Inhibition of Basal and Mitogen-stimulated Pancreatic Cancer Cell Growth by Cyclin D1 Antisense Is Associated with Loss of Tumorigenicity and Potentiation of Cytotoxicity to Cisplatinum

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Abstract

Cyclin D1 belongs to a family of protein kinases that have been implicated in cell cycle regulation. Recent studies have demonstrated that elevated cyclin D1 levels correlate with decreased survival in human pancreatic cancer. In this study we expressed in a stable manner a cyclin D1 antisense cDNA construct in PANC-1 human pancreatic cancer cells. Expression of the antisense construct caused a decrease in cyclin D1 mRNA and protein levels and in cyclin D1–associated kinase activity. Antisense expressing clones displayed significantly increased doubling times, decreased anchorage-dependent and -independent basal growth, and complete loss of tumorigenicity in nude mice. EGF, FGF-2, and IGF-I enhanced mitogen-activated protein kinase activity in antisense expressing clones, but failed to stimulate their proliferation. In contrast, all three growth factors were mitogenic in parental cells. Furthermore, the inhibitory effect of cisplatinum on cell proliferation was enhanced markedly in the antisense expressing clones. These findings indicate that cyclin D1 overexpression contributes to abnormal growth and tumorigenicity in human pancreatic cancer and to the resistance of pancreatic cancer to chemotherapeutic agents. (*J. Clin. Invest.* 1998. 101:344–352.) Key words: cell cycle • chemosensitivity • growth factor • nude mice • kinase activity

Introduction

Cyclins are the regulatory subunits of a protein kinase family. They associate with catalytic cyclin-dependent kinase (CDK)[1] subunits regulating cell cycle progression (1). To date, 11 cyclins and 7 CDKs have been identified, termed cyclins A, B1-2, C, D1-3, E, F, G, and H, and CDK1-7 (2). Cyclin D1 is involved in the regulation of the G_1 phase of the cell cycle (3, 4), an essential period in which cell differentiation is initiated and proliferation is controlled (5). A proposed downstream target of the cyclin D1/CDK complexes is the retinoblastoma tumor suppressor gene product pRb (6–8). Cyclin D1/CDK complexes regulate transcription via the inactivating phosphorylation of pRb that results in the liberation of several nuclear proteins (3, 4). One of these proteins is E2F, which initiates the transcription of genes required for S phase progression (9, 10). In contrast to events that occur after cells enter into the S phase, cell cycle events in the G_1 phase can be influenced by extracellular growth factors (5). Mitogenic growth factors initiate cascades of events during the G_1 phase via specific signaling pathways promoting cell cycle progression by enhancing the assembly of cyclin/CDK complex formation and kinase activities (2–4). Elevated cyclin D1 mRNA levels reduce the dependency of cells on exogenous mitogens, shorten the G_1 phase, and decrease cell size (11–13). Therefore, the overexpression of growth factors in conjunction with deregulated cyclin D1 expression has been proposed to contribute to the loss of cell cycle control and to enhance tumorigenesis (14).

Human pancreatic cancer, an aggressive and devastating disease with poor prognosis (15), is frequently associated with overexpression of growth factors and their receptors (16–19) and with resistance to chemotherapeutic agents (20). Cyclin D1 is overexpressed in a significant proportion of human pancreatic cancers and elevated cyclin D1 levels correlate with decreased postoperative patient survival (21, 22). However, the functional significance of cyclin D1 overexpression in this disorder is not known. In this study, therefore, we overexpressed a cyclin D1 antisense cDNA construct in PANC-1 cells, a human pancreatic cancer cell line that expresses cyclin D1 at relatively high levels. We now report that cyclin D1 antisense expression reduces cyclin D1 protein levels and cyclin D1–associated kinase activity and that these events are associated with significant inhibition of in vitro and in vivo cell proliferation, decreased mitogenic response, and increased chemosensitivity.

Methods

Materials. Random-primed labeling kits were purchased from Ambion (Austin, TX), [γ-^{32}P]ATP, [α-^{32}P]CTP, [α-^{32}P]dCTP, and horseradish-conjugated antibodies from Amersham (Arlington Heights, IL), PANC-1 human pancreatic cancer cells and pRSVneo plasmid from American Type Culture Collection (Rockville, MD), T7 and SP6 RNA polymerase from Boehringer Mannheim (Mannheim, Germany), Noble agar from Difco Laboratories (Detroit, MI), G418 from Gibco Laboratories (Grand Island, NY), lipofectamine reagent from Life Technologies (Gaithersburg, MD), Immobilon-P nitrocellulose membranes from Millipore Corp. (Bedford, MA), mouse monoclonal anti–cyclin D1 antibodies (clones DCS-6 and DCS-11) from

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1. *Abbreviations used in this paper:* CDK, cyclin-dependent kinase; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-methylthiazol-2-yl)-2,5-diaphenyltertrazolium bromide.

Neomarkers Inc. (Fremont, CA), enhanced chemiluminescence substrate from Pierce (Rockford, IL), anti–active mitogen-activated protein kinase (MAPK) rabbit polyclonal antibody from Promega (Madison, WI), anti–ERK-2 antibody and glutathione S-transferase-pRb fusion protein from Santa Cruz Biotechnology (Santa Cruz, CA), cis-platinum (II)-diammine dichloride, 3-(4,5-methylthiazol-2-yl)-2,5-diaphenyltertrazolium bromide (MTT), and protein A–Sepharose from Sigma Chemical Co. (St. Louis, MO), and human IGF-I from United States Biochemical Corp. (Cleveland, OH). COLO-357 human pancreatic cancer cells were a gift from R.S. Metzgar (Duke University, Durham, NC), human EGF from G. Nascimoto (Chiron Co., Emmeryville, CA), and human FGF-2 from J. Abrahams (Scios Nova Co., Mountain View, CA).

Cell culture. PANC-1 and COLO-357 cells were grown in DME, supplemented with 8% FBS, penicillin G (100 U/ml), and streptomycin (100 μg/ml), termed complete medium, and maintained in monolayer culture at 37°C in humidified air with 5% CO_2. The medium for cell lines containing a neomycin resistance gene was supplemented with 1 and 0.5 mg/ml G418 for PANC-1 and COLO-357 cells, respectively. Viability of the cells was determined by trypan blue staining.

