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Prostate-derived factor—a novel inhibitor of drug-induced cell death in colon cancer cells

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Abstract

We investigated the role of the divergent transforming growth factor-β superfamily member, prostate-derived factor (PDF), in regulating response to chemotherapies used in the treatment of colorectal cancer. A clear p53-dependent expression pattern of PDF was shown in a panel of colorectal cancer cell lines following acute exposure to oxaliplatin, 5-fluorouracil, and SN38. PDF gene silencing before chemotherapy treatment significantly sensitized cells expressing wild-type p53, but not p53-null or p53-mutant cells, to drug-induced apoptosis. Similarly, knockdown of PDF expression sensitized HCT116 drug-resistant daughter cell lines to their respective chemotherapies. Inducible PDF expression and treatment with recombinant PDF both significantly attenuated drug-induced apoptosis. Further analysis revealed that PDF activated the Akt but not the extracellular signal-regulated kinase 1/2 signaling pathway. Furthermore, cotreatment with the phosphatidylinositol 3-kinase inhibitor wortmannin abrogated PDF-mediated resistance to chemotherapy-induced apoptosis. Together, these data suggest that PDF may be a novel inhibitor of drug-induced cell death in colorectal cancer cells and that the mature secreted form of the protein activates the phosphatidylinositol 3-kinase/Akt pathway as an acute mechanism of chemoresistance. [Mol Cancer Ther 2009;8(9):2566–74]

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Introduction

Prostate-derived factor (PDF) is a divergent member of the transforming growth factor-β (TGF-β) superfamily. PDF, such as other TGF-β superfamily members, undergoes post-translational modification and cleavage to process its precursor protein to a mature secreted form (1–3). The biological activity of PDF is poorly characterized. Several previous functional studies suggested a proapoptotic role for PDF, with its overexpression resulting in increased levels of cell death in DU145 prostate cell lines (3) and enhanced spontaneous (4) as well as indomethacin-induced apoptosis in HCT116 colorectal cells (5). Knockdown of PDF was shown to result in a marked protection against 12-*O*-tetradecanoylphorbol-13-acetate–induced apoptosis in LNCaP prostate cancer cells (6) and to a decrease in cell death following combined sodium salicylate and phosphatidylinositol 3-kinase (PI3K)/mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase 1/2 inhibitor treatment in A549 lung adenocarcinoma cells (7).

More recent reports have suggested a protumorigenic role for PDF. Increased PDF mRNA expression has been reported in glioblastomas (8). Immunohistochemical staining showed PDF overexpression in tumor compared with adjacent normal tissue in prostate (9, 10), breast (11), colon (12), pancreatic (13), oral carcinomas (14), and highly aggressive metastatic melanoma (15). Furthermore, elevated levels of PDF have been detected in the serum of patients with metastatic colorectal (12), pancreatic (13), breast (16), and prostate carcinomas (17) that positively correlated with the malignant grade of tumor. Taken together, these reports suggest an important role for PDF in the development and progression of these tumors.

PDF is induced in human colon cancer cells by several nonsteroidal anti-inflammatory drugs (5), as well as anticancer agents such as resveratrol (18), genistein (19), trichostatin (20), and pectenotoxin-2 (21), suggesting that PDF expression may be an important regulator of a drug response. Furthermore, PDF overexpression has been shown to correlate with resistance to cisplatin in lung cancer cells (22) and addition of human recombinant PDF (rhPDF) mediated a cytoprotective effect in prostate cancer cells following treatment with docetaxel and mitoxantrone (23). The PDF signaling pathway is not well characterized at present, although PDF was reported to activate ERK1/2 (24) and PI3K/Akt signaling cascades (25). Furthermore, its receptor has not yet been identified; however, there is a suggestion that PDF may act through the classic TGF-β pathway (26).

Colorectal cancer is the second highest cause of cancer mortality in the Western world. The antimetabolite 5-fluorouracil (5-FU) has been the mainstay of colorectal cancer therapy for more than 50 years (27). In advanced disease, 5-FU

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therapy modulated with folinic acid produces response rates of only 20% to 25% (28). Efforts to improve efficacy have led to combinations of 5-FU with the topoisomerase inhibitor irinotecan and the DNA-damaging agent oxaliplatin, resulting in improved response rates of 40% to 50% (29, 30). Despite these improvements, the majority of responding patients relapse, with median survival times of only 22 to 24 months. Clearly, the identification of clinical biomarkers that can predict response to treatment as well as new molecular targets to increase therapeutic efficacy or counteract drug resistance is critical. In this study, we evaluated the role of PDF in regulating the response to chemotherapy in colorectal cancer cells.

Materials and Methods

Materials

5-FU and oxaliplatin were purchased from Sigma Chemical Co. and Sanofi-Synthelabo, respectively. SN38 was purchased from Abatra Technology Co. Ltd. rhPDF was provided by R&D Biosciences. A 200 μg/mL stock solution of rhPDF was prepared in PBS containing 1% bovine serum albumin and 4 mmol/L HCl, aliquoted, and stored at −20°C. Wortmannin was obtained from Calbiochem.

