Enhanced Chemiluminescence detection system (Amersham Pharmacia, Uppsala, Sweden).

**Northern Blot Analysis**

Total RNA from 10^8 cells was isolated with the use of the RNeasy extraction kit (WAK-Chemie, Bad Soden, Germany) according to the manufacturer’s protocol. Purified RNA was quantified by spectrophotometric analysis, and 20 μg of each sample was separated on a 1% agarose/MOPS/formaldehyde gel. After electrophoresis, gels were capillary blotted overnight to Hybond-N filters (Amersham Pharmacia). RNA was then immobilized by cross-linking with UV light.

The vector pRC/CMV-prb, containing full-length human pRB cDNA, was labeled with [α-32P]deoxyctydine triphosphate by random priming with the use of a Megaprime DNA labeling kit (Amersham Pharmacia). The probe was purified on a Sephadex G-50 column to remove unincorporated nucleotides. Hybridization with the labeled probe was performed in the presence of Quick-Hyb buffer (Stratagene, La Jolla, CA) at 65 °C for 2 hours. After hybridization, membranes were washed at 65 °C to a stringency of 0.1% SDS in 0.1x saline sodium citrate and exposed to x-ray films at −70 °C.

**Electrophoretic Mobility Shift Assays**

For the electrophoretic mobility shift assays (EMSAs), double-stranded E2F-1 oligonucleotides (Santa Cruz Biotechnology) were end-labeled with [γ-32P]adenosine triphosphate (Amersham Pharmacia) in a T4 polynucleotide kinase (Promega Corp.) reaction. Unincorporated nucleotides were removed with the use of a nucleotide removal kit (Qiagen, Hilden, Germany). The probe sequences were: 5′-ATTATAAGTTTTCCGGCCCTTTCCTCAA-3′ for the consensus E2F-1 binding site and 5′-ATTATAAGTTTTCCGGATCTCCCTTTTTCTCAA-3′ for the mutated E2F-1 binding motif.

Nuclear extracts were isolated from 10^7 cells by resuspending centrifuged cells in 300 μL of buffer A (10 mM Tris [pH 7.9], 10 mM KCl, 1.5 mM MgCl2, 10 mM K2HPO4, 1 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 10% NP-40). After incubation on ice for 10 minutes, nuclei were collected by centrifugation for 10 minutes at 4 °C at 1000g. Pellets were resuspended in 100 μL of buffer B (same composition as buffer A without NP-40 and with 420 mM NaCl instead of KCl), incubated on ice for 20 minutes, and centrifuged for 10 minutes at 4 °C at 12,000g. Supernatants containing the nuclear extracts were frozen in liquid nitrogen. For the DNA binding reactions, nuclear extracts (3 μg) were incubated with 0.4 ng of radiolabeled oligonucleotide probe and 1 μg of poly[d(I-C)]/poly[(I-C)] (Amersham Pharmacia) in 20 μL of a buffer containing 10 mM Tris/HCl (pH 7.5), 50 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 5% glycerol, and 1 mM dithiothreitol for 20 minutes at 30 °C. For DNA binding reactions that involved competing probes, an excess of unlabeled consensus or mutant oligonucleotides were included in the reactions 10 minutes before the addition of the radiolabeled probe. The protein–DNA complexes were subjected to electrophoresis for 2 hours at room temperature on 5% polyacrylamide gels at 200 V in 0.5x TBE. Gels were dried under vacuum and exposed to x-ray film at −70 °C. For quantification, films were scanned and complexes were analyzed by densitometry using Kodak Digital Science 1D software (Kodak, Rochester, NY).

**Stable Transfections**

Full-length human p16 cDNA was subcloned into the eukaryotic expression vector pRC/CMV, and human E2F-1 cDNA was subcloned into pcDNA3, where each insert was under the control of the CMV promoter. The vectors also contained a gene that, when expressed, would permit selection for resistance to the antibiotic neomycin.

To generate stably transfected cells, we incubated 5 μg of plasmid and 30 μL of lipofectamine (Life Technologies) in 1.6 mL of serum-free medium for 45 minutes at room temperature. Next, 10^6 Capan-1, Panc-1, or DanG cells, growing on 10-cm dishes, were exposed to the formed liposome complexes that had been diluted in 8 mL of serum-free medium for 6 hours, after which they were washed and maintained in fresh complete medium overnight. The cells were detached with trypsin and then

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split at a 1:3 ratio in three 20-cm dishes in complete medium that contained the neomycin analogue G418 at a concentration of 1 mg/mL. After 3 weeks, independent, stable clones were isolated and from then on grown in the presence of 1 mg/mL G418. Cells were also transfected with the vectors pRC/CMV or pcDNA3 without inserts to generate stably transfected control clones.

