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Polyethylene Glycosylated Curcumin Conjugate Inhibits Pancreatic Cancer Cell Growth through Inactivation of Jab1

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ABSTRACT

Jab1 (Jun activation domain binding protein 1), integrated into COP9 signalosome complex (CSN), induces protein instability of many tumor suppressors and cell cycle regulators and is therefore a novel target in cancer therapy. Curcumin, an inhibitor of Jab1/CSN-associated kinase(s), has been reported to suppress tumor growth; however, curcumin is highly hydrophobic, and this feature prevents its usage as an antitumor drug. To increase the solubility and targeted delivery, we generated a water-soluble polyethylene glycol (PEG)-conjugated curcumin system, in which curcumin is covalently linked to PEG_{35kD}. PEGylated curcumin showed much greater reduction of cell growth than free curcumin in pancreatic cancer cells. Cells treated with PEGylated curcumin had increased arrest at the mitotic phase with the formation of abnormal multinucleated cells, indicating that this compound affects cell cycle progression, which may contribute to cell growth inhibition. The stabilities of Jab1 target proteins were also examined. PEGylated curcumin increased protein stability of these proteins in pancreatic cancer cells and directly inhibited the activity of Jab1/CSN-associated kinases. Moreover, the inhibitory effect of PEGylated curcumin on cell proliferation was blunted in pancreatic cancer cells with Jab1 knockdown. The results suggest that PEGylated curcumin inhibits cell proliferation through suppression of Jab1/CSN activity. More importantly, the new compound sensitized pancreatic cancer cells to gemcitabine-induced apoptosis and cell proliferation inhibitory effects. Collectively, the PEGylated curcumin conjugate has much more potent effects on pancreatic cancer cell growth inhibition than free curcumin. The current study provides a biologic rationale to treat patients with pancreatic adenocarcinoma with the nontoxic phytochemical conjugated to PEG for systemic delivery.

Pancreatic ductal adenocarcinoma represents greater than 80% of all pancreatic neoplasms with a death/incidence ratio of approximately 0.99 (Farrow and Evers, 2004; Brand and Mahr, 2005). Although gemcitabine currently is the most commonly used drug for treatment of pancreatic cancer (Burris et al., 1997; Hui and Reitz, 1997), only marginal improvements on survival and tumor response to this drug have been reported. One of the reasons for the low survival rate is the poor responses of pancreatic cancer cells to chemotherapy or radiotherapy. Therefore, the mechanisms responsible for the loss of growth regulation in pancreatic carcinoma cells should be defined, and new treatments based on a better understanding of the biology of pancreatic cancer must be established.

Steps in the genetic basis of pancreatic ductal adenocarcinoma have been identified recently, with the identification of the activation of the oncogene Kras and inactivation of the tumor suppressor genes p16INK4a, p53, and DPC4 (deleted in pancreatic carcinoma locus 4) as characteristic features of invasive pancreatic cancer (Hruban et al., 2001). The protein instability of DPC4, which is also known as Smad4, is a common phenomenon in pancreatic carcinoma cells (Wan et al., 2005) and is strongly correlated with pancreatic tumorigenesis and patient survival (Biankin et al., 2002; Hua et al., 2003). Our previous studies demonstrated that Jab1 (Jun activation domain binding protein 1) plays a key role in inducing protein degradation of DPC4/Smad4 (Wan et al.,

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ABBREVIATIONS: DPC4, deleted in pancreatic carcinoma locus 4; Jab1, Jun activation domain binding protein 1; CSN, COP9 signalosome complex; PEG, polyethylene glycol; siRNA, small interfering RNA; BrdU, 5-bromo-2′-deoxyuridine; GFP, green fluorescent protein; PBS, phosphate-buffered saline; HA, hemagglutinin; MTT, 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PE, phycoerythrin; 7-AAD, 7-amino-actinomycin D; DCC, *N*,*N*′-dicyclohexylcarbodiimide; CK2, casein kinase 2; PKD, protein kinase D.

2002). Jab1 is also known as CSN5 as it is the fifth component of the COP9 signalosome complex (CSN) (Naumann et al., 1999; Seeger et al., 1999). Jab1-integrated CSN also induces degradation of the tumor suppressor p53 (Bech-Otschir et al., 2001) and the cell cycle inhibitors $p27^{kip1}$ (Tomoda et al., 1999) and $p21^{cip1}$ (Peng et al., 2003), all of which are actively involved in suppressing the development of pancreatic cancer. Therefore, Jab1 in the CSN acts as a negative regulator of important pancreatic tumor suppressors by targeting them for degradation. Recent work suggests that the Jab1/CSN possesses kinase activity that phosphorylates proteins such as c-Jun and p53 with consequence for their protein stabilization (Naumann et al., 1999) or ubiquitin-dependent degradation (Bech-Otschir et al., 2001; Sun et al., 2002; Uhle et al., 2003). Curcumin and emodin, two natural plant-derived compounds, have been identified as Jab1/CSN inhibitors as they potently inhibit the kinase activity of Jab1/CSN and have been shown to enhance the stability of the p53 protein (Bech-Otschir et al., 2001; Sun et al., 2002; Uhle et al., 2003). These results indicate that blocking of Jab1/CSN kinase activity causes stabilization of the tumor suppressors and cell cycle regulators and leads to the suppression of pancreatic tumor growth. It is noteworthy that the level of Jab1 expression is significantly elevated in several human malignant cancers, providing additional evidence for the role of Jab1 in tumorigenesis (Sui et al., 2001; Korbonits et al., 2002; Fukumoto et al., 2004).

Curcumin (diferuloylmethane), a derivative of the spice turmeric (*Curcuma longa*), is nontoxic to humans (Maheshwari et al., 2006) and has been widely studied for its anti-inflammatory, antiangiogenic, antioxidant, and anticancer effects and its promotion of wound healing. This compound has antiproliferative and proapoptotic effects in many cancer cell lines and has tumor suppression effects in several tumor animal models. Multiple molecular mechanisms have emerged to elucidate its diverse biological effects. Curcumin has been shown to suppress NF-κB activation (Kunnumakkara et al., 2007), activate caspases, and inhibit the expression of Bcl-2 and Bcl-xL and cellular inhibitor of apoptosis protein-1 (Sharma et al., 2005). Curcumin also affects cell proliferation, angiogenesis, and metastasis by regulating the expression of a variety of related downstream genes (Sharma et al., 2005). The inhibitory effect of curcumin on Jab1/CSN kinase activity leads to stimulation of tumor suppressors, which may be an important pathway for its antitumor effects. Curcumin is highly hydrophobic and cannot be administered systemically. In addition, the bioavailability of oral curcumin is poor. Therefore, better strategies for the systemic delivery of this compound have to be developed. An approach that may solve the above problems of the usage of curcumin as an antitumor drug is the use of its conjugate with polyethylene glycol (PEG) (Greenwald et al., 1996, 2003). PEG is a water-soluble amphiphilic polymer showing excellent biocompatibility and is frequently used in biomedical applications. A low-molecular-weight drug conjugated to higher molecular weight PEG (>20,000) results in high aqueous solubility, slower clearance, reduced systemic toxicity, and efficient accumulation in tumors through enhanced permeability and retention. Several small organic molecules, such as camptothecin, doxorubicin, and paclitaxel, have been conjugated to PEGs and these conjugates especially camptothecin are in clinical trials (Greenwald et al., 2003).

