Inhibition of Cyclin D1 Expression in Human Pancreatic Cancer Cells Is Associated with Increased Chemosensitivity and Decreased Expression of Multiple Chemoresistance Genes[1]

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ABSTRACT

Cyclin D1 belongs to a family of protein kinases that have been implicated in cell cycle regulation. Inhibition of cyclin D1 expression has been recently shown (M. Kornmann, et al., J. Clin. Invest., *101:* 344–352, 1998) to suppress pancreatic cancer cell growth and increase cytotoxic actions of cisplatinum. The aim of the present study was to determine whether inhibition of cyclin D1 expression also modulates the effects of other antineoplastic drugs and whether it is associated with alterations in the level of expression of drug resistance genes. The suppression of cyclin D1 expression after the stable transfection of a cyclin D1 antisense construct in PANC-1 and COLO-357 human pancreatic cancer cells resulted in a significant increase in sensitivity to the fluoropyrimidines 5-fluorouracil and 5-fluoro-2′-deoxyuridine and to mitoxantrone. All of the antisense-expressing clones exhibited a decrease in thymidylate synthase and an increase in thymidine phosphorylase mRNA expression as determined by reverse transcription-PCR analysis and decreased levels of MDR-1 and MRP mRNA as determined by Northern blotting. These findings demonstrate that the inhibition of cyclin D1, in addition to suppressing the growth of pancreatic cancer cells, enhances their responsiveness to multiple chemotherapeutic agents and suggest that this effect may be due to the altered expression of several chemoresistance genes.

INTRODUCTION

In mammalian cells, cell growth is primarily controlled in the G_1 phase, and the factors modulating exit from G_0 and progression through G_1 are critical for determining overall growth rates (1). Cyclin D1, a regulatory kinase subunit that preferentially associates with cyclin-dependent kinase (Cdk) 4, is a critical modulator of G_1 progression (2). Thus, elevated cyclin D1 levels reduce the dependency of cells on exogenous mitogens, shorten the G_1 phase, and decrease cell size (3–5). Conversely, the inhibition of cyclin D1 function by antibody microinjection or suppression of its expression by transfection with a cyclin D1 antisense expression construct markedly attenuates the proliferation of fibroblasts and of colon, esophageal, lung, and pancreatic cancer cells (6–9). The *cyclin D1* gene is located on chromosome *11q13*, a region that harbors translocations and rearrangements in B-cell type neoplasms and parathyroid adenomas, and that is amplified in a number of malignancies (10, 11). Furthermore, elevated cyclin D1 mRNA levels are associated with decreased survival of patients with cancers of the breast, esophagus, colon, and pancreas (12–16). Together, these observations suggest that cyclin D1 may have an important role *in vivo* in neoplastic transformation and disease progression (10).

Human pancreatic cancer is an aggressive and devastating disease with poor prognosis (17) that is often associated with K-*ras* oncogene and *p53* and *DPC4* tumor suppressor gene mutations and often exhibits overexpression of multiple growth factors and their receptors (18). The disease is frequently diagnosed at an advanced stage, precluding operative removal of the tumor, and is generally resistant to chemotherapeutic agents (19). Palliative chemotherapy of pancreatic cancer is sometimes attempted with regional treatment via the celiac arterial axis using mitoxantrone, 5-FU,[3] and cisplatinum as a triple combination (19).

Pancreatic cancers overexpress cyclin D1, and this overexpression correlates with decreased postoperative patient survival (15, 16). Furthermore, the suppression of cyclin D1 by a cyclin D1 antisense strategy inhibits basal and mitogen-stimulated pancreatic cancer cell growth and potentiates cisplatinum cytotoxicity (9). The aim of the present study was to investigate the effects of cyclin D1 suppression on the cytotoxic effects of other commonly used antineoplastic agents in the therapy of human pancreatic cancer and to characterize the expression of several genes that have been implicated in drug-resistance. We now report that suppression of cyclin D1 expression in COLO-357 and PANC-1 human pancreatic cancer cells leads to enhanced chemosensitivity to the fluoropyrimidines 5-FU and 5-FUdR and to mitoxantrone in association with altered expression of drug resistance genes.

MATERIALS AND METHODS

Materials. The following were purchased: (*a*) PANC-1 human pancreatic cancer cells from American Type Culture Collection (Rockville, MD); (*b*) 5-FUdR from Janssen Chimica (New Brunswick, NJ); (*c*) mouse monoclonal antibodies against cyclin D1 (DCS-6 and DCS-11) from Neomarkers Inc. (Fremont, CA); (*d*) oligonucleotides and PCR reagents from Perkin-Elmer (Foster City, CA); (*e*) pRb-GST fusion protein from Santa Cruz Biotechnology (Santa Cruz, CA); and (*f*) 5-FU, mitoxantrone, MTT, and protein A-Sepharose from Sigma Chemical Co. (St. Louis, MO).

COLO-357 human pancreatic cancer cells were a gift from R. S. Metzgar at Duke University (Durham, NC). The human MDR-1 and MRP cDNAs were a gift from Dr. N. Dean at Isis Pharmaceuticals (Carlsbad, CA).