### Transient Transfections and Luciferase Assays

Transient transfection experiments were performed to characterize the transactivation activity of E2F. The vector pE2F-Luc contains a specific E2F cis-acting DNA binding enhancer element located upstream of the TATA-like promoter region from the herpes simplex virus thymidine kinase promoter, which controls the expression of the downstream firefly luciferase reporter gene from Photorinus pylaris. Subconfluent Capan-1 cells (5 x 10^5) were transiently transfected with the vector pE2F-Luc (0.9 µg/35-mm well) with the use of the Effectene transfection reagent (Qiagen) according to the manufacturer’s recommendations. After 24 hours, cells were harvested and lysates were generated to analyze luciferase activity with the dual-luciferase reporter assay system (Promega Corp.) according to the manufacturer’s recommendations. Luciferase measurements were taken in a Lumat BL 9507 luminometer (Berthold, Bad Wildbad, Germany). Emitted luminescence was integrated over a 10-second reaction period and expressed arbitrarily as relative light units.

To normalize for the transfection efficiency, we cotransfected the cells with the vector pRL-Tk-Luc (60 ng) and a Renilla reniformis, where the luciferase gene from Photobacterium phosphoreum is under the control of the herpes simplex virus thymidine kinase promoter. In contrast with firefly luciferase, Renilla luciferase expression is controlled by a promoter not affected by our experimental conditions. Therefore, the Renilla luciferase activity serves as an internal control for differences in transfection efficiency. Values of E2F activities were calculated as E2F/Renilla ratios and expressed as a fold increase of control-transfected cells.

### Cytotoxicity Assay

Cells (1 x 10^5) were seeded in 96-well plates and allowed to grow overnight. They were then incubated continuously with increasing concentrations of chemotherapeutic agents for 20 hours. Cytotoxicity was determined by use of the ApoAlert™ cytotoxicity assay kit (Clontech Laboratories). After being washed with PBS, attached cells were fixed, stained, and solubilized with the dye solution supplied in the cytotoxicity assay kit, and the color intensity was then measured in an enzyme-linked immunosorbent assay plate reader at 540 nm. Values obtained ranged from 0.1 to 1.0 optical density and were within the linear detection range. The intensity of the color reaction is proportional to the number of viable cells and can, therefore, be used as a measure of cell survival or cytotoxicity. Cytotoxicity was expressed as the ratio of the signal obtained under treatment conditions compared with untreated cells.

### Flow Cytometry

For the measurement of the percentage of apoptotic cells in cell-cycle analyses, 10^5 cells were detached with trypsin, washed twice with PBS, and fixed by the dropwise addition of ice-cold ethanol (70% final concentration) while vortexing. Cells were collected by centrifugation at 1000g for 5 minutes at room temperature, resuspended in 0.5 mL of a solution containing 20 µg/mL propidium iodide and 200 µg/mL deoxyribonuclease-free ribonuclease in PBS, and incubated for 30 minutes at 37°C. For flow cytometric analysis, at least 10,000 events were collected with the use of a FACScalibur™ (BD Biosciences). Cell cycle distribution and pre-G1 fraction were determined and quantified with the use of the CellQuest™ program (BD Biosciences).

### DNA Fragmentation Assay

To demonstrate DNA fragmentation, 10^6 Capan-1 cells were lysed in a buffer containing 10 mM Tris/HCl (pH 8.2), 400 mM NaCl, 2 mM EDTA, 1% SDS, and 50 µg/mL proteinase K. An equal volume of 6 M NaCl was added, and samples were vortexed vigorously. After centrifugation for 10 minutes at 2000g, nucleic acids were precipitated from the supernatants by the addition of two volumes of 96% ethanol. The precipitated nucleic acids were collected by centrifugation and, after discarding the supernatant, the pellets were dissolved in TE-buffer (10 mM Tris/HCl [pH 7.5] and 0.2 mM EDTA) and incubated with 20 µg/mL ribonuclease for 30 minutes at 37°C. DNA was separated on a 2% agarose gel and visualized after staining with ethidium bromide.

### PARP–Western Blot

For the assessment of PARP cleavage as a measure of apoptosis, whole-cell lysates, made from 10^6 Capan-1 cells, were generated in a buffer containing 60 mM Tris/HCl (pH 6.8), 6 M urea, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.001% bromphenol blue; sonicated for 15 seconds; and incubated at 65°C for 15 minutes. Proteins from the whole-cell lysates were separated on a 10% SDS–PAGE gel. Full-length PARP and the apoptosis-related cleavage fragment were detected by western blotting analysis with a monoclonal mouse anti-human PARP antibody (diluted 1:100 in PBST) and visualized as described above.