In the present study, we demonstrated that Jab1 overexpression increased pancreatic cancer cell growth and protein degradation of Jab1 target proteins, whereas Jab1 gene silencing by siRNA suppressed pancreatic cancer cell proliferation. PEGylated curcumin inhibited cell proliferation in pancreatic cancer cells and exhibited much greater effect on reduction of proliferation than free curcumin. This compound inhibited the activity of Jab1-associated kinases and changed protein stability of Jab1 target proteins, and the cell-growth inhibitory effect of this compound was blunted in Jab1 knockdown cells. The results suggest that PEGylated curcumin suppresses pancreatic cancer growth at least partially through inactivation of Jab1. In addition, PEGylated curcumin-sensitized pancreatic cancer cells to gemcitabine-induced apoptotic and cell growth inhibitory effects and therefore has the potential to be developed as an antipancreatic cancer drug.

Materials and Methods

Cell Culture, Antibodies, and Reagents. PANC-1, AsPC-1, MiaPaCa-2, and BxPC-3 human pancreatic cancer cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in either Dulbecco's modified Eagle's medium or RPMI 1640 media with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. All cells were maintained in 100-mm tissue culture dishes in a 37°C incubator equilibrated with 5% CO_2 in humidified air. Monoclonal antibodies recognizing human Smad4 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). 5-Bromo-2′-deoxyuridine (BrdU) and monoclonal anti-p53 (clone BP53) were from Calbiochem (San Diego, CA). Monoclonal anti-p27 (clone 1B4) was from Vector Laboratories (Burlingame, CA). Monoclonal anti-BrdU (clone BUZ0a) was from Dako North America, Inc. (Carpinteria, CA). Curcumin was purchased from Sigma Chemical Co. (St. Louis, MO).

Virus Infection. Generation and titration of retroviral supernatants were performed as described previously (Wan et al., 2005). PANC-1 was infected with retrovirus vector containing pMSCVneo-GFP, pMSCVneo/HA-Jab1, pMSCVneo/U6-GFP (siGFP), or pMSCVneo/U6-Jab1 (siJab1) as described above. For infection, the virus-containing supernatant in the presence of 4 μg/ml Polybrene (Sigma) was added to the culture medium. Six days after infection, the efficiency was assayed by immunoblotting.

Western Blot Analysis. Cell lysates in radioimmunoprecipitation assay buffer (10 mM PBS, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 5 mM EDTA) were prepared and all the samples were measured for total protein content using a BCA assay (Pierce, Rockford, IL). Equal amounts of protein samples were loaded onto 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). The blots were blocked in 5% skim milk in 10 mM Tris-HCl, 150 mM NaCl, and 0.5% Tween 20 for 2 h and then incubated overnight at 4°C with primary antibody. The blots were washed and incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After washing, bands were detected on light sensitive film using enhanced chemiluminescence (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

Clonogenic Assays. PANC-1 cells were seeded at 1.0×10^5 cells/well of a six-well plate and infected the next day with either GFP (vector control) or HA-Jab1. In separate experiments, cells were infected with either siGFP (siRNA control) or siJab1. Twenty-four hours after infection, the cells were washed in PBS and trypsinized. Cells were seeded in 6-cm plates at 1000 cells/well for the cells with GFP or Jab1 overexpression and 1500 cells/well for the cells with siGFP or siJab1, and the colonies were grown for 10 days. For compound treatment, cells were seeded in 6-cm plates at 5000 cells/

well and incubated with different concentration of PEGylated curcumin for 7 days. The colonies were stained with 2% crystal violet and counted. Only colonies containing >50 cells were counted. The survival fractions of HA-Jab1- or siJab1-transfected cells were normalized to GFP- or siGFP-transfected cells. For compound treatment, the survival fractions of PEGylated curcumin-treated cells were normalized to vehicle-treated control cells.

MTT Assays. Cellular proliferation was assayed by the 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) method. In brief, after transfection, 50 μl of MTT solution (5 mg/ml) was added to the culture medium. After 4 h at 37°C, the medium was removed, and 50 μl of acidified isopropanol was added to each well. The color was allowed to develop for 5 min, and optical density at 570 nm was determined with a microplate reader (Bio-Rad Laboratories). The mean value and S.E. for each treatment were determined, then converted to a percentage relative to control. IC_{50} was determined by linear interpolation using the formula [(50% − low percentage)/(high percentage − low percentage)] × (high concentration − low concentration) + low concentration.

Apoptosis Assays. Apoptotic cells were detected by dual staining with Annexin V-phycoerythrin (PE) and 7-amino-actinomycin D (7-AAD) using a commercially available kit (BD Pharmingen, San Diego, CA) following the manufacturer's protocol. In brief, cells were washed in PBS and resuspended in binding buffer (0.1 M HEPES/NaOH, pH 7.4, 1.4 M NaCl, and 25 mM $CaCl_2$), and the pellet was resuspended in 5 μl of Annexin V-PE, 5 μl of 7-AAD and 100 μl of binding buffer, followed by incubation for 15 min at room temperature in the dark. Measurement was performed using a FACS Canto II System scanner (BD Biosciences, San Jose, CA). A predetermined count of 10,000 cells was set on forward light scatter. Annexin V-PE was measured in FL-2 detector and 7-AAD in FL-3 detector. Only cells with an intact cell membrane [i.e., 7-AAD (−) and Annexin V-PE (+)] were considered apoptotic.

Immunocytochemistry Analysis. Cells were grown on glass coverslips in six-well plates to 50% confluence, rinsed in PBS, and fixed in neutral-buffered formalin (Thermo Fisher Scientific, Waltham, MA) overnight at 4°C. The cells were permeabilized with acetone for 15 s. Endogenous peroxidases were quenched using an aqueous solution of 3% H_2O_2. Goat serum (3%) was added to block nonspecific immunostaining. The coverslips were incubated with the appropriate primary monoclonal antibody, with p27 antibody at 1:10 and BrdU antibody at 1:60. The antibody-antigen complex was visualized using a 3,3′-diaminobenzidine substrate kit (Biogenex, San Ramon, CA). The coverslips were then counterstained lightly with hematoxylin for the evaluation of p27 and BrdU expression. The positively stained nuclei and negative nuclei on photographs from four random fields of view for each coverslip were counted. The percentage of stained nuclei was estimated by dividing positive nuclei by total nuclei.

Design and Synthesis of PEG-Curcumin Conjugate: PEG_{35kD} Diacid. The diacid was prepared by using a modification of a previously reported procedure. Specifically, PEG_{35kD} (3.5 g, 0.1 mmol) was dried via azeotropic distilllation with toluene (2 × 10 ml) and then dissolved in 20 ml of anhydrous toluene. Potassium *tert*-butoxide (1 M in *tert*-butanol, 0.3 ml) was added to this solution. The mixture was stirred for 1 h at room temperature followed by the addition of ethyl bromoacetate (122 μl, 1.1 mmol). The solution was heated to reflux for 1 h and then cooled to room temperature. Toluene was removed under vacuum. The white precipitate was dissolved in water and extracted with methylene chloride (20 ml × 3). The organic layers were combined, washed with brine (30 ml), and concentrated to afford the PEG_{35kD} diethyl ester (3.5 g, 100%). Characteristic ^{1}H NMR peaks were evaluated: 4.21 (q, J = 7.1 Hz, 4 H), 4.15 (s, 4 H), and 1.29 (t, J = 7.1 Hz, 4 H).

