Curcumin potentiates the antitumor effects of gemcitabine in an orthotopic model of human bladder cancer through suppression of proliferative and angiogenic biomarkers

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ABSTRACT

Little progress has been made in the last three decades in the treatment of bladder cancer. Novel agents that are nontoxic and can improve the current standard of care of this disease are urgently needed. Curcumin, a component of *Curcuma longa* (also called turmeric), is one such agent that has been shown to suppress pathways linked to oncogenesis, including cell survival, proliferation, invasion and angiogenesis. We investigated whether curcumin has potential to improve the current therapy for bladder cancer, using an orthotopic mouse model. Curcumin potentiated the apoptotic effects of gemcitabine against human bladder cancer 253JBV cells in culture. Electrophoretic mobility shift assay revealed that curcumin also suppressed the gemcitabine-induced activation of the cell survival transcription factor NF-κB. In an orthotopic mouse model, bioluminescence imaging revealed that while curcumin alone significantly reduced the bladder tumor volume, maximum reduction was observed when curcumin was used in combination with gemcitabine ($P < 0.01$ versus vehicle; $P < 0.01$ versus gemcitabine alone). Curcumin also significantly decreased the proliferation marker Ki-67 and microvessel density (CD31) ($P < 0.01$ versus vehicle; $P < 0.01$ versus gemcitabine alone), but maximum reduction occurred when it was combined with gemcitabine ($P < 0.01$ versus vehicle; $P < 0.01$ versus gemcitabine alone). Curcumin abolished the constitutive activation of NF-κB in the tumor tissue; induced apoptosis, and decreased cyclin D1, VEGF, COX-2, c-myc and Bcl-2 expression in the bladder cancer tissue. Overall our results suggest that curcumin alone exhibits significant antitumor effects against human bladder cancer and it further potentiates the effects of gemictabine, possibly through the modulation of NF-κB signaling pathway.

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1. Introduction

Bladder cancer is the fifth most common cancer in the United States (with an estimated 68,810 new cases and 14,100 deaths in 2007), and is a significant cause of morbidity and mortality all over the world [1]. While bladder cancer consists of a broad spectrum of histological tumors, transitional cell carcinoma (TCC) represents nearly 95% of all bladder cancers. Although chemotherapy does prolong life in some patients, the duration is short and only small percentages of the patients respond to the treatment. Because of lack of effective chemotherapy, the death rate from bladder cancer has remained constant over the last few decades. With an increasing cost burden of bladder cancer, it is the single most expensive human tumor from time of diagnosis to death, thus there is an urgent need for novel therapeutic agents to improve outcomes.

Anticancer drugs that are currently used to treat bladder cancer include cisplatin, methotrexate, vinblastine, and doxorubicin. For metastatic bladder cancer, a combination of gemcitabine and cisplatin are used [2]. As a single agent, gemcitabine is being studied in a national South West Oncology Group (SWOG) trial against superficial bladder cancer. Gemcitabine (2′-deoxy-2′,2′-difluorocytidine; dFdC) is a novel deoxycytidine analogue, a pyrimidine antimetabolite related to cytarabine, exerting its cytotoxic effects through inhibition of DNA synthesis. However, at doses required for efficacy, there are numerous toxic side effects including myelosuppression, neutropenia, fever, infection, anemia,

nausea, and vomiting [3]. Additionally, the median age at diagnosis is 73 years, and the 5-year survival is 38% for regional and 9% for distant cancer [1]. Thus agents which are well tolerated in an older population and can improve the outcome of current therapy are highly desirable.

Here we report that curcumin is such agent, commonly consumed in Southeast Asia, may have potential against bladder cancer. Curcumin (diferuloylmethane), derived from the spice turmeric (*Curcuma longa*), is a nontoxic agent that has been shown to inhibit the cell survival, proliferation, invasion, and characteristic angiogenesis of a wide variety of tumor cells, including bladder cancer, through the modulation of various cell signaling pathways [4,5]. Numerous clinical trials have indicated that curcumin is quite safe when administered even at very high doses [6]. It is currently in clinical trials in our institute, the University of Texas M. D. Anderson Cancer Centre, for the treatment of multiple myeloma [7] and pancreatic cancer [8]. In this study we determined whether curcumin has any potential against bladder cancer, either alone or in combination with gemcitabine *in vitro* and in an orthotopic human bladder cancer mouse model. The mechanism by which curcumin mediates any effect against bladder cancer was also explored.

2. Materials and methods

2.1. Materials

Curcumin (77.5% curcumin; 4.21% bisdemethoxy curcumin, 18.27% demethoxycurcumin; also called C3 complex) was kindly supplied by Sabinsa (Piscataway, NJ). The following polyclonal antibodies against p65 (recognizing the epitope within the N-terminal domain of human NF-κB p65), cyclin D1, and monoclonal antibodies against VEGF, COX-2, c-myc and Bcl-2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The liquid DAB+ substrate chromogen system-horse radish peroxidase used for immunocytochemistry was obtained from DakoCytomation (Carpinteria, CA). Penicillin, streptomycin, modified Eagle's MEM, amino acid, vitamin, and fetal bovine serum were obtained from Invitrogen (Grand Island, NY). Tris, glycine, NaCl, sodium dodecyl sulphate, and bovine serum albumin were obtained from Sigma Chemical (St. Louis, MO). Gemcitabine (Gemzar; kindly supplied by Eli Lilly, Indianapolis, IN) was stored at 4 °C and dissolved in sterile PBS on the day of use. D-Luciferin potassium salt (Xenogen, Hopkinton, MA) was dissolved in sterile PBS at 40 mg/mL concentration.

2.2. Cell lines and culture conditions

Human bladder cancer 253JBV cell line was generously provided by Dr. Colin P.N. Dinney (Department of Urology, The University of Texas M. D. Anderson Cancer Center, Houston, TX). This cell line was selected for this study because it develops a poorly differentiated and highly metastatic human bladder tumor that represents transitional cell carcinoma *in vivo*. This cell line was established from 253J human bladder cancer cell line by orthotopic implantation and *in vivo* recycling [9]. The cells were grown as a monolayer in modified Eagle's MEM supplemented with 10% fetal bovine serum, vitamins, sodium pyruvate, L-glutamine, nonessential amino acids, and penicillin-streptomycin.

2.3. Animals

Male *nu/nu* mice were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). The animals were housed four per cage in a specific pathogen-free animal facility and fed with regular chow diet with water *ad libitum*. Our experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee.

2.4. Antiproliferative assay

To determine whether curcumin or gemcitabine inhibit the proliferation of cells, was examined by the ability of mitochondria to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye in bladder cancer cell line 253JBV [10]. The cells (5000 cells per well) were incubated with curcumin and/or gemcitabine in triplicates in a 96-well plate for 48 h at 37 °C. MTT (5 mg/mL in PBS) solution was added to each well. After 2 h of incubation, the medium was replaced with dimethyl sulphoxide (40 μL/well) and MTT precipitates were dissolved before quantification of optical densities (570 nm). This experiment was repeated thrice. Cell viability was expressed as a percentage: (absorbance of the experiment samples/absorbance of the control) × 100.