Cell Culture and Establishment of Cyclin D1 Antisense-expressing Clones. PANC-1 and COLO-357 cells were grown in DMEM, supplemented with 8% fetal bovine serum, penicillin G (100 units/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 establishment and the selection of PANC-1 and COLO-357 clones that express the full-length 1.1-kb human cyclin D1 antisense cDNA construct (pMV7D1AS) was carried out as described previously (9). The pRSVneo plasmid containing the G418 resistance gene was used to establish control clones expressing vehicle vector alone—termed sham. The medium for cell lines containing a neomycin resistance gene

[3] The abbreviations used are: 5-FU, 5-fluorouracil; 5-FUdR, 5-fluoro-2′-deoxyuridine; 5-FdUMP, 5-fluoro-dUMP; MTT, 3-(4,5-methylthiazol-2-yl)-2,5-diaphenyltetrazolium bromide; MDR, multidrug resistance; MRP, MDR protein; TS, thymidylate synthase; TP, thymidine phosphorylase; pRb, retinoblastoma protein; pRb-GST, glutathione *S*-transferase-pRb.

was supplemented with 1 mg/ml and 0.5 mg/ml G418 for PANC-1 and COLO-357 cells, respectively. After clonal expansion, cells from each independent clone were screened for cyclin D1 levels and cyclin D1 antisense expression as described previously (9).

Cell Growth Assay. Cell growth was determined by the MTT colometric growth assay as described recently (9, 20). Cells (10,000/well) were seeded in 96-well plates and incubated for 24 h in complete medium before the addition of increasing concentrations of the antineoplastic agents for the indicated times. Cells were then again incubated with complete medium, and the MTT assay was initiated 48 h after the start of drug treatment. 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 (21, 22).

Quantitation of Relative Gene Expression Using Real-Time Fluorescence Detection. The quantitation of relative mRNA expression was carried out using a real-time fluorescence detection method as described recently (23), based on the method originally described by Horikoshi *et al.* (24). This method has been used to quantitate gene expression in cell lines and tumor biopsy specimens (25–28). In brief, after RNA isolation and cDNA preparation from each sample as described previously (25), the specific cDNA of interest and β-actin reference cDNA were separately PCR-amplified using a fluorescent oligonucleotide probe with a 5′ reporter dye (6FAM) and a downstream 3′ quencher dye (TAMRA; Ref. 29). During PCR, the 5′ to 3′ nuclease activity of the Taq DNA polymerase releases the reporter, whose fluorescence can be detected by the laser detector of the ABI Model 7700 Sequence Detection System (Perkin-Elmer, Foster City, CA). After crossing a fluorescence threshold, the PCR-amplification results in linear curves of the corresponding PCR products (23). Using β-actin as a reference gene avoids the need to directly quantitate the RNA, which could be a major source of error for analysis. Moreover, the relative gene expression values using β-actin as denominator correlate closely with the protein content (26). After establishment of the threshold fluorescence intensity, the cycle at which this first occurred was defined as the threshold cycle. Relative gene expression was determined based on the threshold cycles of the gene of interest and of the internal standard β-actin, respectively. Internal positive (samples of known value) and negative controls (samples without polymerase) were performed in parallel during different determinations to assure equivalent assay conditions.

The following oligonucleotides were used: (*a*) forward TS primer, 5′-CCGCTCGGTGTGCCTTT; (*b*) reverse TS primer, 5′-GATGTGCGCAA-TCATGTACGT; (*c*) TS probe, 6FAM-5′-AACATCGCCAGCTACGC-CCTGC-TAMRA; (*d*) forward TP primer, 5′-CCTGCGGACGGAATCCT; (*e*) reverse TP primer, 5′-TCCACGAGTTTCTTACTGAGA; (*f*) TP probe, 6FAM-5′-CAGCCAGAGATGTGAACAGCCACCG-TAMRA; (*g*) forward β-actin primer, 5′-TGAGCGCGGCTACAGCTT; (*h*) reverse β-actin primer, 5′-CCTTAATGTCACACACGATT; and (*i*) β-actin probe, 6FAM-5′-AC-CACCACGGCCGAGCGG-TAMRA. The PCR amplification was performed using a 96-well optical tray and caps with a final reaction mixture of 25 μl consisting of 600 nM each primer; 200 nM probe; 5 units Ampli-Taq Gold; 200 μM each dATP, dCTP, and dGTP; 400 μM dUTP; 5.5 mM MgCl2; 1 unit AmpErase uracil *N*-glycosylase; and 1× TaqMan Buffer A containing a reference dye at the following cycling conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

Northern Blot Analysis. Northern blot analysis of total RNA (10 μg) from exponentially growing cells was carried out as described previously (9). Blots were hybridized under high stringency conditions with cyclin D1 sense and antisense cRNA probes as described previously (9), with a mouse 7S cDNA (20), and with human MDR1 and MRP cDNA probes (30, 31).

Cyclin D1 Immunoblotting and Kinase Activity Assay. Cyclin D1 immunoblot and cyclin D1-associated kinase activity assays were performed as described previously (9). For the determination of kinase activity, cell lysates (250 μg/sample) were incubated with a highly specific anti-cyclin D1 antibody (DCS-11) and protein A-Sepharose. Captured immunocomplexes were washed, resuspended in kinase buffer containing [γ-³²P]ATP and pRb-GST fusion protein as substrate, and incubated for 20 min at 30°C before being subjected to SDS-PAGE and film exposure.