### Statistical Analysis

Student’s t tests were performed with the use of the Statistical Package for Social Sciences 9.0 software package (Statistical Package for Social Sciences Inc., Chicago, IL). All tests were two-sided, and data were considered to be statistically significant at P<.05. Data represent the mean and 95% confidence intervals from three independent experiments unless otherwise specified.

### RESULTS

#### Expression of pRB in Nontransformed Human Pancreas and Pancreatic Malignancies

To assess the role of pRB in pancreatic cancer, we first performed immunohistochemical analyses of pRB expression in a large panel of surgically resected pancreatic tissues, including samples of nontransformed human pancreas, chronic pancreatitis, and ductal adenocarcinoma. We used a monoclonal antibody against human pRB antibody, which recognizes the hypophosphorylated and hyperphosphorylated configuration of pRB (31). In parallel, serial sections from the same tissues were stained with a monoclonal antibody against the nuclear antigen Ki67 to assess the...
proportion of proliferative cells. Predicted from its function as a nuclear phosphoprotein, pRB staining was predominantly and reproducibly confined to the nucleus (Fig. 1). In the nontransformed pancreatic tissues, all three functional compartments (acinar, ductal, and islet cells) demonstrated very few (<2%) Ki67-positive or pRB-positive cells (Fig. 1, A and B, respectively). Similarly, only a small fraction of pancreatic duct cells stained positive for Ki67 or pRB in 25 samples of chronic pancreatitis (representative samples are shown in Fig. 1, C and D, respectively). By contrast, there was a pronounced nuclear overexpression of pRB in malignant transformed duct cells of adenocarcinoma specimens. Comparison between serial sections revealed that the number of cells expressing the Ki67 antigen was greatly exceeded by the number of cells overexpressing pRB (Fig. 1, E and F). An additional interesting observation was made in a separate case of an intraductal papillary carcinoma (Fig. 1, G and H). Examination of the transition zone from phenotypically normal duct epithelial cells to neoplastic, transformed cells revealed a pronounced overexpression of nuclear pRB in the malignant duct cells.

Additional analysis of four acinar cell carcinomas and six neuroendocrine tumors of the pancreas revealed that pRB overexpression was specific for the ductal adenocarcinoma phenotype (Fig. 2). Although acinar cell carcinomas had a relatively high proliferative index, a very small fraction of acinar tumor cells stained positive for pRB (Fig. 2, A and B, respectively). Similarly, neuroendocrine tumors, which are characterized by low mitotic indices, also had a very small fraction of cells staining positive for pRB (Fig. 2, C and D). Quantitative analysis of all tissues investigated is presented in Fig. 2, E, and supports the observation that overexpression of pRB is specifically and selectively restricted to ductal adenocarcinoma of the human pancreas.

Expression of pRB and p16 in Human Pancreatic Cancer Cell Lines

To address the functional significance of pRB overexpression in pancreatic cancer, we needed to characterize a suitable in vitro system. We therefore analyzed pRB expression in a panel of eight different human pancreatic ductal carcinoma cell lines. By western blot analysis, all the cell lines expressed the pRB protein, although there was a slightly different distribution pattern between the hypophosphorylated (p105) and the hyperphosphorylated (p110) isoform of pRB (Fig. 3, A).

Because expression of pRB and expression of p16 are thought to be inversely related and mutually exclusive in transformed human epithelial cells, we then examined the same cell lines for expression of p16. In seven of the eight pancreatic cancer cell lines, we could not detect p16 by western blot analysis, using a monoclonal anti-p16 antibody (Fig. 3, A). Although the p16 protein was detected in Capan-2 cells, it is known to be functionally inactive because of an intragenetic mutation (32). These data indicate that pRB expression in human pancreatic cancer cells is maintained at the expense of p16; this is a pattern similar to the situation described previously in other cell types and tissues (33,34).

