Characterization of programmed cell death 4 in multiple human cancers reveals a novel enhancer of drug sensitivity

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Abstract

Programmed cell death 4 (Pdcd4), originally identified as an inhibitor of murine cellular transformation, inhibits protein synthesis by directly interacting with eukaryotic initiation factor 4A (eIF4A) of the translation initiation complex. The relevance of Pdcd4 to a broad range of human cancers derived from multiple tissue sites is unknown. Protein expression patterns from the National Cancer Institute drug-screening panel of 60 human cancer cells (NCI60) were analyzed by Western blot methods and revealed frequent reduction of Pdcd4 protein levels in renal-, lung-, and glia-derived tumors. Greater than mean Pdcd4 protein levels correlated with the antitumor activity of geldanamycin and tamoxifen. Stable expression of antisense *PDCD4* significantly reduced the sensitivity of MCF-7 breast cancer cells to geldanamycin and to tamoxifen. Sensitivity to geldanamycin significantly increased in UO-31 renal cancer cells expressing sense *PDCD4* cDNA. Increased geldanamycin sensitivity was accompanied by enhanced cell cycle arrest and apoptosis. One primary mode of inactivation of Pdcd4 in human cancers appears to involve down-regulated expression, and this down-regulation causes a decreased sensitivity to geldanamycin cytotoxicity. Thus, up-regulating Pdcd4 expression may be promising for geldanamycin-based combination therapy. [Mol Cancer Ther. 2004;3(2):103–110]

Introduction

The progression of a tumor from an early benign lesion to metastatic cancer is not fully understood. Hence, molecular signatures for stages of progression are being sought to identify and validate new molecular targets for cancer prevention and treatment. In the murine JB6 cell model system, programmed cell death 4 (Pdcd4) inhibits tumor promoter-induced neoplastic transformation and transformation-required transactivation of the activator protein 1 (AP-1) transcription factor complex, but does not inhibit other transformation-required events such as NF-κB or ornithine decarboxylase (ODC) activation (1). A number of AP-1 target genes have been implicated in cellular invasion and metastatic progression, including urokinase plasminogen activator receptor (uPAR) and various matrix metalloproteinase family members (2–4). Although Pdcd4 prevents murine cellular transformation, the function of *PDCD4* in human cancer is unknown.

PDCD4, cloned after differential display analysis of murine JB6 variants, is highly expressed in transformation resistant (P−) but not in transformation susceptible (P+) cells or transformed (Tx) cells (5, 6). Reducing Pdcd4 protein levels in P− cells by antisense RNA expression is accompanied by acquisition of a transformation susceptible phenotype, indicating that reduction of Pdcd4 protein is sufficient to permit neoplastic transformation (5). Ectopic expression of *PDCD4* in stably transfected P+ cells renders them resistant to tumor promoter-induced transformation, indicating that elevated expression of Pdcd4 protein inhibits neoplastic transformation (1). Pdcd4 down-regulation is also necessary to maintain the tumor phenotype, since elevation of Pdcd4 expression suppresses Tx tumor phenotype (6).

Pdcd4 directly interacts with and inhibits the helicase activity of eIF4A and inhibits cap-dependent translation both *in vitro* and *in vivo*, while competing for binding of eIF4A to the scaffold protein eIF4G (7). Pdcd4 also binds eIF4G, independently of interacting with eIF4A (7). Although the consequence of eIF4G binding remains to be determined, Pdcd4 is the first example of a protein that inhibits translation through attenuation of eIF4A activity. Inhibition of AP-1 is sufficient to inhibit tumor promotion in the JB6 cell model, in a human keratinocyte progression series, and in mice (1, 5, 8). A Pdcd4 mutant that fails to bind and inhibit eIF4A helicase activity also fails to inhibit tumor promoter-induced AP-1 activity (7). The lack of eIF4A binding by a mutant Pdcd4 releases the Pdcd4-specific inhibition of translation and the inhibition of AP-1 activation required for neoplastic transformation. Several human tumors and tumor cell lines show elevated levels of translation initiation factors including eIF4A (9), eIF4E (10), and eIF4G (11). Therefore, Pdcd4-based inhibitory regulation of translation factors may negatively regulate human cancer development.

The cellular transformation inhibition function of *PDCD4* (1, 5) renders *PDCD4* a candidate tumor suppressor gene. Activation of oncogenes and inactivation of tumor suppressor genes are necessary for the development of cancer (12–14). In addition to alterations in upstream regulators or downstream effectors, tumor suppressor genes can be

directly inactivated by alteration of the genomic sequence or down-regulation of the tumor suppressor gene product expression. To ascertain the relevance of *PDCD4* to human cancer, protein levels were assessed in the National Cancer Institute drug-screening panel of 60 human cancer cells (NCI60). The hypothesis tested was that Pdcd4 protein levels would be prognostic or causal for antitumor activity of current or exploratory chemotherapeutic compounds. Measurement of Pdcd4 protein levels supported the hypothesis. Pdcd4 was found to contribute to the sensitivity to geldanamycin of MCF-7 breast cancer cells and UO-31 renal cancer cells. Thus, Pdcd4 levels are predictive for and contributory to geldanamycin responsiveness.