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

RESULTS

Effects of Cyclin D1 Antisense Expression on Chemosensitivity. Cyclin D1 antisense-expressing clones of COLO-357 and PANC-1 human pancreatic cancer cells display markedly attenuated growth properties and increased sensitivity to cisplatinum (9). To determine the effects of cyclin D1 suppression on chemosensitivity, parental and sham-transfected (sham-C) COLO-357 cells, cyclin D1 antisense-expressing COLO-357 cells (clones CC7 and CC9), parental and sham-transfected (sham) PANC-1 cells, and cyclin D1 antisense-expressing PANC-1 cells (clones C5 and C34) were incubated with increasing concentrations of 5-FU for 48 h. Incubation with 5-FU resulted in significant dose-dependent growth-inhibitory effects in all of the cell lines with increased 5-FU sensitivity in the antisense-expressing COLO-357 and PANC-1 clones (Fig. 1). At 5-FU concentrations ≥25 μg/ml, clone CC7 displayed significantly increased growth inhibition compared with parental and sham-transfected COLO-357 cells (Fig. 1*A*). Similarly, the cyclin D1 antisense-expressing PANC-1 clones C5 and C34 displayed a significant increase in growth inhibition compared with parental and sham-transfected
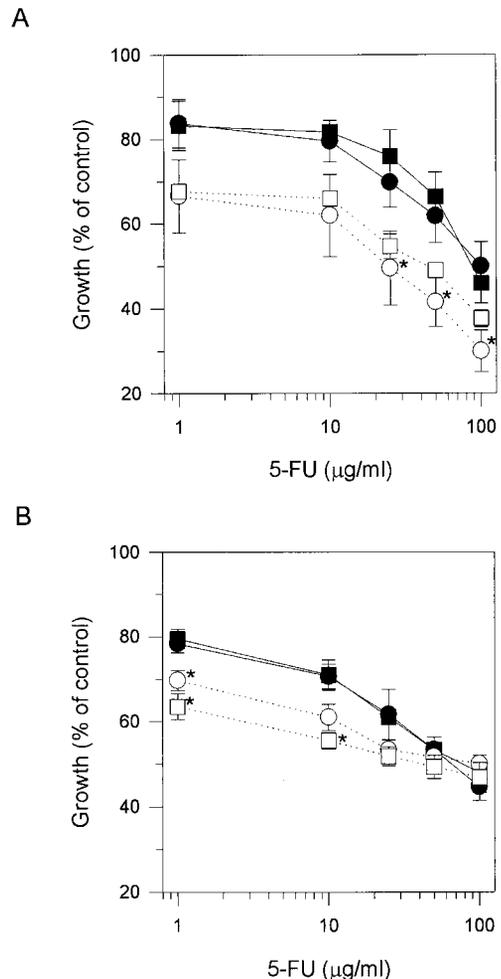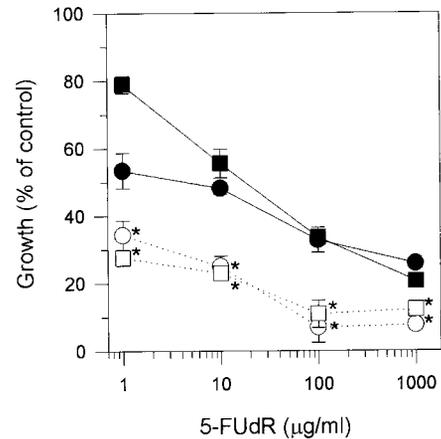

Fig. 1. Effects of 5-FU on cell growth. (*A*) parental (●) and sham-transfected (■) COLO-357 cells and cyclin D1 antisense-expressing COLO-357 clones CC7 (○) and CC9 (□) and (*B*) parental (●) and sham-transfected (■) PANC-1 cells and cyclin D1 antisense-expressing PANC-1 clones C5 (○) and C34 (□) were seeded in 96-well plates (10,000 cells/well), incubated for 24 h in complete medium, and then incubated for 48 h in complete medium in the absence or presence of increasing 5-FU concentrations. Growth was determined by the MTT assay. Results are expressed as growth in % of control and are the means (±SE) of quadruplicate determinations of at least three separate experiments. ∗, significant increase of 5-FU toxicity compared with parental and sham-transfected cells (all *P*s < 0.05).

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PANC-1 cells (Fig. 1B). Parental and sham-transfected COLO-357 cells displayed ID_{25} of 15 μg/ml and 30 μg/ml, respectively, whereas the cyclin D1 antisense-expressing COLO-357 clones displayed at least 15-fold lower ID_{25} values <1 μg/ml (Table 1). The differences in 5-FU inhibitory actions were less pronounced in PANC-1 clones. Thus, parental and sham-transfected PANC-1 cells displayed LD_{25} of 3 μg/ml and 4 μg/ml, respectively, whereas the cyclin D1 antisense expressing PANC-1 clones displayed 3- to 4-fold lower ID_{25} values <1 μg/ml (Table 1).