Tissue Culture

The p53 wild-type HCT116 and HCT116 p53-null human colon cancer cell line were kindly provided by Professor Bert Vogelstein (Johns Hopkins University, Baltimore, MD). The 5-FU–resistant and oxaliplatin-resistant HCT116 sublines were generated in our laboratory as previously described (31, 32). The SN38-resistant HCT116 subline was generated in our laboratory by repeated exposure to stepwise increasing concentrations of SN38 over a period of ~9 mo and added every 4 wk to 5 nmol/L SN38 to maintain the resistant phenotype. All HCT116-derived cell lines were maintained in McCoy's 5A medium supplemented with 10% dialyzed FCS, 50 μg/mL penicillin-streptomycin, 2 mmol/L L-glutamine, and 1 mmol/L sodium pyruvate. To avoid acute drug-inducible effects, resistant sublines were cultured in the absence of drug for 1 wk before subsequent analysis. HT29 and H630 cells (obtained from the National Cancer Institute, Bethesda, MD) were maintained in DMEM and supplemented as above. LoVo cells (kindly provided by AstraZeneca) were maintained in DMEM supplemented as above minus sodium pyruvate (all medium and supplements from Invitrogen Life Technologies Corp.). All cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Generation of PDF-Inducible Cell Line

The HCT116 tetracycline (Tet) repressor cell line was generated by stable transfection of the pcDNA6/TR plasmid (Invitrogen) into p53 wild-type HCT116 parental cells using the GeneJuice (Novagen) method and selection in blasticidin (5 μg/mL)–containing medium. The HCT116 Tet-regulated PDF-inducible cell line was generated by stable transfection of the pcDNA4/TO plasmid (Invitrogen) containing the full-length PDF coding sequence into the HCT116 Tet repressor cell line using GeneJuice. Stable clones were selected in blasticidin (2.5 μg/mL)–containing and zeocin (20 μg/mL)–containing media. The HCT116 Tet-regulated HLacZ-inducible control cell line was generated by stable transfection of the pENTR/HI/TO vector containing the *HLacZ* gene into the HCT116 Tet repressor cell line under selection in blasticidin and zeocin as described above. Both cell lines were maintained in HCT116 culture medium and periodically added to blasticidin (2.5 μg/mL) and zeocin (20 μg/mL). To induce the expression of exogenous PDF, cells were incubated in growth medium containing 0.5 or 5 ng/mL of Tet.

Real-time Reverse Transcription-PCR Analysis

Details are in Supplementary Materials and Methods.

Preparation of Concentrated Medium

Conditioned culture medium was centrifuged in an Amicon Ultra-15 PL-10 concentration column (Millipore Corp.) at 4,000 rpm/4°C for 30 min. The resulting concentrated medium was syringed 10 times using a 19-gauge needle and centrifuged at 13,000 rpm/4°C for 30 min. The supernatant was analyzed for mature PDF expression using immunoblotting as described below. Equal lane loading was determined by staining the nitrocellulose membrane with Ponceau S solution (Sigma Chemical) before blocking.

Immunoblotting

Full details are in Supplementary Materials and Methods. The antibodies anti–NAG-1 rabbit polyclonal antibody (Upstate Biotechnology) and anti-ERK1/2 (K-23; Santa Cruz Biotechnology) rabbit polyclonal antibodies and anti–phospho-Akt (Ser^{473}; Cell Signaling), anti-Akt (Cell Signaling), anti–phospho-ERK1/2 (Thr^{202}/Tyr^{204}; Cell Signaling), and poly(ADP-ribose) polymerase (PARP; eBioscience) mouse monoclonal antibodies were used in conjunction with horseradish peroxidase–conjugated anti-rabbit and anti-mouse secondary antibodies (Amersham). Equal loading was assessed using β-tubulin (Sigma Chemical) or glyceraldehyde-3-phosphate dehydrogenase (Biogenesis) mouse monoclonal primary antibodies. The SuperSignal chemiluminescent system (Pierce) or enhanced chemiluminescence Plus (Amersham) was used for detection.

Flow Cytometry

Details are in Supplementary Materials and Methods.

Small Interfering RNA Transfection

The PDF small interfering RNA (siRNA) construct and scrambled sequence were purchased from Qiagen Sciences, Inc. The PDF target sequences used were GUUAUUUAUUAUUAAUUUA, GGGAAGAUUCGAACACCGA, AGCUGUCUGAACUGUUAAA, and GGAACUGUGUAUUUAUUUA. The nontargeting scrambled control (SC) sequences used were UUCUCCGAACGUGUCACGU (sense) and ACGUGACACGUUCGGAGAA (antisense). siRNA transfections were done as previously described (33).

Clonogenic Survival Assay

The HCT116 wild-type cells were transfected using Amaxa nucleofection kit according to the manufacturer's instructions. Electroporated cells were seeded in 24-well plates at a density of 500 per well. After 24 h, cells were treated with oxaliplatin, 5-FU, or SN38. After 72 h, the growth

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medium was replaced with fresh medium containing the same concentrations of drugs, and the cells were allowed to grow for another 5 d. Colonies were visualized by crystal violet staining. The dye was dissolved in 250 μL of 0.2 mol/L sodium citrate and ethanol solution (50%-50% mix); 200 μL from each well were transferred to a 96-well plate and absorbance was read at 570 nm.

Results

Drug-Induced PDF Expression in Colorectal Cancer Cell Lines with Different p53 Status

The HCT116 p53 wild-type and p53-null HCT116 cell lines were treated with ~$IC_{60(72\ h)}$ doses (Table 1) of oxaliplatin, 5-FU, and SN38 (the active metabolite of irinotecan) for 0, 6, 12, 24, and 48 hours, and the expression level of *PDF* mRNA was analyzed using real-time reverse transcription-PCR. As shown in Fig. 1A, a time-dependent increase in *PDF* mRNA levels in p53 wild-type HCT116 cells was observed following treatment with all three chemotherapies compared with the time-matched control; however, the extent of *PDF* up-regulation was significantly lower in response to SN38 than for the other two drugs. *PDF* mRNA expression in LoVo cells (p53 wild-type) followed the same expression pattern, with time-dependent up-regulation of *PDF* mRNA observed after treatment with oxaliplatin and 5-FU, but not SN38 (Fig. 1A). In contrast, no significant increase in *PDF* mRNA level was detected in HCT116 p53-null cells in response to chemotherapy compared with untreated control (Fig. 1A); similar results were obtained in the p53-mutant HT29 and H630 cell lines (data not shown).