Constructs. The 1.1-kb human cyclin D1 cDNA that contains the entire coding sequence was subcloned in its antisense orientation into the retroviral expression vector pMV7, termed pMV7D1AS (23). The pMV7 vector contained a 5′ moloney murine leukemia virus (Mo-MuLV) LTR, followed by the 1.1-kb antisense cyclin D1 sequence, a herpes simplex thymidine kinase promoter linked to the G418 resistance gene, and a 3′ MoMuLV LTR. The pRSVneo plasmid containing the G418 resistance gene was used to establish control clones expressing vehicle vector alone, termed sham.

Transfection. PANC-1 and COLO-357 cells were transfected in a stable manner with the pMV7D1AS plasmid and the pRSVneo vector control plasmid with lipofectamine reagent using the conditions described by the supplier (Life Technologies). In brief, 4×10^5 cells were seeded per 10-cm dish and grown in complete medium for 24 h. Plasmid DNA (15 μg) and lipofectamine (40 μl) were mixed in 6 ml of serum-free medium and incubated for 30 min at 23°C. Cells were then incubated for 5 h at 37°C with the DNA lipofectamine mixture and for an additional 19 h after adding 6 ml of DME containing 20% FBS. Selection of individual independent clones was performed as described previously (24). Briefly, cells were plated at a 1:10 dilution in selection medium containing 1 and 0.5 mg/ml G418 for PANC-1 and COLO-357 cells, respectively. After 2–3 wk, single, independent clones of cells were randomly isolated, and each individual clone was plated separately. After clonal expansion, cells from each independent clone were tested for cyclin D1 levels and cyclin D1 antisense expression as described in the following paragraphs.

Immunoblotting. Exponentially growing cells (∼ 40–50% confluent) were washed twice with ice-cold PBS and lysed in buffer containing 125 mM Tris (pH 6.8) and 1% SDS. Cell lysates were subjected to SDS-PAGE and electrotransferred to Immobilon-P membranes. After blocking, the membranes were blotted with a highly specific anti–cyclin D1 antibody (DCS-6) and with a secondary horseradish-conjugated antibody (22, 25, 26). Bound antibodies were visualized using enhanced chemiluminescence. To confirm equal loading, membranes were stripped for 30 min at 50°C in buffer containing 2% SDS, 62.5 mM Tris (pH 6.7), and 100 mM 2-mercaptoethanol and blotted with an anti–ERK-2 antibody.

Cyclin D1 kinase activity assay. The cyclin D1 kinase activity assay was performed as described previously (23, 25, 26). Exponentially growing cells were washed twice with ice-cold PBS and lysed in buffer containing 10% glycerol, 50 mM Hepes (pH 7.5), 150 mM sodium chloride, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 0.1% Tween 20, 10 mM β-glycerophosphate, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 50 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml benzamidine, and 1 mM PMSF. After sonicating and clarifying by centrifugation, the cell lysate solutions were incubated for 2 h with 2 μg of a highly specific anti–cyclin D1 antibody (DCS-11) at 23°C and for 1 h after adding 25 μl of slurry protein

A–Sepharose (27). Immunocomplexes were captured by centrifugation and washed four times with lysis buffer and twice with 50 mM Hepes (pH 7.5) containing 1 mM DTT. The beads were suspended in 30 μl of kinase buffer containing 50 mM Hepes (pH 7.5), 10 mM magnesium chloride, 1 mM DTT, 2.5 mM EGTA, 10 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM sodium fluoride, 20 mM ATP, and 5 μCi [γ-^{32}P]ATP (3,000 Ci/mmol), and 0.3 μg of soluble glutathione S-transferase-pRb fusion protein as a substrate. After incubation for 20 min at 30°C, samples were suspended in 5× Laemmli buffer, boiled for 5 min at 100°C, and subjected to SDS-PAGE. The dried gels were exposed to Kodak XAR-5 films at −80°C using intensifying screens.

Northern blot analysis. Total RNA (10 μg) from exponentially growing cells was prepared by the acid guanidinium thiocyanate method, size-fractionated on 0.8% agarose/2.2% formaldehyde gels, electrotransferred, and ultraviolet cross-linked onto nylon membranes. The blots were then prehybridized, hybridized, and washed under high stringency conditions as described, depending on whether sense and antisense cyclin D1 cRNA or 7S cDNA probes were used (16, 23). The mouse 7S fragment was used to document equivalent RNA loading (16). Blots were exposed to Kodak XAR-5 films at −80°C using intensifying screens. The full-length cyclin D1 antisense cDNA was subcloned into pGEM-7Zf and used to generate antisense riboprobes for cyclin D1 and cyclin D1 antisense cDNAs. cRNA probes were labeled with [α-^{32}P]CTP (3,000 Ci/mmol) using T7 and SP6 RNA polymerase, respectively, and cDNA probes were random-primed labeled with [α-^{32}P]dCTP.

Cell growth assays. Anchorage-dependent cell growth was determined by the MTT colorimetric growth assay (28). Briefly, cells were cultured in 96-well plates and incubated for 4 h with 0.625 μg/ml MTT. After removal of the medium, the dye crystals were dissolved in acidified isopropanol and the optical density was measured at 570 and 650 nm with an ELISA plate reader as described (29). We have demonstrated previously that cell proliferation of pancreatic and colon carcinoma cell lines determined by this assay closely correlates with cell counting and [H³]thymidine incorporation (19, 30, 31).

To determine basal growth, 1.5×10^4 cells/well were plated and incubated for 48 h in 200 μl of complete medium. To determine doubling times, 3.0×10^3 cells/well were plated and incubated in complete medium. The optical densities on one plate were determined daily. Doubling times were also assessed by cell counting with a hemacytometer, after plating 5×10^4 cells/well in 6-well plates.

To assess the mitogenic effects of EGF, FGF-2, and IGF-I, 1.5×10^4 cells/well were plated and incubated for 24 h in complete medium and for 48 h in serum-free medium containing antibiotics, 0.1% BSA, 5 mg/liter transferrin, and 5 μg/liter selenious acid in the absence or presence of the ligands. Medium was replaced with fresh serum-free medium containing the respective additions after 24 h.