Incubation with 5-FUdR for 48 h also resulted in a significant dose-dependent growth-inhibitory effect in COLO-357 and PANC-1 cells (Fig. 2). Furthermore, Cyclin D1 antisense expression caused a significant potentiation of 5-FUdR-mediated growth inhibition at all of the tested concentrations in COLO-357 cells (Fig. 2A). Thus, parental and sham-transfected COLO-357 cells displayed an ID_{50} of 10 μg/ml and 20 μg/ml, respectively. In contrast, the ID_{50} in the cyclin D1 antisense-expressing COLO-357 clones was <1 μg/ml (Table 1). Although cyclin D1 antisense expression also increased 5-FUdR sensitivity in PANC-1 cells, this effect was less pronounced than in COLO-357 cells. Thus, in clone C5, cyclin D1 antisense expression was associated with enhanced sensitivity at 1 μg/ml, 10 μg/ml, and 1,000 μg/ml 5-FUdR (Fig. 2B; Table 1). In contrast, in clone C34, 5-FUdR-mediated growth inhibition was significant only at 1000 μg/ml 5-FUdR (Fig. 2B; Table 1). Nonetheless, although parental and sham-transfected PANC-1 cells displayed an ID_{50} of 400 μg/ml and 200 μg/ml, respectively, cyclin D1 antisense-expressing PANC-1 clones displayed at least 40-fold lower ID_{50} values (Table 1).

We next sought to determine whether the suppression of cyclin D1 expression alters the cytotoxic effects of mitoxantrone, a drug that may be used in combination with 5-FU for pancreatic cancer chemotherapy (19). After a 60-min incubation, mitoxantrone caused a significant dose-dependent inhibitory effect in both cell lines. However, the inhibitory effect of mitoxantrone effects was enhanced in the cyclin D1 antisense-expressing clones. Thus, significantly increased growth inhibition compared with parental and sham-transfected cells was observed at concentrations ≤10 μg/ml in both COLO-357 clones (Fig. 3A) and ≤1 μg/ml in both of the PANC-1 clones (Fig. 3B). In parental and sham-transfected COLO-357 cells, the ID_{50} values were 0.8 μg/ml and 1.0 μg/ml, respectively. In contrast, in COLO-357 clones CC7 and CC9, the ID_{50} values were at least 8-fold lower (Table 1). Furthermore, in PANC-1 clones C5 and C34, the ID_{50} values were 3.0 μg/ml and 1.5 μg/ml, respectively, whereas the ID_{50} value of sham-transfected PANC-1 cells was >25 μg/ml (Table 1). Both of the cyclin D1 antisense-expressing PANC-1 clones also displayed significantly increased toxicity at high mitoxantrone concentrations (10 μg/ml and 25 μg/ml) compared with sham cells (Fig. 3B). In contrast, mitoxantrone exerted similar growth-inhibitory effects in parental PANC-1 cells and clones C5 and C34 at these concentrations (Fig. 3B). The ID_{25} and ID_{50} of 5-FU, 5-FUdR, and mitoxantrone are summarized in Table 1.
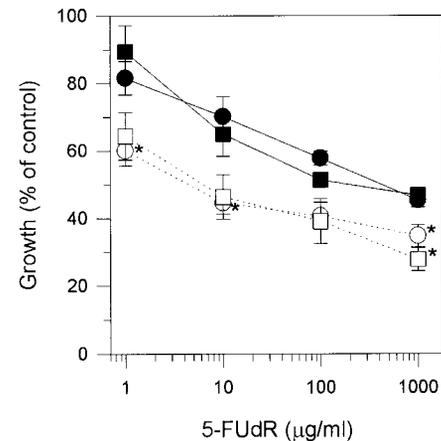
Fig. 2. Effects of 5-FUdR on cell growth. *(A)* parental (●) and sham-transfected (■) COLO-357 cells and cyclin D1 antisense-expressing COLO-357 clones CC7 (○) and CC9 (□) and *(B)* parental (●) and sham-transfected (■) PANC-1 cells and cyclin D1 antisense-expressing PANC-1 clones C5 (○) and C34 (□) were seeded in 96-well plates (10,000 cells/well), incubated for 24 h in complete medium, and then incubated for 48 h in complete medium in the absence or presence of increasing 5-FUdR concentrations. Growth was determined by the MTT assay. Results are expressed as growth in % of control and are the means (±SE) of quadruplicate determinations of at least three separate experiments. ∗, significant increase of 5-FUdR toxicity compared with parental and sham-transfected cells (all *P*s < 0.05).

Effects of Cyclin D1 Suppression on Expression of Chemoresistance Genes. Resistance to 5-FU and 5-FUdR has been associated with high TS mRNA and protein levels in several *in vitro* and *in vivo* studies (24–28, 32, 33). Sensitivity to fluoropyrimidines is also affected by the intracellular TP levels. Thus, the transfection of TP into cancer cells or the induction of TP by IFN or other cytokines increased their sensitivity to 5-FU and 5-FUdR (34–38) because of the

Table 1 *Effects of cyclin D1 suppression on fluoropyrimidine and mitoxantrone toxicity*

