Notch-1 Down-Regulation by Curcumin Is Associated with the Inhibition of Cell Growth and the Induction of Apoptosis in Pancreatic Cancer Cells

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BACKGROUND. Notch signaling plays a critical role in maintaining the balance between cell proliferation, differentiation, and apoptosis, and thereby may contribute to the development of pancreatic cancer. Therefore, the down-regulation of Notch signaling may be a novel approach for pancreatic cancer therapy. It has been reported that curcumin down-regulates many genes that are known to promote survival and also up-regulates genes that are known promoters of apoptosis in pancreatic cancer cells in vitro. It also has been reported that there is cross-talk between Notch-1 and another major cell growth and apoptotic regulatory pathway, the nuclear factor κB (NF-κB) pathway, which is down-regulated by both curcumin and reduction of Notch-1 levels. However, to the authors' knowledge to date, no studies have determined whether the down-regulation of Notch-1 signaling, resulting in the inactivation of NF-κB activity, contributes to curcumin-induced cell growth inhibition and apoptosis in pancreatic cancer cells.

METHODS. The authors used multiple molecular approaches, such as the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, an apoptosis assay, gene transfection, real-time reverse transcriptase-polymerase chain reaction analysis, Western blot analysis, and an electrophoretic mobility shift assay to measure the DNA binding activity of NF-κB.

RESULTS. Curcumin inhibited cell growth and induced apoptosis in pancreatic cancer cells. Notch-1, Hes-1, and Bcl-X_L expression levels concomitantly were down-regulated by curcumin treatment. These results correlated with the inactivation of NF-κB activity and increased apoptosis induced by curcumin. The down-regulation of Notch-1 by small-interfering RNA prior to curcumin treatment resulted in enhanced cell growth inhibition and apoptosis.

CONCLUSIONS. The current results provide the first demonstration to the authors' knowledge that the Notch-1 signaling pathway is associated mechanistically with NF-κB activity during curcumin-induced cell growth inhibition and apoptosis of pancreatic cells. These results suggest that the down-regulation of Notch signaling by curcumin may be a novel strategy for the treatment of patients with pancreatic cancer. ***Cancer* 2006;106:2503–13.** *© 2006 American Cancer Society.*

KEYWORDS: apoptosis, Notch-1 signaling pathway, curcumin, nuclear factor κB.

Pancreatic cancer remains the fourth leading cause of cancer-related death in the U.S.[1] This may be because patients present with late-stage disease at diagnosis, it has high metastatic potential, and there is a lack of effective therapies available, resulting in high mortality for patients diagnosed with pancreatic cancer. This disappointing outcome strongly suggests that the evaluation of novel, targeted therapeutic agents is needed urgently to improve the outcome of patients who are diagnosed with this deadly disease.

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Among many other genes, the Notch genes play important roles in the carcinogenesis of human cancers, including pancreatic cancer. Notch genes encode proteins that can be activated by interacting with a family of its ligands.[2,3] On activation, Notch is cleaved, releasing intracellular Notch (ICN), which translocates into the nucleus. The ICN associates with transcriptional factors, regulating the expression of target genes and modulating the development and growth of cells.[4,5] To date, 4 vertebrate Notch genes have been identified (Notch-1-Notch-4). In addition, 4 ligands (Dll-1, Dll-4, Jagged-1, and Jagged-2) have been identified in vertebrates.[6,7]

Increased expression of Notch genes and their ligands has been detected in human pancreatic cancer tissues. Miyamoto et al. reported that Notch pathway components and Notch target genes are up-regulated in invasive human pancreatic cancer.[8] Terris et al. found that the Notch gene was over-expressed in human pancreatic cancer.[9] Fukushima et al. also reported that Jagged-1 ligand and Hes-1, which is 1 of the Notch downstream genes, were over-expressed in pancreatic cancer.[10] All of those reports clearly suggested a possible link between Notch gene over-expression and pancreatic cancer. Therefore, Notch may be an important target for the treatment of patients with pancreatic carcinoma.

Another mechanism that may cause resistance to apoptosis in pancreatic carcinoma is the activation of nuclear transcription factor κB (NF-κB). It has been reported that there is cross-talk between Notch-1 and the NF-κB pathway. Specifically, Notch-1 strongly induced NF-κB promoter activity in reporter assays[11] and induced expression of several NF-κB subunits.[12] Levels of basal and stimulation-induced NF-κB activity were decreased significantly in mice that had reduced Notch levels.[13,14] Constitutive levels of Notch activity are essential to maintain NF-κB activity in various cell types. The Notch and NF-κB pathways are key regulators of numerous cellular events, such as proliferation, differentiation, and apoptosis. Therefore, the inactivation of Notch-1-mediated cell growth inhibition and the induction of apoptosis may be mediated in part through the inactivation of NF-κB activity.

Epidemiologic and animal studies have shown that many dietary constituents play a role in cancer prevention.[15–18] In particular, curcumin (diferuloylmethane), which is a phenolic compound from the plant *Curcuma longa* (Linn), is a widely used flavoring agent in food.[15] Curcumin has shown antitumor activity in many cancers, including pancreatic cancer.[16–20] Plummer et al. reported that curcumin consistently suppressed NF-κB activation by inhibiting IκB kinase, which induces IκBα phosphorylation, leading to the inhibition of NF-κB downstream gene expression in pancreatic cancer cells.[21] NF-κB downstream gene over-expression is associated with apoptosis resistance, angiogenesis, enhanced invasion, and metastasis. Although there have been extensive investigations to elucidate the mechanism of action of curcumin as an antitumor agent, the exact mechanism has yet to be established.

Because Notch-1 down-regulation showed antineoplastic effects in vivo and in vitro,[22–25] the potential for treating certain cancers may be realized by inhibiting Notch signal transduction. Therefore, for the current report, we tested our hypothesis whether down-regulation of Notch-1 gene expression by curcumin could inhibit cell growth and induce apoptosis, which may be associated mechanistically with the down-regulation of NF-κB in pancreatic cancer cells.