Effect of p16 Reconstitution on pRB Expression in Capan-1 Cells

Because loss of p16 was associated with high pRb expression levels, we hypothesized that reintroduction of p16 might suppress pRB gene expression in pancreatic cancer cells. The exceedingly low transfection efficiency achieved in pancreatic cancer cell lines precludes a transient transfection approach (unless sensitive reporter systems can be used); thus, we stably transfected the human p16 cDNA into Capan-1 cells and selected a set of 20 different independently isolated, p16-positive clones that were resistant to neomycin. All 20 clones expressed comparable levels of p16 protein by western blot analysis (data not shown). Three p16-positive clones, named p16/1, p16/2, and p16/3, were selected at random for further investigations. Reconstitution of p16 resulted in a pronounced decrease in the expression of pRB compared with wild-type parental and control transfected cells (Fig. 3, B). Decreased pRB expression by the
re-expression of p16 did not result from a nonspecific inhibition of gene expression because the expression level of proliferating cell nuclear antigen (PCNA), which was used as an internal control, was unchanged by the transfection of p16 (Fig. 3, B). These results were further confirmed by immunocytochemical staining for pRB and p16 (Fig. 3, C). Control transfected Capan-1 cells demonstrated strong nuclear staining for pRB but not for p16, which was virtually absent. By contrast, p16-transfected Capan-1 cells showed strong nuclear staining for p16 but not for nuclear pRB. Thus, upon reconstitution of p16 expression, the phenotype of Capan-1 cells changed from a p16-+/pRB+ expression pattern to a p16+/pRB– expression pattern.

We next analyzed whether the p16-mediated decrease in pRB expression was reflected in changes in the steady-state pRB messenger RNA (mRNA) levels. A northern blot of total RNA was probed with a human pRB cDNA and revealed that pRB mRNA steady-state levels were decreased in p16-transfected cell clones compared with the levels in wild-type or control transfected cells (Fig. 4, A). These results confirm and extend previous studies (22–24) that described a p16-induced inhibitory effect detectable at the pRB mRNA level, depending on the cellular and tissue context examined.

To demonstrate the specificity of p16-dependent pRB regulation, we then examined whether expression of the pRB-related pocket protein family members RB2 (also known as p130) and p107 were also affected by p16 re-expression. A representative western blot analysis of RB2 and p107 is shown in Fig. 4, B. Although stable transfection of p16 resulted in a pronounced decrease in pRB expression levels, no substantial changes in RB2 and p107 expression levels could be detected. Similar to pRB, p107 and RB2 migrate as double bands that represent different forms of phosphorylation (35). These results suggest that the p16-mediated inhibition of pRB expression is highly specific.

E2F family transcription factors represent the best characterized, biologically relevant target of pRB activity. Their association with hypophosphorylated pRB results in E2F inactivation or conversion into transcriptional repressors. Accordingly, E2F activity could serve to evaluate the functional consequence of the loss of pRB expression. Therefore, we characterized the activity of E2F by two different experimental approaches. First, we assessed the ability of E2F to recognize and bind sequence-specific DNA by EMSAs. Nuclear extracts prepared from p16-transfected and control transfected Capan-1 cells were incubated with E2F-1-specific oligonucleotides and subsequently analyzed for protein–DNA interactions (Fig. 5, A). Capan-1 cells stably transfected with p16 formed sixfold to eightfold more DNA–protein complexes than the control transfected cells, suggesting that loss of pRB expression is associated with the liberation of

Fig. 2. Expression of pRB and Ki67 in acinar and neuroendocrine pancreatic tumors. Serial sections of formalin-fixed, paraffin-embedded pancreatic tissues were stained with monoclonal antibodies, which were detected with the use of the alkaline phosphatase/anti-alkaline phosphatase method. Sections were counterstained with hematoxylin. Representative examples of immunostaining for Ki67 (A) and pRB (B) in acinar cell carcinomas of the pancreas and for Ki67 (C) and pRB (D) in a neuroendocrine pancreatic tumor (original magnification x20) are shown. Immunohistochemical staining for Ki67 (open bars) and pRB (filled bars) expression was quantified in 500 cells from representative areas of 106 different paraffin-embedded tissue specimens (E). Numbers of tissue specimens per group and percentages of positive stained cells are given. Bars represent the mean and upper 95% confidence interval. P values derived with the two-sided Student’s t test for pRB expression in pancreatic carcinoma compared with normal pancreas or chronic pancreatitis are shown. n.d. = not determined.
the sequestered E2F transcription factor. The specificity of the E2F DNA–protein complexes was confirmed when the E2F-specific complexes disappeared completely when incubated with an excess of unlabeled wild-type E2F oligonucleotides but not when incubated with an excess of unlabeled mutant oligonucleotides that are deficient for E2F binding (Fig. 5, A) (36). Second, we transiently transfected an E2F-promoter luciferase reporter construct containing four copies of the cis-acting enhancer element (37), which is induced by E2F as part of a positive autoregulatory loop (Fig. 5, B). If loss of pRB releases sequestered E2F, then E2F would be free to bind to the enhancer element and induce the expression of the firefly luciferase reporter gene. A fourfold to sixfold increase in E2F promoter-dependent luciferase activity was detected in p16-transfected cells compared with wild-type and control transfected cells. This indicated that the decrease in pRB expression resulted in a functionally relevant increase in E2F transactivation activity.
with the expression in control transfected cells, we observed increased expression of cyclin A in p16-transfected cells that were depleted of pRB (Fig. 5, C).