The PEG_{35kD} diester (3.5 g, 0.1 mmol) was dissolved in 15 ml of sodium hydroxide (1 N) and stirred at room temperature for 4 h. The solution was adjusted to pH 3 to 4 with HCl (2 M) and extracted with

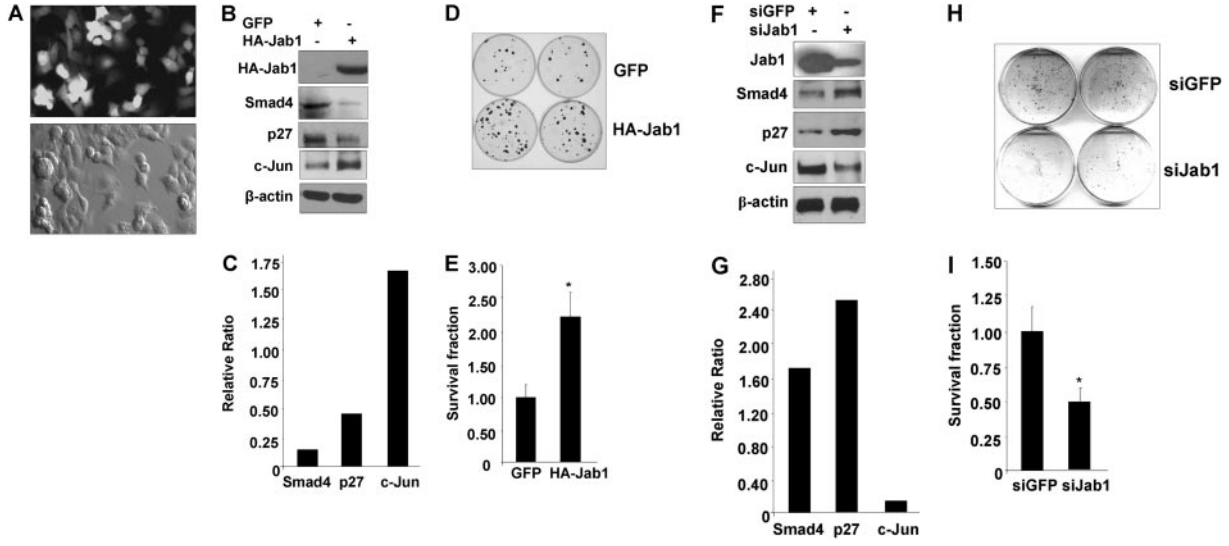

Fig. 1. Jab1 overexpression increases and Jab1 gene silencing by siRNA suppresses pancreatic cancer cell growth. A, GFP is efficiently overexpressed in PANC-1 cells. PANC-1 cells were infected with a retrovirus containing pMSCVneo-GFP. Green light (top) representing GFP expression. Bottom, cell density. B and C, Jab1 overexpression decreases endogenous Smad4 and p27 expression and increases c-Jun level. PANC-1 cells were infected with virus containing GFP or HA-Jab1. Cells were harvested and expression levels of Jab1, Smad4, p27, c-Jun, and β-actin were measured by Western blot analysis with antibodies against HA, Smad4, p27, c-Jun, or β-actin (B). The intensity of the bands in B was quantified by filmless autoradiographic analysis, and the ratio of protein level in Jab1 overexpressed cells to that in control cells was calculated (C). D and E, Jab1 overexpression increases colony numbers. PANC-1 cells were infected with virus containing GFP or HA-Jab1. Cells were reseeded in 6-cm plates at 1000 cells/well and colony numbers were photographed (D) and counted (E). The survival fractions of HA-Jab1-transfected cells were normalized to GFP-transfected cells. The values represent means ± S.D. from three independent experiments. *, $p < 0.005$, compared with control. F and G, suppression of Jab1 by retroviral delivery of siRNA increases endogenous Smad4 and p27 expression and decreases c-Jun level. PANC-1 cells were infected with virus containing siGFP or siJab1. Cells were harvested and expression levels of Jab1, Smad4, p27, c-Jun, and β-actin were measured by Western blot analysis with antibodies against HA, Smad4, p27, c-Jun, or β-actin (F). The intensity of the bands in F was quantified by filmless autoradiographic analysis, and the ratio of protein level in cells with Jab1 knockdown to that in control cells was calculated (G). H and I, Jab1 knockdown reduces colony numbers. PANC-1 cells were infected with virus containing siGFP or siJab1. Cells were reseeded in 6-cm plates at 1500 cells/well, and colony numbers were photographed (H) and counted (I). The survival fractions of siJab1-transfected cells were normalized to siGFP-transfected cells. The values represent means ± S.D. from three independent experiments. *, $p < 0.005$ compared with control.

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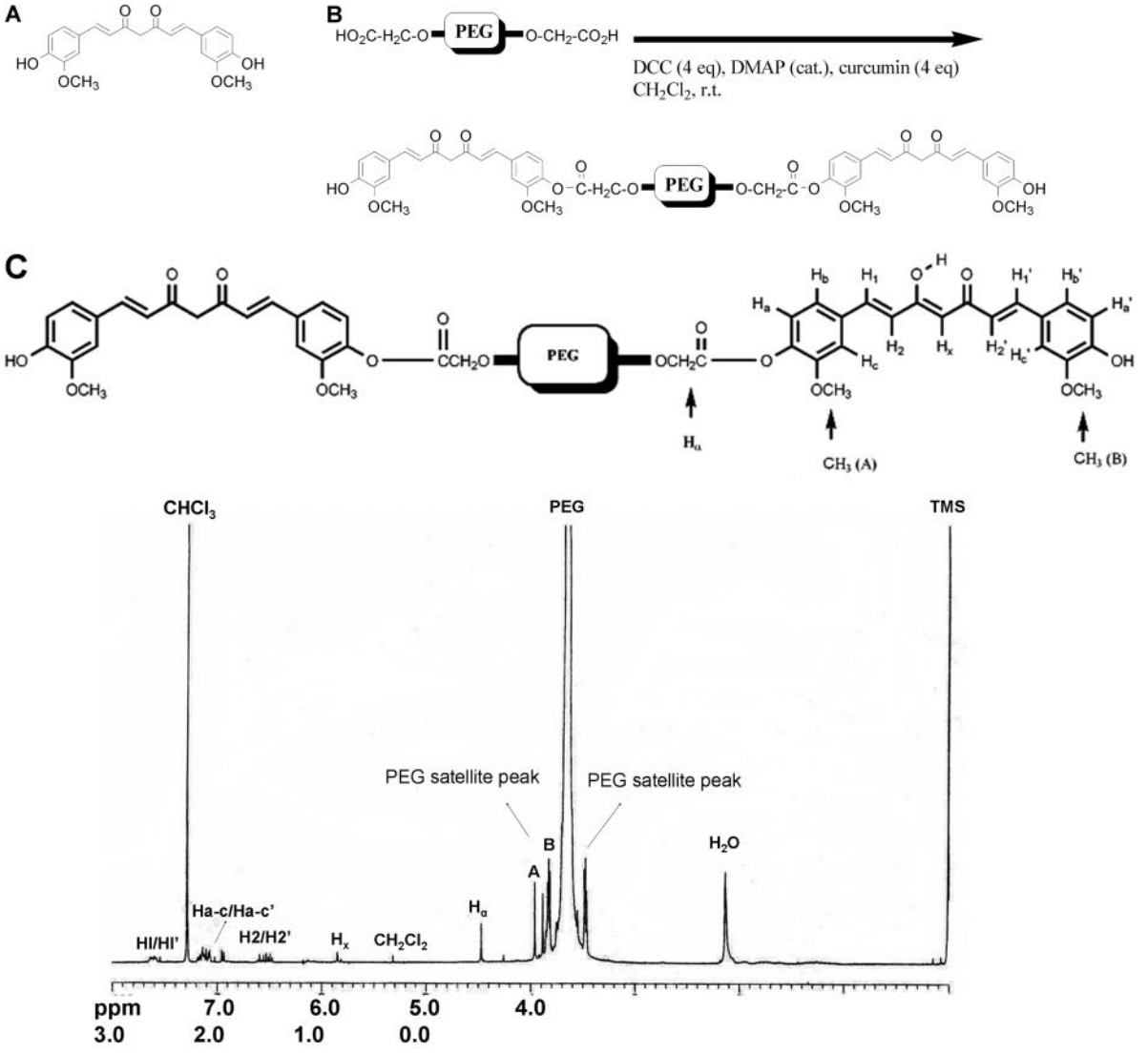