MATERIALS AND METHODS

Cell Culture and Experimental Reagents

The human pancreatic cancer cell lines BxPC-3 (wild-type k-Ras) and PANC-1 (mutant k-Ras) (American TypeCulture Collection, Rockville, MD) were cultured in RPMI-1640 media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum/1% penicillin and streptomycin in a 5% carbon dioxide atmosphere at 37°C. A cell death enzyme-linked immunoadsorbent assay (ELISA) kit was obtained from Roche (Indianapolis, IN). Primary antibodies for Notch-1, Hes-1, cyclin D1, and Bcl-X_L were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All secondary antibodies were obtained from Pierce (Rockford, IL). Notch-1 small-interfering RNA (siRNA) and siRNA control were obtained from Santa Cruz Biotechnology. Lipofectamine 2000 was purchased from Invitrogen. Chemiluminescence detection of proteins was performed using a kit from Amersham Biosciences (Amersham Pharmacia Biotech, Piscataway, NJ). Curcumin was obtained from Sigma Chemical Company (St. Louis, MO). Protease inhibitor cocktail, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and all other chemicals, were obtained from Sigma Chemical Company.

Cell Growth Inhibition Studies by MTT Assay

BxPC-3 and PANC-1 cells (5×10^3) were seeded in a 96-well culture plate and subsequently were treated with 5 μM, 10 μM, and 15 μM curcumin for 24 hours, 48 hours, and 72 hours. Control cells were treated with 0.1% dimethyl sulfoxide (DMSO) in culture medium. After treatment, the cells were incubated with MTT reagent (0.5 mg/mL; Sigma Chemical Company) at 37°C for 2 hours and then with isopropanol at room temperature for 1 hour. The spectrophotometric absorbance of the samples was measured using an Ultra Multifunctional Microplate Reader (Tecan, Durham, NC). Results were plotted as the mean ± standard deviation of 3 separate experiments from 6 determinations per experiment for each experimental condition.

Histone DNA ELISA for Detection of Apoptosis

The Cell Death Detection ELISA Kit was used for assessing apoptosis in BxPC-3 and PANC-1 cells that were treated with curcumin according to the manufacturer's protocol. Briefly, BxPC-3 cells were treated with 5 μM, 10 μM, and 15 μM curcumin or with 0.1% DMSO (control) for 72 hours. After treatment, the cells were lysed, and the cell lysates were overlaid and incubated in microtiter plate modules coated with antihistone antibody. Samples were then incubated with anti-DNA peroxidase followed by color development with ABTS™ substrate. The optical densities of the samples were determined by using the Ultra Multifunctional Microplate Reader (Tecan) at 405 nanometers (nm).

Two-Step, Real-Time, Quantitative Reverse Transcriptase-Polymerase Chain Reaction Analysis for Gene Expression Studies

BxPC-3 and PANC-1 cells were seeded in 100-mm dishes and allowed to attach for 24 hours, followed by the addition of 5 μM, 10 μM, 15 μM curcumin or 0.1% DMSO (control), and incubation for 72 hours. After incubation, the total RNA from each sample was isolated by Trizol (Invitrogen) and purified by using the RNeasy Mini Kit and the RNase-free DNase Set (Qiagen, Valencia, CA) according to the manufacturer's protocols. One microgram of total RNA from each sample was subjected to first-strand combinational DNA (cDNA) synthesis by using the TaqMan reverse transcriptase (RT) reagent kit (Applied Biosystems, Foster City, CA) in a total volume of 50 μL, including 6.25 U MultiScribe RT and 25 pmol random hexamers. RT reaction was performed at 25°C for 10 minutes, followed by 48°C for 30 minutes, and 95°C for 5 minutes.

The primers used in the polymerase chain reaction (PCR) analysis were as follows: For Notch-1, the forward primer was 5′-CAC TGT GGG CGG GTC C-3′, and the reverse primer was 5′-GTT GTA TTG GTT CGG CAC CAT-3′. For β-actin, the forward primer was 5′-CCA CAC TGT GCC CAT CTA CG-3′, and the reverse primer was 5′-AGG ATC TTC ATG AGG TAG TCA GTC AG-3′. The primers were checked by running a virtual PCR, and primer concentration was optimized to avoid primer dimer formation. In addition, dissociation curves were checked to avoid nonspecific amplification. Real-time PCR amplications were undertaken with the Mx4000 Multiplex QPCR System (Stratagene, La Jolla, CA) using a 2 × SYBR Green PCR Master Mix (Applied Biosystems). One microliter of RT reaction was used for a total volume of 25-μL quantitative PCR reactions. The thermal profile for SYBR real-time PCR was 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute. A comparative C_T method ($2^{-\Delta\Delta CT}$) was used to analyze the relative changes in gene expression.

Western Blot Analysis

Cells were lysed in lysis buffer (50 mmol/L Tris [pH 7.5], 100 mmol/L NaCl, 1 mmol/L ethylenediamine tetraacetic acid [EDTA], 0.5% NP40, 0.5% Triton X-100, 2.5 mmol/L sodium orthovanadate, 10 μL/mL protease inhibitor cocktail, and 1 mmol/L phenyl methyl sulfonyl fluoride [PMSF]) by incubating for 20 minutes at 4°C. The protein concentrations were determined using the Bio-Rad assay system (Bio-Rad, Hercules, CA). Total proteins were fractionated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the gels were transferred onto nitrocellulose membrane. The membranes were blocked with 5% nonfat dried milk or bovine serum albumin in 1 × Tris buffered saline containing 0.1% Tween-20 and then incubated with appropriate primary antibodies. Horseradish peroxidase-conjugated antirabbit or antigoat immunoglobulin G was used as the secondary antibody, and the protein bands were detected using the enhanced chemiluminesence detection system (Amersham Pharmacia Biotech). Western blot analyses were quantified by using laser densitometry, and the results are presented as the mean of 3 independent experiments with error bars representing the standard deviation. For reprobing, membranes were incubated for 30 minutes at 50°C in buffer that contained 2% SDS, 62.5 mmol/L Tris (pH 6.7), and 100 mmol/L 2-mercaptoethanol. The membranes then were washed and incubated with the desired primary antibody.