Materials and Methods

Chemicals

Tamoxifen and geldanamycin were purchased from Sigma Chemical (St. Louis, MO). Ascomycin was purchased from BIOMOL Research Labs (Plymouth Meeting, PA).

Cell Lines

Dr. D. Scudiero of the Developmental Therapeutics Program (DTP), NCI, provided exponentially growing NCI60 cells (15). To produce MCF-7 cells with reduced Pdcd4 protein levels, the MCF-7 cells were stably transfected with pMM-antisense *PDCD4* plasmid or pMM-control as described previously (5) and independently derived antisense clones 29as, 40as, and 57as and control clone 23c were generated. Pooled clones of UO-31 cells ectopically expressing pCMV-*PDCD4* or pCMV-control were generated for cell proliferation assays, cycle analysis, and TUNEL assays.

Protein Extraction and Western Blot Analysis

Cell pellets from the NCI60 cells were lysed in 10 mM Tris/0.1% SDS lysis buffer plus protease inhibitors. Cellular extracts were quantitated in triplicate using the BCA Protein Assay Kit (Pierce, Rockford, IL). Samples were randomized and loaded onto gels with the melanoma line, SK-MEL-2, used as a reference for Pdcd4 expression compensating for variations in film exposure. Protein extracts (10 μg/lane) were fractionated (10% SDS-PAGE). After transfer to nitrocellulose membrane, immunoblot analysis was performed with rabbit Pdcd4 antibody (1:5000). A Pdcd4 carboxyl-terminal domain peptide was used to generate rabbit antiserum (CFVSEGDGGRLKPESY-OH). Anti-rabbit secondary antibody conjugated to horseradish peroxidase was visualized with ECL (Amersham Biosciences, Piscataway, NJ) after multiple exposures to XAR5 Scientific Imaging Film (0, Rochester, NY). The density of the bands was determined by using 1D Image Analysis Software (Kodak). The mean intensity of Pdcd4 expression for all the cell lines was assigned a value of zero.

The linear range of the X-ray film was determined by immunoblotting GST-Pdcd4 recombinant protein (0.1, 1.0, 5.0, 10, and 50 ng). If linearity was observed, the sum intensity of pixels from the X-ray film was determined. At least two blots were performed per tissue group to confirm the findings.

COMPARE Analysis

A molecular targets version of the COMPARE program (16–20) was used to analyze relationships between basal patterns of Pdcd4 protein levels and drug sensitivity in the NCI60 cells. The Pdcd4 protein levels were represented as a mean-graph pattern, which was used as a seed to derive correlations between Pdcd4 protein levels and growth inhibitory properties of compounds in multiple databases, including the Standard Agent database, which comprises 170 chemical compounds including both preclinical and clinical drugs with antitumor properties. Agents were ranked according to Pearson correlation coefficient (PCC) and two-tailed P value. Using Bonferroni adjustment for multiple comparisons in the 170-compound Standard Agent database, statistical significance was conferred to P values less than 0.0006.

Cell Proliferation Assay

Cell proliferation was assessed using colorimetric XTT assay (Roche Diagnostics, Indianapolis, IN) according to manufacturer's instructions. Cells were plated in 96-well plates at 2500 cells/well. After 24 h to allow for cell adherence, cells were treated with drug or solvent control for 72 h as described for individual experiments. Colorimetric change was measured in a scanning multi-well spectrophotometer.

Flow Cytometry

Cell cycle distribution conferred by DNA histograms was generated by fluorescence-activated cell sorting analysis (21) after propidium iodide staining with a Coulter XL flow cytometer (Beckman Coulter, Fullerton, CA) and analyzed with MultiPlus (Phoenix Flow System, San Diego, CA). Determination of fluorescently labeled TUNEL-positive cells (Roche Diagnostics) was assayed 72 h after drug treatment using flow cytometry.