Cell lines	5-FU (μg/ml)			5-FUdR (μg/ml)			Mitoxantrone (μg/ml)		
	ID_{25}	ID^{50}	100[a]	ID_{25}	ID_{50}	1000[a]	ID_{25}	ID_{50}	1[a]
COLO-357	15	100	50 ± 5.7	<1	10	26 ± 1.5	<0.1	0.8	69 ± 9.0
Sham-C	30	90	46 ± 4.7	2	20	21 ± 1.2	0.1	1.0	72 ± 8.6
CC7	<1	25	30 ± 4.9[b]	<1	<1	7.7 ± 0.7[b]	<0.1	<0.1	44 ± 0.9[b]
CC9	<1	50	38 ± 2.0	<1	<1	12 ± 0.9[b]	<0.1	<0.1	44 ± 4.3[b]
PANC-1	3	75	45 ± 3.2	4	400	45 ± 2.0	1.3	4.0	77 ± 6.0
Sham	4	90	48 ± 1.2	4	200	47 ± 1.4	4.0	>25	86 ± 5.8
C5	<1	100	50 ± 2.1	<1	4	35 ± 3.4[b]	<1	3.5	54 ± 1.9[b]
C34	<1	50	47 ± 3.5	<1	5	37 ± 7.0[b]	<1	1.5	55 ± 4.1[b]

[a] Data are expressed as percent growth by comparison with the respective untreated controls and are the means ± SE of quadruplicate determinations from at least three separate experiments.
[b] Significant difference (*P* < 0.05) by comparison with the corresponding parental and sham-transfected cells.
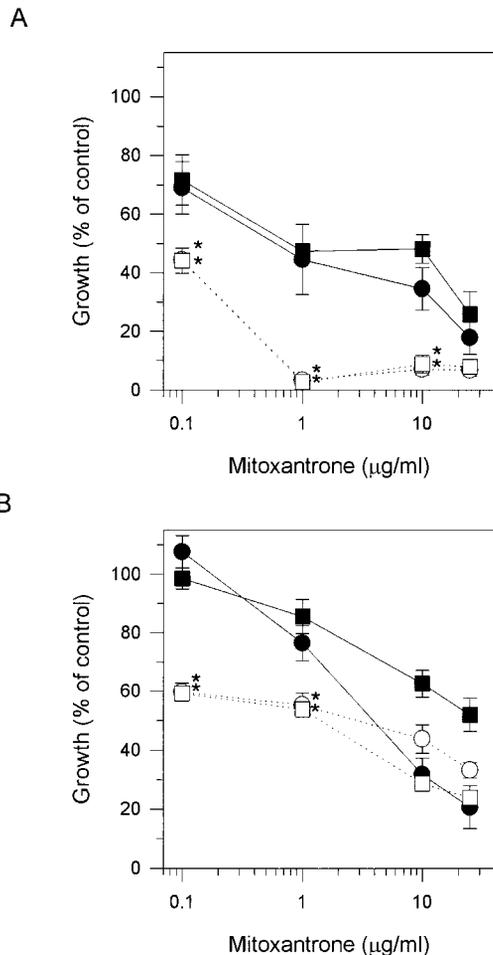
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A

B

Fig. 3. Effects of mitoxantrone on cell growth. (*A*) parental (●) and sham-transfected (■) COLO-357 cells and cyclin D1 antisense-expressing COLO-357 clones CC7 (○) and CC9 (□) and *(B)* parental (●) and sham-transfected (■) PANC-1 cells and cyclin D1 antisense-expressing PANC-1 clones C5 (○) and C34 (□) were seeded in 96-well plates (10,000 cells/well), incubated for 24 h in complete medium, and then incubated for 60 min in complete medium in the absence or presence of increasing mitoxantrone concentrations. After the removal of mitoxantrone, cells were incubated for an additional 47 h before the initiation of the MTT assay. Results are expressed as growth in % of control and are the means (± SE) of quadruplicate determinations of at least three separate experiments. ∗, significant increase of mitoxantrone toxicity compared with parental and sham-transfected cells (all *P*s < 0.05).

greater metabolic activation to the TS-inhibitory metabolite 5-FdUMP (35). Therefore, relative TS and TP gene expression was quantitated next using a real-time fluorescence reverse transcription-PCR procedure, illustrated in Fig. 4. TS mRNA levels were significantly reduced by at least 52 and 36% in the antisense-expressing COLO-357 clones CC7 and CC9, respectively, in comparison with parental and sham-transfected COLO-357 cells (Table 2). In contrast, the suppression of cyclin D1 resulted in an at least 1.7-fold and 1.6-fold increase in TP mRNA levels in clones CC7 and CC9, respectively, compared with parental and sham-transfected COLO-357 cells ($P < 0.05$; Table 2). In the cyclin D1 antisense-expressing PANC-1 clones C5 and C34 TS, mRNA levels were decreased by 33 and 41%, respectively, in comparison with sham but only by 12 and 21%, respectively, in comparison with parental PANC-1 cells (Table 2). The reduction of TS levels was significant in C34 in comparison with parental and sham-transfected PANC-1 cells ($P \leq 0.003$) but in C5, only in comparison with sham cells ($P = 0.0015$). Cyclin D1 suppression significantly increased TP mRNA levels in C5 and C34 by at least 2.3-fold and 4.9-fold, in comparison with parental and sham-transfected PANC-1 cells (Table 2).