Preparation of Nuclear Extract

BxPC-3 and PANC-1 cells that were exposed to curcumin or kept as controls were washed with cold phosphate buffered saline and suspended in 0.15 mL lysis buffer (10 mM N-2-hydroxyethyl piperazine-N′-2-ethane sulphonate [HEPES] [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM ethylene guanine tetraacetic acid [EGTA], 1 mM dithiothrietol [DTT], 1 mM PMSF, 2 μg/mL leupeptin, 2 μg/mL aprotinin, and 0.5 mg/mL benzamidine). The cells were allowed to swell on ice for 20 minutes; then, 4.8 μL of 10% Nonidet P-40 were added. Next, the tubes were mixed vigorously on a

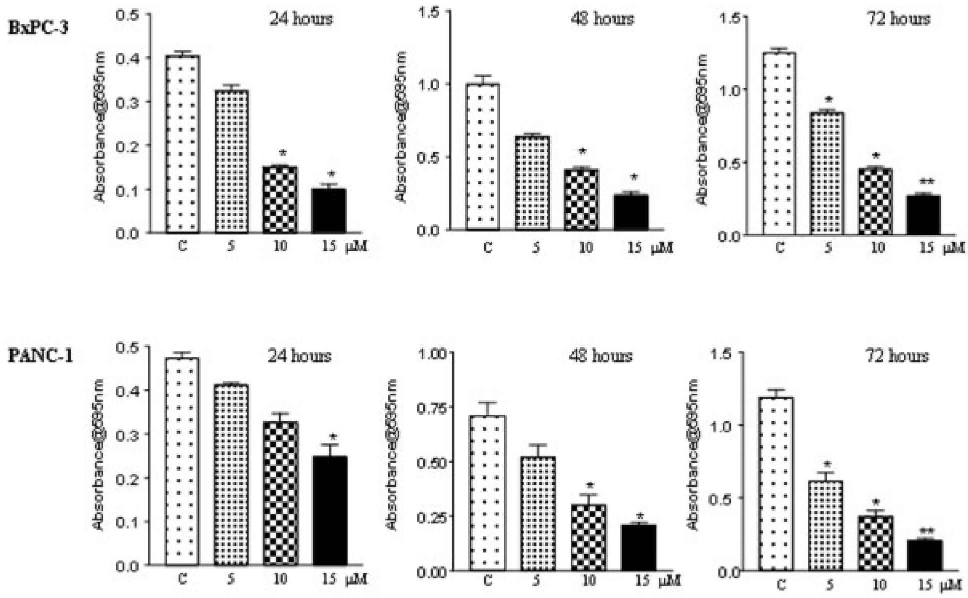


FIGURE 1. The inhibitory effect of curcumin on the growth of BxPC-3 and PANC-1 pancreatic cancer cells was tested by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. BxPC-3 and PANC-1 cells treated with 5 μmol/L, 10 μmol/L, and 15 μmol/L curcumin for 24 hours, 48 hours, or 72 hours. The control lane (C) depicts cells that were treated with dimethyl sulfoxide. Single asterisk: $P < .05$; double asterisks: $P < .01$.) The treatment of BxPC-3 and PANC-1 pancreatic cancer cells with curcumin resulted in cell growth inhibition. The inhibition of cell growth was dose-dependent and time-dependent.

vortex mixer for a few seconds and centrifuged for 120 seconds in a microfuge. The nuclear pellet was resuspended in 30 μL of ice-cold nuclear extraction buffer (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2 μg/mL leupeptin, 2 μg/mL aprotinin, and 0.5 mg/mL benzamidine) and incubated on ice with intermittent mixing. Then, the tubes were centrifuged for 5 minutes in a microfuge at 4°C, and the supernatant fluid (nuclear extract) was collected in a cold Eppendorf tube and stored at − 70°C for later use. The protein content was measured by using the bichloroacetic acid method.

Electrophoretic Mobility Shift Assay for Measuring NF-κB Activity

An electrophoretic mobility shift assay (EMSA) was performed by incubating 10 μg of nuclear protein extract with IRDye™ 700-labeled NF-κB oligonucleotide (LI-COR, Lincoln, NE). The incubation mixture included 2 μg of poly(dI-dC) in a binding buffer. The DNA-protein complex formed was separated from free oligonucleotide on 8.0% native polyacrylamide gels by using buffer that contained 50 mM Tris, 200 mM glycine [pH 8.5], and 1 mM EDTA and then was visualized with the Odyssey Infrared Imaging System by using Odyssey software (release 1.1; LI-COR). Supershift assay using NF-κB p65 antibody also was conducted to confirm the specificity of NF-κB DNA-binding activity. For loading control, 10 μg of nuclear proteins from each sample were subjected to Western blot analysis for retinoblastoma protein, which demonstrated no alternation after curcumin treatment.

Plasmids and Transfections

The Notch-1 cDNA plasmid encoding the Notch-1 intracellular domain was a kind gift from L. Miele (Department of Biopharmaceutical Sciences and Cancer Center, University of Illinois at Chicago, Chicago, IL[26]). BxPC-3 cells were transfected with Notch-1 siRNA and siRNA control, respectively, using Lipofectamine 2000. BxPC-3 cells were transfected stably with human Notch-1 ICN or vector alone (pcDNA3) and were maintained under neomycin selection. The transfected cells were treated with 10 μM curcumin for 72 hours or were kept as controls. The proteins were extracted and measured by Western blot analysis. In addition, the cell growth and apoptotic cells in transfected cells with treatments were detected by using the MTT assay and the Cell Apoptosis ELISA Detection Kit, respectively, according to the procedures described above. NF-κB activity was measured by EMSA as described earlier.