The propeptide form of PDF was also potently up-regulated in HCT116 p53 wild-type following treatment with oxaliplatin, 5-FU, and, to a lesser extent, SN38 (Fig. 1B), with a concomitant increase in the secreted mature PDF. No induction of the PDF propeptide was observed after treatment of HCT116 p53-null cells with IC_{60} doses of chemotherapy, and only moderate increases (2- to 3-fold) in PDF propeptide expression were detected in H630 and HT29 cell lines following treatment with 5-FU, but not oxaliplatin or SN38 (Fig. 1B). In LoVo cells, robust induction of PDF propeptide following treatment with oxaliplatin and 5-FU, but not SN38, was observed (Fig. 1C); however, no secreted mature PDF was detected (data not shown).

Effect of PDF Silencing on Chemosensitivity in Colorectal Cancer Cell Lines

To assess the role of PDF in modulating the cellular response to cytotoxic drug treatment, we initially used PDF-targeted siRNA. To confirm the specificity of PDF-targeted siRNAs, all sequences were tested for potential off-target effects on other TGF-β family members. Real-time PCR showed no changes in TGF-β mRNA expression levels after transfection with PDF siRNA (data not shown). Pretreatment of HCT116 p53 wild-type cells with PDF siRNA led to a significant decrease of both constitutive and drug-induced expression of PDF (Fig. 2A, *inset*). PDF silencing resulted in a significant increase in apoptosis following treatment with each chemotherapeutic agent (Fig. 2A). Furthermore, Western blot analysis showed increased PARP cleavage (a hallmark of apoptosis) after combined PDF siRNA and chemotherapy treatment (Fig. 2A, *inset*; data not shown). PDF silencing resulted in a significant increase in apoptosis in RKO cells following exposure to oxaliplatin and 5 μmol/L 5-FU, but not SN38, and these results were confirmed by Western blot analysis of PARP cleavage (Fig. 2B, *inset*; data not shown). In contrast, no change in chemotherapy-induced apoptosis was observed following PDF silencing in HCT116 p53-null cells or the p53-mutant cell lines H630 and HT29 (Fig. 2C; data not shown). These results suggest that functional p53 is necessary not only for PDF induction following chemotherapy treatment but also for PDF-mediated protection against drug-induced cell death.

LoVo cells are known to harbor a recessive mutation in furin, an enzyme that seems to be necessary for processing of TGF-β to its mature form (34, 35). Despite the potent up-regulation of PDF propeptide in this cell line in response to chemotherapy (Fig. 1C), no mature secreted PDF was detected (data not shown), suggesting that furin is also important for processing of PDF. Notably, no significant changes in chemotherapy-induced apoptosis were observed following PDF gene silencing in this cell line (Fig. 2D). This suggests that it is the mature form of the protein and not the propeptide that plays an important role in regulating the response to chemotherapy.

To further assess the effect of PDF silencing on chemotherapy-mediated cell death, we used clonogenic survival assays. Colony formation was significantly inhibited in cells transfected with PDF siRNA and cotreated with low doses of oxaliplatin, 5-FU, and, to a lesser extent, SN38 compared

Table 1. IC_{60} doses (72 h) of chemotherapy agents in colorectal cancer cell line panel as determined by MTT assay

	Oxaliplatin (μmol/L)	5-FU (μmol/L)	SN38 (nmol/L)
HCT116 p53 wild-type	1	5	5
HCT116 p53 null	2	20	5
H630	1	5	30
HT29	1	2.5	7.5
LoVo	2	5	20
RKO	2.5	1	7

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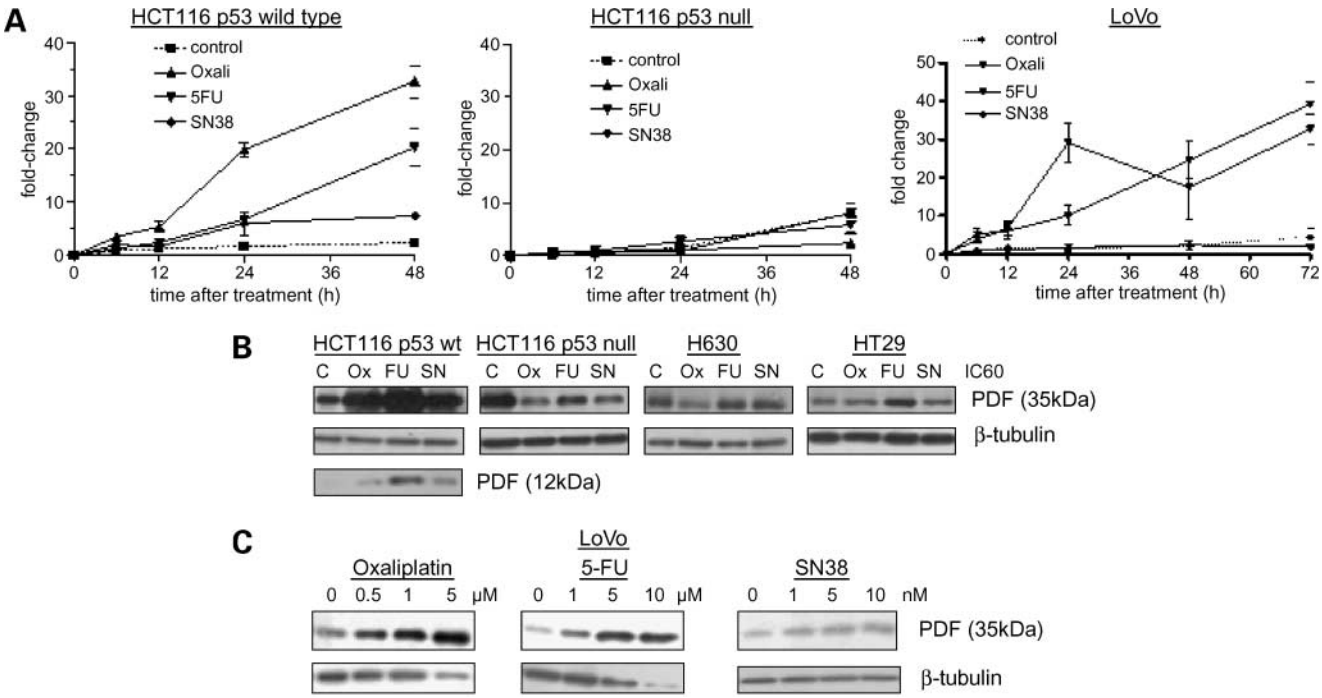