Results

Pdcd4 Protein Levels in the NCI Panel of Tumor Cell Lines

Inactivation of tumor suppressor genes occurs not only through gene mutation or deletion but alternatively as a result of reduced gene expression (22, 23). To determine phenotypic alterations associated with loss of Pdcd4 function, the protein expression pattern of the *PDCD4* gene in multiple human tumors was examined by Western blot analysis of the NCI60 human cancer cell panel. Pdcd4 localized predominantly to the cytoplasm under conditions of asynchronous cell growth, confirmed by immunohistochemical analysis of JB6 cells (7). The Pdcd4 band was determined to be M_r 54,000 and was competed by prior incubation of the Pdcd4 antibody with a peptide based on the Pdcd4 carboxyl-terminal domain, which was originally used to generate rabbit antiserum (Fig. 1A). In a small subset of cells, a band at M_r 46,000–48,000 kDa was also competed by the Pdcd4-specific peptide. Despite the lack of direct evidence, this band had been speculated to be a degradation product (24). However, there was no correlation between loss of the M_r 54,000 species of Pdcd4 and presence of the putative degradation product.
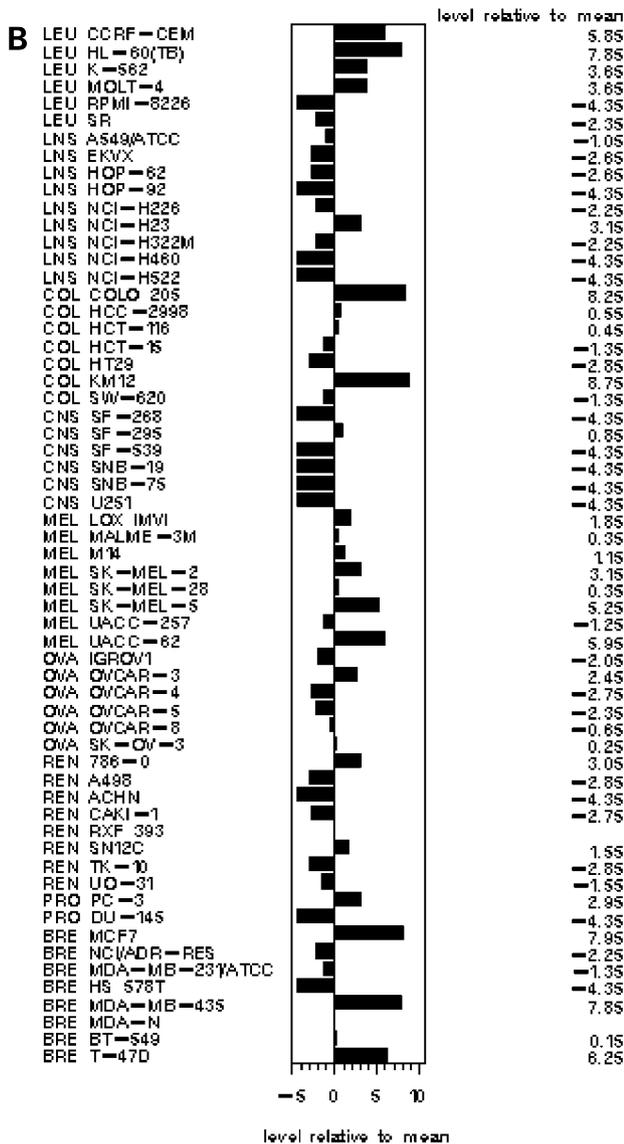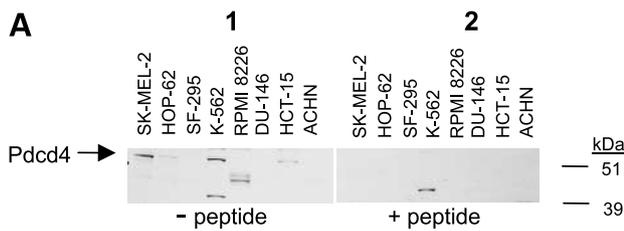
Figure 1. Pdcd4 protein levels in the NCI panel of tumor cell lines. **A,** *panel 1* is a representative example of immunoblot data from whole cell extract for several human tumor cell lines using rabbit polyclonal Pdcd4 antibody. *Panel 2* is an anti-Pdcd4 immunoblot with the same extracts and the antibody previously incubated with Pdcd4 carboxyl-terminal peptide (+ *peptide*) used to generate antiserum. See Materials and Methods for peptide sequence. Each well contains equal protein. **B,** graphical summary of Pdcd4 protein levels in the form of a mean graph of the NCI60 cells. Expression above the mean Pdcd4 protein levels is drawn to the *right* of the centerline and below the mean is drawn to the *left*. The melanoma line, SK-MEL-2, was loaded onto every gel for normalization. The protein expression patterns were determined in duplicate.