Densitometric and Statistical Analysis

The bidimensional optical densities of Notch-1 and β-actin proteins on the films were quantified and analyzed with Molecular Analyst software (Bio-Rad). The ratios of Notch-1 against β-actin were calculated. The cell growth inhibition by transfection or curcumin treatment was evaluated statistically by using StatMate software (GraphPad Software, Inc., San Diego, CA). Comparisons were made between control and transfection or curcumin treatment. $P<.05$ was used to indicate statistical significance.

RESULTS

Cell Growth Inhibition by Curcumin in BxPC-3 and PANC-1 Cells

The treatment of BxPC-3 and PANC-1 pancreatic cancer cells for 1–3 days with 5 μM, 10 μM, and 15 μM of curcumin resulted in cell growth inhibition. The results indicated that the inhibition of cell growth was dose-dependent and time-dependent in both pancreatic cancer cell lines (Fig. 1). Next, we explored whether the inhibition of cell growth also was accompanied by the induction of apoptosis by curcumin. DNA-histone fragmentation analysis was employed to investigate the degree of apoptosis induced by curcumin.

Curcumin-Induced Apoptosis in Pancreatic Cancer Cell Lines

BxPC-3 and PANC-1 cells were treated either with 5 μM, 10 μM, and 15 μM curcumin or with 0.1% DMSO (control) for 72 hours. After treatment, apoptosis was observed in the 2 cell lines. The induction of apoptosis was dose-dependent (Fig. 2). These results provided convincing data that curcumin induced apoptosis in BxPC-3 and PANC-1 cells. To understand further the molecular mechanism involved in curcumin-induced apoptosis of pancreatic cancer cells, alterations in the cell survival pathway were investigated.

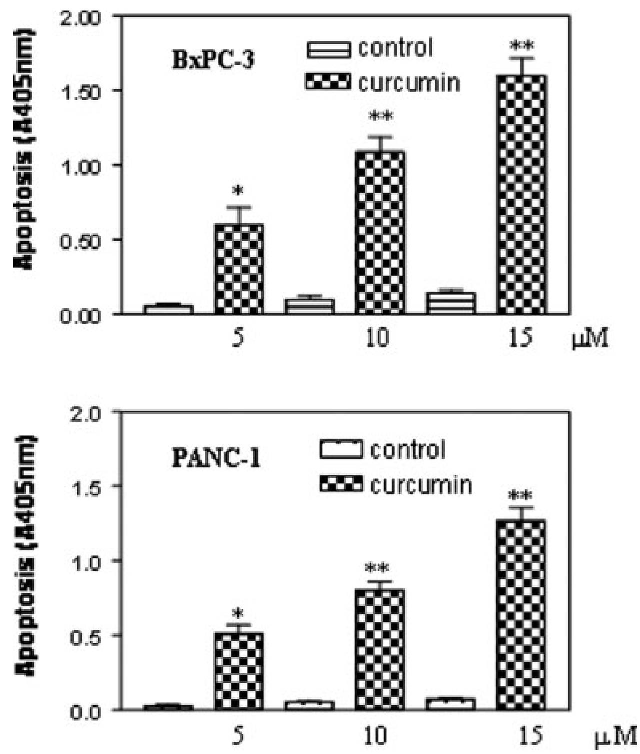


FIGURE 2. Curcumin-induced apoptosis in BxPC-3 and PANC-1 pancreatic cancer cells was measured with the histone/DNA fragment analysis by using an enzyme-linked immunoadsorbent assay. Control lane: Control cells (C) were treated with dimethyl sulfoxide. Cells from the pancreatic cancer cell lines were treated with 5 μmol/L, 10 μmol/L, and 15 μmol/L curcumin for 72 hours. Single asterisk: $P<.05$; double asterisks: $P<.01$.

Down-Regulation of Notch-1 mRNA Expression by Curcumin

Notch-1 mRNA expression in BxPC-3 and PANC-1 cells that were treated with curcumin was assessed by using real-time RT-PCR analysis. Expression of the Notch-1 gene at the mRNA level was down-regulated after curcumin treatment, suggesting that there was transcriptional inactivation of Notch-1 gene expression in pancreatic cancer cells (Fig. 3A,B).

Down-Regulation of Notch-1, Hes-1, Bcl-X_L, and Cyclin D1 Protein Expression by Curcumin

To verify whether the alternation of Notch-1 gene at the level of transcription ultimately results in alternations at the level of translation, we conducted Western blot analysis for detection of Notch-1. Western blot analysis showed that protein levels of Notch-1 were down-regulated in both BxPC-3 cells and PANC-1 cells that were treated with curcumin in a time-dependent manner (Fig. 3C and D). These results are in direct agreement with the RT-PCR data, which indicated that curcumin regulates the transcription and translation of the Notch-1 gene. In addition, we observed that the expression of Notch-1 downstream target genes, including Hes-1, Bcl-X_L, and cyclin D1, also were down-regulated in curcumin-treated cells (Fig. 3C,D).

Inhibition of NF-κB Activation by Curcumin

We investigated whether the downstream effect of curcumin induced by down-regulation of Notch-1 was associated mechanistically with the NF-κB pathway. Nuclear extracts from control and curcumin-treated BxPC-3 and PANC-1 cells were subjected to analysis for NF-κB DNA-binding activity as measured by EMSA. The results showed that 10 μM and 15 μM curcumin inhibited NF-κB DNA-binding activity significantly in BxPC-3 and PANC-1 cells compared with control cells, respectively (Fig. 4). The specificity of NF-κB DNA binding to the DNA consensus sequence was confirmed by supershift. These results indicated