2.5. Live/Dead assay

To determine whether curcumin can potentiate the apoptotic effects of gemcitabine in bladder cancer cells, we used a Live/Dead assay kit (Invitrogen). This two-color assay identifies live versus dead cells on the basis of membrane integrity and esterase activity. This assay uses calcein, a polyanionic, green fluorescent dye that is retained within live cells, and a red fluorescent ethidium homodimer dye that can enter cells through damaged membranes and bind to nucleic acids but is excluded by the intact plasma membranes of live cells [10]. Briefly, cells (5000 cells/well) were incubated in chamber slides, treated with gemcitabine for 24 h and curcumin for 4 h. Cells were then stained with the assay reagents for 30 min at room temperature. Cell viability was determined under a fluorescence microscope by counting live (green) and dead (red) cells.

2.6. Cell cycle analysis

To determine the effect of curcumin and/or gemcitabine on the cell cycle, cells were treated with curcumin and/or gemcitabine for 48 h. Cells were trypsinized, washed with PBS, and centrifuged. Supernatants were removed and the cells were resuspended in 500 μL of a cold (4 °C) propidium iodide solution (50 μg/mL propidium iodide, 0.1% Triton X-100, and 0.1% sodium citrate, in PBS) and incubated for at least 2 h at 4 °C before analysis. PI fluorescence was measured by fluorescence-activated cell sorting (FACS) analysis (FACScan FL-3 channel, Becton Dickinson, Mountain View, CA). Cells were scored as apoptotic (% Sub-G_0/G_1) as described previously [11].

2.7. Annexin V assay

An early indicator of apoptosis is the rapid translocation and accumulation of the membrane phospholipid phosphatidylserine from the cytoplasmic interface of membrane to the extracellular surface. This loss of membrane asymmetry can be detected by using the binding properties of Annexin V. This staining was done with the use of a kit (Annexin V apoptosis detection kit I, BD Biosciences). Cells were treated with gemcitabine and curcumin for 48 h. Then the cells were harvested by trypsinization, washed with PBS, and resuspended in 100 μL of binding buffer containing 5 μL Annexin V antibody, which was conjugated with the FITC fluorescence dye and propidium iodide. Then the cells were incubated for 15 min on ice, and 400 μL of binding buffer was added to each sample before analysis by flow cytometry.

2.8. Orthotopic implantation of 253JBV-Luc cells

To make it possible to image the tumor, bladder cancer cell line 253JBV was stably transfected with the luciferase reporter as described [12]. 253JBV-Luc cells were harvested from 70% to 80% confluent cultures by exposure to trypsin. Proteolysis was stopped with medium containing 10% fetal bovine serum, and the cells were washed once in serum-free medium and resuspended in HBSS. Only single-cell suspensions with >90% viability was used for injections. Mice were anesthetized with isoflurane, a small, lower abdominal incision was made, and the bladder was exteriorized. Tumor cells (0.5×10^6 cells/50 μL HBSS) were injected into the bladder wall muscle using a 30-gauge needle and a calibrated push button-controlled device. To prevent leakage, a cotton swab was pressed against for 30 s over the site of injection. The incision was closed in one layer with wound clips.

2.9. Experimental protocol

After 1 week of implantation, mice were randomized into the following treatment groups ($n = 8$) based on the bioluminescence measured after the first IVIS imaging: (a) untreated control (corn oil, 100 μL daily); (b) curcumin alone (1 g/kg), once daily, orally (c) gemcitabine alone (25 mg/kg), thrice weekly, i.p.; and (d) combination of curcumin (1 g/kg), once daily, orally and gemcitabine (25 mg/kg), thrice weekly, i.p. Tumor volumes were monitored weekly by the bioluminescence IVIS Imaging System 200 using a cryogenically cooled imaging system coupled to a data acquisition computer running Living Image software (Xenogen Corp., Alameda, CA). Before imaging, animals were anesthetized in an acrylic chamber with 2.5% isoflurane/air mixture and injected i.p. with 40 mg/mL D-Luciferin potassium salt in PBS at a dose of 150 mg/kg body weight. After 10 min of incubation with luciferin, mice were placed in a right lateral decubitus position, and a digital grayscale animal image was acquired followed by acquisition and overlay of a pseudocolor image representing the spatial distribution of detected photons emerging from active luciferase within the animal. Signal intensity was quantified as the sum of all detected photons within the region of interest per second. Mice were imaged on the 0, 7th, 14th, 21st, and 28th days of treatment. Therapy was continued for 4 weeks. On the 28th day, mice in all groups were sacrificed (this time point was chosen based on our previous experience in orthotopic bladder cancer models showing that beyond this time, control tumors grow to a size which causes bladder obstruction in the animals leading to uremia and death). Tumor volumes were compared among groups using unpaired Student's *t*-test. Half of the tumor tissue was formalin fixed and paraffin embedded for immunohistochemistry. The other half was snap frozen in liquid nitrogen and stored at −80 °C.

2.10. Preparation of nuclear extracts from tumor samples and cell lines

Tumor tissues from control and treated groups of mice were minced and incubated on ice for 30 min in 0.5 mL of ice-cold buffer A [10 mM HEPES (pH 7.9), 1.5 mM KCl, 0.5 mM DTT, 0.5 mM phenylmethylsulphonyl fluoride (PMSF)]. The minced tissue was homogenized using a Dounce homogenizer and centrifuged at $16,000 \times g$ at 4 °C for 10 min. The resulting nuclear pellet was suspended in 0.2 mL of buffer B [20 mM HEPES (pH 7.9), 25% glycerol, 420 mM NaCl, 0.5 mM DTT, 0.2 mM EDTA, 0.5 mM PMSF, 2 μg/mL leupeptin] and incubated on ice for 2 h with intermittent mixing. The suspension was then centrifuged at $16,000 \times g$ at 4 °C for 30 min. The supernatant (nuclear extract) was collected and stored at −80 °C until use. Protein concentration was measured by the Bradford assay with BSA as the standard.

2.11. Electrophoretic mobility shift assay

To assess NF-κB activation, nuclear extract was prepared from tumor samples and electrophoretic mobility shift assays (EMSA) carried out essentially as described previously [13]. Briefly, nuclear extracts prepared from tumor tissues/bladder cancer cells (1×10^6 c/mL) were incubated with ^{32}P-end-labeled 45-mer double-stranded NF-κB oligonucleotide (4 μg of protein with 16 fmol of DNA) from the HIV long terminal repeat (5′-TTGTTA-CAA**GGGACTTTC**CGCTG**GGGACTTTC**CAGGGGGAGGCGTGG-3′; bold-face indicates NF-κB-binding sites) for 30 min at 37 °C. The resulting DNA-protein complex was separated from free oligonucleotide on 6.6% native polyacrylamide gels. A double-stranded mutant oligonucleotide (5′-TTGTTACAACT**CACTTTC**CGCTGC**TCACTTTC**-CAGGGAGGCGTGG-3′) was used to examine the specificity of binding of NF-κB to the DNA. For supershift assays, nuclear extracts prepared from gemcitabine-treated cells were incubated with antibodies against either p50 or p65 subunit of NF-κB for 30 min at 37 °C before the complex was analyzed by EMSA. Preimmune serum was included as a negative control. Specificity of binding was also determined by using an excess of either unlabeled or mutated oligonucleotide for competition. The dried gels were visualized, and radioactive bands were quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and ImageQuant software.