**Effect of pRB Reduction on Chemotherapy-Induced Apoptosis**

Overexpression of pRB has been shown to protect against apoptosis in certain cell types in vivo and in vitro (28,29,39). To address the association between pRB expression and chemoresistance in human pancreatic cancer cells, we compared the effects of the clinically most relevant chemotherapeutic agents currently used for the treatment of pancreatic cancer (gemcitabine, 5-FU, and mitomycin C) on wild-type and pRB-depleted Capan-1 cell clones. Cell-cycle analysis and growth studies revealed that depletion of pRB by the re-expression of p16 resulted in increased cell proliferation by decreasing the mean doubling time from 33.4 ± 2.1 hours in p16-negative control cells to 21.8 ± 2.2 hours in p16-transfected clones. In addition, re-expression of p16 resulted in an increase in the percentage of cells in the S-phase fraction from 15.2 ± 0.6% in p16-negative control cells to 24.1 ± 1.0% in p16-transfected clones (data not shown).

Capan-1 control clones were almost resistant to treatment with mitomycin C and 5-FU (Fig. 6, A). Therapeutically relevant concentrations induced less than 25% cytotoxicity. Even at maximal concentrations of 1 μg/mL mitomycin C and 250 μg/mL 5-FU, chemotherapy-induced cytotoxicity did not exceed 40% in the wild-type parental or control transfected cells (Fig. 6, A). In contrast, mitomycin C and 5-FU chemotherapy-induced cytotoxicity was profound and dose dependent in the pRB-depleted, p16-transfected cells. Similar results were obtained after treatment with gemcitabine (data not shown).

Induction of apoptosis constitutes one major mechanism of chemotherapeutic action (40,41). To determine whether the pRB-regulated increase in cytotoxicity was the result of reconstituted apoptosis, we performed cell-cycle analyses. After exposure to 5-FU for 20 hours, pRB-depleted p16-transfected Capan-1 cells demonstrated a pronounced pre-G1 peak suggestive of apoptotic cells (data not shown). The induction of apoptosis in response to gemcitabine and 5-FU in pRB-depleted cells was confirmed by assessing additional specific apoptosis markers (Fig. 6, B), such as cleavage of the mitochondrial PARP protein, which results in the appearance of the 85-kd PARP cleavage product (Fig. 6, B, upper panel) and the occurrence of DNA fragmentation (Fig. 6, B, lower panel). In contrast, wild-type and control-transfected cells showed negligible signs of apoptosis after exposure to either gemcitabine or 5-FU.