Figure 1. **A,** real-time reverse transcription-PCR analysis of PDF mRNA level in HCT116 p53 wild-type and HCT116 p53-null cells following treatment with IC_{60} doses of oxaliplatin, 5-FU, and SN38 for 0, 6, 12, 24, and 48 h. Real-time PCR analysis of PDF mRNA level in LoVo cells after treatment with 5 μmol/L oxaliplatin, 10 μmol/L 5-FU, and 10 nmol/L SN38 for 0, 6, 12, 24, 48, and 72 h. Data represent mean of three independent experiments. *, $P < 0.05$; **, $P < 0.01$, calculated using a two-tailed unpaired *t* test (GraphPad Prism v4.02) compared with time-matched control. **B,** Western blot analysis of PDF propeptide and mature form expression in HCT116 p53 wild-type, HCT116 p53-null, H630, and HT29 cells following treatment with IC_{60} doses of oxaliplatin, 5-FU, and SN38 for 72 h. The table represents IC_{60} values for each chemotherapeutic agent at 72 h. Values from three independent experiments are shown. **C,** Western blot analysis of PDF protein expression in LoVo cells following treatment with different concentrations of oxaliplatin, 5-FU, and SN38 for 72 h. The results were reproduced in four sets of independent experiments.

with control siRNA–transfected cells (Fig. 2E). In contrast, no significant difference in clonogenic potential was detected in cells transfected with PDF siRNA in the absence of chemotherapeutic agents, suggesting that PDF is only an important regulator of cell survival during the response to treatment with cytotoxic drugs.

Effect of PDF Silencing on Chemosensitivity in HCT116 Drug-Resistant Cells

Next, we examined whether PDF silencing could resensitize a panel of HCT116 drug-resistant cell lines to their respective chemotherapies. As shown in Fig. 3A, the oxaliplatin- and SN38-resistant daughter lines showed an approximate 2-fold decrease in the basal levels of PDF mRNA and protein compared with parental cells, whereas PDF mRNA and propeptide expression in the 5-FU–resistant cell line was comparable with that in parental cells. Following siRNA-mediated PDF knockdown, oxaliplatin-resistant cells were significantly resensitized to oxaliplatin (Fig. 3B). In the 5-FU–resistant cells, a significant increase in apoptosis was observed following PDF silencing in the absence of drug treatment (Fig. 3C). Treatment with increasing concentrations of 5-FU resulted in a moderate increase in apoptosis in the 5-FU–resistant cells; however, the fold change was similar in the SC and PDF siRNA–transfected cells. In the SN38-resistant cell line, no significant changes in apoptosis were observed following exposure to SN38 (Fig. 3D). These results show that PDF silencing can partially resensitize oxaliplatin-resistant cells to oxaliplatin but is not sufficient to reverse the drug resistance in the SN38-resistant model. Interestingly, selection for 5-FU resistance seems to have generated a cell line that is sensitive to loss of PDF expression.

Effect of Inducible PDF Expression on Chemosensitivity in HCT116 Parental Cells

To complement the siRNA studies, we examined the effect of PDF overexpression on chemosensitivity in HCT116 p53 wild-type cells. Following exposure to 0.5 and 5 ng/mL of Tet, expression levels of the PDF propeptide increased by approximately 2- and 20-fold, respectively, in PDF-inducible cell line compared with the HLacZ control cell line (Fig. 4A). The mature form of PDF was also Tet inducible, and similar to the proform, expression levels could be titrated depending on the concentration of Tet (Fig. 4A). No significant change in apoptosis was observed in the PDF-inducible cells following PDF induction (Fig. 4A), indicating that even relatively high levels of PDF overexpression had no effect on spontaneous apoptosis in HCT116 cells. To examine the effect of inducible PDF overexpression on chemotherapy-induced cytotoxicity, control- and PDF-inducible cells were treated with IC_{60} doses of chemotherapy for 72 hours in the

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presence or absence of Tet. Basal PDF expression was low in both the control- and PDF-inducible cell lines in the absence of Tet; however, following treatment with 1 μmol/L oxaliplatin (Fig. 4B), 5 μmol/L 5-FU (Fig. 4C), and 5 nmol/L SN38 (Fig. 4D), PDF was acutely induced in both cell lines by ~5-fold compared with untreated controls. Following treatment with each drug, PARP cleavage was observed, with comparable levels in both the control- and PDF-inducible cell lines. When 5 ng/mL Tet was added to the culture medium, an approximate 10- to 15-fold increase in PDF propeptide expression levels was observed in the PDF-inducible cells compared with control cells, and this induction was enhanced following exposure to each drug. In the presence of 5 ng/mL Tet, PDF-inducible cells were less sensitive to drug treatment compared with HLacZ control cells as shown by significantly attenuated PARP cleavage (Fig. 4B–D).