Resistance to mitoxantrone has been reported to be associated with cross-resistance to Adriamycin and vinblastine in multidrug-resistant cell lines that overexpress the *MDR-1* gene product P-glycoprotein (39–41). Moreover, transfection of a MDR-1 cDNA resulted in decreased sensitivity to mitoxantrone (41). Therefore, we next compared the expression of cyclin D1 antisense and sense in relation to the mRNA expression of MDR-1 and MRP, a related protein, that has also been implicated in MDR (30, 31). As expected, the transfected clones, but not parental or sham transfected clones, exhibited the cyclin D1 antisense transcript (Fig. 5). As reported previously (9), cyclin D1 antisense-transfected clones also expressed low levels of cyclin D1 mRNA and protein levels and exhibited attenuated cyclin D1-associated kinase activity toward the pRb-GST substrate. Northern blot analysis with human MDR1 and MRP cDNA fragments revealed the presence of the 8-kb MRP mRNA transcript (31) in parental and sham-transfected COLO-357 and PANC-1 cells (Fig. 5). In contrast, MRP transcript levels were markedly decreased in all of the cyclin D1 antisense-expressing COLO-357 and PANC-1 clones (Fig. 5). High levels of a 4.5-kb MDR1 mRNA transcript (30) were also present in parental and sham-transfected COLO-357 cells, whereas only low levels of the MDR1 transcript were present in the cyclin D1 antisense expressing COLO-357 cells (Fig. 5*A*). MDR1 mRNA transcripts were below the level of detection by Northern blotting in all of the PANC-1 cells, even after long exposure of the autoradiographs (data not shown).

DISCUSSION

Resistance of tumor cells to various cytotoxic drugs is a major impediment to cancer chemotherapy. It may occur via a number of mechanisms, including overexpression of the *MDR1* and *MRP* gene products, alterations in expression of enzymes required for activation of the cytotoxic drugs, and modulation of DNA repair (30–34, 42). Previously, overexpression of cyclin D1 in a human fibrosarcoma cell line has been shown to confer resistance to methotrexate (43), which suggests that cyclin D1 overexpression can contribute to the resistance of cancer cells to chemotherapeutic agents. Conversely, suppression of cyclin D1 levels has been shown to potentiate the response of human pancreatic cancer cells to cisplatinum (9).

It is well established that pancreatic cancers are often resistant to standard chemotherapy (19). Nonetheless, palliative chemotherapy is sometimes performed with regional treatment via the celiac arterial axis using mitoxantrone, 5-FU, and cisplatinum as a triple combination (19). This regional chemotherapeutic protocol is based on *in vitro* concentration and time-response studies and *in vitro* Phase II trials (44). In view of the fact that cisplatinum chemosensitivity is enhanced followed suppression of cyclin D1 levels (9), we elected to study drugs and related agents that are currently used for regional chemotherapy of pancreatic cancer via the celiac arterial axis (19). Similarly, the incubation times chosen for these drugs were 48 h for 5-FU and 5-FUdR and 60 min for mitoxantrone because these exposure times are recommended for *in vivo* chemotherapy via the celiac arterial axis (44). Moreover, we used low concentrations of each drug in the *in vitro* assay because these levels are achievable by celiac arterial axis infusion *in vivo* (44). Using these clinically based protocols as a guideline in the present study, we demonstrated for the first time that suppression of cyclin D1 expression in human pancreatic cancer cells is associated with a marked increase in chemosensitivity to 5-FU, 5-FUdR, and mitoxantrone.