**Functional Relevance of pRB Depletion for Chemotherapy-Induced Apoptosis**

We then attempted to dissect whether the chemotherapy-induced apoptosis in p16-expressing Capan-1 cells was a result of decreased pRB expression or a consequence of other, undefined biologic roles for p16 that were unrelated to pRb. We reintroduced p16 in two other human pancreatic tumor cell lines, Panc-1 and DanG. Stable transfection with p16 CDNA resulted in the expression of p16 protein in both cell lines at levels comparable with those achieved in Capan-1 cells (compare Fig. 3, B, and Fig. 7). However, in contrast to p16-transfected Capan-1 cells, inhibition of pRB expression could not be detected...
Fig. 6. Effects of decreased pRB expression on chemotherapy-induced cytotoxicity and apoptosis. A) Wild-type (open bars), control-transfected (mock; hatched bars), and p16-transfected (solid bars) Capan-1 clones were incubated for 20 hours with increasing concentrations of mitomycin C and 5-fluorouracil (5-FU). Cells were processed according to the ApoAlert cytotoxicity assay protocol, in which the amount of viable, attached cells is measured on the basis of a colorimetric reaction. Higher cytotoxicity corresponds to weaker color intensity, resulting from the detachment of dead cells. Cytotoxicity is expressed as a percentage of the total signal in untreated controls. The means and upper 95% confidence intervals from three separate experiments are shown. There was a statistically significant difference between the cytotoxicity of wild-type or control-transfected cells and that of p16-transfected cells when treated with mitomycin C at concentrations of 0.1 μg/mL or higher or with any concentration of 5-FU (P<.05, Student's t test). Solid black bars represent two independent p16-overexpressing clones. For each graph, the order of the bars is the same (wild-type, mock, p16/1, and p16/2). B) Wild-type (open bars), control-transfected (mock; hatched bars), and p16-transfected (solid bars) Capan-1 clones were incubated for 20 hours with gemcitabine (1 μg/mL) or 5-FU (50 μg/mL), and apoptosis was determined by poly-adenosine diphosphate-ribose-polymerase (PARP) cleavage (upper panel) and DNA fragmentation (lower panel). For PARP cleavage analysis, cell lysates were immunoblotted with a murine monoclonal anti-PARP antibody. The indicated molecular mass of the full-length PARP protein (116 kilodaltons [kD]) and that of its apoptotic cleavage fragment (85 kD) were deduced from a molecular size marker subjected to electrophoresis in parallel. For DNA fragmentation analysis, genomic DNA was extracted from wild-type (wt), control-transfected (mock 1), or p16-transfected (p16/1 and p16/2) Capan-1 cells after treatment with (+ lanes) or without (− lanes) chemotherapeutic drug and were subjected to electrophoresis on a 1% agarose gel. The gel was stained with ethidium bromide to visualize the DNA. The unmarked lane shows the fragment sizes of the 100-base pair (bp) marker.
Fig. 7. Effects of p16 reconstitution in Panc-1 and DanG cells. Cell lysates from Panc-1 or DanG wild-type, control-transfected (mock 1), and p16-transfected (p16/1 and p16/2) clones were immunoblotted with murine monoclonal antibodies specific for p16 and pRB (upper panel). Panc-1 and DanG cell clones were incubated for 20 hours with increasing concentrations of gemcitabine (middle panel) and 5-fluorouracil (5-FU) (lower panel) as indicated. Cells were processed according to the ApoAlert cytotoxicity assay protocol. Cell survival is expressed as the percentage of viable cells after treatment with chemotherapeutic agents as compared with untreated control cells. The means and the upper 95% confidence intervals from three separate experiments are shown. Open bars = wild-type cells; hatched bars = mock-transfected cells; solid bars = two independent p16-overexpressing clones. For each graph, the order of the bars is the same (wild-type, mock, p16/1, and p16/2).
in either Panc-1 or DanG cells. We then incubated these clones with increasing concentrations of gemcitabine and 5-FU and determined cytotoxicity. Cytotoxicity was low unless drug concentrations of 100 µg/mL gemcitabine or 50 µg/mL 5-FU and higher were used (Fig. 7). However, there was no statistically significant difference in cytotoxicity between control and p16-transfected cells in either cell line. These data indicate that the loss of pRB expression rather than reconstitution of p16 is responsible for induction of chemotherapy-induced apoptosis.

Effect of Overexpression of E2F-1 on Chemotherapy-Induced Apoptosis

The presumed protective function of pRB against apoptosis may be mediated by the pRB-sequestered E2F transcription factor family, the main target of pRB. To test our hypothesis that the loss of pRB expression reconstitutes chemosensitivity via the liberation of E2F, we stably overexpressed E2F-1 in Capan-1 cells. We generated three independent cell clones that stably overexpressed functional E2F-1 (Fig. 8, A). By this approach, we obtained a similar magnitude of E2F transactivation compared with p16-mediated loss of pRB expression (compare Fig. 5, B, and Fig. 8, A). We then treated the E2F-1-transfected cell clones with increasing concentrations of 5-FU and gemcitabine and quantified the induction of apoptosis by flow cytometric analysis of the cell-cycle distribution. Compared with wild-type or control-transfected cells, overexpression of E2F-1 restored chemotherapy-induced apoptosis in response to 5-FU and gemcitabine (Fig. 8, B and C). A similar induction of chemosensitivity was also observed when E2F-1 was stably overexpressed in the human pancreatic cell line AsPC-1 (data not shown).

DISCUSSION

This is the first study, to our knowledge, that describes the selective overexpression of the retinoblastoma-susceptibility gene product pRB in the malignant transformation in human pancreatic ductal cancer cells. Several interesting aspects regarding the pRB expression pattern can be derived from our immunohistochemical studies. First, nuclear overexpression of pRB was not observed in chronic inflammatory diseases of the pancreas, such as chronic pancreatitis, but was strictly confined to the presence of malignant transformation. Because longstanding chronic pancreatitis has been identified as a risk factor for the development of pancreatic carcinoma (42), this observation suggests that pRB overexpression is most likely not an early event during pancreatic carcinogenesis. Second, the degree of pRB overexpression is independent of the proliferation index and is, therefore, unlikely to merely reflect the proliferative status of a given pancreatic cancer cell. Similarly, we were unable to detect an association between the tumor grade and the extent of pRB expression (data not shown). Third, pRB overexpression was demonstrated only in adenocarcinomas with a ductal phenotype, which clinically represent more than 90% of all human pancreatic malignancies (2). This implies that the molecular mechanisms responsible for pRB overexpression are cell type specific for and restricted to duct cell carcinogenesis in the human pancreas.