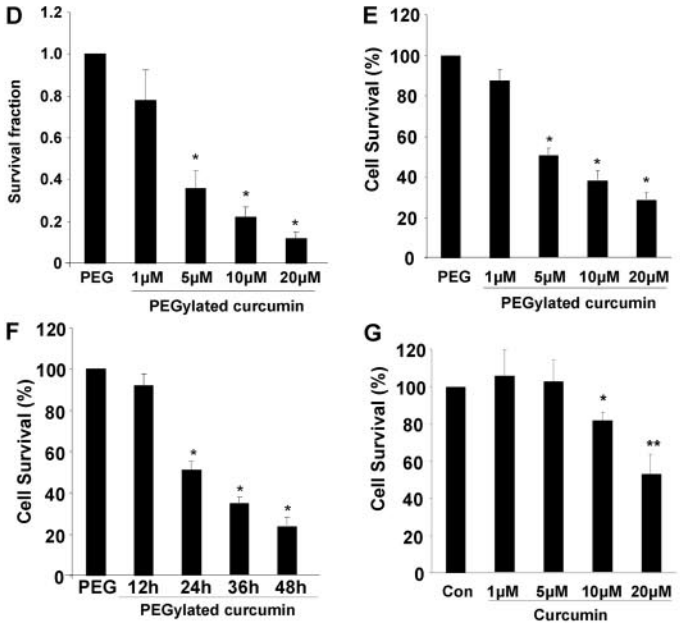

methylene chloride (20 ml × 3). The combined organic solutions were washed with water, brine, and concentrated to 5 ml of residue, to which diethyl ether was added to crystallize the PEG_{35kD} diacid (3.1 g, 88%). Characteristic ^1H NMR peak: 4.16 (s, 4 H).

Curcumin-PEG_{35kD} Conjugate. The PEG_{35kD} diacid (3.2 g, 0.09 mmol) and curcumin (135 mg, 0.36 mmol) in a flame-dried reaction flask were dried via azeotropic distillation with anhydrous toluene (10 ml × 2). 4-(*N*,*N*-Dimethylamino)pyridine (1 mg) was then added, followed by 20 ml of freshly distillated methylene chloride. To the resulting yellow suspension, DCC (76 mg, 0.36 mmol) was added at room temperature, and the mixture was stirred for 2 h. The reaction mixture was concentrated to 5 ml of residue and recrystallized with methylene chloride/ethyl ether (three times) to provide the curcumin-PEG_{35kD} conjugate (2.9 g, >90%). Characteristic ^1H NMR peaks were evaluated: 7.62 (d, J = 15.6 Hz, 2 H), 7.61 (d, J = 15.6 Hz, 2 H), 6.93 (d, J = 8.2 Hz, 2 H), 6.56 (d, J = 15.8 Hz, 2 H), 6.50 (d, J = 15.8 Hz, 2 H), 5.84 (s, 2 H), 4.46 (s, 4 H), 3.96 (s, 6 H), and 3.88 (s, 6 H). The purity of the PEG-conjugate was assessed by proton NMR and known reactions. More than 90% of the carboxyl groups from the PEG-diacid were linked to curcumin, and the other of the COOH groups reacted with the coupling reagent *N*,*N*′-dicyclohexylcarbodiimide (DCC) to form a urea type moiety. The PEGylated curcumin has a characteristic peak at 4.5 ppm (labeled as Hα on NMR), whereas the corresponding proton at the end bonded to DCC has a chemical shift at 4.2 ppm). The ratio was estimated on the basis of integration. For ^1H NMR, chemical shifts are expressed in parts per million (δ scale) downfield from tetramethylsilane and are referenced to residual protium in the NMR solvent ($CHCl_3$: δ 7.26). Data were presented as follows: chemical shift, multiplicity, coupling constant (in Hertz), integration.

Statistical Analysis. Results are presented as the mean ± S.D. Data were analyzed by Student's t test, and statistical significance was accepted at $P < 0.05$. Each experiment was repeated independently at least three times.

Results

Jab1 Is Both Sufficient and Necessary for Pancreatic Cancer Cell Growth. To determine the function of Jab1 in pancreatic cancer cells, we overexpressed Jab1 by infection of PANC-1 cells with a retrovirus containing pMSCVneo-HA-Jab1 and used pMSCVneo-GFP (control). Two stable cell lines (PANC-1-Jab1 and PANC-1-GFP) have been generated by infecting PANC-1 cells with these two viruses individually. The infection efficiency was determined to be approximately 90% (Fig. 1A). We examined whether the activity of Jab1 is enhanced in Jab1-overexpressed cells. Studies from other researchers and from our own laboratory demonstrated that Jab1/CSN plays an important role in inducing the protein degradation of p27 (Tomoda et al., 1999) and Smad4 (Wan et al., 2002) but enhancing protein stabilization of c-Jun (Naumann et al., 1999). We then assessed the levels of Smad4, p27, and c-Jun in the cells with Jab1 overexpression. Overexpression of Jab1 resulted in a significant reduction in the levels of Smad4 and p27 but caused elevation of c-Jun level (Fig. 1, B and C). As PANC-1 cells contain mutant allele of *p53*, the level of p53 was not affected by Jab1 overexpression (data not shown). We then assessed whether overexpression of Jab1 would affect cancer cell growth by clonogenic assay. Jab1 overexpression in PANC-1 cells resulted in a significant elevation of colony formation compared with control (Fig. 1, D and E).

We also developed retroviral siRNA delivery vector pMSCVneo/U6-GFP (siGFP, irrelevant siRNA control) and pMSCVneo/U6-Jab1 (siJab1) to determine the effects of a reduction in the levels of Jab1 in PANC-1 cells. siJab1 successfully reduced Jab1 expression, elevated the levels of

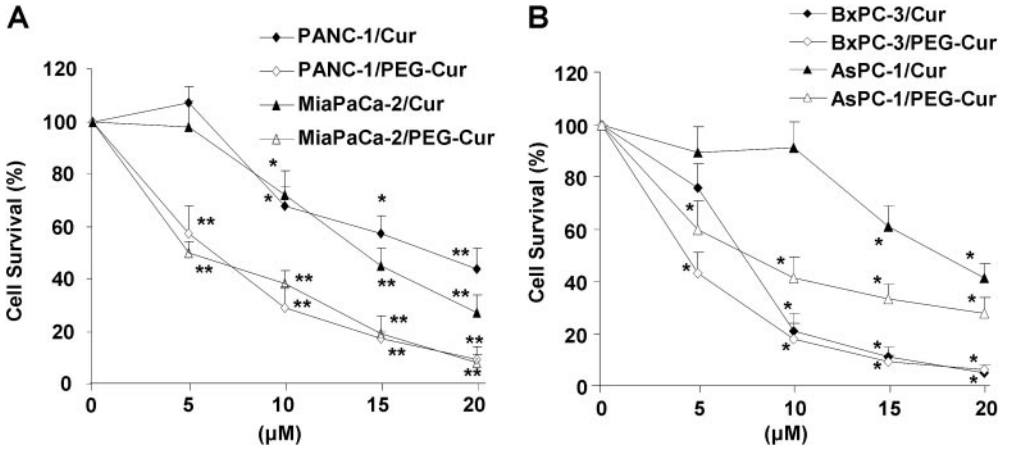

Fig. 3. Comparison of the effects of curcumin and PEGylated curcumin on cell proliferation in different pancreatic cancer cell lines. A, PANC-1 or MiaPaCa-2 cells were treated with different doses of curcumin or PEG-curcumin for 24 h. MTT assays were performed. The values represent means ± S.D. from three independent experiments. *, $p < 0.01$; **, $p < 0.001$ compared with vehicle control-treated cells. B, BxPC-3 or AsPC-1 cells were treated with different doses of curcumin or PEG-curcumin for 24 h. MTT assays were performed. The values represent means ± S.D. from three independent experiments. *, $p < 0.001$ compared with vehicle control-treated cells.