Pdcd4 protein levels, displayed in mean-graph format (16), showed patterns in a number of tissue types (Fig. 1B). Five of six cancer cell lines derived from the central nervous system (CNS) tumors showed complete loss of Pdcd4 protein. Immmunohistochemical analysis demonstrated that Pdcd4 was readily detected in brain tissue under normal conditions (data not shown). A pattern of less than mean Pdcd4 protein levels was seen in eight of nine non-small cell lung cancer lines and five of seven renal cancer cell lines. However, seven of eight melanoma lines displayed a pattern of greater than mean expression of Pdcd4 protein. Thus, the human cancer sites in which the cell lines showed most frequent reduction in Pdcd4 protein expression were derived from lung, glia, and the CNS.

Greater Than Mean Levels of Pdcd4 Are Predictive for Specific Drug Sensitivity in the NCI60 Cancer Cell Panel

We hypothesized that Pdcd4 expression level status may be a marker in the response of some human cancers to current and potential chemotherapeutic drugs. The protein levels of Pdcd4 in NCI60 cells were compared with *in vitro* chemosensitivity data for over 100,000 compounds using the NCI Developmental Therapeutics Program COMPARE statistical algorithm. The COMPARE program was used to create Wilcoxon rank-order analyses to determine whether either positive or negative correlations exist for the 50% growth inhibition pattern of drugs compared to molecular target of interest, Pdcd4. In the Standard Agents category consisting of 170 compounds under current clinical and investigative use as chemotherapeutic drugs, expression of greater than mean levels of Pdcd4 protein in the NCI60 cells resulted in significantly positive rank-order correlations to geldanamycin and tamoxifen (Table 1). Analysis of the Selected 3000 Agent

Table 1. Correlation of Pdcd4 protein levels with 50% growth inhibition data from the NCI60 human tumor panel

Rank	Drug Name	Pearson	P (Two-Tail)
1	**Geldanamycin**	**0.47**	**0.00019**
2	**Tamoxifen**	**0.44**	**0.00047**
10	Vinblastine sulfate	0.35	0.00665
14	Paclitaxel	0.31	>0.01
15	Bleomycin	−0.31	>0.01
28	6-Mercaptopurine	0.26	>0.01
30	BCNU	0.25	>0.01
33	CCNU	0.25	>0.01
39	Thioguanine	0.23	>0.01
40	Methotrexate	0.23	>0.01
76	5-Fluorouracil	0.15	>0.01
132	Doxorubicin	0.06	>0.01
140	VP-16	0.05	>0.01
146	Cisplatin	−0.04	>0.01

Note: Comparison of Pdcd4 protein levels and drug sensitivity data for the NCI cancer panel resulted in rank-order correlation. Positive Pearson Correlation Coefficient (Pearson) indicates that the greater abundance of the target may associate with sensitivity to the drug, whereas a negative correlation is indicative of more target conferring cellular resistance to the given drug. Utilizing Bonferroni adjustment, a two-tail P value of less then 0.0006 was considered significant. Drugs considered significantly correlated with Pdcd4 protein levels are in bold.

Table 2. Relative levels of Pdcd4 protein and mRNA in NCI60

Cancer Type	Cell Line	Pdcd4 Protein	Pdcd4 Array
Leukemia	CCRF-CEM	++++	++
	HL-60(TB)	++++	+++
	K-562	+++	++
	MOLT-4	+++	nd
	RPMI-8226		++++
	SR	+	+
Lung	A549/ATCC	+	++
	EKVX	+	+
	HOP-62	+	+
	HOP-92	−	++
	NCI-H226	+	+
	NCI-H23	+++	+++
	NCI-H322M	+	+
	NCI-H460	−	nd
	NCI-H522	−	++
Colon	COLO 205	++++	++++
	HCC-2998	++	++
	HCT-116	++	++
	HCT-15	+	++
	HT29	+	+
	KM12	++++	++++
	SW-620	+++	+++
CNS	SF-268	−	++++
	SF-295		nd
	SF-539	−	++++
	SNB-19	−	−
	SNB-75	−	++
	U251	−	+
Melanoma	LOX IMVI	++	++++
	MALME-3M	++	+
	M14	++	+
	SK-MEL-2	+++	++
	SK-MEL-28	++	++
	SK-MEL-5	++++	++
	UACC-257	+	+
	UACC-62	++++	++
	IGROV1	+	++
Ovary	OVCAR-3	++	++++
	OVCAR-4	+	+
	OVCAR-5	+	+
	OVCAR-8	++	+
	SK-OV-3	++	+
	786-0	+++	+
Kidney	A498	+	+++
	ACHN	−	++
	CAKI-1	+	++++
	SN12C	++	+++
	TK-10	+	+
	UO-31	+	+
	PC-3	++	+++
Prostate	DU-145	−	+++
	MCF7	++++	++++
Breast	MCF7/ADR-R	+	++
	MDA-MB-231	+	+
	HS 578T	−	++
	MDA-MB-435	++++	++++
	BT-549	++	+++
	T-47D	++++	++++

Note: Relative Pdcd4 protein levels as follows: −, no detection; +, −4.35 to −2.00; ++, −1.99 to 0; +++, 0.01 to 2.0; ++++, >2.00. Conversion of Pdcd4 microarray data from color key as follows: +, −2.00 to −1.20 (green); ++, −1.00 to −0.20; +++, 0 to 1.00; ++++, 1.20 to 2.00 (red). All microarray values were log base 2. Pdcd4 microarray data were adapted from Ross *et al.* (25) and Scherf *et al.* (26).