2.12. Ki-67 immunohistochemistry

Formalin-fixed, paraffin-embedded sections were stained with anti-Ki-67 (rabbit monoclonal clone SP6; NeoMarkers, Fremont, CA) antibody as described previously [14]. Results were expressed as percentage of Ki-67$^+$ cells ± S.E. per 40× magnification. A total of ten 40× fields was examined and counted from each groups. The values were statistically compared by one-way analysis of variance (ANOVA) and Dunnett multiple comparison post-test.

2.13. Tumor microvessel density

Microvessel density was used as a marker for tumor angiogenesis. Frozen sections were fixed in formalin and stained with an antibody to CD31 (PECAM-1, PharMingen, San Diego, CA) as described previously [14]. The density of vessels was determined by counting the number of vessels per high-powered field (100×) in four areas of each tumor section. Results were expressed as the mean number of vessels ± S.E. per high-power field (100×). A total of 20 high-power fields was examined and counted from three tumors of each of the treatment groups. The values were compared using by one-way analysis of variance (ANOVA) and Dunnett multiple comparison post-test.

2.14. Quantification of apoptosis in tumor sections

Apoptotic cells in tumor samples were quantified by fluorescent terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) using a commercial kit (DeadEnd Fluorometric TUNEL System, Promega, Madison, WI) according to the manufacturer's instructions with the following modifications. TUNEL-positive cells from 10 independent fields were quantified manually. Samples were fixed with 4% paraformaldehyde (methanol-free) for 10 min at room temperature, washed with PBS, and permeabilized by incubating with 0.2% Triton X-100 in PBS (v/v) for 15 min. The samples were incubated with equilibration buffer (from the kit). Reaction buffer containing equilibration buffer (45 μL), nucleotide mix (5 μL), and Tdt (1 μL) was added to the sections and incubated in a humidified chamber for 1 h at 37 °C protected from light. The reaction was terminated by immersing the samples in 2× SSC [30 mM NaCl, 3 mM sodium citrate (pH 7.2)]

for 15 min followed by three washes to remove unincorporated fluorescein-dUTP. Background reactivity was determined by processing the slides in the absence of Tdt (negative control). Immunofluorescence microscopy was performed using a Zeiss Plan-Neofluar lens on an epifluorescence microscope equipped with narrow band pass excitation filters mounted in a filter wheel (Ludl Electronic Products, Hawthorne, NY) to individually select for green, red, and blue fluorescence. Images were captured using a cooled CCD camera (Photometrics, Tucson, AZ). DNA fragmentation was detected by localized green fluorescence within the nucleus of apoptotic cells. For total TUNEL expression, apoptotic events were quantified manually [15]. The values were compared using by one-way analysis of variance (ANOVA) and Dunnett multiple comparison post-test.

2.15. Immunohistochemical analysis for VEGF, COX-2, p65 and cyclin D1 in tumor tissues

Tissue sections of formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene followed by treatment with a graded series of alcohol [100%, 95%, and 80% ethanol/double-distilled H_2O (v/v)] and rehydrated in PBS (pH 7.5) [16]. Antigen retrieval for paraffin-embedded tissues was performed with sodium citrate 0.01 mol/L (pH 6.0) for 98 °C for 5 min. Endogenous peroxidase was blocked by the use of 3% hydrogen peroxide in methanol for 10 min. The samples were washed thrice with PBS and incubated for 20 min at room temperature with a protein blocking solution containing 5% normal horse serum and 1% normal goat serum in PBS. Excess blocking solution was drained, and the samples were incubated overnight at 4 °C with one of the following: 1:100 dilution of rabbit polyclonal anti-VEGF (Santa Cruz Biotechnology), 1:400 dilution of rabbit polyclonal anti-human p65 (Abcam), 1:100 dilution of anti-cyclin D1, or 1:100 dilution of anti-COX-2 antibodies. The samples were then rinsed thrice with PBS and incubated for 1 h at room temperature with the appropriate dilution of the secondary antibody: peroxidase-conjugated anti-rabbit IgG, $F(ab')_2$ fragment (Jackson ImmunoResearch Laboratory, West Grove, PA), anti-mouse IgG1 (BD PharMingen), or anti-mouse IgG (Jackson ImmunoResearch Laboratory). Positive reactions were visualized by incubating the slides with stable 3,3'-diaminobenzidine (Research Genetics, Huntsville, AL) for 5–10 min. The sections were then washed thrice with PBS, counterstained with Gill's hematoxylin for 14–20 s (Biogenex, San Ramon, CA), washed thrice with distilled water, and treated with PBS for 1 min. The slides were mounted with a universal mounting medium (Research Genetics) and dried at 56–60 °C for 5–10 min. Slides were examined under the microscope. The values were compared using by one-way analysis of variance (ANOVA) and Dunnett multiple comparison post-test.

2.16. Western blot analysis

Tumor tissues from each group were minced and incubated on ice for 30 min in 0.5 mL of ice-cold whole-cell lysate buffer (10% NP-40, 5 mM NaCl, 1 mM HEPES, 0.1 mM EGTA, 0.5 mM EDTA, 0.1 mM PMSF, 0.2 mM sodium orthovanadate, 1 mM NaF, 2 μg/mL aprotinin, 2 μg/mL leupeptin). The minced tissue was homogenized using a Dounce homogenizer and centrifuged at $16,000 \times g$ at 4 °C for 10 min. The proteins were then fractionated by SDS-PAGE, electrotransferred to nitrocellulose membranes, blotted with each antibody, and detected by enhanced chemiluminescence (GE Healthcare, Piscataway, NJ).

3. Results

The goals of this study were first, to determine whether curcumin potentiates the antitumor effects of gemcitabine in vitro;

second, to determine whether curcumin improves the antitumor effects of gemcitabine in an orthotopic nude mouse model; and third, to delineate the mechanism by which curcumin mediates its effects. Since 95% of all bladder cancers encountered in the United States are transitional cell carcinoma, we selected highly metastatic human transitional cell carcinoma 253JBV cells for this investigation. To facilitate the imaging of the tumor in animals, luciferase-transfected 253JBV cells were employed.