To assess the chemosensitivity to cisplatinum, 1.5×10^4 cells/well were plated and incubated for 48 h in complete medium. After 24 h, cisplatinum was added as indicated for 6 h or 60 min. These assay settings were chosen because they include cisplatinum exposure times that are recommended for regional chemotherapy via the celiac arterial axis in vivo (32). The effects of cisplatinum (10 μg/ml) on cell number and viability were also determined by cell counting and trypan blue staining with a hemacytometer, after seeding 10^5 cells/well in 12-well plates.

Basal anchorage-independent growth was assessed by a double-layer soft agar assay (33). Briefly, 4×10^3 viable cells were suspended in complete medium containing 0.3% agar and seeded in triplicate in 6-well plates onto a base layer of complete medium containing 0.5% agar. 1 ml of complete medium containing 0.3% agar was added every 5 d. After 14 d, colonies > 100 μm in diameter were counted by microscopy (Inverted Diaphot 300; Nikon Inc., Melville, NY) and stained with MTT solution (300 μg/well) for 24 h.

In vivo tumorigenicity assay. To assess the effect of antisense cyclin D1 on tumorigenicity, 3×10^6 cells expressing vector alone or cyclin D1 antisense were injected subcutaneously into multiple sites of

4–6-wk old, female, athymic (nude) mice. The animals were monitored for tumor formation every week and killed 8–12 wk after injection.

MAPK assay. Cells were cultured in 6-well plates for 24 h in complete medium to 50% confluency and then for 18 h in serum-free medium before stimulation with ligands. The cells were lysed in a modified RIPA buffer as described previously (29). Cell extracts (25 μg/lane) were subjected to 12% SDS-PAGE. Western blot analysis was carried out with anti–active MAPK antibodies and secondary horseradish-conjugated antibodies according to the protocol of the manufacturer (Promega).

Statistics. Statistical analysis was performed with SigmaStat software (Jandel Scientific, San Raphael, CA). Results are expressed as mean±SD or as mean±SEM and the Student's t test and the Fisher exact test were used for statistical analysis (two-sided). $P < 0.05$ was taken as level of significance.

Results

Expression of antisense cyclin D1 in PANC-1 human pancreatic cancer cells. To investigate the functional significance of cyclin D1 expression in pancreatic cancer cells, we overexpressed a cyclin D1 antisense cDNA construct in PANC-1 human pancreatic cancer cells. Overall, 48 independent clones were selected after 3 wk of growth in medium supplemented with G418. Four clones were dropped during the subsequent expansion process since they were growing too slowly. After clonal expansion, the remaining 44 clones of cells were subjected to immunoblotting to determine the cyclin D1 protein level in each clone. 12 of the 44 clones displayed a marked and
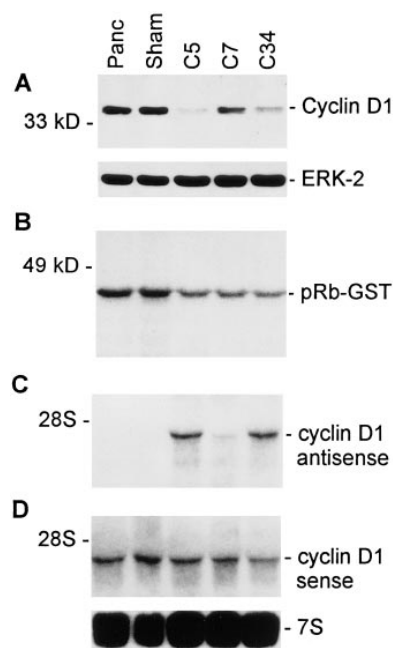
5 a moderate decrease in cyclin D1 protein levels, respectively. In contrast, 27 clones did not exhibit reduced cyclin D1 protein levels. A representative Western blot analysis of two clones with markedly decreased cyclin D1 levels (C5 and C34) and one with moderately reduced cyclin D1 levels (C7) is shown in comparison to PANC-1 and sham transfected cells (Fig. 1 *A*). Equal levels of a 36-kD cyclin D1 band were seen in PANC-1 and sham transfected cells. In contrast, in the three selected clones there was a decrease in cyclin D1 protein levels of 35–81% in comparison with parental PANC-1 and sham transfected cells ($n = 3$, $P < 0.05$). The same clones exhibited attenuated cyclin D1–associated kinase activities (Fig. 1 *B*).

To examine whether the reduced cyclin D1 protein levels and reduced cyclin D1–associated kinase activities were due to the expression of cyclin D1 antisense, Northern blot analysis was performed next. Since the transcripts of the endogenous cyclin D1 and the exogenous cyclin D1 antisense are similar in size (22, 23), the blot was hybridized with specific cRNA probes that distinguished the sense and antisense cyclin D1 transcripts. When hybridized with the probe detecting exogenous cyclin D1 antisense (Fig. 1 *C*), a 4.8-kb transcript was detected in all clones transfected with the cyclin D1 antisense construct. In contrast, no signal was obtained in PANC-1 or sham transfected cells. The level of cyclin D1 antisense expression in each clone correlated with the decrease in the cyclin D1 protein level (Fig. 1, *A* and *C*). When hybridized with the probe detecting endogenous cyclin D1 sense mRNA (Fig. 1 *D*), a 4.4-kb transcript was present in PANC-1 and sham transfected cells and in cyclin D1 antisense transfected clones. Densitometric analysis with normalization to 7S revealed that cyclin D1 mRNA levels were reduced by 39–70% in the anti-
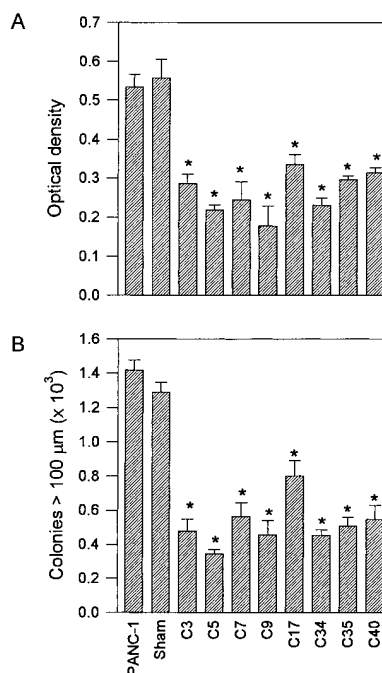