Database revealed a clustering of elevated Pdcd4 protein levels and the activity of compounds with mechanisms that included inhibition of Hsp90 or inhibition of *cis/trans* peptidyl prolyl isomerase (data not shown).

Table 2 provides a comparison of Pdcd4 protein levels to the published *PDCD4* gene expression levels determined by microarray analysis of the NCI60 cells (25, 26). Note that gene expression levels and protein levels correlate highly in tissues such as lung, colon, and breast. However, in the renal- or CNS-derived cell lines, there is a lack of correlation between transcript levels and protein levels. This suggests that the regulation of Pdcd4 is primarily transcriptional or pretranslational in lung, colon, and breast cancer, but both transcriptional and translational regulation may occur in renal and CNS cancers.

Pdcd4 Protein Level Contributes to Cellular Sensitivity to Tamoxifen and Geldanamycin, but not to Ascomycin

To test the causal hypothesis that above-mean Pdcd4 protein level not only predicted but also contributed to sensitivity to the drugs predicted by the above COMPARE analysis, we derived MCF-7 cells having stable expression of *PDCD4* antisense RNA, and consequently down-regulated Pdcd4 protein levels. *PDCD4* antisense clones 29 (29as), 40 (40as), and 57 (57as) displayed reductions in endogenous Pdcd4 protein levels of 50%, 80%, and 90%, respectively, compared to either the parental MCF-7 or vector control MCF-7 clone (23c) (Fig. 2A). No changes in cell growth or global protein expression were noted (data not shown). The IC_{50} of tamoxifen in whole serum, in which the cytotoxicity of tamoxifen was not reversed by increasing estrogen levels, was increased in a small but reproducible manner from 12.5 μM in parental and control cells to 15 μM in *PDCD4* antisense expressing cells (Fig. 2B). The observation of no additional shift in sensitivity with 80–90% reduction in Pdcd4 levels suggests that a reduction of endogenous Pdcd4 protein levels by 50% (seen in clone 29as) may be a threshold for decreased sensitivity to tamoxifen. The IC_{50} of geldanamycin was increased from 100 nM as determined in parental and control MCF-7 cells to greater than 1 μM in *PDCD4* antisense expressing lines (Fig. 2C). Doses above 500 nM were utilized to determine actual IC_{50} for the *PDCD4* antisense clones (Data not shown). However, for geldanamycin, in contrast to tamoxifen, 90% reduction of Pdcd4 had a greater effect than 50% reduction of Pdcd4 protein levels. No change in sensitivity to ascomycin, an inhibitor of peptidyl prolyl isomerase, was seen (Fig. 2D). The reduction of Pdcd4 caused a significant decrease in the sensitivity to geldanamycin.

To further test whether Pdcd4 protein levels altered sensitivity to geldanamycin, the UO-31 renal cancer cell line, which contained low Pdcd4 protein amount and showed insensitivity to geldanamycin, was chosen to