3.1. Curcumin potentiates the antitumor effects of gemcitabine against bladder cancer cell line 253JBV in vitro

We first examined the effect of curcumin, gemcitabine and the combination on the viability of bladder cancer cells by the MTT method. The results show that both curcumin and gemcitabine inhibited the growth of bladder cancer cells in a dose-dependent manner, but maximum inhibition was observed when two agents were employed in combination (Fig. 1A). The Live/Dead showed that curcumin increased the gemcitabine-induced apoptosis from 3% to 76% (Fig. 1B). FACS quantification of Sub-G_0/G_1 population demonstrated that curcumin increased gemcitabine-induced apoptosis from 20% to almost 60% (Fig. 1C). Annexin V staining also revealed that curcumin enhanced apoptosis induced by gemcitabine, from 20% to almost 70% (Fig. 1D). Thus all these methods reveal that curcumin enhanced the efficacy of gemcitabine against bladder cancer cells.

Next, we also analyzed the effect of curcumin and gemcitabine on the expression of tumor cell survival, inflammatory, proliferative and angiogenetic gene products by western blotting. The results show that curcumin or gemcitabine alone had minimal effect on tumor cell survival (Bcl-2), proliferative (c-myc and cyclin D1), proinflammatory (COX-2), and angiogenetic (VEGF) gene products (Fig. 1E), however, when both were combined together showed significant downregulation of the expression of those gene products.

3.2. Curcumin inhibits NF-κB activation induced by gemcitabine
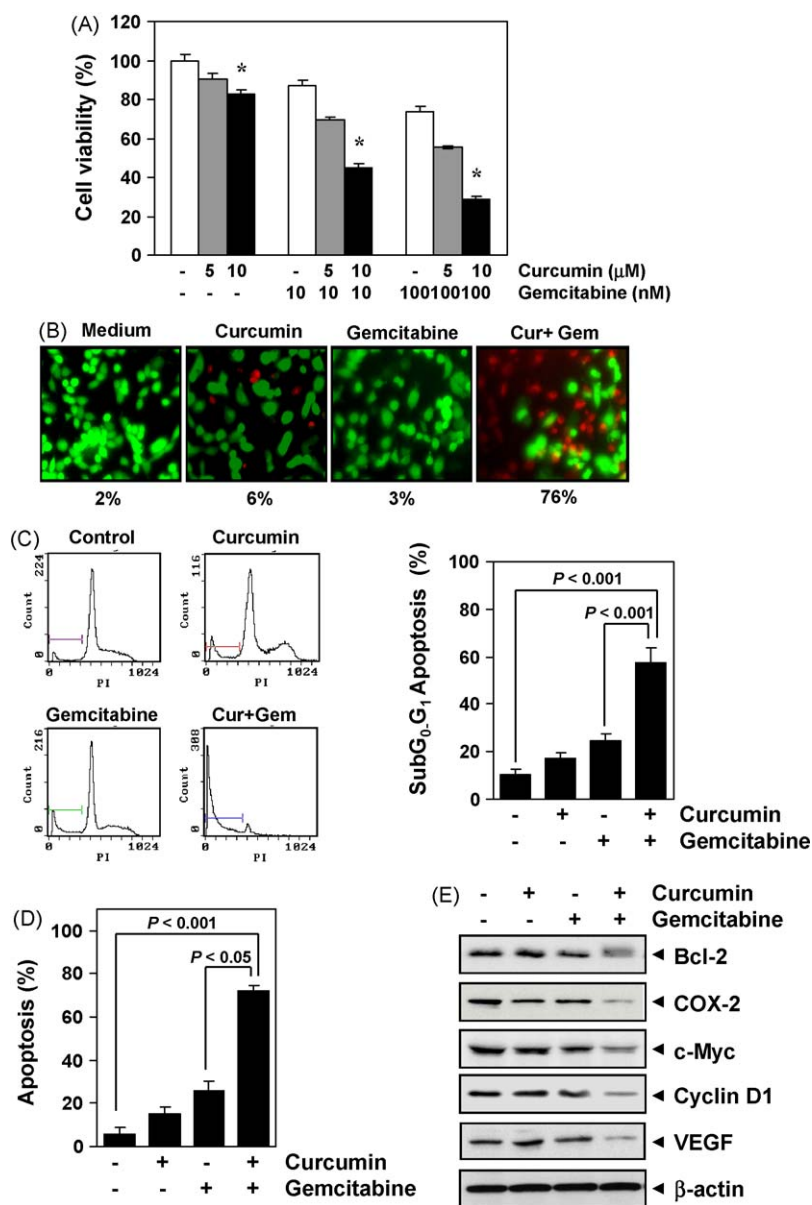
How curcumin potentiates the effect of gemcitabine against bladder cancer cells was investigated. Since NF-κB has been shown to mediate antiapoptotic effects, we investigated if curcumin mediates its effects through suppression of NF-κB activation. We found that gemcitabine-induced NF-κB in a time-dependent manner in 253JBV cells (Fig. 2A), and curcumin suppressed gemcitabine-induced NF-κB activation in a dose-dependent manner (Fig. 2B).

To determine the specificity of NF-κB activated by gemcitabine, the band was analyzed by supershift assay. Nuclear extracts from gemcitabine-stimulated 253JBV, when treated with antibodies against the p50 (NF-κB1) or p65 (RelA) subunits of NF-κB, the major band was shifted to a higher molecular mass (Fig. 2C), thus suggesting that the gemcitabine-induced complex consisted of p50 and p65 subunits. Preimmune serum (PIS) and mutant oilgonucleotide of NF-κB had no effect on this band, and excess (100-fold) unlabeled NF-κB caused complete disappearance of the band.