Cell sensitivity to the antimetabolites 5-FU and 5-FUdR is known to be modulated by alterations in the expression of TS and TP. TS (EC 2.1.1.45) regulates a rate-limiting step in DNA synthesis (45) by catalyzing the methylation of deoxyurdine-5′-monophosphate to TMP
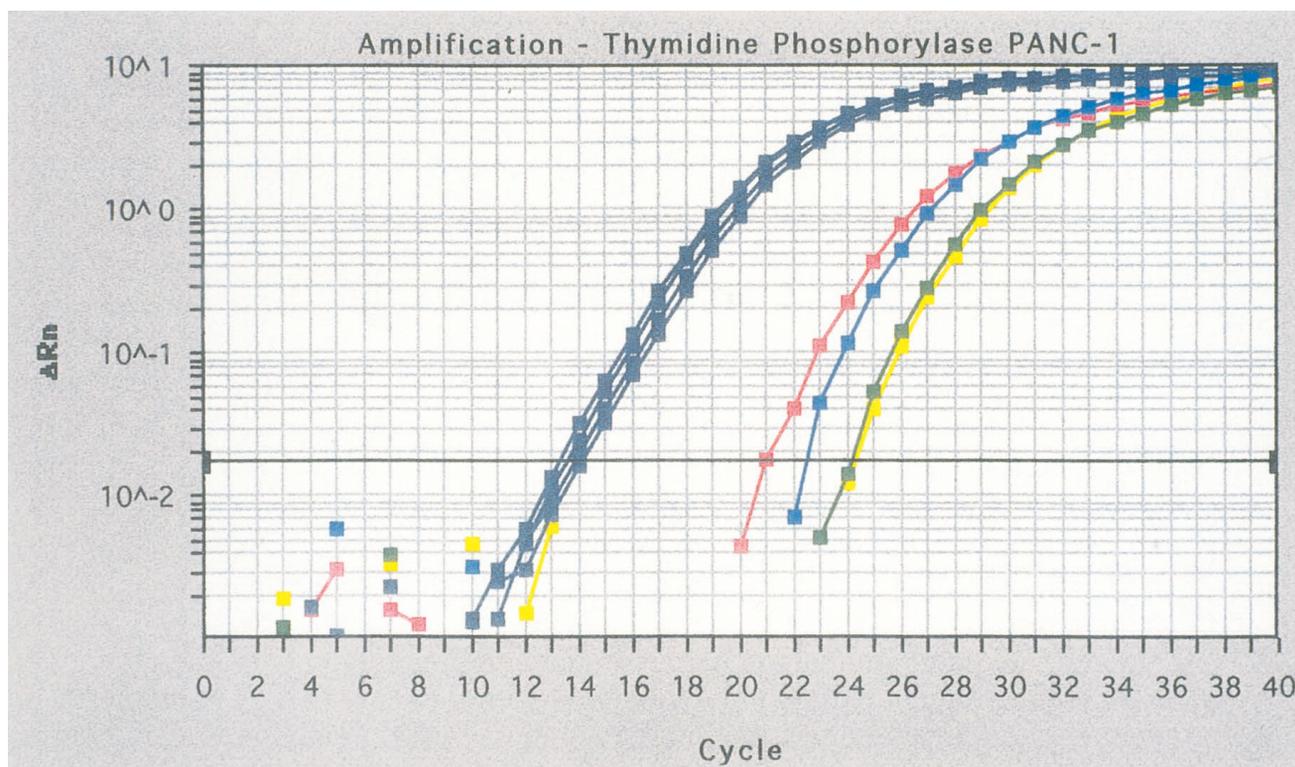
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Fig. 4. Quantitation of relative TP gene expression using real-time fluorescence PCR in PANC-1 cells. PCR amplification was separately carried out with specific primers and specific fluorescent probes for TP and β-actin. The relative normalized fluorescence change (ΔRn) *versus* time (PCR *cycle number*) is plotted for each TP sample [parental (*yellow*); sham (*green*); C5 (*light blue*); C34 (*pink*)] and each β-actin sample [all (*purple*)]. After the establishment of the threshold fluorescence intensity (*horizontal black line*), the cycle at which each sample reached the fluorescence threshold—termed threshold cycle—was determined. Relative gene expression was determined based on the threshold cycles of the gene of interest and of the internal standard β-actin, respectively.

with 5,10-methyltetrahydrofolate as a cofactor, thereby providing the sole intracellular *de novo* source of thymidylate. The increased expression of TS has been associated with resistance to the fluoropyrimidines 5-FU and 5-FUdR (24–28, 32, 33). In contrast, TP exerts a dual effect (TP, EC 2.4.2.4). It catalyzes the reversible interconversion of thymine to thymidine using deoxyribose-1-phosphate and inorganic phosphate as second substrates and also catalyzes a deoxyribosyl transfer reaction from one nucleotide to another (46, 47). TP may control the intracellular levels of thymidine, which at higher concentrations becomes toxic to cells and causes replication errors in DNA (48). Conversely, the reverse reaction could be used to convert 5-FU to 5-FUdR in the presence of deoxyribosyl-donating compounds (49), and 5-FUdR can be activated to 5-FdUMP, which blocks TS activity (32, 33). Overexpression of TP by transfection into cancer cells increased their sensitivity to both 5-FU and 5-FUdR (34, 35). Moreover, the induction of TP by IFN and other cytokines produces a

synergistic potentiation of 5-FU (36, 37) because of greater metabolic activation to the TS inhibitor 5-FdUMP (35). Thus, our findings suggest that the increased sensitivity to 5-FU and 5-FUdR is due to decreased TS and increased TP expression in the cyclin D1 antisense-expressing clones. To our knowledge, this is the first report to show that the alteration of cyclin D1 expression can modulate TS and TP expression in association with enhanced fluoropyrimidine sensitivity.

It has been demonstrated previously in human cell lines that TS mRNA expression correlates well with TS enzyme activity (50) and with TS protein levels (26). Furthermore, the inhibition of TS mRNA with TS antisense oligonucleotides or stable expression of a TS antisense cDNA reduces TS protein and TS activity levels in association with increased sensitivity to fluoropyrimidines (51). This increased sensitivity to fluoropyrimidines occurs only when TS protein or activity levels are reduced in parallel to TS mRNA (51). In our study, we demonstrated that expression of cyclin D1 antisense reduces TS mRNA levels and increases fluoropyrimidine sensitivity. Taken together, these observations suggest that the suppression of cyclin D1 expression leads to decreased TS protein levels.