Figure 2. Pdcd4 protein levels contribute to sensitivity of MCF-7 cells to tamoxifen and geldanamycin. **A,** antisense expression reduces Pdcd4 protein levels in independent MCF-7 cell line clones (*29AS*, *40AS*, *57AS*) compared to parental and vector control (*23C*) cell lines. Immunoblotting was performed as described in Fig. 1. **B – D,** evaluation of chemosensitivity of MCF-7 clones expressing Pdcd4 antisense construct. All cell lines were incubated with indicated concentrations for 72 h with tamoxifen, geldanamycin, ascomycin, or solvent alone. Cell proliferation determined by XTT assay was expressed as a percentage of the vehicle-treated control. Each value is the mean ± SD of eight replicate treatments in a 96-well tissue culture format. Graphs are representative of three independent experiments for tamoxifen and geldanamycin treatments and two independent experiments for ascomycin treatment. ♦, MCF-7; ■, 23C; △, 29AS; □, 40AS; ◇, 57AS.
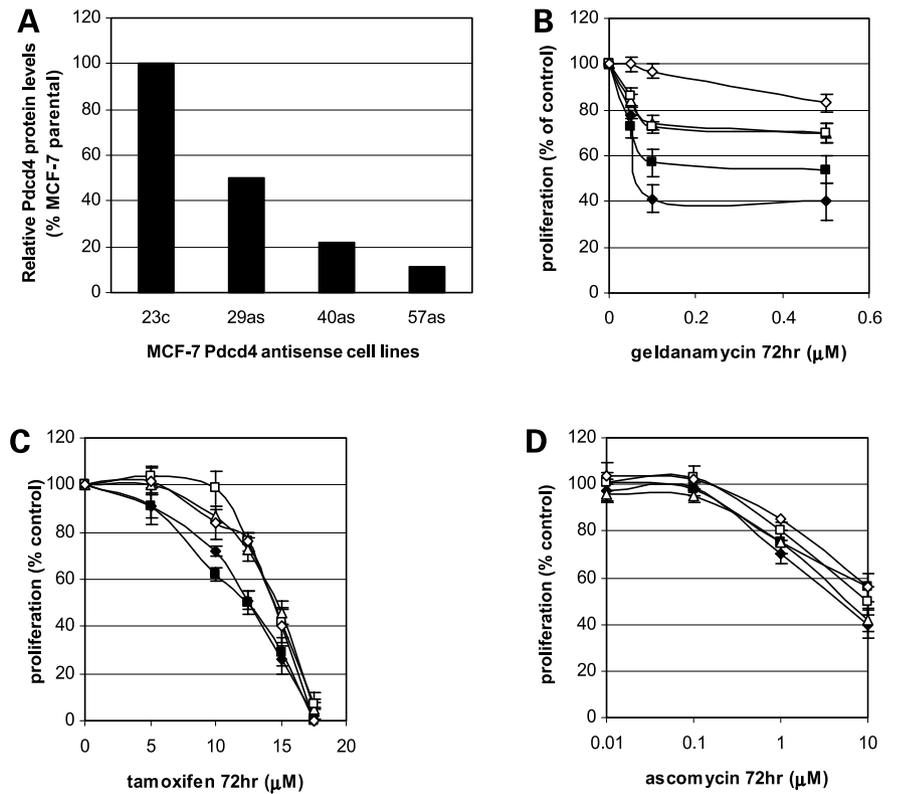
express sense *PDCD4* cDNA. Pooled clones were generated and Pdcd4 protein levels were increased by 2.4 fold in the pooled clones compared to either parental or vector alone UO-31 cells (Fig. 3A). No changes in cell growth or global protein expression were noted (data not shown). The IC_{50} of geldanamycin was decreased from 400 nM as determined in parental and control UO-31 cells to 140 nM in *PDCD4* sense expressing pooled clones (Fig. 3B). The data further confirmed the causal contribution of Pdcd4 protein levels to geldanamycin sensitivity.

Pdcd4 Enhances Geldanamycin-Induced G_2-M Arrest in the Low Rb Expressing Renal Cancer Cell Line UO-31

To determine whether the enhancement of geldanamycin-induced growth inhibition by Pdcd4 was attributable to inhibition of cell proliferation alone or to a combination of both growth arrest and induction of cell death, DNA content and cell cycle status were determined in UO-31 renal carcinoma cells. Pdcd4-expressing pooled clones responded to 24 h geldanamycin treatment (250 nM) with an accumulation of cells in G_2-M phase of the cell cycle (DMSO: 16.1%; geldanamycin: 33.7%). Vector alone expressing clones were unresponsive to geldanamycin and continued to proliferate (DMSO: 9.9%, geldanamycin: 15.0%) (Fig. 4A). Pdcd4 expression increased hypodiploid cells in sub-G_1 fraction and increased TUNEL-positive cells 72 h after geldanamycin treatment compared to vector alone expressing clones (Fig. 4B). Thus, together, Pdcd4 and geldanamycin induced G_2-M arrest accompanied by apoptosis.

Discussion

The present study demonstrates that the murine transformation inhibitor and candidate human tumor suppressor gene, *PDCD4*, is down-regulated at the protein level in a number of human cancers. This down-regulation not only is a marker of drug sensitivity, but also contributes to the decreased sensitivity of MCF-7 breast cancer cells to tamoxifen and geldanamycin and of UO-31 renal cancer cells to geldanamycin. Therapeutic strategies to up-regulate Pdcd4 expression in combination with geldanamycin treatment may offer promise.

With the characterization of the NCI60 tumor cell panel, this report establishes that in human cancer, one mode of inactivation of the candidate tumor suppressor gene *PDCD4* involves down-regulated protein levels. *PDCD4* may be a candidate for haplo-insufficiency or dominant loss of allele and function. Thresholds of protein function appear to be important for at least two tumor suppressor genes, *p27^{Kip1}* and *p53*. Evidence suggests that mice hemizygous for either *p27^{Kip1}* (27) or *p53* (28) are predisposed to tumor development compared to wild-type mice. To directly answer whether haplo-insufficiency of Pdcd4 protein causes a greater susceptibility to tumor development *in vivo*, the generation *PDCD4* hemizygous and nullizygous mice is currently being pursued.