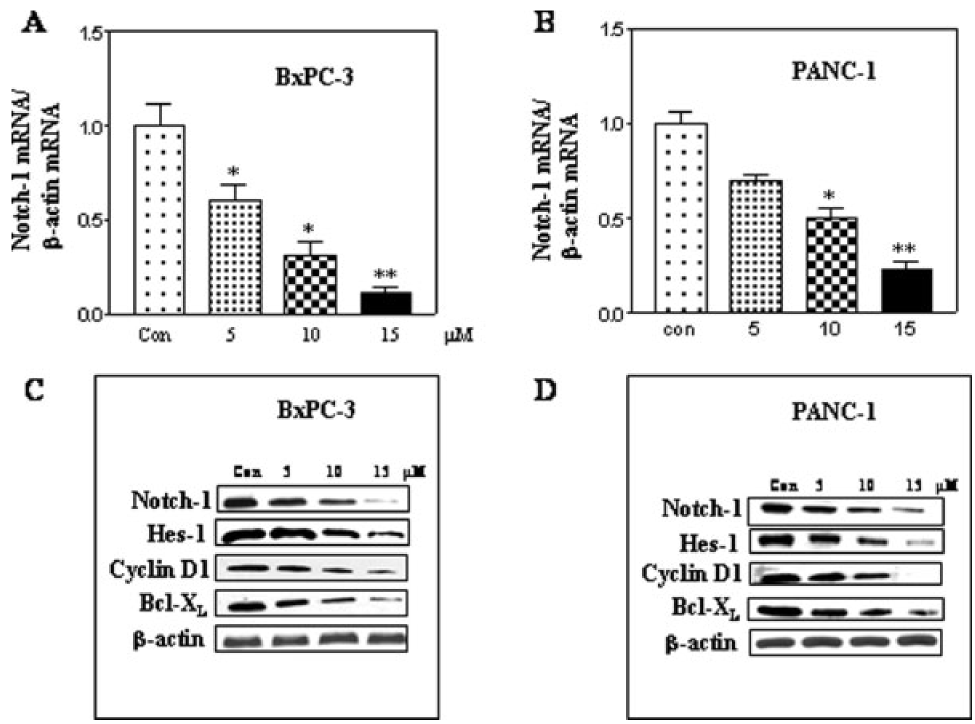


FIGURE 3. (A,B) Inhibition of Notch-1 mRNA is illustrated after 72 hours of curcumin treatment in pancreatic cancer cells. Control lane: Control cells (C) were treated with dimethyl sulfoxide (DMSO). The mRNA levels in BxPC-3 and PANC-1 pancreatic cancer cells treated with curcumin were assessed by using real-time reverse transcriptase-polymerase chain reaction analysis. Expression of the Notch-1 gene at the mRNA level was down-regulated after curcumin treatment. Single asterisk: $P < .05$; double asterisks: $P < .01$. (C,D) Inhibition of Notch-1, Hes-1, cyclin D1, and Bcl-X_L protein expression is illustrated after 72 hours of curcumin treatment in pancreatic cancer cells. Control lane: Control cells were treated with DMSO.

that curcumin decreases NF-κB DNA-binding activity in pancreatic cancer cells.

Down-Regulation of Notch-1 Expression by siRNA Promotes Curcumin-Induced Cell Growth Inhibition and Apoptosis in BxPC-3 Cells

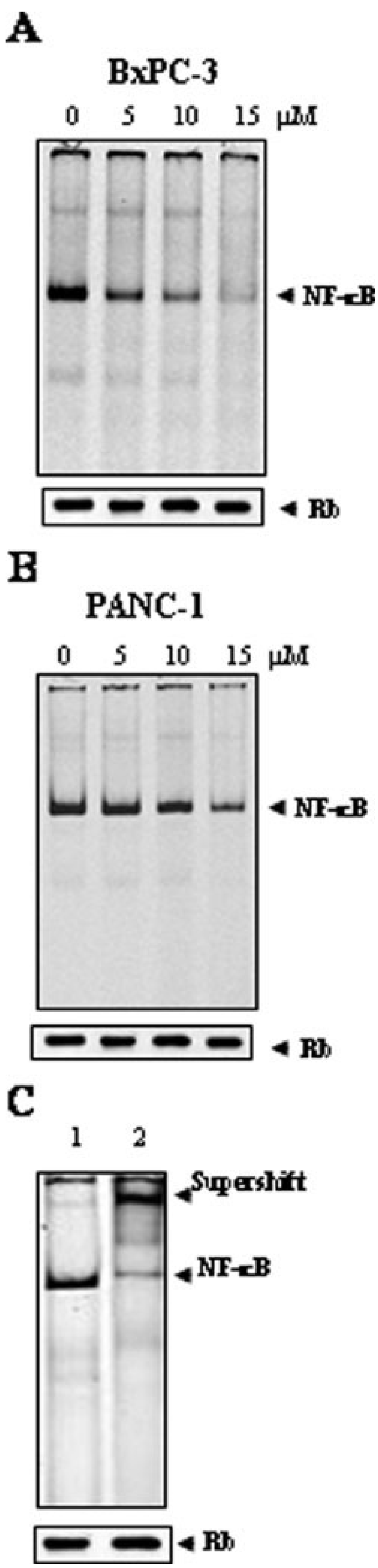
Because we observed similar effects of curcumin in both BxPC-3 cells and PANC-1 cells, we conducted a gene knock-down experiment only in BxPC-3 cells. We used Western blot analysis to detect the protein level of Notch-1 and found that intracellular Notch-1 was down-regulated in Notch-1 siRNA-transfected BxPC-3 cells compared with siRNA control-transfected cells (Fig. 5A). Down-regulation of Notch-1 expression significantly inhibited cell growth induced by curcumin (Fig. 5B). Notch-1 siRNA-transfected BxPC-3 cells were significantly more sensitive to spontaneous and curcumin-induced apoptosis (Fig. 5C). Nuclear extracts from Notch-1 siRNA-transfected BxPC-3 cells with different treatments were subjected to analysis for NF-κB DNA-binding activity, as measured by EMSA. The results showed that Notch-1 siRNA also inhibited NF-κB DNA-binding activity. Curcumin plus Notch-1 siRNA inhibited NF-κB activity to a greater degree compared with curcumin alone (Fig. 5D).