Figure 1. Characterization of cyclin D1 antisense expression in PANC-1 cells. (*A*) Immunoblotting. Total cell lysates (25 μg/lane) were subjected to 12% SDS-PAGE, transferred to a membrane, and blotted with anti–cyclin D1 antibodies (1:400, 5-min exposure, *upper panel*) and reprobed with anti–ERK-2 antibodies to confirm equal loading (1:2,000, 1-min exposure, *lower panel*). (*B*) Cyclin D1–associated kinase activity. Total cell lysates (350 μg) were incubated with anti–cyclin D1 antibodies. Immunocomplexes were captured with protein A–Sepharose, resuspended in kinase buffer containing glutathione *S*-transferase-pRb (pRb-GST) fusion protein as substrate, and subjected to 12% SDS-PAGE after incubation for 20 min at 30°C (2-h exposure). (*C* and *D*) Northern blotting. Total RNA (10 μg/lane) was size-fractionated, electrotransferred to a membrane, and hybridized with cyclin D1 riboprobes (250,000 cpm/ml) that can distinguish between cyclin D1 antisense (*C*) and sense (*D*, *upper panel*) transcripts (7-d exposures). A 7S cDNA probe (40,000 cpm/ml; 12-h exposure, *D*, *lower panel*) was used as loading control. Molecular mass markers and rRNA positions are indicated on the left.

Figure 2. Characterization of the basal cell growth in cyclin D1 antisense expressing PANC-1 cells. (*A*) Anchorage-dependent growth. Indicated cells (15,000/well) were seeded in 96-well plates and incubated for 48 h in complete medium before initiation of the MTT assay. Results are shown as mean optical density values (±SEM) of quadruplicate determinations from three separate experiments for each cell line. *$P < 0.015$ compared with PANC-1 and sham transfected cells. (*B*) Anchorage-independent growth. Indicated cells (4,000/well) were seeded in 6-well plates in complete medium containing 0.3% agar. After 14 d, colonies > 100 μm in diameter were counted by an inverted light microscope. Data are expressed as mean colony number (±SD) of triplicate determinations for each cell line. *$P ≤ 0.002$ compared with PANC-1 and sham transfected cells.
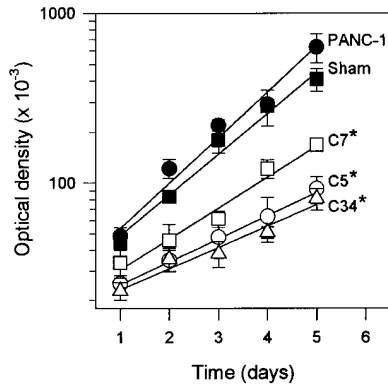
Figure 3. Determination of doubling times in PANC-1 cells. Indicated cells (3,000/well) were seeded in 96-well plates and incubated for the indicated time in complete medium. Results are shown as mean optical density values ($\pm$SD) of quadruplicate determinations of each test point. *$P <$ 0.01 compared with PANC-1 and sham transfected cells.

sense expressing clones in comparison with PANC-1 and sham transfected cells.

Growth properties of cyclin D1 antisense expressing PANC-1 cells. Anchorage-dependent growth of parental PANC-1 cells, sham transfected cells, six cyclin D1 antisense expressing clones with markedly reduced cyclin D1 levels (C3, C5, C9, C34, C35, and C40), and two clones exhibiting moderately reduced cyclin D1 levels (C7 and C17) was compared using the MTT assay. The anchorage-dependent growth of all eight tested antisense expressing clones was reduced by 37–68% in comparison to PANC-1 and sham transfected cells (all *P* values < 0.015; Fig. 2 *A*). Furthermore, PANC-1 and sham transfected cells displayed exponential doubling times of 26.4 and 28.8 h, respectively (Fig. 3). In contrast, the cyclin D1 antisense transfected clones C5, C7, and C34 displayed longer doubling times of 38.4–55.2 h. Doubling times were also determined by cell counting with similar results (data not shown). The cyclin D1 antisense expression also influenced the morphology of the cells. Thus, antisense transfected cells were larger, had more cytoplasm, and displayed a higher cytoplasmic to nuclear ratio compared with PANC-1 and sham transfected cells, while the size of the nucleus remained the same (Fig. 4).

The anchorage-independent growth of parental PANC-1 cells, sham transfected cells, and the above eight cyclin D1 antisense expressing clones was assessed by monitoring colony formation in soft agar (Fig. 2 *B*). PANC-1 and sham transfected cells displayed colony forming efficiencies of 35 and 32%, respectively. In contrast, the eight clones displayed 1.6–3.9-fold lower colony forming efficiencies ranging from 9 to 20% (all *P* values ≤ 0.002). Clones C7 and C17, which showed only moderate reduction of their cyclin D1 levels by Western blot analysis, displayed the highest colony forming efficiencies with 14 and 20%, respectively, while the clones with marked reduction of their cyclin D1 levels displayed colony forming efficiencies of 9–12%. In addition to the lower colony number, the colonies formed by the cyclin D1 antisense transfected cells were generally smaller (Fig. 5), probably reflecting the longer doubling times.

Next, the tumorigenicity of antisense cyclin D1 expressing cells was compared with sham transfected cells in athymic mice. To this end, 3×10^6 cells were subcutaneously injected at each site. All sites injected with sham transfected cells (4/4) developed visible multinodular tumors that measured 1.2–4.0 cm in largest diameter after 8 wk (Fig. 6). In contrast, none of the sites that were injected with cyclin D1 antisense transfected cells (0/12) developed tumors, even after 12 wk ($P = 0.014$).

Growth properties of cyclin D1 antisense expressing COLO-357 cells. To confirm the specificity of the cyclin D1 antisense construct and to exclude the possibility of nonspecific effects due to clonal variation, we next established clones that express antisense cyclin D1 in a stable manner in COLO-357 cells. The anchorage-dependent growth of parental COLO-357 cells, COLO-357 cells transfected with the G418 resistance plasmid (sham-C), and two cyclin D1 antisense expressing clones (CC7 and CC9) that exhibited markedly reduced cyclin D1 levels (Fig. 7 *A*) was compared using the MTT assay. The basal growth of clones CC7 and CC9 was inhibited by 45–56% in comparison with the growth of parental COLO-357 cells and sham-C transfected cells (all *P* values < 0.0005; Fig. 7 *B*). Furthermore, clones CC7 and CC9 displayed doubling times of 57.6 and 52.8 h, respectively, while parental COLO-357 and sham-C transfected cells displayed doubling times of 33.6 and 34.0 h, respectively.