Cyclin D1 suppression enhanced the cytotoxic effects of mitoxantrone, an anthracenedione derivate related to the anthracycline antibiotics that inhibit DNA topoisomerase II (42, 52). Transfection and multidrug selection experiments have demonstrated that resistance to mitoxantrone can be associated with MDR1 and/or MRP overexpression (40, 41, 53). Indeed, subsequent analysis of MDR1 and MRP expression revealed that cyclin D1 suppression decreased MDR1 and MRP mRNA levels. The MDR-encoding gene *MDR1* plays a major role in the acquisition of simultaneous cellular resistance toward several drugs that results in a failure of chemotherapy (30) and encodes a M_r 170,000 transmembrane protein, P-glycoprotein, which acts as a drug-transporting efflux pump and decreases drug

Table 2 *Effects of cyclin D1 suppression on relative TS and TP gene expression*

Cell lines	Relative gene expression	
	TS[a]	TP[a]
COLO-357	52 ± 0.3	0.17 ± 0.04
Sham-C	38 ± 4.0	0.27 ± 0.01
CC7	19 ± 4.2[b]	0.45 ± 0.02[b]
CC9	25 ± 4.1[b]	0.43 ± 0.07[b]
PANC-1	35 ± 0.1	0.57 ± 0.01
Sham	46 ± 2.6	0.62 ± 0.03
C5	31 ± 2.2	1.45 ± 0.05[b]
C34	27 ± 0.6[b]	2.38 ± 0.06[b]

[a] Data are expressed as the ratio of the threshold cycle of the internal standard β-actin and the threshold cycle of the gene of interest and are the means ± SD from at least three separate determinations.
[b] Significant difference ($P < 0.05$) by comparison with the corresponding parental and sham-transfected cells.
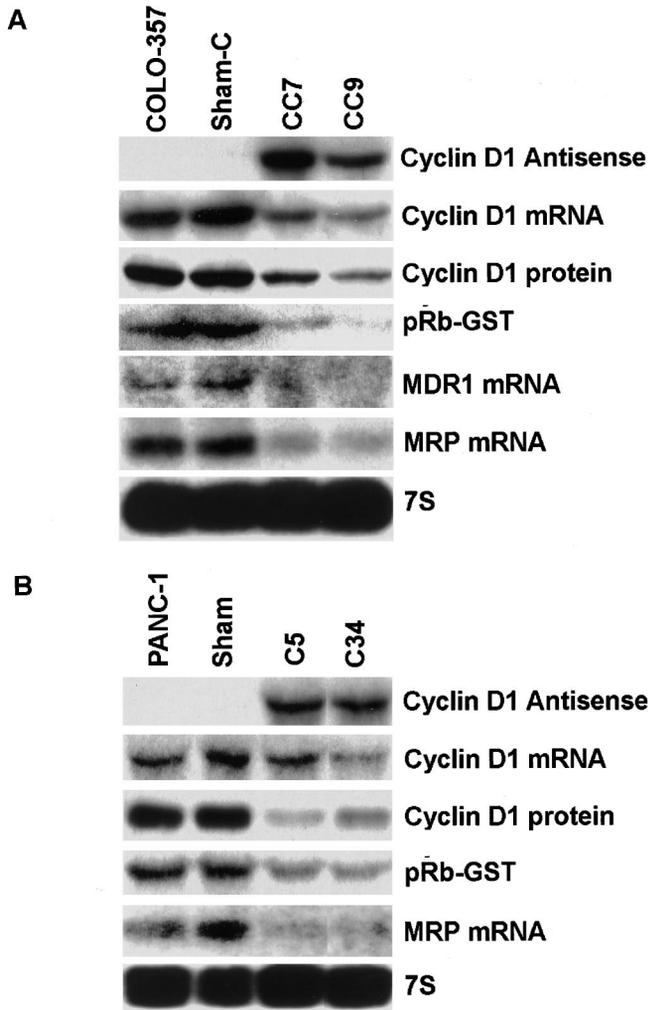
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Fig. 5. Effects of cyclin D1 antisense expression on MDR-1 and MRP mRNA levels. Northern blot analysis of total RNA (10 μg/lane) from COLO-357 (*A*) and PANC-1 (*B*) cells was carried out with human MDR1 and MRP cDNA fragments and human cyclin D1 riboprobes that specifically detect endogenous cyclin D1 mRNA and exogenous cyclin D1 antisense cDNA. A *7S* probe served as a loading control for Northern blotting. For immunoblot analysis, total cell lysates (25 μg/lane) were subjected to SDS-PAGE, transferred to immobilon-P membranes, and blotted with a monoclonal mouse anti-cyclin D1 antibody (DCS-6). To assess cyclin D1-associated kinase activity, total cell lysates (350 μg) were incubated with a monoclonal mouse anti-cyclin D1 antibody (DCS-11) and protein A-Sepharose. Captured immunocomplexes were resuspended in kinase buffer containing pRb-GST fusion protein as substrate, incubated for 20 min at 30°C, and subjected to SDS-PAGE.

accumulation in resistant cells (54). In a multidrug-resistant small cell lung cancer cell line, another transporter gene, *MRP*, has been identified (31). The drug-resistance profiles of cells overexpressing MRP or MDR1 mRNA are similar but not identical (55). Thus, it is possible that the increased sensitivity to mitoxantrone is due, in part, to the decreased expression of MRP and MDR1.

The exact molecular mechanisms by which the suppression of cyclin D1 leads to alterations in the levels of expression of multiple drug resistance genes are not known and require further investigation. However, 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* (19). The present observations suggest that cyclin D1 plays an important role in the pathobiology and aggressiveness of human pancreatic cancer. Our findings also 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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Inhibition of Cyclin D1 Expression in Human Pancreatic Cancer Cells Is Associated with Increased Chemosensitivity and Decreased Expression of Multiple Chemoresistance Genes

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