3.3. Curcumin potentiates the antitumor effects of gemcitabine in orthotopic bladder cancer tumors in nude mice

Next, we examined the effects of curcumin and gemcitabine, alone and in combination in vivo (Fig. 3A). The mice were randomized into four groups, and therapy was initiated 7 days after tumor implantation. IVIS imaging was done once a week, on days 7, 14, 21 and 28 after the start of treatment. The bioluminescence imaging results (Fig. 3B) showed a greater increase in tumor volume in the control group than the three
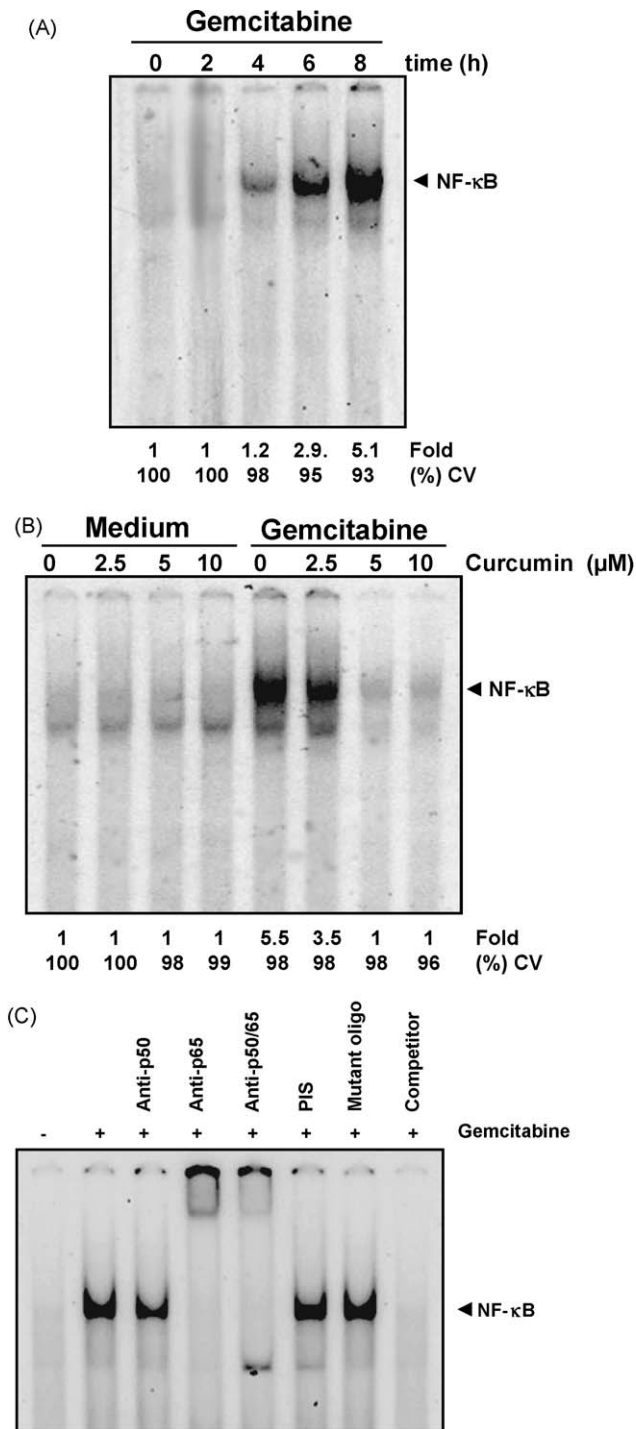
Fig. 1. Curcumin potentiates the effects of gemcitabine against human bladder cancer cells *in vitro*. (A) 253JBV cells (5000 cells per 0.1 mL) were incubated with indicated concentrations of curcumin and gemcitabine at 37 °C for 48 h and then cell viability was examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent. Data are the representative of three independent experiments. *Columns*, mean (n = 3); *bars*, S.E. $^*P < 0.001$, compared with untreated cells. (B) Curcumin potentiates the cytotoxic effects of gemcitabine as determined by the Live/Dead assay. Cells were treated with curcumin (5 μM), gemcitabine (100 nM), or the combination. After 24 h incubation, cells were stained with the assay reagents and cell viability was determined under a fluorescence microscope. *Percentages*, apoptotic bladder cancer cells. *Values* are mean of triplicates. (C) Curcumin potentiates the cell cycle arrest in human bladder cancer cells. 253JBV (1×10^6) cells were incubated with curcumin (5 μM), gemcitabine (100 nM) or both for 48 h, then stained with propidium iodide and analyzed using FACS analysis. Percentage of Sub-G_0/G_1 apoptosis in each group was plotted. Data are the representative of three independent experiments. *Columns*, mean (n = 3); *bars*, S.E. (D) Curcumin potentiate the gemcitabine-induced apoptosis in bladder cancer cells. 253JBV (1×10^6) cells were incubated with curcumin (5 μM), gemcitabine (100 nM) or both for 48 h. Thereafter, cells were incubated with anti-Annexin V antibody with FITC and analyzed with a flow cytometer for early apoptotic events. *Columns*, mean (n = 3); *bars*, S.E. (E) Curcumin potentiates the effect of gemcitabine on the expression of gene products linked to tumor cell survival, inflammatory, proliferation and angiogenesis. 253JBV (0.5×10^6) cells were incubated with curcumin (5 μM), gemcitabine (100 nM) or both for 48 h. Whole-cell extracts were prepared, separated on SDS-PAGE, and subjected to Western blot analysis using the indicated proteins. The same blots were stripped and reprobed with β-actin antibody to show equal protein loading.

treated groups. All animals were sacrificed on the 28th day. The final tumor volumes on day 28 after the start of treatment showed significant decrease in the curcumin + gemcitabine group as compared with vehicle-treated control group ($P < 0.01$ versus vehicle) or with gemcitabine alone ($P < 0.0001$ versus gemcitabine) (Fig. 3B, left panel). The tumor volumes in curcumin alone and gemcitabine alone groups were not statistically significant as compared with control group (P = 0.643 and 0.247, respectively).

When examined by tumor weight, control groups showed consistently larger tumors than the treated groups (Fig. 3C). When compared to the vehicle group, single agent gemcitabine-treated ($P < 0.01$) or curcumin-treated mice showed inhibition of the growth of the tumors to the same extent. Curcumin and gemcitabine together group showed remarkable inhibition of tumor growth ($P < 0.001$ versus vehicle; $P < 0.05$ versus gemcitabine): the tumors were significantly smaller than those exposed to vehicle or to single agents.

(A) Gemcitabine

0 2 4 6 8 time (h)

◄ NF-κB

| 1 | 1 | 1.2 | 2.9. | 5.1 | Fold |
| 100 | 100 | 98 | 95 | 93 | (%) CV |

(B) Medium   Gemcitabine

0 2.5 5 10 0 2.5 5 10 Curcumin (μM)

◄ NF-κB

| 1 | 1 | 1 | 1 | 5.5 | 3.5 | 1 | 1 | Fold |
| 100 | 100 | 98 | 99 | 98 | 98 | 98 | 96 | (%) CV |

(C)

Anti-p50 Anti-p65 Anti-p50/65 PIS Mutant oligo Competitor

- + + + + + + + Gemcitabine

◄ NF-κB

Fig. 2. Gemcitabine induces NF-κB activation in human bladder cancer cells and curcumin inhibits it. (A) Bladder cancer cells (253JBV cells, 1×10^6) were treated with gemcitabine 100 μM for indicated time intervals. Nuclear extracts were prepared and then analyzed for NF-κB activation using EMSA. Fold activation is indicated. (B) Bladder cancer cells (253JBV cells, 1×10^6) were exposed to gemcitabine 100 μM for 8 h and then treated with different concentrations of curcumin for 4 h. Nuclear extracts were prepared and were analyzed for NF-κB activation using EMSA. CV (%) indicates cell viability of the cells. (C) NF-κB induced by gemcitabine is composed of p65 and p50 subunits. Nuclear extracts from untreated 253JBV cells or 100 μM gemcitabine-treated were incubated with the indicated antibodies, an unlabeled NF-κB oligonucleotide probe, or a mutant oligonucleotide (*Mutant oligo*) probe. They were then assayed for NF-κB activation by EMSA.