Comparison of our data with the analysis of pRB expression in other human neoplasms reveals a heterogeneous picture. High levels of pRB expression have been associated with some benign tumors and early invasive cancers (43,44), whereas low to undetectable levels of pRB have been reported in some but not all end-stage, high-grade invasive cancers (43–46). These inconsistent observations are reflected by our own results, in which biologically aggressive acinar cell carcinomas had low to undetectable pRB expression levels, whereas ductal adenocarcinomas had high pRB expression levels. Therefore, it appears likely that pRB expression is regulated in a highly tissue-specific manner. Furthermore, without evaluating each malignancy individually, it will be difficult to make general statements about the role of pRB in different cancers.

The molecular mechanisms underlying pRB overexpression in ductal pancreatic adenocarcinomas are unclear. Genetic analysis of pRB in a variety of primary human adenocarcinomas has revealed mutational abnormalities in only a small percentage (<5%) and no evidence for gene amplification (19,47). Although the primary regulation of pRB during the cell cycle seems to occur post-translationally through phosphorylation, pRB mRNA levels can also be drastically manipulated by a variety of stimuli, such as cellular differentiation (48), cytokines [e.g., transforming growth factor-β (49) and interferon gamma (50)], or by autoregulation through pRB itself (51). Furthermore, p16, which is functionally inactivated in virtually all pancreatic carcinomas (19), is able to transcriptionally inhibit pRB expression (22–24). It appears conceivable that several of the above-mentioned mechanisms might be involved in the increased expression of pRB in pancreatic cancer. Of these potential mechanisms, an attractive hypothesis would involve the loss of p16-mediated transcriptional inhibition of pRB through the functional inactivation of p16 during carcinogenesis that would result in the overexpression of pRB.

On the basis of the expression pattern in human pancreatic cancer tissue and its previously documented protective function against apoptosis (28,29), we evaluated the role of pRB overexpression in the regulation of apoptosis in human pancreatic cancer cells. To address this problem, we initially chose the human pancreatic cancer cell line Capan-1 as a representative in vitro model because the genetic background of Capan-1 cells is similar, if not identical, to that of primary human pancreatic cancers. Capan-1 cells express activated K-ras (52), mutated DPC4 (53), and mutated p53, but they do not express p16 because of a homozygous gene deletion (19). This genetic background is of considerable importance because all of these alterations have been implicated in the regulation of apoptosis in other tissues (54).

Reconstitution of p16 expression has been described to inhibit pRB gene transcription in a variety of cell types, although this inhibitory effect was dependent on the cell and tissue type examined (22–24). Therefore, we initially evaluated whether reconstitution of p16 would yield similar effects on pRB expression in pancreatic cancer cells by generating several stably p16-transfected Capan-1 cell clones. Subsequent analysis of these independent clones revealed a profound and specific decrease of pRB expression without any effect on other members of the pRB pocket protein family. The loss of pRB protein expression by the reconstitution of p16 was accompanied by a decrease in pRB mRNA levels, suggesting that p16 may act at a transcriptional level to inhibit pRB expression (22). However, the variance of p16-mediated inhibition of pRB expression previously observed in other tissues (22–24) was also observed between individual human pancreatic carcinoma cell lines. For example, by contrast with Capan-1 cells, reconstitution of p16 had no effect on pRB expression in Panc-1 or DanG cells. The reasons for these cell type-specific effects remain unclear, although one possible ex-
planation might be the difference in the genetic background of each individual cell line (53). Despite extensive trials, we were unable to characterize additional pancreatic carcinoma cell lines in more detail because of technical limitations, such as the resistance of different cell lines to the various transfection protocols. However, we successfully transfected hepatic carcinoma cells with p16; this transfection also resulted in decreased pRB expression and increased chemosensitivity (data not shown).

Aside from the cell type specificity, reconstitution of p16 with consequent loss of pRB expression in Capan-1 cells offers an excellent model to study the role of pRB in the regulation of apoptosis. To further characterize the functional consequences of p16-mediated inhibition of pRB, we performed EMSA and transactivation assays. Inhibition of pRB expression resulted in the liberation of sequestered transcription factors of the E2F family, which was detected by increased DNA binding to an E2F consensus oligonucleotide in EMSA and increased E2F-mediated transactivation of an E2F-sensitive reporter construct. Furthermore, these data were supported by the analysis of endogenous cyclin A levels, a well-known downstream target of
pRB and E2F (38), which were increased as a consequence of pRB depletion.