Over-Expression of Notch-1 by cDNA Transfection Reduced Curcumin-Induced Cell Growth Inhibition and Apoptosis in BxPC-3 Cells

The over-expression of Notch-1 by cDNA transfection showed over-expression of Notch-1 protein, as confirmed by Western blot analysis (Fig. 6A), and this over-expression in Notch-1 rescued curcumin-induced cell growth inhibition and abrogated curcumin-induced apoptosis to a certain degree (Fig. 6B,C). The over-expression of Notch-1 by cDNA transfection abrogated inactivition of NF-κB DNA-binding activity by curcumin (Fig. 6D). These results provide evidence for potential cross-talk between Notch-1 and the NF-κB signaling pathway during curcumin-induced cell growth inhibition and apoptosis in BxPC-3 cells.

DISCUSSION

In the current study, we observed that curcumin elicited a dramatic effect on growth inhibition and induction of apoptotic processes in BxPC-3 cells, as dem-



onstrated by the MTT assay and DNA-histone fragmentation analysis, respectively. To elucidate the mechanism of increased apoptosis induced by curcumin, we investigated the activity of Notch-1, which plays a key role in inhibition of the apoptotic response.

Notch signaling is an important cell signaling pathway that is needed critically for regulation of the balance between cell proliferation, differentiation, and apoptosis.[27] The Notch gene is known to suppress apoptosis and promote cell proliferation through a growth factor-mediated survival pathway.[24] There is evidence that Notch-1 prevents apoptosis in erythroleukemia cells.[25] Nair et al. reported that Notch-1 induced cyclin D1 and CDK2 activity and inhibited p53-dependent apoptosis in cervical cancer cells.[28] Recent results have shown that Notch-1 expression regulates cell death through both apoptosis and cell cycle pathways in erythroleukemia cells and was associated in part with the regulation of Bcl-X_L, $p21^{cip1/waf1}$, $p27^{kip1}$, and NF-κB.[12] It has been reported that Notch pathway components and Notch target genes are up-regulated in human pancreatic cancer.[8] Therefore, the down-regulation of Notch signaling may be a novel approach for pancreatic cancer therapy. Because of the central role of Notch in cell survival and proliferation, we explored this transcription factor as a target for curcumin. In the current study, we demonstrated for the first time that curcumin down-regulates the transcription and translation of Notch-1 and its downstream gene, Hes-1, cyclin D1, and Bcl-X_L. We also observed that the down-regulation of Notch-1 by siRNA, together with curcumin treatment, inhibited cell growth and induced apoptosis to a greater degree in pancreatic cancer cells compared with curcumin alone. These results indicate that the synergy between Notch-1 siRNA and curcumin are obvious in their effects on apoptosis induction rather than growth inhibition (Fig. 5), suggesting that curcumin may have other ways to induce apoptosis. Conversely, the over-

FIGURE 4. Curcumin inhibits nuclear factor κB (NF-κB) DNA binding activity. (A,B) Dose response of curcumin-induced inhibition of NF-κB DNA binding activity is illustrated. BxPC3 and PANC-1 pancreatic cancer cells were treated with 5 μM, 10 μM, and 15 μM curcumin for 72 hours. Nuclear extracts were prepared from control and curcumin-treated cells and were subjected to analysis for NF-κB DNA binding activity, as measured by using an electrophoretic mobility shift assay. The retinoblastoma (Rb) protein level served as a nuclear protein-loading control. (C) A supershift assay showed that the NF-κB band was shifted because of the formation of a bigger complex after the addition of anti-NF-κB p65 antibody. This assay confirmed the specificity of NF-κB binding to the DNA consensus sequence.