Effect of Mature Recombinant PDF on Drug-Induced Apoptosis

To further examine the role of PDF in regulating the response of colorectal cancer cells to chemotherapy treatment, we assessed the apoptotic phenotype of LoVo and HCT116 p53-null cells following cotreatment with rhPDF and chemotherapy. These cell lines were selected because LoVo cells are unable to generate mature PDF despite being p53 wild-type, and HCT116 p53-null cells do not up-regulate PDF in response to chemotherapy (Fig. 1). Moreover, chemotherapy-induced apoptosis was not affected by PDF silencing in these cell line models (Fig. 2). As shown in Fig. 5A, addition of 20 ng/mL rhPDF led to a significant decrease in the levels of apoptosis in LoVo cells treated with each chemotherapeutic agent. Similarly, cotreatment of HCT116 p53-null cells with 20 ng/mL rhPDF and each chemotherapeutic agent resulted in a significant decrease in apoptosis compared with chemotherapy only–treated cells (Fig. 5B). Thus, these results further support a role for PDF in blocking chemotherapy-induced apoptosis.

To gain insight into downstream signaling involved in PDF-mediated protection from drug-induced cell death, we evaluated activation of Akt and ERK1/2 in HCT116 p53 wild-type cells following exposure to 20 ng/mL rhPDF, as PDF has been previously shown to activate these key antiapoptotic signaling molecules (11, 23, 24). As shown in Fig. 5C, Akt phosphorylation was detected as early as 30 minutes after treatment and continued to increase up to 6 hours after administration of rhPDF. No change in total Akt level was induced by rhPDF, indicating that Akt was activated following rhPDF treatment. In contrast, no change in ERK1/2 phosphorylation was observed in rhPDF-treated samples (Fig. 5C). To obtain further evidence that Akt is

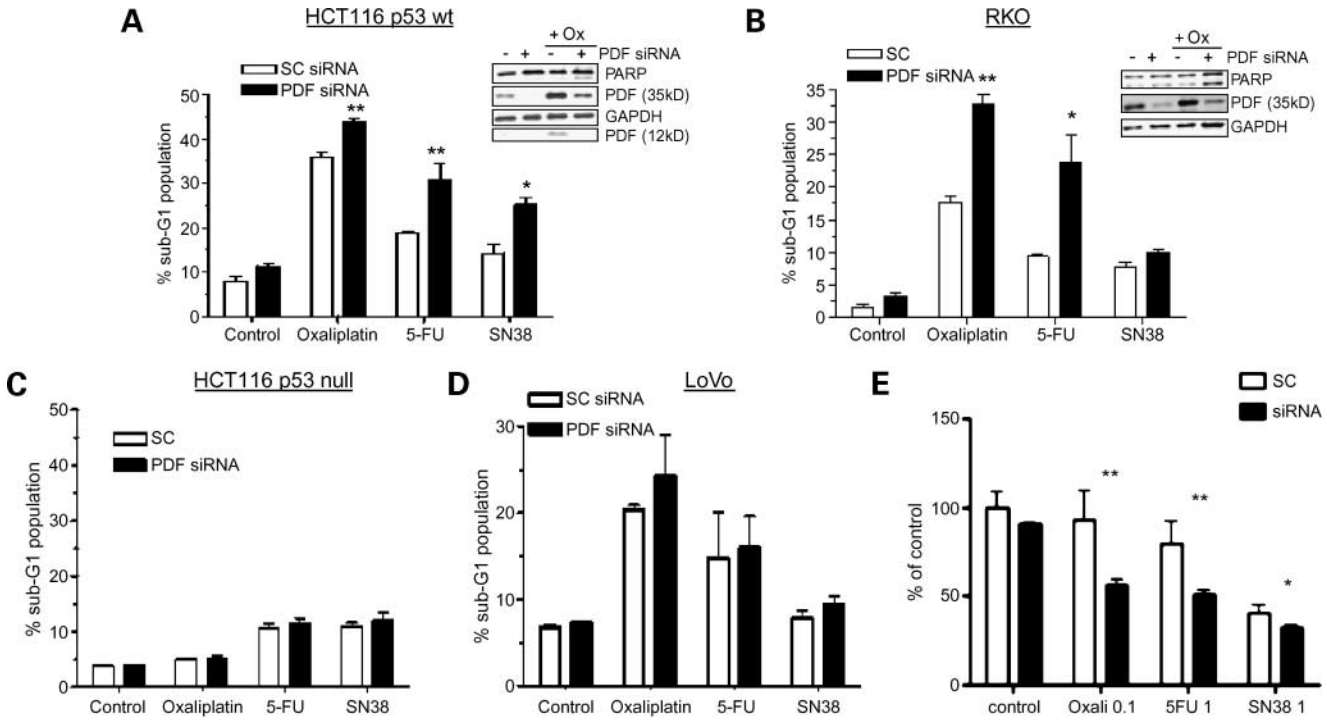

Figure 2. Flow cytometric analysis of apoptosis in HCT116 p53 wild-type (**A**), RKO (**B**), HCT116 p53-null (**C**), and LoVo (**D**) cells following treatment with 1 μmol/L oxaliplatin, 5 μmol/L 5-FU, and 5 nmol/L SN38 for 48 h in combination with 5 nmol/L PDF (*black columns*) or SC (*white columns*) siRNA. Data represent mean of three independent experiments. *, $P < 0.05$; **, $P < 0.01$, calculated using a two-tailed unpaired *t* test (GraphPad Prism v4.02). Western blot insets in **A** and **B** assess PARP cleavage and PDF knockdown after siRNA transfection alone or in combination with 1 μmol/L oxaliplatin for 48 h in HCT116 p53 wild-type (**A**) and RKO cells (**B**). The data are representative of three independent experiments. **E,** clonogenic cell survival assay following SC (*white columns*) or PDF siRNA (*black columns*) transfection and chemotherapy treatment in HCT116 p53 wild-type cells. The data are plotted as a percentage of SC without chemotherapeutic agent. The results are representative of three separate experiments.