Fig. 2. PEGylated curcumin inhibits cell growth. A, general chemical structure of curcumin. B, synthetic route of PEG-curcumin conjugation. C, ^1H NMR spectrum of a synthesized PEGylated curcumin. D, dose-dependent inhibitory effect of PEGylated curcumin on colony formation in PANC-1 cells. Cells were seeded and incubated with different concentration of PEGylated curcumin or 20 μM PEG (control) for 7 days. The survival fractions of PEGylated curcumin-transfected cells were normalized to PEG-treated cells. The values represent means ± S.D. from three independent experiments. *, $p < 0.001$ compared with PEG-treated cells. E, dose-dependent inhibitory effect of PEGylated curcumin on cell proliferation in PANC-1 cells. Cells were treated with different doses of PEGylated curcumin or 20 μM PEG for 24 h. MTT assays were performed. The cell survival rates were normalized to PEG-treated cells as a percentage. The values represent means ± S.D. from three independent experiments. *, $p < 0.001$ compared with PEG-treated cells. F, time-dependent effects of PEGylated curcumin on cell proliferation. PANC-1 cells were treated with 5 μM PEGylated curcumin or PEG for different time periods. MTT assays were performed. The cell survival rates were normalized to PEG-treated cells as a percentage. The values represent means ± S.D. from three independent experiments. *, $p < 0.001$ compared with PEG-treated cells. G, dose-dependent inhibitory effect of curcumin on cell proliferation in PANC-1 cells. Cells were treated with different doses of free curcumin or vehicle control (Con) for 24 h. MTT assays were performed. The cell survival rates were normalized to vehicle control-treated cells as a percentage. The values represent means ± S.D. from three independent experiments. *, $p < 0.05$; **, $p < 0.001$ compared with vehicle control-treated cells.

Smad4 and p27, and decreased the level of c-Jun in PANC-1 cells (Fig. 1, F and G), indicating that the activity of Jab1 was inhibited in Jab1 knockdown cells. It is noteworthy that cell growth as represented by colony formation was suppressed (Fig. 1, H and I). Taken together, these results support the concept that Jab1 activity in human pancreatic cancer cells is associated directly with cell growth.

TABLE 1

Growth inhibitory effects of curcumin and PEGylated curcumin on a panel of human pancreatic cancer cell lines

Mean of triplicate experiments (MTT assay after 24 h of exposure to curcumin or PEG-curcumin) was calculated.

	IC_{50}			
	PANC-1	MiaPaCa-2	BxPC-3	AsPC-1
		μM		
Curcumin	17	15	7	18
PEG-Curcumin	6	5	4	8

PEGylated Curcumin Inhibits Cell Growth in Pancreatic Cancer Cells. Curcumin was previously reported to inhibit cell proliferation in pancreatic cancer cells (Hidaka et al., 2002; Li et al., 2004; Lev-Ari et al., 2005; Li et al., 2005; Wang et al., 2006). This natural compound is a potent inhibitor of Jab1/CSN-associated kinase(s) (Bech-Otschir et al., 2001; Sun et al., 2002; Uhle et al., 2003). To increase its solubility, we generated a water-soluble PEG-conjugated curcumin system in which curcumin (Fig. 2A) is covalently linked to PEG_{35kD} (Fig. 2B). Figure 2C shows the ^1H NMR spectrum of PEGylated curcumin. The solubility of PEGylated curcumin in water is much higher than that of curcumin because PEGylated curcumin is easily dissolved in