The mechanism by which Pdcd4 is down-regulated remains unknown. However, at least one mechanism of regulation appears to occur at a pretranslational, possibly
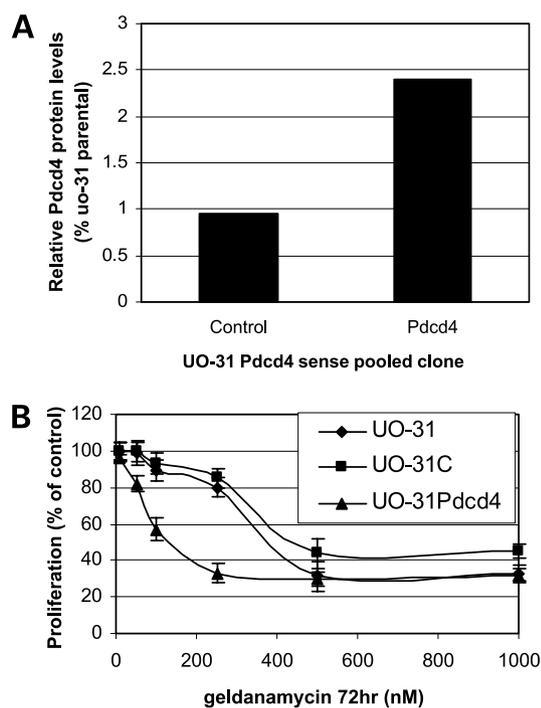
A

UO-31 Pdcd4 sense pooled clone

B

Figure 3. Pdcd4 protein levels contribute to sensitivity of UO-31 cells to geldanamycin. **A,** ectopic sense *PDCD4* expression increases relative Pdcd4 protein levels in UO-31 pooled clones compared to parental and vector control cell lines. Immunoblotting was performed as described in Fig. 1. **B,** evaluation of chemosensitivity of UO-31 pooled clones expressing *PDCD4* sense construct. All lines were incubated with indicated concentrations for 72 h with geldanamycin or solvent alone. Cell proliferation determined by XTT assay was expressed as a percentage of the vehicle-treated control. Each value is the mean ± SD of eight replicate treatments in a 96-well tissue culture format. The graph is representative of two independent experiments for geldanamycin treatment.

transcriptional level. *PDCD4* transcript levels were altered in a number of physiological circumstances in both mouse and human cells (29–32). Conflicting evidence exists as to whether the *PDCD4* transcript is up- or down-regulated in response to differing physiological stimuli, especially apoptotic induction. Expression of the *PDCD4* transcript is up-regulated on induction of apoptosis in a number of model systems (29) and in senescent human diploid fibroblasts (33). However, treatment of mouse lymphoma cells with topoisomerase inhibitors, which induce apoptosis, down-regulates the *PDCD4* transcript (34). No causal relationship has been attributed to the changes in *PDCD4* transcript levels and apoptosis.

Interestingly, the heat shock protein 90 (Hsp90) plays a part in the therapeutic mechanisms of both geldanamycin and tamoxifen, suggesting that Pdcd4 may play a role in regulating targets of Hsp90. Geldanamycin binds to a conserved pocket of Hsp90 (35–37). The binding releases its chaperone protection allowing the degradation of a number of oncogenes or mutated tumor suppressors, including receptor tyrosine kinases (38), steroid receptors (39), Raf (40), and p53 (41). Tamoxifen is an antiestrogenic drug widely used for adjuvant chemotherapy for estrogen receptor positive breast cancer. The concentration of