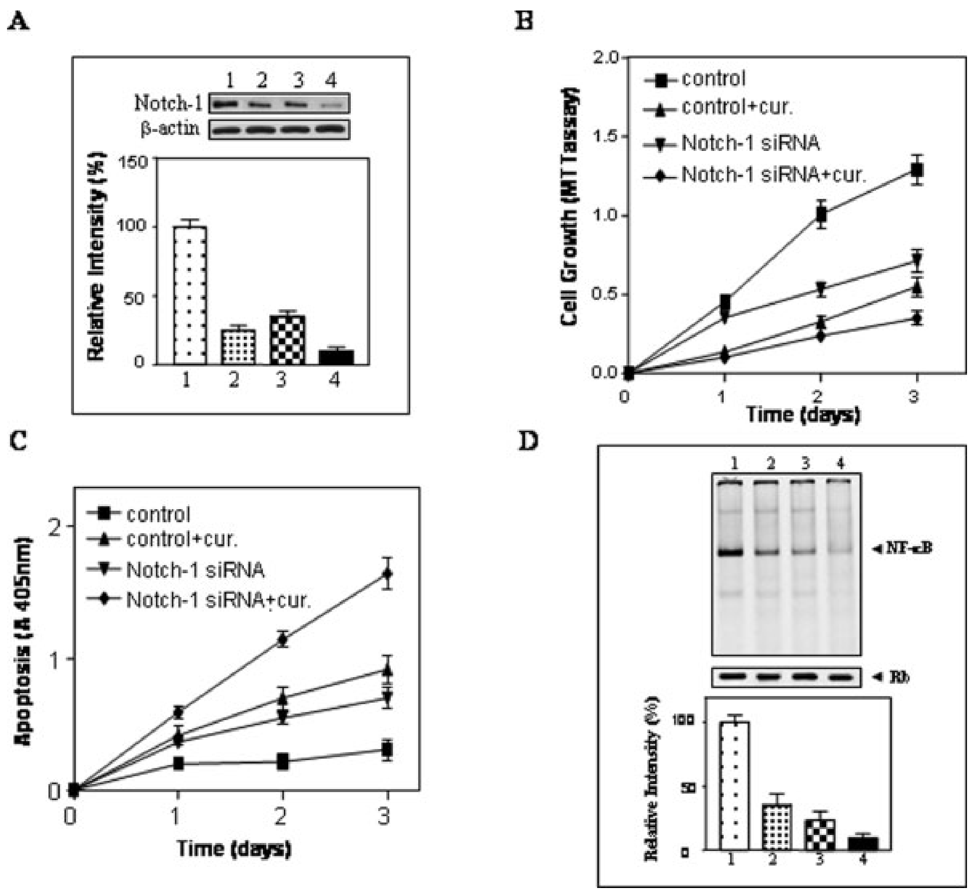


FIGURE 5. BxPC-3 pancreatic cancer cell growth inhibition and cell death were induced by Notch-1 small-interfering RNA (siRNA) and curcumin. (A) Notch-1 expression was down-regulated by curcumin and Notch-1 siRNA. Western blot analysis was used to detect the protein level of Notch-1. Intracellular Notch-1 was barely detectable in Notch-1 siRNA-transfected BxPC-3 cells compared with control cells: 1) control, 2) 10 μmol/L curcumin, 3) Notch-1 siRNA, and 4) Notch-1 siRNA plus 10 μmol/L curcumin. (B) The down-regulation of Notch-1 expression significantly inhibited cell growth. Curcumin plus siRNA Notch-1 inhibited cell growth to a greater degree compared with curcumin alone. (C) BxPC-3 pancreatic cancer cell death was induced by Notch-1 siRNA and curcumin. Down-regulation of Notch-1 expression significantly increased apoptosis induced by curcumin. Notch-1 siRNA-transfected BxPC-3 cells were significantly more sensitive to spontaneous and curcumin-induced apoptosis. (D) NF-κB DNA binding activity was measured by electrophoretic mobility shift assay (EMSA). Top: Curcumin and Notch-1 siRNA significantly inhibited NF-κB DNA-binding activity. Curcumin plus Notch-1 siRNA inhibited NF-κB DNA binding activity to a greater degree compared with curcumin alone. The retinoblastoma (Rb) protein level served as a nuclear protein-loading control. Control cells were treated with dimethyl sulfoxide: 1) control, 2) 10 μmol/L curcumin, 3) Notch-1 siRNA, and 4) Notch-1 siRNA plus 10 μmol/L curcumin. Bottom: A densitometric quantification is provided of the data presented at the top. The histogram indicates the relative band intensity, in arbitrary densitometric units, derived from densitometric scans of 3 independent experiments. Results are expressed as the percentage of NF-κB activity in the control. Columns and bars indicate the mean and standard deviation, respectively ($n = 3$ experiments).

expression of Notch-1 by Notch-1 cDNA transfection abrogated curcumin-induced apoptosis to a certain degree. Therefore, we believe strongly that the down-regulation of Notch-1 by curcumin is linked mechanistically to apoptotic processes because of the inactivation of Notch-1 and its downstream target genes.

Because NF-κB plays important roles in many cellular processes, research on the interaction of NF-κB activation with other cell signal-transduction pathways, including the Notch pathway, has received increased attention in recent years. Reportedly, there is cross-talk between Notch-1 and the NF-κB pathway. Notch-1 strongly induces NF-κB-promoter activity and induces the expression of several NF-κB subunits and NF-κB DNA-binding activity.[12,29] Activation of NF-κB leads to the up-regulation of several downstream target genes, including cyclin D1 and Bcl-X_L. Thus, the down-regulation of Notch-1 results in lower NF-κB activity and its downstream targets. Levels of basal and stimulation-induced NF-κB activity were decreased significantly in mice that had reduced Notch levels.[14] Therefore, it is possible that Notch-1-