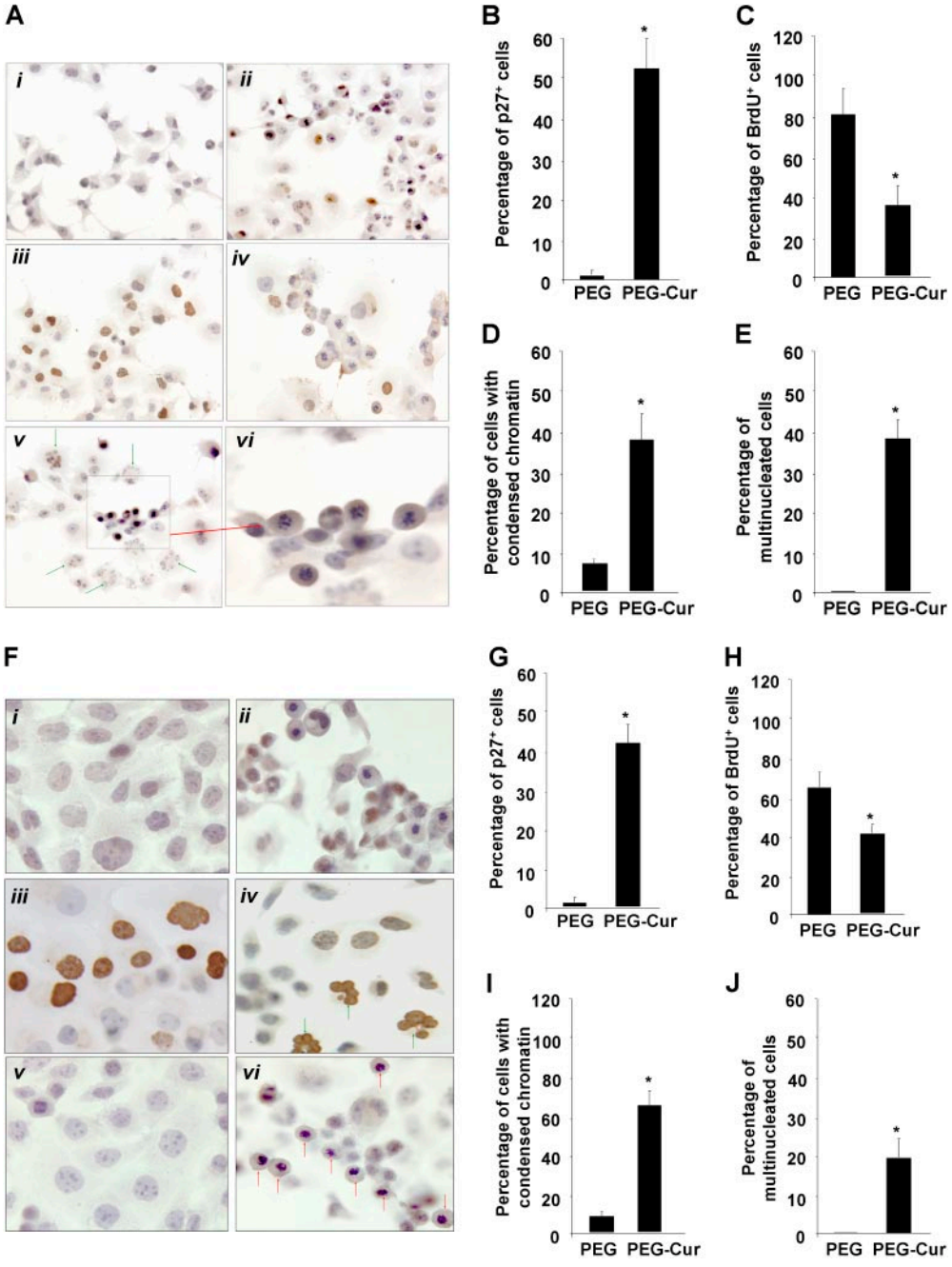

Fig. 4. Immunohistochemical analysis of the effects of PEGylated curcumin on cell proliferation. A to E, responses of PANC-1 cells to PEGylated curcumin. Representative immunostaining image of PANC-1 cells to PEGylated curcumin (A). p27 was elevated in PEG-curcumin-treated PANC-1 cells (ii) compared with PEG control treated cells (i). BrdU incorporation was reduced in PEG-curcumin-treated PANC-1 cells (iv) compared with PEG control (iii). Hematoxylin and eosin staining of PEG-curcumin-treated PANC-1 cells (v and vi). The inset in v, enlarged in vi, indicates abnormal mitotic cells. Green arrows indicate abnormal multinuclear cells. p27-positive (B) and BrdU-positive (C) cells and cells with condensed chromatin (D) and multinuclei (E) were counted in three different fields in one slide. Three different slides for each treatment were counted. F to J, responses of MiaPaCa-2 cells to PEG-curcumin. Representative immunostaining image of PANC-1 cells to PEGylated curcumin (F). p27 protein levels were elevated in PEG-curcumin-treated MiaPaCa-2 cells (ii) compared with PEG control (i). BrdU incorporation was reduced in PEG-curcumin-treated MiaPaCa-2 cells (iv) compared with PEG control (iii). Green arrows indicate positive BrdU-labeling in abnormal multinuclear cells. Hematoxylin and eosin staining of PEG control (v) and PEG-curcumin-treated PANC-1 cells (vi). Red arrows indicate abnormal mitotic cells. p27-positive (G) and BrdU-positive (H) cells and cells with condensed chromatin (I) and multinuclei (J) were counted in three different fields in one slides. Three different slides for each treatment were counted. *, $p < 0.001$ compared with PEG-treated cells.

PBS at 1 mM, whereas curcumin cannot be dissolved in PBS at much lower concentrations. To determine whether PEGylated curcumin affects cell proliferation, we performed colony formation assays in PANC-1 cells. PEGylated curcumin inhibited cell proliferation in a dose-dependent manner (Figs. 2D). The inhibitory effects of PEGylated curcumin were not very great at concentrations of 1 μM, but there was a profound effect at concentrations of 5 μM and higher. Similar results were obtained using MTT assays in which PEGylated curcumin inhibited cell proliferation in a dose- and time-dependent manner (Fig. 2, E and F). The growth inhibitory effects of the compound required a minimum of 24 h treatment (Fig. 2F). The effect of free curcumin on cellular proliferation was also examined. Twenty micromolar curcumin exhibited inhibitory effect on cell proliferation similar to that of 5 μM PEGylated curcumin (Fig. 2G). Thus, PEGylated curcumin has greater inhibitory effects than that of free curcumin on cell proliferation.

PEGylated Curcumin Has Greater Effects Than Free Curcumin on Inhibition of Cell Growth in Pancreatic Cancer Cells. We then compared the effects on cellular proliferation of PEGylated curcumin with those of free curcumin in four human pancreatic cancer cell lines, PANC-1, MiaPaCa-2, BxPC-3, and AsPC-1, treated with increasing doses of both compounds. MTT assays showed that a dose-dependent effect of free curcumin on cell proliferation was detected; curcumin started to exhibit its inhibitory effects on cell proliferation at 10 μM. The antiproliferative effects of PEGylated curcumin were much stronger than free curcumin at equimolar concentrations in PANC-1 (approximately 2.64-fold at a concentration of 10 μM, Fig. 3A), MiaPaCa-2 (approximately 2.28-fold at a concentration of 10 μM; Fig. 3A), and AsPC-1 (approximately 2.38-fold at concentration of 10 μM; Fig. 3B). The effect of PEGylated curcumin on cell proliferation in BxPC-3 cells was greater than that of free curcumin at lower concentration (5 μM) but was similar to that of free curcumin at higher concentrations (Fig. 3B). PEG alone as a control did not affect cell growth in these four cell lines tested (data not shown). The IC_{50} values of PEGylated curcumin are much lower than those of free curcumin in four cell lines (Table 1). These results suggest that the activity of PEGylated curcumin conjugates in vitro was much greater than that of free curcumin at equimolar concentrations.

To confirm the inhibitory effects of PEGylated curcumin on cell proliferation at the cellular level, we examined the changes in cellular proliferation by BrdU labeling and p27 expression by immunohistochemical analysis in pancreatic cancer cells treated with 5 μM PEGylated curcumin or PEG control. PEGylated curcumin elevated p27 protein levels in both PANC-1 cells (Fig. 4, A ii and B) and MiaPaCa-2 cells (Fig. 4, F ii and G) compared with controls (Fig. 4, A i and F i). BrdU staining was consistently and dramatically reduced