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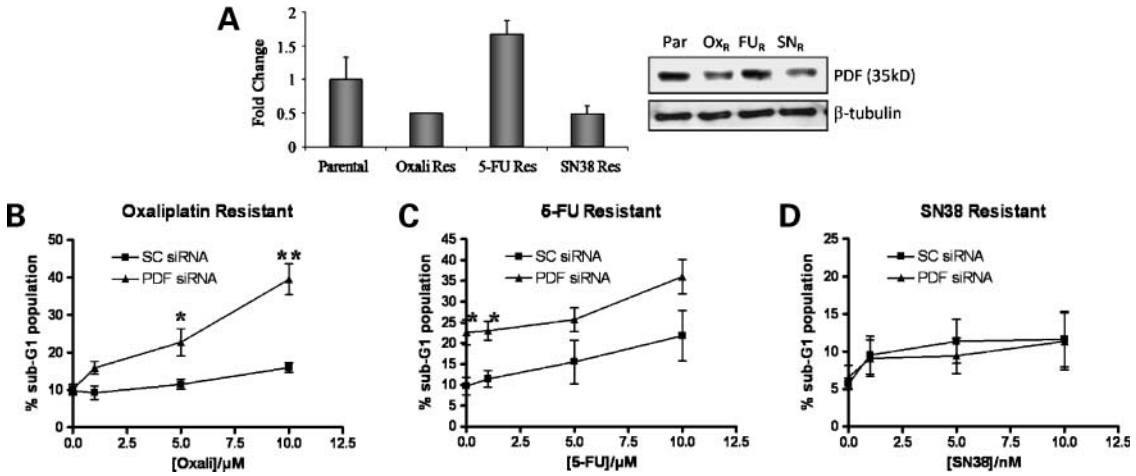

Figure 3. **A,** real-time reverse transcription-PCR analysis of PDF mRNA and Western blot analysis of basal PDF protein (proform) expression levels in HCT116 oxaliplatin–resistant, 5-FU–resistant, and SN38-resistant cells. *Line graphs,* fraction of apoptotic cells (as measured by flow cytometry) following treatment of HCT116 oxaliplatin–resistant (**B**), 5-FU–resistant (**C**), and SN38-resistant cells (**D**) with oxaliplatin, 5-FU, and SN38, respectively, for 48 h in combination with PDF or SC siRNA. Data represent mean of five independent experiments. *, $P < 0.05$; **, $P < 0.01$, calculated using a two-tailed unpaired *t* test (GraphPad Prism v4.02).

involved in PDF downstream signaling, we used the PI3K inhibitor wortmannin. As expected, addition of rhPDF resulted in a significant decrease in oxaliplatin-induced apoptosis in LoVo cells (Fig. 5D). Cotreatment with wortmannin enhanced oxaliplatin-induced apoptosis, suggesting that the PI3K pathway plays an important role in regulating sensitivity to oxaliplatin in this cell line. Notably, in the presence of wortmannin, rhPDF was no longer able to abrogate oxaliplatin-induced apoptosis. These findings were supported by Western blot analysis, which revealed increased Akt phosphorylation following oxaliplatin treatment that was enhanced on cotreatment with PDF. Addition of wortmannin abrogated the activation of Akt induced by both oxaliplatin and rhPDF alone and the activation induced by combined treatment with both agents (Fig. 5D). These results suggest that PDF-induced resistance to apoptosis is mediated by its activation of the PI3K/Akt survival pathway.

Discussion

The divergent TGF-β superfamily member, PDF, was recently identified in a DNA microarray screen carried out in our laboratory designed to identify novel panels of genes that may regulate resistance to 5-FU and oxaliplatin in colon cancer cells (36). A clear p53-dependent expression pattern was shown in a panel of colorectal cell lines. Time course experiments showed that PDF mRNA and propeptide levels were highly inducible in p53 wild-type cells (HCT116 p53 wild-type, RKO, and LoVo) following acute exposure to oxaliplatin, 5-FU, and SN38. In contrast, drug-induced PDF expression was shown to be completely abrogated in the isogenic p53-null HCT116 daughter cell line and the p53-mutant cell lines H630 and HT29. These observations agree with several published studies showing p53-dependent PDF induction following treatment with several antitumorigenic compounds (5, 18, 19, 37, 38). The modest up-regulation of PDF protein expression in p53-mutant cell lines (H630 and HT29) following treatment with 5-FU suggests that 5-FU may induce PDF expression in these cell lines via p53-independent mechanisms, as has been reported for other compounds (5, 39, 40), or that some p53-mutant proteins can retain some ability to induce PDF expression in response to certain drugs.

In our study, PDF silencing in the absence of chemotherapy did not significantly alter the basal apoptotic phenotype of HCT116 cells. However, PDF silencing before treatment with oxaliplatin, 5-FU, and, to a lesser extent, SN38 significantly sensitized HCT116 cells to chemotherapy-induced apoptosis. These data suggest that although basal PDF expression is not critical for maintaining cell viability, acute up-regulation of PDF may protect cells from drug-induced apoptosis. In contrast, no sensitization to drug-induced apoptosis was detected in p53-mutant or p53-null cell lines following PDF silencing. Data from the furin-deficient cell line LoVo pointed to the importance of mature secreted form of the protein in mediating drug resistance because PDF silencing had no effect on chemotherapy-induced death in this cell line despite up-regulation of the PDF propeptide following drug treatment.