Effects of EGF, FGF-2, and IGF-I in cyclin D1 antisense expressing PANC-1 cells. EGF, FGF-2, and IGF-I (1 or 5 nM) alone or in triple combination significantly increased the
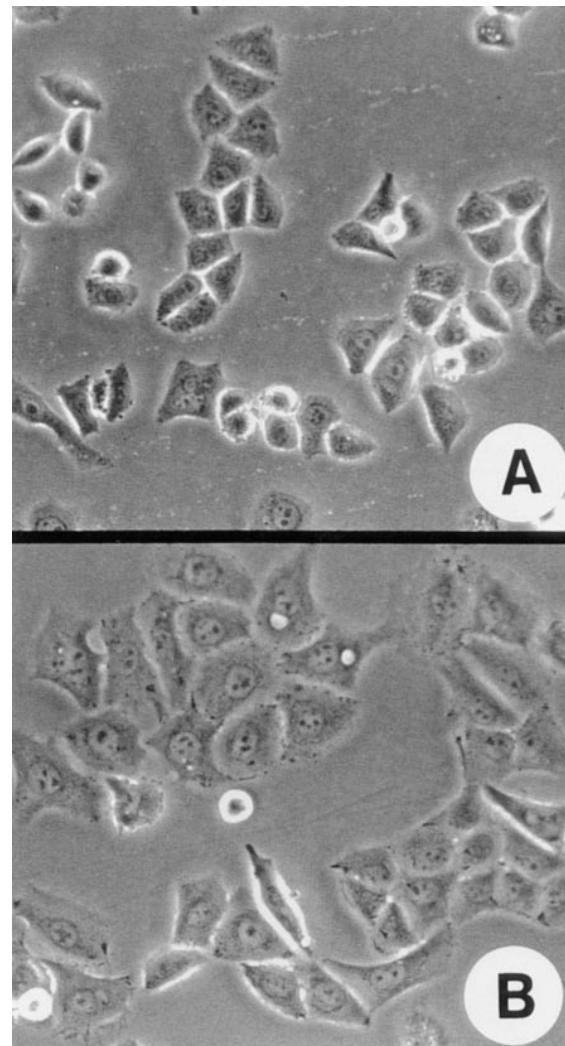

Figure 4. Cell morphology of PANC-1 cells. Exponentially growing sham transfected (*A*) and cyclin D1 antisense transfected cells (*B*) were photographed. Generally, antisense expressing cells were larger in size and displayed a higher cytoplasmic to nuclear ratio. ×900.
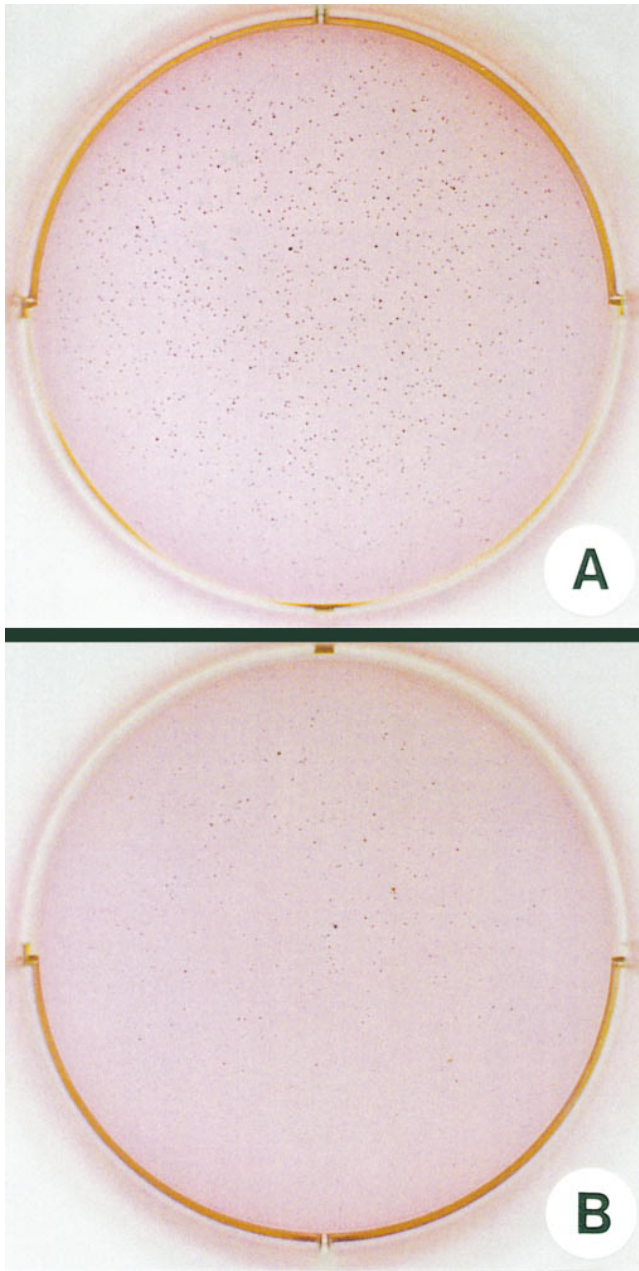
Figure 5. Soft agar assay for PANC-1 cells. Cells (4,000/well) were seeded in triplicate in 6-well plates in a 0.3% agar solution as described above. After 2 wk, the colonies were stained by adding 300 µg MTT/well for 24 h. Representative wells for sham transfected (*A*) and cyclin D1 antisense transfected (*B*) clones (C5) are shown. ×2.5.

Figure 6. In vivo tumorigenicity of PANC-1 cells. 3×10^6 cells were subcutaneously injected at each site in athymic nude mice. Sites injected with sham transfected cells (*left mouse*) developed multinodular tumors (*outlined by arrowheads*) within 8 wk. No tumors were visible at sites that were injected with cyclin D1 antisense transfected cells (*arrows*, *right mouse*), even after 12 wk.

growth of PANC-1 and sham transfected cells. These increases ranged from 30 to 46% above growth of corresponding untreated controls (Table I). In contrast, the mitogenic effects of EGF, FGF-2, and IGF-I were completely blocked in cyclin D1 antisense expressing clones (Table I, all *P* values < 0.04). In Fig. 8, the growth stimulatory effects of 5 nM EGF, FGF-2, and IGF-I are displayed for all tested cell lines.