3.4. Curcumin inhibits biomarkers of proliferation and angiogenesis in the tumors

To investigate the mechanisms by which curcumin manifests its effects against bladder cancer, we examined by immunohistochemistry the expression of Ki-67, a biomarker for growth of tumors and CD31, a biomarker for microvessel density in tumor tissues. Results in Fig. 4A and B show that both curcumin and gemcitabine, when compared with the untreated control, decreased the expression of Ki-67 to a similar extent, but the maximum decrease was noted when two agents were used in combination ($P < 0.01$ versus vehicle; $P < 0.01$ versus gemcitabine alone). Curcumin was more effective than gemcitabine in decreasing CD31 expression, but the two in combination were maximally effective (Fig. 4C and D) ($P < 0.01$ versus vehicle; $P < 0.01$ versus gemcitabine alone).

3.5. Curcumin potentiates gemcitabine-induced apoptosis in the tumor tissue

As shown in Fig. 5A, TUNEL staining showed that curcumin and gemcitabine alone induced 26% and 10% apoptosis, respectively; but the two agents together, induced 88% apoptosis ($P < 0.01$ versus vehicle; $P < 0.01$ versus gemcitabine alone) in the tumor tissue, indicating curcumin is highly effective in potentiating the apoptotic effects of gemcitabine.

3.6. Curcumin decreases cyclin D1 expression in the tumor tissue

Whether curcumin can affect the cyclin D1 expression, another proliferation marker, in the tumor tissue, was examined by IHC. As shown in Fig. 5B, curcumin and gemcitabine alone reduced the expression of cyclin D1 to 16% and 48% of control, respectively. The two agents together, however, reduced the expression by 98% ($P < 0.01$ versus vehicle; $P < 0.01$ versus gemcitabine alone) in the tumor tissue.

3.7. Curcumin decreases COX-2 expression in the tumor tissue

Whether curcumin can affect the COX-2 expression, a marker for inflammation, in the tumor tissue was examined by IHC. As shown in Fig. 5C, curcumin and gemcitabine alone reduced the expression of COX-2 to 30% and 56% of control, respectively. The two agents together, however, reduced the expression by 92% ($P < 0.01$ versus vehicle; $P < 0.01$ versus gemcitabine alone) in the tumor tissue.

3.8. Curcumin decreases VEGF expression in the tumor tissue

Whether curcumin can affect the VEGF expression, another marker for angiogenesis, in the tumor tissue was examined by IHC. As shown in Fig. 5D, curcumin and gemcitabine alone reduced the expression of VEGF to 15% and 80% of control, respectively. The two agents together, however, reduced the expression by 94% ($P < 0.01$ versus vehicle; $P < 0.01$ versus gemcitabine alone) in the tumor tissue.

3.9. Curcumin inhibits NF-κB and downregulates the NF-κB-regulated gene products in tumor tissues

Whether curcumin can affect NF-κB expression, linked with survival, proliferation and angiogenesis, was examined by IHC. As shown in Fig. 6A, curcumin alone reduced the expression of NF-κB by 82%; whereas gemcitabine alone had no significant effect. Two agents together reduced the expression by 98% in the tumor tissue.

We also analyzed the effect of curcumin and gemcitabine on NF-κB in tumor tissues by EMSA. The results show that curcumin
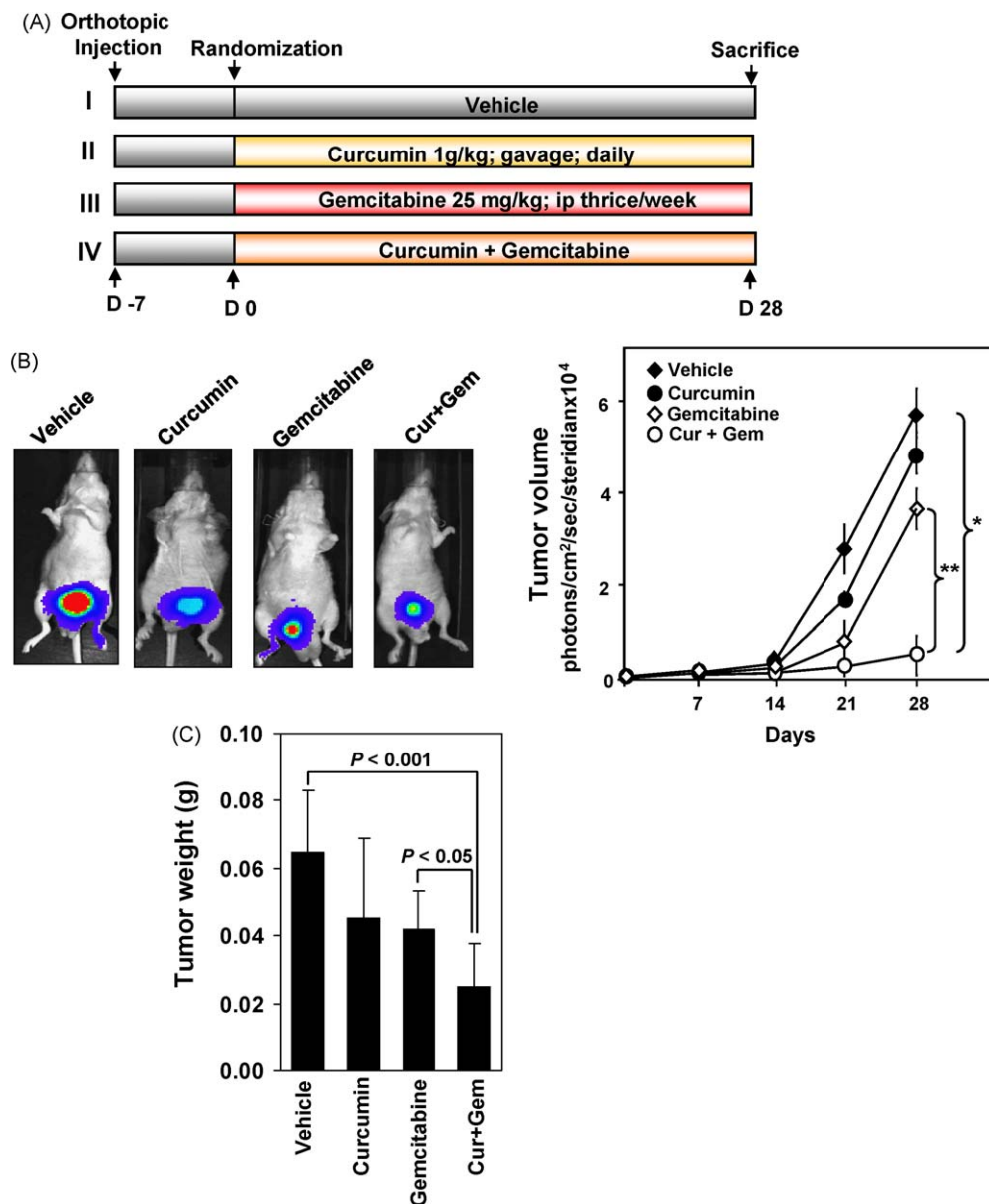
Fig. 3. Curcumin potentiates the antitumor effects of gemcitabine against human bladder cancer growth in nude mice. (A) Schematic representation of experiment protocol described in materials and methods. Animals were divided into four groups. (a) untreated control (corn oil, 100 μL daily); (b) curcumin alone (1 g/kg), once daily, orally; (c) gemcitabine alone (25 mg/kg), thrice weekly, i.p.; and (d) combination of curcumin (1 g/kg), once daily, orally and gemcitabine (25 mg/kg), thrice weekly, i.p. (B) (Left) Bioluminescence IVIS images of orthotopically implanted bladder tumor in live, anesthetized mice. (Right) Measurements of photons per second depicting the tumor volumes of mice using the IVIS imaging at indicated time intervals ($n = 8$). *Points*, mean; *bars*, S.E. $^*P < 0.01$, vehicle vs. Cur + Gem; $^{**}P < 0.0001$, gemcitabine vs. Cur + Gem. (C) Tumor weights measured on the last day of the experiment ($n = 8$); *bars*, S.E. $P < 0.001$, vehicle vs. Cur + Gem; $P < 0.05$, gemcitabine vs. Cur + Gem.