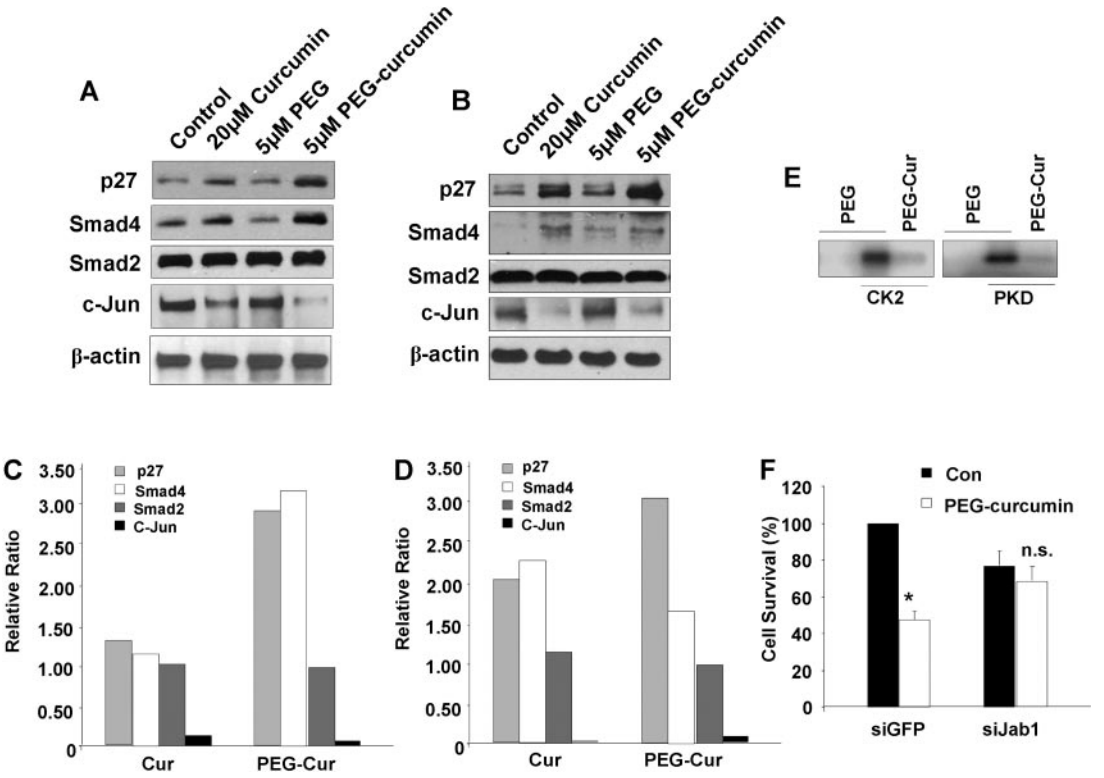

Fig. 5. PEGylated curcumin inhibits cell growth through inactivation of Jab1. A to D, PEGylated curcumin changes protein levels of Jab1 target proteins in pancreatic cancer cells. PANC-1 (A) and AsPC-1 (B) cells were treated with indicated compounds for 24 h and lysed. Cell lysates were subjected to Western blotting using specific antibodies against Smad4, Smad2, p27, c-Jun, and β-actin. The intensity of the bands in (A and B) was quantified by filmless autoradiographic analysis, and the ratio of protein level in cells treated with curcumin or PEGylated curcumin to that in control cells was calculated (C and D). E, PEGylated curcumin inhibits the phosphorylation of c-Jun by Jab1/CSN-associated kinases. Recombinant c-Jun was incubated with either recombinant CK2 or PKD with [γ-^{32}P]ATP in the presence of 5 μM PEG or PEGylated curcumin. After 1 h at 37°C, the reaction mix was separated by SDS-PAGE and the dried gel was autoradiographed. F, inhibitory effect of PEGylated curcumin on cell proliferation was blunted in Jab1 knockdown cells. PANC-1 cells were infected with virus containing siGFP or siJab1 and treated with PEG (control) or PEGylated curcumin for 24 h, and MTT assays was performed. The values represent means ± S.D. from three independent experiments. *, $p < 0.001$ compared with PEG treatment of siGFP group; n.s., no significance compared with PEG treated siJab1 group.

after treatment of PEGylated curcumin in both cell lines (PANC-1, Fig. 4, A iv and C; MiaPaCa-2, Fig. 4, F iv and H) in comparison with controls (Fig. 4, A iii and F iii). It is noteworthy that most of the cells treated with PEGylated curcumin exhibited mitotic features in a prometaphase or metaphase-like state with condensed chromatin (PANC-1 cells, Fig. 4, A v, A vi, and D; MiaPaCa-2 cells, Fig. 4, F vi and I). Another unique feature was that numerous large, multinucleated cells were visible in each microscopic field of PEGylated curcumin-treated cells (Fig. 4, A v, E, F iv, and J). The aberrant mitosis and multinucleated cells were not seen in equimolar concentration of free curcumin-treated cells (data not shown). These results suggest that the inhibitory effects of PEGylated curcumin on cell proliferation may be through arresting cells at mitosis.

PEGylated Curcumin Inhibits Cell Growth via Suppression of Jab1 Activity in Pancreatic Cancer Cells. We then investigated whether this PEGylated curcumin compound affects Jab1 activity. We first examined whether this compound affects the protein levels of Smad4, p27, and c-Jun, which are Jab1 target proteins, in pancreatic cancer cells. As shown in Fig. 5, A to D, treatment with 5 μM PEGylated curcumin for 24 h resulted in a significant elevation of the protein levels of Smad4 and p27 and reduction of c-Jun in both PANC-1 and AsPC-1 cells. As a control, the level of Smad2, which is not a Jab1 target protein, was not affected by the treatment of this compound, indicating that the effect of PEGylated curcumin was Jab1-specific. To assess whether PEGylated curcumin directly inhibits Jab1/CSN-associated kinase activity, we performed an in vitro kinase assays in which CK2 and PKD, previously identified Jab1/CSN-associated kinases, were incubated with the protein target c-Jun. As expected, PEGylated curcumin dramatically inhibited CK2- and PKD-induced phosphorylation of c-Jun (Fig. 5E). To further examine whether Jab1 is required in PEGylated curcumin-induced cell growth inhibition, we treated the cells with 20 μM free curcumin and 5 μM PEGylated curcumin in PANC-1 cells with Jab1 knockdown by siRNA introduction. The inhibitory effect of free curcumin and PEGylated curcumin on cell growth was blunted in Jab1 knockdown cells (Fig. 5F). The results suggest that the inhibitory effect of PEGylated curcumin on cell growth occurs, at least in part, through suppression of Jab1 activity.

Synergistic Effects of PEGylated Curcumin with Gemcitabine on Cell Growth Inhibition and Apoptosis in PANC-1 and AsPC-1 Cells. Gemcitabine (2′,2′-difluoro-