To determine whether the abrogation of mitogenic response in antisense expressing clones was due to alterations upstream of cyclin D1, we next investigated the effects of EGF, FGF-2, and IGF-I on MAPK activity. ERK-1 and ERK-2

were activated in a similar pattern in PANC-1 and sham transfected cells and in antisense transfected clones (Fig. 9), suggesting that cyclin D1 antisense expression did not inhibit signal transduction pathways upstream of the MAPK cascade.

Impact of cyclin D1 antisense expression on in vitro chemosensitivity. To determine whether cyclin D1 antisense expression altered the sensitivity of PANC-1 cells to chemotherapeutic agents, cells were incubated with various concentrations of cisplatinum. In a first set of experiments, cells were incubated for the maximal recommended time (6 h) for intraarterial infusion of cisplatinum (32; Fig. 10 *A*). At the maximal cisplatinum concentration shown (25 µg/ml), the growth of PANC-1 and sham transfected cells was inhibited by 15 and 17%, respectively. In contrast, the same concentration of cisplatinum inhibited the growth of cyclin D1 antisense transfected clones by 34–41%. The most significant differences (all *P* values < 0.014) were observed at a concentration of 10 µg/ml cisplatinum, irrespective of whether growth was assayed by MTT (Fig. 10 *A*) or by cell counting (data not shown).

Next, PANC-1 cells were incubated with the same cisplatinum concentrations, but for only 60 min (Fig. 10 *B*). This time
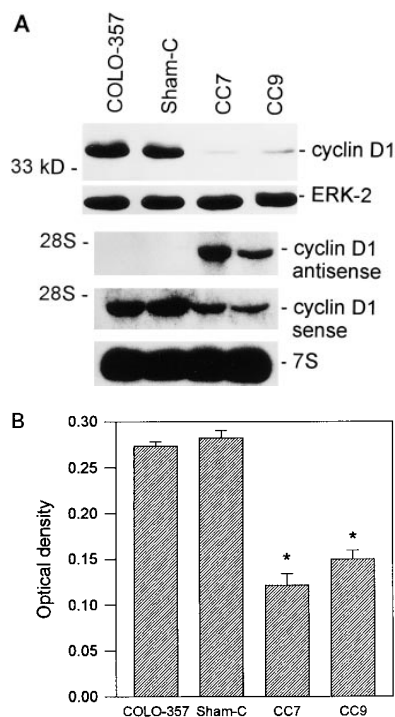
Figure 7. Characterization of cyclin D1 antisense expression and anchorage-dependent growth in COLO-357 cells. (A) Immunoblotting and Northern blotting. Western and Northern blot analysis for COLO-357 cells was carried out as described above for PANC-1 cells. Molecular mass markers and rRNA positions are indicated on the left. (B) Anchorage-dependent growth. Indicated cells (15,000/well) were seeded in 96-well plates and incubated for 48 h in complete medium before initiation of the MTT assay. Results are shown as mean optical density values ($\pm$SEM) of quadruplicate determinations from three separate experiments for each cell line. *$P < 0.0005$ compared with parental COLO-357 and sham-C transfected cells.
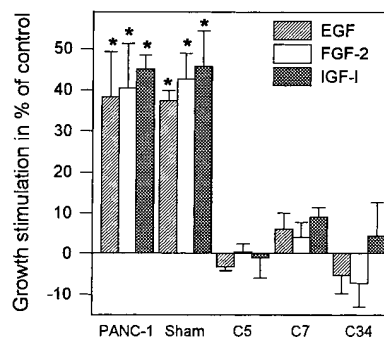

Figure 8. Effects of EGF, FGF-2, and IGF-I on the growth of PANC-1 cells. Indicated cells (15,000/well) were seeded in 96-well plates, incubated for 24 h in complete medium, and then in serum-free medium for 48 h in the absence or presence of 5 nM of the indicated growth factors. Growth was determined by the MTT assay. Results are expressed as percent growth stimulation compared with corresponding untreated controls and are shown as mean ($\pm$SEM) of quadruplicate determinations of three separate experiments. *$P < 0.05$ compared with corresponding untreated control.

period is currently used for cisplatinum celiac artery infusion in pancreatic cancer patients in vivo (20). After 60 min, cisplatinum exerted similar inhibitory effects in the cyclin D1 antisense expressing clones that had also been observed after the 6-h incubation period (Fig. 10, A and B). In contrast, cisplatinum did not inhibit the growth of parental PANC-1 and sham transfected cells that were incubated for 60 min (Fig. 10 B). The LD_{25} for cisplatinum in the antisense expressing clones ranged from 2 to 4 μg/ml and were > 25 μg/ml in PANC-1 and sham transfected cells, respectively (Table II).

We next used the 60-min protocol to test the cisplatinum chemosensitivity of parental COLO-357, sham-C cells, and cyclin D1 antisense transfected clones CC7 and CC9. Cisplatinum (25 μg/ml) did not inhibit the growth of COLO-357 and sham-C cells (Fig. 10 C). In contrast, the same concentrations of cisplatinum inhibited the growth of CC7 and CC9 by 56 and

38%, respectively (Fig. 10 C). The LD_{25} for cisplatinum were < 1 μg/ml in both antisense expressing COLO-357 clones and > 25 μg/ml in parental COLO-357 and sham-C cells.

Discussion

Cyclin D1 with its catalytic partners CDK4 and CDK2 is a critical modulator of cell cycle progression through G_1 (1) and appears to have an important role in neoplastic transformation. Thus, the cyclin D1 gene is located on chromosome 11q13, in a region that contains several potential oncogenes and that is amplified in a number of malignancies, including carcinomas of the breast, esophagus, bladder, and lung, and squamous cell carcinomas of the head and neck (14, 34). This region is also the site of chromosomal translocations and rearrangements in B cell type neoplasms and parathyroid adenomas (14, 34). Elevated cyclin D1 mRNA levels have been reported in several of these malignancies (34) in association with shortened G_1, decreased cell size, and reduced dependency on mitogens (11–13). Cyclin D1 inhibition by antibody microinjection or by transfection with a cyclin D1 antisense expression construct prevents normal fibroblasts from entering the S phase of the cell cycle and markedly inhibits the proliferation of human esophageal and colon cancer cells (23, 25, 35) and murine lung cancer cells (36). Clinically, increased cyclin D1 levels have been correlated with decreased survival of patients with cancers of the pancreas (21, 22), esophagus (37), and breast (38).