Further evidence for the role of PDF in modulating the response to chemotherapy treatment was obtained using a PDF-inducible expression system. No change in the levels of spontaneous cell death was observed following inducible PDF expression in the absence of chemotherapy cotreatment. This contrasts with an earlier study, which reported enhanced apoptosis in HCT116 cells overexpressing PDF (5). However, this earlier study used a mixed population of transfected HCT116 cells rather than an inducible model, and the difference in apoptosis between PDF- and control-transfected cells was small (<4%). Moreover,

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another study reported a significant increase in growth rate as well as clonogenic potential in PDF-overexpressing prostate cancer cells (24). Notably, inducible PDF overexpression was shown to significantly attenuate chemotherapy-induced apoptosis. Consistent with our findings from the inducible PDF models, we found that addition of exogenous recombinant PDF led to significantly decreased chemotherapy-induced apoptosis, indicating that it is the mature form of PDF that contributes to acute drug resistance. Similar results were reported by Huang and colleagues (23), who showed that rhPDF induced a cytoprotective effect in mitoxantrone- and docetaxel-treated prostate cancer cells. However, our findings contrast with those reported by Shim and Eling (6), who found that PDF knockdown protected LNCaP prostate cells from 12-*O*-tetradecanoylphorbol-13-acetate–induced apoptosis, suggesting that the effects of PDF silencing on apoptosis may be context specific.

To identify the possible downstream factors involved in PDF signaling, we analyzed the activation of Akt and ERK1/2, two major prosurvival signaling molecules. Drug-induced and constitutive activation of Akt and ERK1/2 has been implicated in acquired and intrinsic multidrug resistance in several malignancies (reviewed in refs. 41, 42). We found that PDF seems to act primarily through the PI3K/Akt pathway. This finding is consistent with reports from other groups showing that PDF can activate the PI3K/Akt pathway (43, 44). Additionally, PDF has recently been shown to transactivate the ErbB2 receptor, which could subsequently activate the PI3K pathway (25). In contrast to other published studies, we did not find any evidence of ERK1/2 activation after treatment with rhPDF (11, 24, 45). It cannot be excluded, however, that PDF activates other prosurvival signaling cascades, and experiments are currently under way to identify other possible pathways involved in modulating PDF-induced chemoresistance.

In the present study, we found that PDF silencing induced spontaneous cell death in HCT116 5-FU–resistant cells in the absence of drug and partially resensitized oxaliplatin-resistant cells to drug-induced apoptosis. These data suggest that as a result of selection in chemotherapy, the 5-FU–resistant cells may have become reliant (at least in part) on PDF expression to survive. These data further suggest a protective role for PDF in regulating drug response and, moreover, that PDF may be targeted to resensitize or induce apoptosis in drug-resistant cells. Consistent with our findings, PDF overexpression is involved in resistance to cisplatin (22) as well as in docetaxel and mitoxantrone resistance (23). In addition, PDF was reported as one of two genes consistently up-regulated in biopsies from

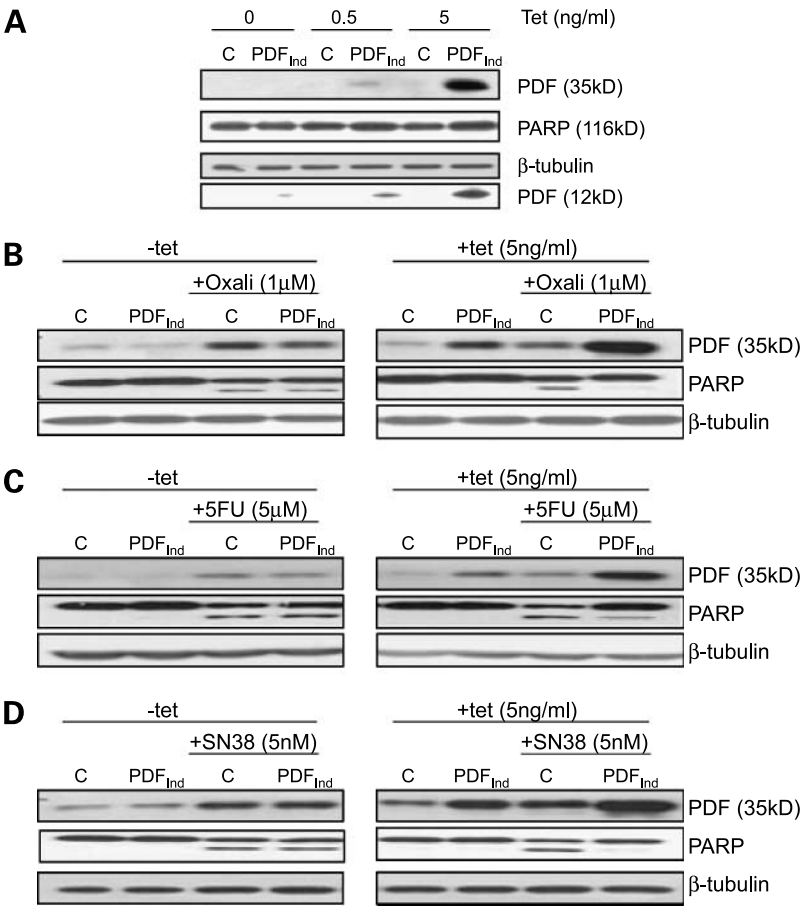

Figure 4. **A,** Western blot analysis of PDF propeptide and PARP expression and mature PDF levels following treatment of HCT116 HLacZ (*C*) and PDF-inducible (PDF_{ind}) cells with 0, 0.5, and 5 ng/mL of Tet for 72 h. Western blot analysis of PDF propeptide and PARP expression levels following treatment of HCT116 HLacZ (*C*) and PDF-inducible (PDF_{ind}) cells with or without 5 ng/mL Tet for 72 h in the presence or absence of 1 μmol/L oxaliplatin (**B**), 5 μmol/L 5-FU (**C**), and 5 nmol/L SN38 (**D**). The results were reproduced in three sets of independent experiments.