completely downregulated the NF-κB in tumor tissue and gemcitabine had minimal effect (Fig. 6B). NF-κB is known to regulate the expression of genes involved in survival (Bcl-2), proliferation (cyclin D1 and c-myc), inflammation (COX-2) and angiogenesis (VEGF) [17]. Western blot results confirmed that curcumin downregulated the expression of all these proteins, but gemcitabine alone had minimal effect (Fig. 6C). These data are in agreement with the IHC analysis (Fig. 5).

4. Discussion

The aim of the current study was to find a non-toxic agent that enhances the therapeutic effect of gemcitabine, against bladder cancer. For various reasons, we selected curcumin to examine its potential as such an agent *in vitro* and *in vivo*. *In vitro* results using various methods showed that curcumin and gemcitabine alone are quite effective in suppressing the proliferation and inducing apoptosis but two agents together are much more effective. When examined for the mechanism, we found that gemcitabine activates NF-κB, and curcumin downmodulate the NF-κB activation. Orthotopic animal models for bladder cancer also revealed that curcumin alone had significant antitumor activity against bladder cancer and this activity was further enhanced when combined with gemcitabine. When examined for biomarkers of survival, proliferation, inflammation, and angiogenesis, curcumin significantly downregulated all of them.

This is the first report to indicate that curcumin has a potential to enhance the effect of gemcitabine against human bladder

cancer. We found that curcumin alone also had significant effect against the growth of bladder cancer. Using another bladder cancer cell line (KU-7), Chadalapaka et al. recently showed that curcumin could suppress its growth in athymic nude mice [18]. Several mechanisms may explain how curcumin mediates its effects on bladder cancer cells. We showed that curcumin's effects on NF-κB and NF-κB-regulated gene products represent one of the major mechanisms. Curcumin downregulated gemcitabine-induced NF-κB in vitro; and it also inhibited NF-κB activation in the tumor tissue in vivo. Several gene products that have been linked with survival, proliferation, invasion, and angiogenesis are known to be regulated by NF-κB activation [17]. Chemoresistance has also been linked with NF-κB activation [19]. How curcumin inhibits NF-κB activation, has also been investigated. We have shown that inhibition of IκBα kinase, IκBα phosphorylation and IκBα degradation [20,21] are the major mechanisms by which curcumin suppresses NF-κB activation. The downregulation of NF-κB-regulated genes such as Bcl-2, survivin and cyclin D1 by curcumin was recently linked to the downregulation of specific protein (Sp)-1, Sp3 and Sp4 [18]. The downregulation of Sp has been demonstrated in the bladder tumors by curcumin.

We found that curcumin significantly downregulated the proliferation marker Ki-67 in the bladder tissue. This correlated with downregulation of other markers of proliferation including cyclin D1, c-myc and COX-2. The overall apoptosis in the tissue, as indicated by the TUNEL staining, was synergistically upregulated by curcumin and gemcitabine. Besides proliferation and apoptosis markers, we found that curcumin also downregulated microvessel density marker CD31 and angiogenesis marker VEGF in the tumor tissue. Curcumin has been shown by various groups to suppress angiogenesis in vitro and in vivo [22]. Both VEGF and VEGFR1 have been shown to be downregulated by curcumin [18,21]. The downregulation of angiogenic markers by curcumin is also most likely due to downregulation of NF-κB and Sp.