Table I. Effect of Cyclin D1 Antisense on the Mitogenic Response of PANC-1 Cells to EGF, FGF-2, and IGF-I

Cell line	EGF		FGF-2		IGF-I		EGF + FGF-2 + IGF-1	
	1 nM	5 nM	1 nM	5 nM	1 nM	5 nM	1 nM	5 nM
PANC-1	31.0±3.1*	38.3±10.9*	32.7±6.2*	40.3±10.9*	34.7±3.5*	45.0±3.5*	47.5±6.5*	51.0±4.0*
Sham	29.7±2.7*	37.3±2.4*	30.0±4.0*	42.7±6.2*	44.0±4.7*	45.7±8.7*	40.0±5.0*	42.7±5.2*
C5	2.7±6.9‡	−3.3±0.9‡	−1.7±5.5‡	0.3±2.0‡	−0.7±6.3‡	−1.0±5.0‡	2.0±3.5‡	−2.3±4.1‡
C7	2.0±1.7‡	6.0±4.0‡	9.7±0.3‡	4.0±3.6‡	−2.3±7.0‡	9.0±2.3‡	19.5±3.8‡	18.4±5.7‡
C34	−4.7±4.2‡	−5.3±4.5‡	−1.3±8.6‡	−7.3±5.7‡	7.3±2.6‡	4.3±8.2‡	11.0±6.8‡	7.0±5.5‡

Results are expressed as growth stimulation in percentages above corresponding untreated controls. Data are shown as mean ($\pm$SEM) of quadruplicate determinations from three separate experiments. *Significant growth stimulation compared with corresponding untreated controls ($P < 0.05$). ‡Significantly reduced mitogenic response compared with sham transfected cells ($P < 0.04$).
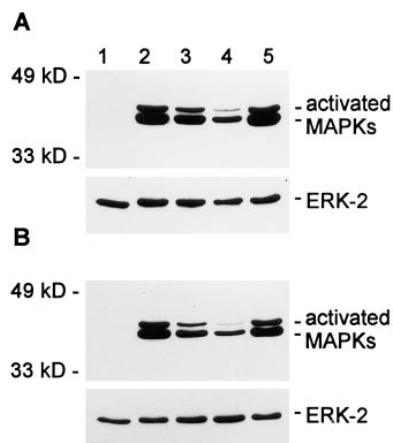
Figure 9. Effect of growth factors on the activation of ERK-1 and ERK-2 (MAPK) in PANC-1 cells. Sham transfected (*A*) and cyclin D1 antisense transfected (*B*) cells (C5) were stimulated for 10 min in the absence (lane *1*) or presence of 5 nM EGF (lane *2*), FGF-2 (lane *3*), IGF-I (lane *4*), or the triple combination (lane *5*). Cell lysates (25 μg/lane) were subjected to 12% SDS-PAGE, electrotransferred to membranes, and blotted with a specific anti–active MAPK antibody (1:2,000, 1-min exposure, *upper panels*). Membranes were reprobed with an anti–ERK-2 antibody (1:2,000, 1-min exposure, *lower panels*) to confirm equivalent loading. Molecular mass markers are shown on the left. Similar activation patterns were observed for parental PANC-1 cells and clones C7 and C34 (data not shown).

Figure 10. Effect of cisplatinum on cell growth. Indicated cells (15,000/well) were seeded in 96-well plates and incubated for 48 h in complete medium. (*A* and *B*) Parental PANC-1 cells (*filled circles*), sham transfected cells (*filled boxes*), clone C5 (*open circles*), clone C7 (*open boxes*), and clone C9 (*open triangles*). (*C*) Parental COLO-357 cells (*filled circles*), sham-C cells (*filled boxes*), clone CC7 (*open circles*), and clone CC9 (*open boxes*). Cisplatinum was added for 6 h (*A*) or for 60 min (*B* and *C*) after 24 h. Growth was determined by the MTT assay. Results are expressed as percent growth stimulation compared with corresponding untreated controls and are shown as mean ($\pm$SEM) of quadruplicate determinations of three separate experiments. *$P <$ 0.014 compared with parental cells and sham transfected cells.

Furthermore, a significant proportion of human pancreatic cancers harbor a homozygous deletion or mutations of the *p16* (*MTS-1*) tumor suppressor gene that encodes a specific cyclin D/CDK4 binding protein inhibiting CDK4 activity (39–41). Presumably, the *p16* mutations or deletions have the capacity to potentiate the oncogenic drive from overexpressed cyclin D1 in pancreatic cancer.

The aim of this study was to investigate the biological effects of a full-length cyclin D1 antisense cDNA construct that decreases cyclin D1 levels in pancreatic cancer cells. We preferred to use an antisense expression vector for the inhibition of cyclin D1 expression to the use of antisense oligodeoxynucleotides, because of the availability of a cyclin D1 antisense vector that has been used successfully in other carcinoma types (23, 25) and its potential practical advantages for in vivo cancer therapy (42). The same studies indicate that the integration of this vector into the genome does not result in nonspecific inhibitory effects (23, 25). In contrast to the several day half-life of oligodeoxynucleotides, the duration of action with intracellular expression vectors is up to several months (42, 43). Furthermore, suitable systems that specifically deliver the vector to the target cells with greater efficiency than antisense oligodeoxynucleotides may soon become available (42).