Inhibition of pRB expression increased the sensitivity of Capan-1 cells to chemotherapy-induced cytotoxicity. We analyzed three chemotherapeutic drugs commonly used in the clinical treatment of advanced human pancreatic cancer: the nucleoside analogue gemcitabine, the antimetabolite 5-FU, and the DNA-intercalating agent mitomycin C. pRB-expressing wild-type and control-transfected Capan-1 cells were resistant to the chemotherapeutic drugs even at high concentrations; this resistance reflects the clinical observation that human pancreatic cancers are often chemoresistant. We observed a dose-dependent increase in the chemotherapy-induced cytotoxicity in pRB-depleted, p16-transfected Capan-1 cells. Because induction of apoptosis has been identified as one major mechanism for the antiproliferative effects of chemotherapeutic agents (55,56), we tested the hypothesis that the decreased expression of pRB sensitized pancreatic cancer cells for chemotherapy-induced apoptosis by assessing three independent analyses for apoptosis (cell-cycle distribution, DNA fragmentation, and PARP cleavage). All assays consistently confirmed that loss of pRB expression resulted in increased chemotherapy-induced apoptosis compared with pRB-expressing controls, independent of the chemotherapeutic drug investigated. These observations indicate that, because the therapeutic effects of these drugs have different molecular modes of action, pRB may function late in the apoptotic induction pathway (i.e., at a point beyond which all the drug-activated pathways converge) or pRB may have multiple functions, depending on the pathway activated by a particular drug.

Although the molecular mechanisms underlying the apoptosis-protective effect of pRB overexpression have not been completely elucidated, a previous report (28) has shown that the amount of sequestered E2F transcription factors might account for most of the observed apoptosis-preventive effects of pRB. E2F-1 can induce either cell-cycle progression, by accelerating entry into S phase, or cell death, through the induction of apoptosis (54). Furthermore, overexpression of E2F-1 enables induction of apoptosis in a p53-dependent and p53-independent manner [reviewed in (57)]. Moreover, pRB can specifically inhibit E2F-1-induced apoptosis (58). These observations agree with our data on pancreatic cancer cells that stable overexpression of E2F-1 and decreased pRB expression, which results in the liberation of E2F, may be responsible for the demonstrated increase in chemotherapy-induced apoptosis.

It is interesting that the apoptosis-protective effect of pRB appears to operate independent of p53 because Capan-1 cells express a mutated p53 protein. Although p53 plays a central role in the regulation of apoptosis in response to a variety of stimuli (56), the role of p53 in mediating chemotherapy-induced apoptosis is particularly dependent on the cellular context and has been shown to inhibit or promote chemosensitivity, depending on the cell type investigated [reviewed in (59)]. Therefore, our observation of the p53-independent apoptosis-protective effect of pRB supports previous in vivo and in vitro studies, which also demonstrated that expression of pRB can determine apoptosis sensitivity in a p53-independent manner (28,60).

Although reconstitution of p16 offers an attractive model to study the apoptosis-protective effects of pRB, it appears essential to dissect the putative intrinsic biologic role of p16 in regulating apoptosis from any indirect effects elicited through the transcriptional decrease in pRB expression. This is further underscored by the notion that p16 alone can either stimulate apoptosis (9) or inhibit chemotherapy-induced apoptosis (59). To address this problem, we stably transfected p16 in two additional human pancreatic cell lines (Panc-1 and DanG). In contrast to Capan-1 cells, reconstitution of p16 did not result in decreased pRB expression, confirming previous observations that the genetic background of the cell type and tumor tissue specificity are crucial for the functional interdependence between p16 and pRB observed in other tissues (61). In both cell lines, expression of reconstituted p16 did not effect expression of pRB and did not increase chemotherapy-induced cytotoxicity. These data argue strongly against a direct effect of p16 in chemotherapy-induced apoptosis and support the critical role of pRB expression in guarding the sensitivity and response of pancreatic cancer cells in response to chemotherapy.

In summary, we provide strong evidence that cell type-selective overexpression of pRB during human pancreatic duct cell carcinogenesis may contribute to the resistance to apoptosis commonly observed in response to chemotherapy for the treatment of human pancreatic cancer. On the basis of these findings, pRB represents a novel therapeutic target to interfere with chemoresistance in pancreatic cancer patients.

REFERENCES

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NOTES

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