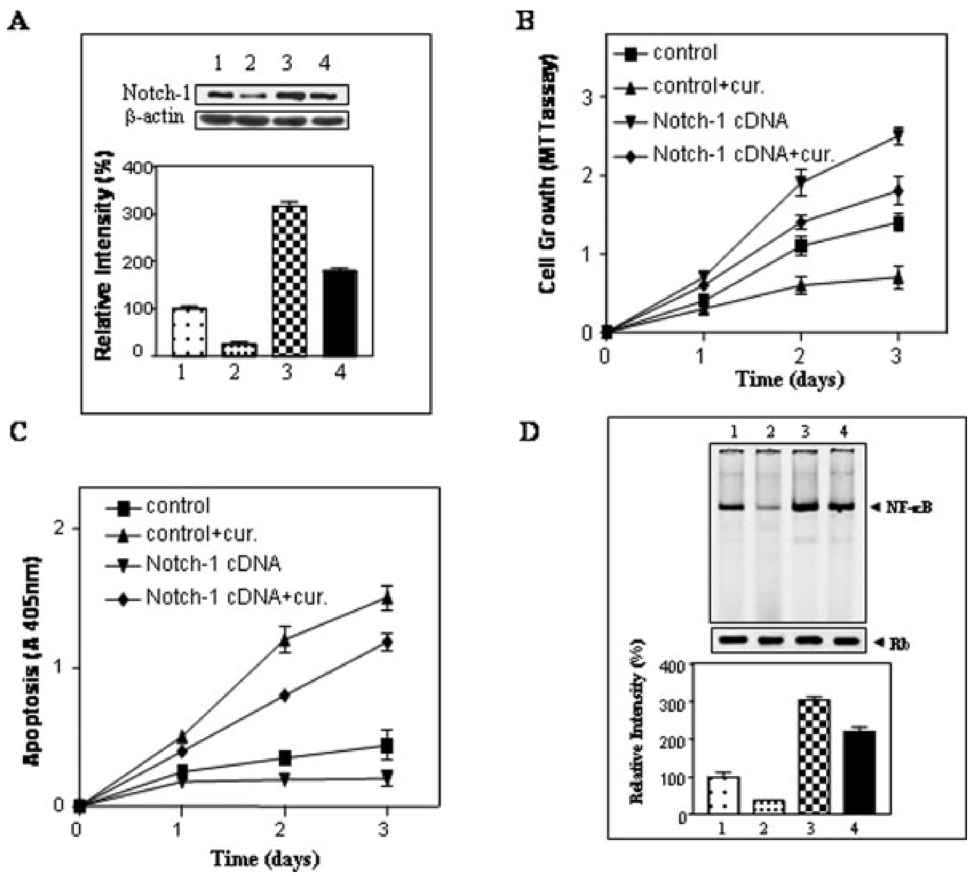


FIGURE 6. (A) Notch-1 expression was up-regulated by Notch-1 combinational DNA (cDNA). Top: Intracellular Notch-1 was increased in Notch-1 cDNA-transfected BxPC-3 cells compared with control transfected cells; 1) control, 2) control plus 10 μmol/L curcumin (cur.), 3) Notch-1 cDNA, and 4) Notch-1 cDNA plus 10 μmol/L curcumin. Bottom: Each histogram indicates the relative band intensity, in arbitrary densitometric units, derived from densitometric scans of 3 independent experiments. Results are expressed as percentage of control of Notch-1/β-actin. Columns and bars indicate the mean and standard deviation, respectively ($n = 3$ experiments). (B) Over-expression of Notch-1 significantly promoted cell growth. Over-expression of Notch-1 rescued curcumin-induced cell growth inhibition. (C) Over-expression of Notch-1 by Notch-1 cDNA transfection abrogated curcumin-induced apoptosis to a certain degree. (D) Notch-1 cDNA increased nuclear factor κB (NF-κB) DNA-binding activity in BxPC-3 pancreatic cancer cells. Top: Over-expression of Notch-1 by cDNA transfection abrogates inactivation of NF-κB DNA-binding activity by curcumin. The retinoblastoma (Rb) protein level served as a nuclear protein-loading control; 1) control, 2) control plus 10 μmol/L curcumin, 3) Notch-1 cDNA, and 4) Notch-1 cDNA plus 10 μmol/L curcumin. Bottom: This chart presents a densitometric quantification of the data presented in the top.

induced cell growth and inhibition of apoptosis, in part, are because of the activation of the NF-κB pathway. It was shown previously that curcumin is a potent inhibitor of NF-κB DNA-binding activity in pancreatic cancer cells.[30,31] Therefore, the results obtained from our gene transfection studies and NF-κB activity assays, together with published data, clearly suggest that there is cross-talk between Notch and the NF-κB pathway during curcumin-induced cell growth inhibition and apoptosis. The activation of NF-κB appears to be mediated by Notch-1, and the inhibition of NF-κB activity by curcumin treatment may be mediated in part by inhibition of the Notch-1 pathway. Furthermore, we observed that curcumin abrogated the NF-κB activation stimulated by Notch-1 cDNA transfection, suggesting that curcumin inhibits NF-κB activity through the Notch-1 signaling pathway: which also may be attributable to the inactivation of cyclin D1. Previous studies from our laboratory showed for the first time that cyclin D1 cDNA transfection may lead to the activation of NF-κB.[32] Therefore, it appears that NF-κB transcriptionally activates cyclin D1, and this leads to the perpetual activation of NF-κB in cancer cells. The current results, therefore, contribute immensely to the field, showing that Notch-1 leads to the activation of NF-κB and its target genes, such as cyclin D1, and that this pathway may be inactivated by curcumin.

Several other potential mechanisms also may explain why curcumin inhibits NF-κB activity through the Notch-1 signaling pathway. It has been reported that activated Notch-1 is mediated through the acti-

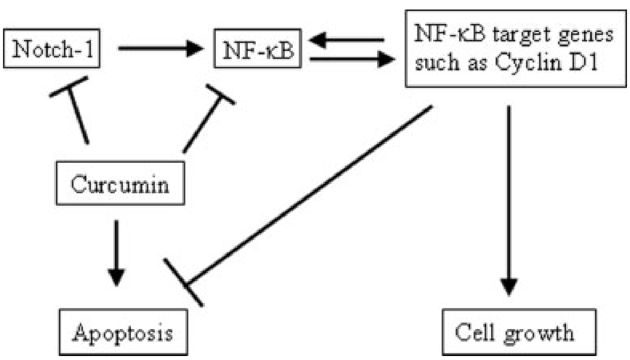


FIGURE 7. This is a schematic representation of curcumin-induced inhibition of nuclear factor κB (NF-κB) DNA-binding activity through Notch-1 signaling pathway.

vation of protein kinase B/Akt.[28,33] Activated Akt also reportedly activates IκB kinase (IKK), which regulates the NF-κB transcription factor.[34] Based on these observations, it would be logical to assume that the reduction in activity of NF-κB, in part, is because of the reduction in IKK expression. Indeed, we recently found that down-regulation of Notch-1 blocked NF-κB activation by inhibiting the expression of IKK.[35] NF-κB inhibition was correlated with suppression of IKK and IκBα phosphorylation by the down-regulation of Notch-1. It has been demonstrated that the suppression of NF-κB was mediated by curcumin-induced attenuation of IKK, and curcumin decreased the expression of NF-κB-regulated gene products, including cyclooxygenase 2 (COX-2) and prostaglandin E2, in pancreatic cancer.[30] Based on our results, along with the results reported by other investigators, we speculate that a possible mechanism by which curcumin may induce apoptosis is through the down-regulation of Notch-1, leading to the down-regulation of Akt, IKK, and NF-κB regulated genes, such as COX-2, survivin, Bcl-2, Bcl-X_L, and cyclin D1, during curcumin-induced cell growth inhibition and induction of apoptosis. On the basis of our results, we propose a hypothetical pathway by which curcumin may induce apoptosis in BxPC-3 cells, demonstrating possible cross-talk between Notch-1 and the NF-κB signaling pathway (Fig. 7).

The molecular mechanism(s) by which curcumin exerts its inhibitory effects on pancreatic cancer cells, as revealed in the current study, has opened up exciting new avenues for devising novel therapeutic strategies. The finding that curcumin regulates the transcription and translation of Notch-1 gene, which is known for its critical involvement in the control of angiogenesis, tumor cell invasion, and metastasis, may be exploited for devising therapeutic strategies. In addition, the down-regulation of Notch-1 and NF-κB by curcumin may be a useful strategy for chemosensitization of metastatic pancreatic cancer cells to standard therapeutics. However, further in-depth investigations together with preclinical animal studies are needed to establish the cause-and-effect relation of Notch-1 gene regulation and curcumin-induced cell growth inhibition and apoptosis in pancreatic cancer to test this novel hypothesis in future clinical trials.

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