In this study we demonstrated that cyclin D1 antisense expression reduced cyclin D1 protein levels and cyclin D1–associated kinase activity and that these events were associated with significant inhibition of in vitro and in vivo cell proliferation in human pancreatic cancer cells. Furthermore, cyclin D1 antisense expression completely abrogated the mitogenic activity of EGF, FGF-2, and IGF-I. However, cyclin D1 antisense expressing cells still exhibited a marked increase in MAPK activation after addition of each of these growth factors. To our knowledge, this is the first demonstration that cyclin D1 antisense expression can block the mitogenic actions of ligands that activate transmembrane tyrosine kinase receptors. The fact that all three growth factors were still able to activate MAPK suggests that cyclin D1 may represent a final common pathway for mitogenic signaling via tyrosine kinase receptors
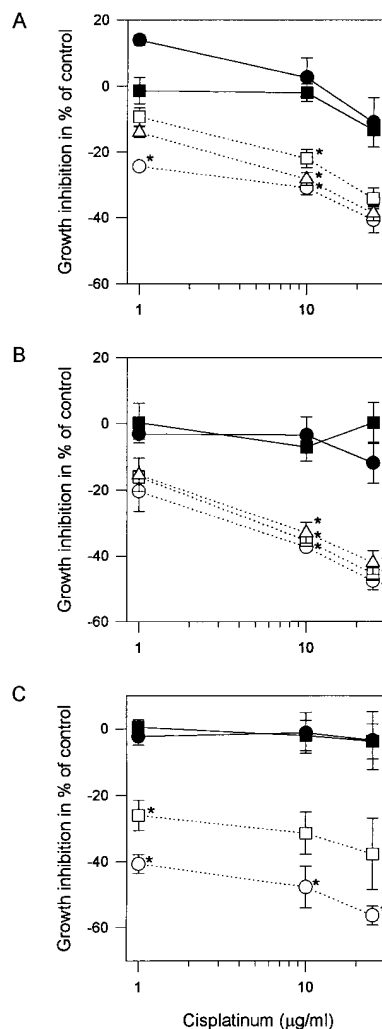
in pancreatic cancer. This hypothesis is consistent with recent findings based on microinjection of cyclin D1 neutralizing antibodies that diverse mitogenic signals are strictly dependent on cyclin D1/CDK activity to induce cell cycle progression (26). In addition, our findings demonstrate that growth factor–induced mitogenesis can be abrogated in spite of MAPK activation.

PANC-1 cells express 400,000 EGF receptors per cell, produce TGF-α and amphiregulin (44–46), and harbor K-*ras* on-

Table II. Summary of the Growth Properties of Cyclin D1 Antisense Transfected Clones in Comparison with Sham Transfected PANC-1 Cells

	Sham	C5	C7	C34
Cyclin D1 protein level (% of sham)	100	21	65	28
Anchorage-dependent growth (% of sham)	100	39	44	41
Doubling times (h)	28.8	52.8	38.4	55.2
Colony forming efficiency (% of seeded cells)	32.2	8.6	14.1	11.4
Tumorigenicity in nude mice	4/4	0/4	0/4	0/4
LD$_{25}$ of cisplatinum (μg/ml) at 60 min	> 50	2	4	3

cogene and *p53* tumor suppressor gene mutations and a homozygous deletion of the *p16* tumor suppressor gene (39, 47, 48). Human pancreatic cancers overexpress multiple growth factors and their tyrosine kinase receptors that activate the MAPK pathway (16–19). Additionally, they harbor K-*ras* and *p53* mutations, as well as *p16* mutations or deletions (39–41, 49) that may result in a decreased ability to turn off these excessively activated receptors and to slow down cell cycle progression. Therefore, the ability of cyclin D1 antisense to markedly suppress the growth of these cells suggests that cyclin D1 may be of paramount importance for pancreatic cancer cell growth. Although PANC-1 cells harbor a deletion of *p16* (39, 48), the presence of this mutation is not essential for the growth inhibitory effect of the cyclin D1 antisense construct. Thus, the cyclin D1 antisense construct also reduced cyclin D1 levels in COLO-357 cells that have wild-type *p16* (41), and attenuated the growth of the antisense transfected COLO-357 clones. Taken together, these observations raise the possibility that specifically blocking cyclin D1 activity may provide an efficient mechanism for simultaneously attenuating diverse mitogenic signaling pathways in pancreatic cancer.

One of the hallmarks of pancreatic cancer is the failure of standard chemotherapeutic agents to suppress tumor spread in vivo and cancer cell growth in vitro (20). Recently, it has been reported that cyclin D1 overexpression in a human fibrosarcoma cell line is associated with increased resistance to methotrexate (50), indicating that cyclin D1 overexpression can contribute to the resistance of cancer cells to chemotherapeutic agents. Indeed, in this study we found that the reduction of the cyclin D1 level was associated with a marked increase in chemosensitivity to cisplatinum in PANC-1 and COLO-357 cells. The in vivo achievable cisplatinum concentration that is not associated with significant clinical toxicity during an infusion time of 60 min in humans is at or below 10 µg/ml (32). When these conditions were recapitulated in vitro in the present study, cisplatinum significantly inhibited the growth of the antisense expressing clones, whereas no effects were observed in cells with normal cyclin D1 levels. Therefore, the therapeutic use of a cyclin D1 antisense strategy in parallel with the currently used celiac arterial axis cisplatinum chemotherapy protocol (20) could result in higher response rates to chemotherapy in vivo.

To our knowledge, this is the first report describing an increase in chemosensitivity that is associated with reduced cyclin D1 levels. The alkylating agent cisplatinum inhibits the growth of rapidly dividing cells in a cell cycle phase nonspecific manner by causing inter- and intrastrand DNA cross-links (51). It is not clear whether the increase in cisplatinum sensitivity of cyclin D1 antisense expressing cells is caused by a decrease in substrate or enzyme activities involved in DNA repair or by a decrease of P-glycoprotein activity resulting in intracellular accumulation of cisplatinum (51). Recently, we reported that abrogating mitogenic signaling through EGF receptor by transfecting PANC-1 cells with a truncated EGF receptor expression vector caused a modest increase in the growth suppressive actions of cisplatinum (52). In this study we blocked the mitogenic signaling of several growth factors by cyclin D1 antisense expression. Therefore, it is possible that the blockade of autocrine mitogenic signals might also be a mechanism for the increase in cisplatinum chemosensitivity.

Irrespective of the mechanisms, our observations suggest that cyclin D1 plays an important role in the pathobiology and aggressiveness of human pancreatic cancer and raise the possibility that future therapeutic strategies aimed at cyclin D1 may have the dual advantage of suppressing pancreatic cancer growth while enhancing the chemosensitivity of the tumor cells.

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