tamoxifen necessary for the antiproliferation effect appears to be in the range that is not reversible by adding estrogen. This further suggests that the alteration of tamoxifen sensitivity by Pdcd4 may be through an Hsp90 pathway rather than through an estrogen receptor pathway. It appears unlikely that geldanamycin may bind to Pdcd4. However, we cannot distinguish between the two possibilities that Pdcd4 may act on the Hsp90 pathway or another pathway that may synergize the geldanamycin activity, possibly transforming geldanamycin from a cytostatic agent to a cytotoxic agent. It will be of interest
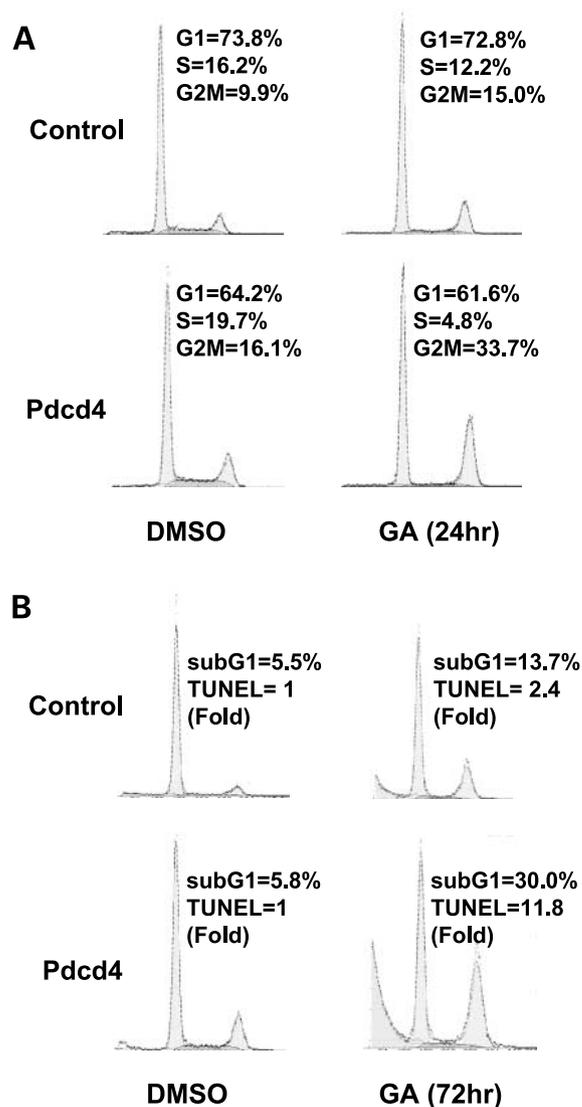
A

B

Figure 4. Elevated Pdcd4 protein levels enhance geldanamycin-induced G_2-M arrest and apoptosis in UO-31 cells. **A,** cell cycle analysis by fluorescence-activated cell sorter after 24 h of geldanamycin (250 nM) treatment with UO-31 control and *PDCD4* pooled clones. Data are representative of three experiments. **B,** analysis of sub-G_1 fraction from cell cycle analysis by fluorescence-activated cell sorter after 72 h of geldanamycin (1000 nM) treatment. Fold increase in apoptotic cells was determined after fluorescently labeled TUNEL and analysis by flow cytometry. *X* axis: DNA content; *Y* axis: cell number. Data are representative of two experiments.

to determine whether Pdcd4 may be a useful molecular marker to better identify breast cancer patients who will respond to tamoxifen with prolonged survival and whether the loss of Pdcd4 alters response to geldanamycin or tamoxifen *in vivo*. Recently, the loss of Pdcd4 expression in human lung adenocarcinoma has been reported and correlates with tumor progression and poor patient prognosis (42). A similar study of breast cancer tissue may be warranted.

We chose to follow up the down-regulation findings of geldanamycin in the MCF-7 cells with a tumor cell line that matched two criteria: (*a*) deficiency in Pdcd4 expression and (*b*) decreased sensitivity to geldanamycin in comparison to the other NCI60 cells. UO-31 renal cancer cells met both requirements. We also chose to further study geldanamycin because of the correlation between compounds that inhibited Hsp90 activity and Pdcd4 protein levels. Because renal cell cancer is refractory to standard chemotherapeutic agents, novel experimental chemotherapeutics are being sought to address deficiencies in current chemotherapy regimens against renal cancer. geldanamycin is known to cause RB-dependent G_1 cell cycle arrest (43). However, in breast cancer cells deficient for RB function, geldanamycin causes G_2-M arrest (44). UO-31 cells have relatively low levels of RB protein compared to other cell lines of the NCI60 drug screening database (http://dtp.nci.nih.gov) and are resistant to geldanamycin. However, when Pdcd4 is introduced, the UO-31 cells become more sensitive to G_2-M-associated arrest and present evidence of apoptosis after geldanamycin treatment. Thus, the combination of Pdcd4 expression and geldanamycin treatment causes UO-31 cells to respond. Pdcd4 may play a role in driving apoptosis in cells with inhibited Hsp90 chaperone activity. The relationship between Hsp90, Pdcd4, and apoptosis remains unclear. However, better understanding of the function of Pdcd4 itself may lead to development of new drugs that take advantage of the molecular mechanism(s) by which Pdcd4 inhibits tumor development.

In conclusion, these findings establish that Pdcd4 protein levels are significantly correlated and specifically contribute to the antitumor activities of tamoxifen and especially to geldanamycin. At least one primary mode of inactivation of Pdcd4 involves down-regulation of constitutive protein levels. Further understanding of the role Pdcd4 plays in tumor development may provide new tools for cancer prevention and control.

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