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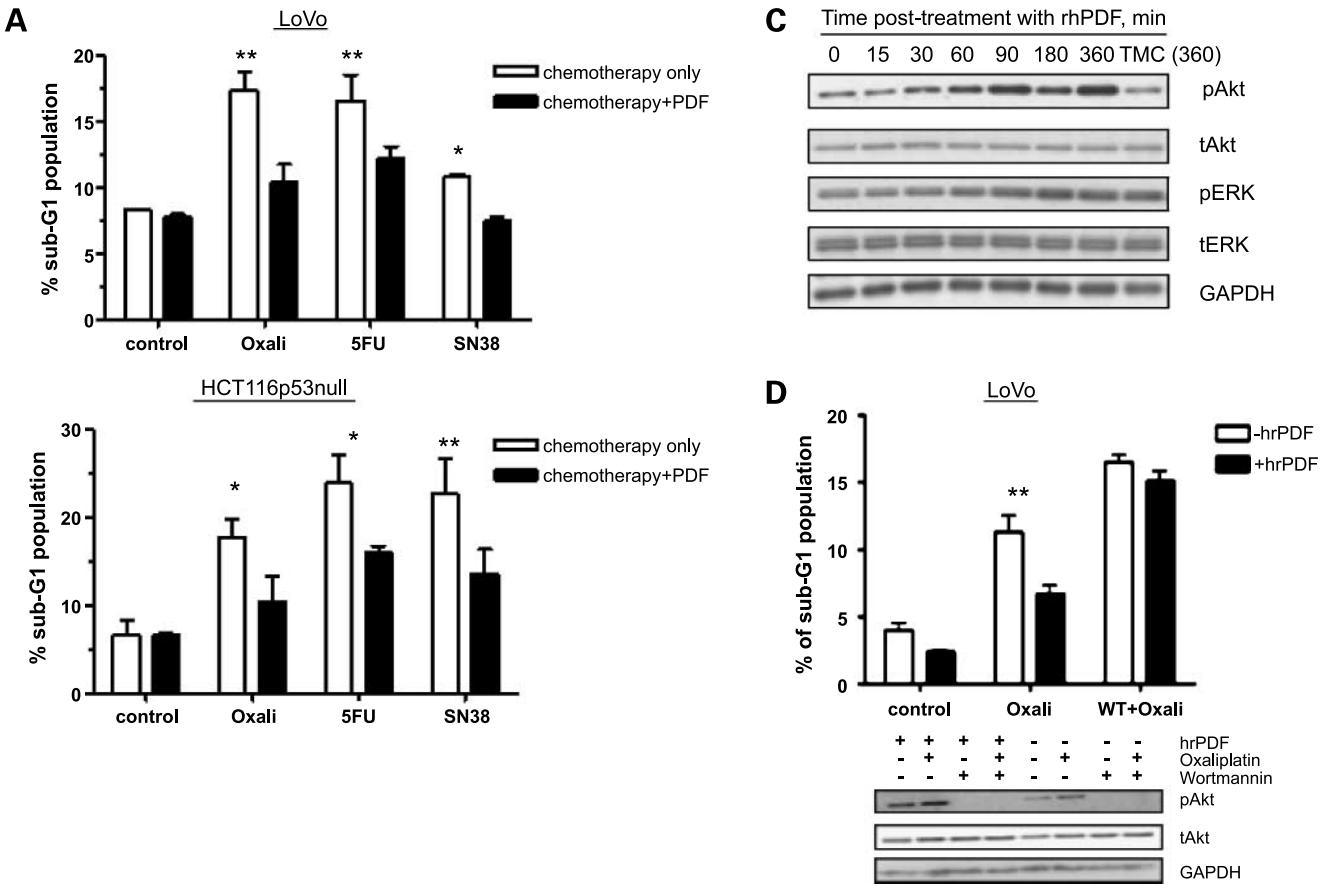

Figure 5. Levels of apoptosis, as measured by the percentage of cells in sub–G_0-G_1, were analyzed by flow cytometry following treatment of LoVo (**A**) and HCT116 p53-null (**B**) cells with IC_{60} doses of oxaliplatin, 5-FU, and SN38 only (*white columns*) or in combination with 20 ng/mL rhPDF (*black columns*) for 24 h. Data represent mean of three independent experiments. *, $P < 0.05$; **, $P < 0.01$, calculated using a two-tailed unpaired *t* test (GraphPad Prism v4.02). **C,** Western blot of cell lysates from HCT116 p53 wild-type cells stimulated with 20 ng/mL rhPDF for 0 h, 15 min, 30 min, 1 h, 1.5 h, 3 h, and 6 h in serum-free medium and untreated cells (time-matched control). The results were reproduced in three sets of independent experiments. **D,** flow cytometry assay in LoVo cells after exposure to oxaliplatin or oxaliplatin in combination with wortmannin treatment in the presence (*black columns*) or absence (*white columns*) of 20 ng/mL rhPDF for 24 h. The drug concentrations were oxaliplatin (1 μmol/L) and wortmannin (100 nmol/L). Data represent mean of three independent experiments. **, $P < 0.01$, calculated using a two-tailed unpaired *t* test (GraphPad Prism v4.02). Western blot analysis of phospho-Akt (*pAkt*) and total Akt (*tAkt*) in LoVo cells following treatment with 20 ng/mL rhPDF, 1 μmol/L oxaliplatin, and 100 nmol/L wortmannin for 24 h.

breast cancer patients after treatment with epirubicin/cyclophosphamide and epirubicin/Taxol (46).

In conclusion, the results presented in this study indicate that PDF may be a novel inhibitor of drug-induced cell death in colon cancer cells. Thus, PDF may represent a biomarker of chemoresistance and/or represent a novel therapeutic target in this disease. Future studies will be directed to further elucidate the mechanism by which PDF enhances drug resistance.

Disclosure of Potential Conflicts of Interest

P.G. Johnston: founder and Director, Almac Diagnostics; grant support, Amgen; consultant, Pfizer, Amgen, GlaxoSmithKline, and Roche. No other potential conflicts of interest were disclosed.

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