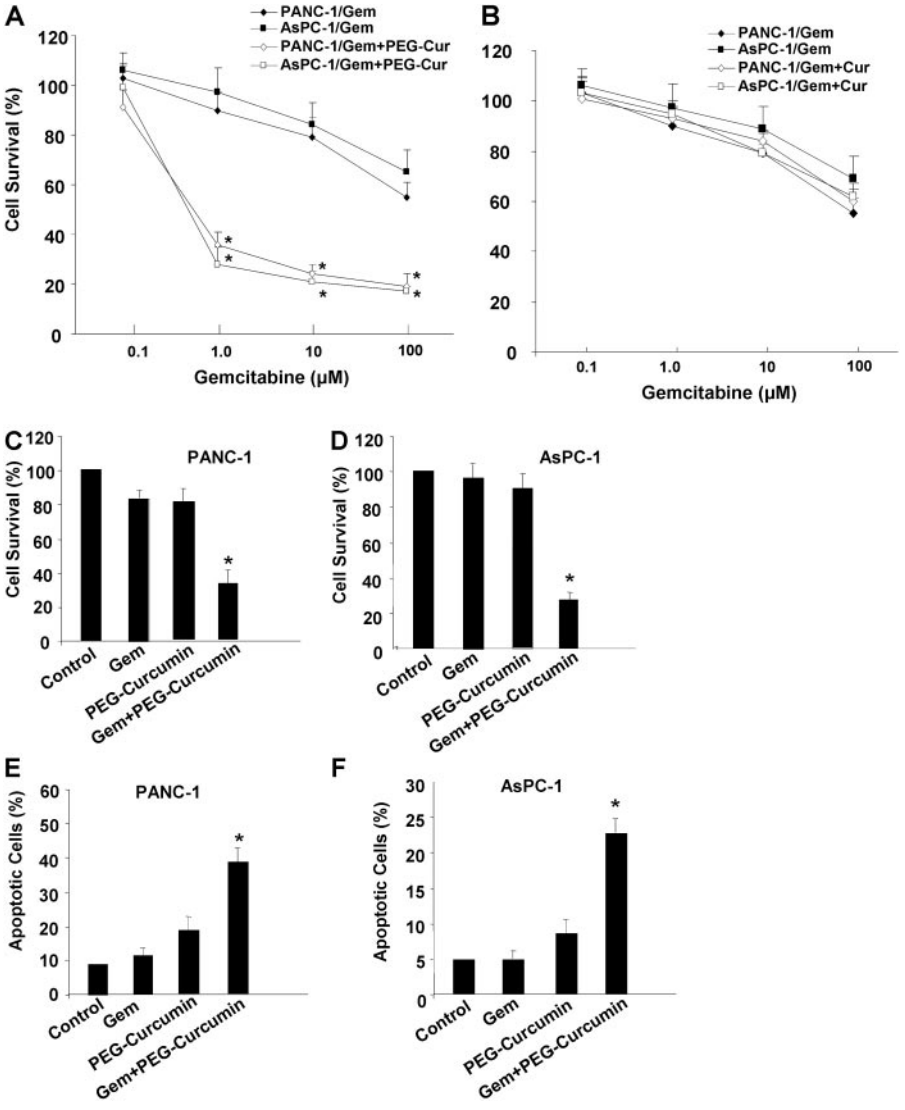

Fig. 6. Synergistic effects of PEGylated curcumin with gemcitabine on cell growth. A, enhancement of gemcitabine-induced cytotoxicity by PEG-curcumin cotreatment in PANC-1 and AsPC-1 cells. Cells were exposed to 1 μM PEGylated curcumin with increasing concentrations (0.1–100 μM) of gemcitabine for 24 h, after which cell survival was determined by MTT assays. *, $p < 0.001$ compared with group treated with gemcitabine alone. B, failure of free curcumin to increase gemcitabine-induced cytotoxicity in PANC-1 and AsPC-1 cells. Cells were exposed to 1 μM free curcumin with increasing concentrations (0.1–100 μM) of gemcitabine for 24 h, after which cell survival was determined by MTT assays. C and D, enhancement of gemcitabine-induced cytotoxicity by PEG-curcumin cotreatment in PANC-1 (C) and AsPC-1 (D) cells. Cells were treated with PEG-curcumin (1 μM) and/or gemcitabine (Gem, 10 μM) for 24 h, after which cell survival was determined by MTT assays. The values represent means ± S.D. from three independent experiments. *, $p < 0.001$ compared with both gemcitabine-alone and PEG-curcumin alone treatment groups. E and F, enhancement of gemcitabine-induced cell apoptosis by PEGylated curcumin cotreatment in PANC-1 (E) and AsPC-1 (F) cells. Cells were treated with PEGylated curcumin (1 μM) and/or gemcitabine (Gem, 10 μM) for 24 h, after which apoptosis cells were determined by flow cytometry using an Annexin-V-PE/7-AAD apoptosis detection kit. The values represent means ± S.D. from three independent experiments. *, $p < 0.001$ compared with both gemcitabine-alone and PEG-curcumin-alone treatment groups.

deoxycytidine; Gemzar) is the FDA-approved chemotherapeutic agent for the treatment of pancreatic adenocarcinoma (Burris et al., 1997; Hui and Reitz, 1997). However, gemcitabine resistance occurs in most patients and limits its effectiveness. We examined whether PEGylated curcumin can act to sensitize pancreatic tumor cells to the effects of gemcitabine by analysis of growth inhibition and apoptosis. As shown in Fig. 6, gemcitabine inhibited cell proliferation of PANC-1 and AsPC-1 cells dose dependently at concentrations lower than 100 μM by MTT assay; the AsPC-1 cells were less sensitive (Fig. 6, A and B). But when 1 μM PEGylated curcumin was added, cell growth inhibition was significantly induced in both cell types (Fig. 6A). One micromolar curcumin, however, did not exert the same effect (Fig. 6B). Treatment of the cells with a combination of 10 μM gemcitabine and 1 μM PEGylated curcumin for 24 h resulted in a markedly enhanced inhibition of proliferation (Fig. 6, C and D) and increased apoptosis with Annexin V staining (Fig. 6, E and F), suggesting that PEGylated curcumin sensitizes pancreatic cells to respond to gemcitabine.

Discussion

The present study demonstrates that water-soluble PEGylated curcumin conjugate that we generated increases the effects of curcumin on inhibition of cellular growth in pancreatic cancer cells. More importantly, the new compound sensitized pancreatic cancer cells to gemcitabine-induced cell apoptosis and growth inhibitory effects. These results provide evidence supporting the potential entry of this PEGylated curcumin into preclinical animal studies and clinical trials for treatment of patients with pancreatic cancer. Curcumin has been widely used as an anti-inflammatory and anticancer drug. This compound is nontoxic and virtually devoid of side effects in animals as well as in humans (Hsu and Cheng, 2007) and therefore may be a potential therapeutic agent for cancer. However, even though free curcumin has antitumor effects against a variety of cancer cells, including pancreatic cancer cells (Hidaka et al., 2002; Li et al., 2004, 2005; Lev-Ari et al., 2005; Wang et al., 2006; Kunnumakkara et al., 2007), this agent is highly hydrophobic and cannot be administered systemically. In addition, the bioavailability and circulation half-life of oral curcumin are poor. Thus, the clinical application of curcumin has been limited. To overcome these problems, we developed a strategy that conjugates curcumin to a water-soluble high-molecular-weight PEG_{35kD} and examined the effects of this conjugate on pancreatic cancer cell proliferation and apoptosis. PEG-curcumin inhibited cell proliferation and apoptosis in all four human pancreatic cancer cell lines, and its effects were much more potent than those of free curcumin, perhaps because the covalent conjugation of curcumin with PEG (PEGylation) increases its water solubility and its molecular size, and steric hindrance may improve its cellular permeability and proteolytic stability, there enhancing its half-life in cells.

The current work demonstrated that PEGylated curcumin-induced cell growth inhibition is caused, at least in part, by inhibition of Jab1/CSN-associated kinase activity, providing a molecular mechanism for the antipancreatic cancer effect of this compound. Jab1, integrated into CSN complex, controls protein stability of several key mediators controlling cell proliferation, cell cycle progression and apoptosis such as the cyclin-dependent kinase inhibitors $p27^{kip1}$ (Tomoda et al., 1999), $p21^{cip1}$ (Peng et al., 2003), p53 (Bech-Otschir et al., 2001), Smad4 (Wan et al., 2002), and Smad7 (Kim et al., 2004). We also found that Jab1 is a critical factor governing pancreatic cancer cell proliferation by regulating the stability of these key regulatory proteins, consistent with previous results by others (Fukumoto et al., 2004, 2006; Kouvaraki et al., 2006). It is noteworthy that PEGylated curcumin-treated cells exhibited effects similar to those of Jab1 knockdown on elevating protein levels of Jab1 target proteins as well as inhibiting pancreatic cancer cell proliferation. This effect was blunted in Jab1 knockdown cells. Thus, the effect of PEGylated curcumin is Jab1-dependent. We also found that most of the cells were arrested in mitosis and abnormally large multinucleated cells were observed after PEGylated curcumin treatment. Previous study by Fukumoto et al. (2006) demonstrated that Jab1 knockdown also caused $S/G_2/M$ phase arrest, providing indirect evidence that this effect of PEGylated curcumin might also be due to Jab1 inactivation. Further identification of the mechanism of the effect of this conjugate on cellular mitosis is needed and is under way. Curcumin possesses a wide range of pharmacological properties including anti-inflammatory, anti-infectious, and anticarcinogenic activities (Goel et al., 2008), and a number of different targets have been proposed to mediate these different effects, including transcription factors, enzymes, hormones, growth factors, apoptotic and mitochondrial signaling molecules, and their associated receptors (Goel et al., 2008). Therefore, PEGylated curcumin may also affect these signaling pathways in cancer cells. However, the fact that the effect of PEGylated curcumin on cell growth inhibition was antagonized by knockdown of Jab1 in cells indicates that Jab1/CSN is one of the major targets of PEGylated curcumin in pancreatic cancer.

Evaluation of the effects of PEGylated curcumin on tumor growth and survival in mouse pancreatic tumor models is, of course, the major subject for further studies. PEG has been used widely to increase the circulation half-life of rapidly eliminated drugs, to impart enhanced permeability and retention (Maeda, 2001), and to increase passive targeting of anticancer drugs. Many studies have demonstrated that the residence time in the bloodstream of PEG-protein conjugates increases according to the molecular size (Caliceti and Veronese, 2003). It is believed that size enlargement promotes the accumulation into tumor tissues by the passive enhanced permeability and retention mechanism. In addition, optimal PEGylation of drugs could selectively improve their in vivo therapeutic potency and reduce side effects (Kamada et al., 2000; Tsutsumi et al., 2000). The in vitro results presented in the current study demonstrate that PEG-conjugated curcumin exhibited much higher ability to suppress cell proliferation and induce apoptosis of pancreatic carcinoma cells, which provides a basis for the further investigation of this drug in preclinical animal studies and clinical trials.

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