The bladder cancer 253JBV cells used in the current study is a variant of the human 253J bladder cancer cell line selected via orthotopic "recycling" in vivo for aggressive growth and metastasis [9]. Tumors derived from these cells secrete high levels of angiogenic factors (VEGF, IL-8, and active MMP-9) and tend to be refractory to gemcitabine-induced apoptosis [23]. These features mirror the properties of advanced bladder cancer in patients; thus providing a clinically relevant experimental tool for
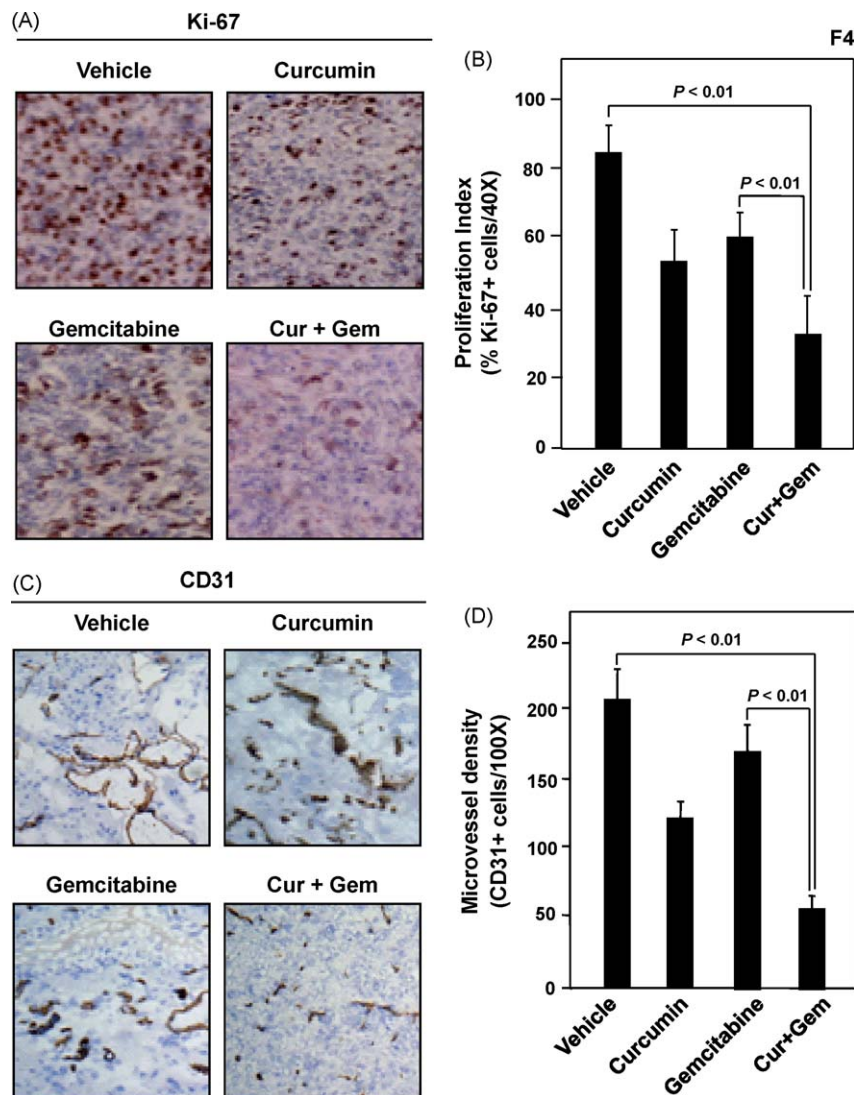
Fig. 4. Curcumin potentiates the effect of gemcitabine by inhibiting markers of proliferation (Ki-67) and microvessel density (CD-31). (A) Immunohistochemical analysis of proliferation marker Ki-67 in bladder tumors indicates the inhibition of cell proliferation in curcumin alone and in combination with gemcitabine-treated mice. (B) Quantification of Ki-67$^+$ cells as described in materials and methods. Columns, mean (n = 3); bars, S.E. (C) Immunohistochemical analysis of CD31 for microvessel density indicates inhibition of angiogenesis by curcumin alone and in combination with gemcitabine-treated mice. (D) Quantification of CD31$^+$ for microvessel density as described in Section 2. Columns, mean (n = 3); bars, S.E.
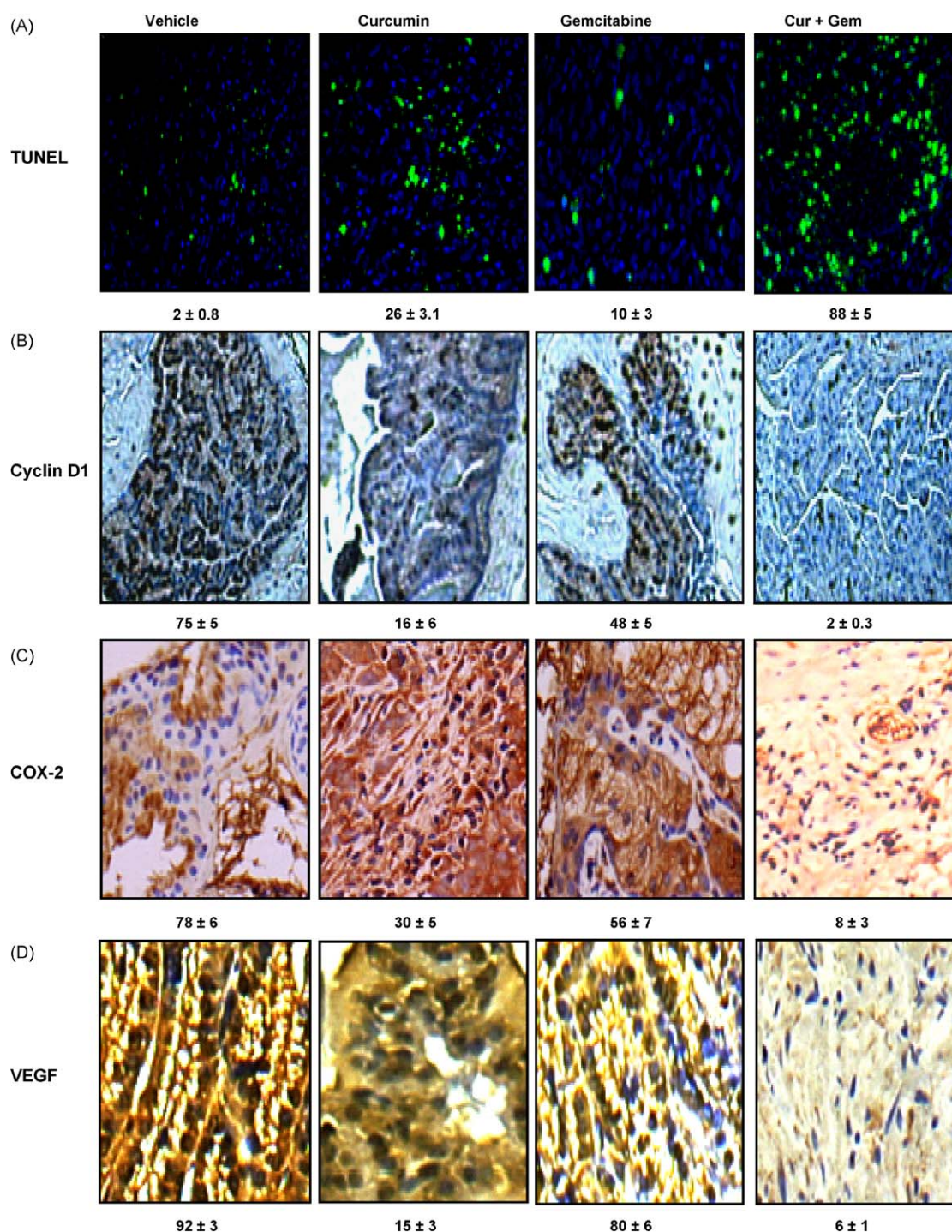
Fig. 5. Curcumin potentiates the effect of gemcitabine by inducing apoptosis and inhibiting cyclin D1, COX-2, and VEGF. (A) TUNEL assay showed that Curcumin potentiates the effect of gemcitabine by inducing apoptosis in mouse bladder tissues. (B) Immunohistochemical analysis of Cyclin D1. The percentage inhibition cyclin D1 by curcumin alone and in combination with gemcitabine is indicated. (C) Immunohistochemical analysis of COX-2. The percentage inhibition COX-2 by curcumin alone and in combination with gemcitabine is indicated. (D) Immunohistochemical analysis of VEGF. The percentage inhibition VEGF by curcumin alone and in combination with gemcitabine is indicated.

assessing the activity of curcumin. We found that the combination therapy resulted in significant antitumor activity. Bladder tumors initially respond well to multi-drug cytotoxic chemotherapeutic regimens, but eventually most patients succumb to chemoresistant metastasis, after a relatively short disease interval. At the current time, there is considerable interest in chemotherapy regimens based upon gemcitabine in this group of patients. Gemcitabine doses currently used have numerous side effects including myelosuppression, neutropenia, fever, infection, anemia, nausea and vomiting [3]. Thus therapies are being developed which can enhance the efficacy of gemcitabine. We have shown previously that treatment with a proteasome inhibitor bortezomib can enhance gemcitabine-induced apoptosis of bladder cancer [23]. However, bortezomib too has significant toxicity, which limits its clinical use. On the other hand, curcumin has minimal systemic side effects, and thus is a likely candidate for clinical use,
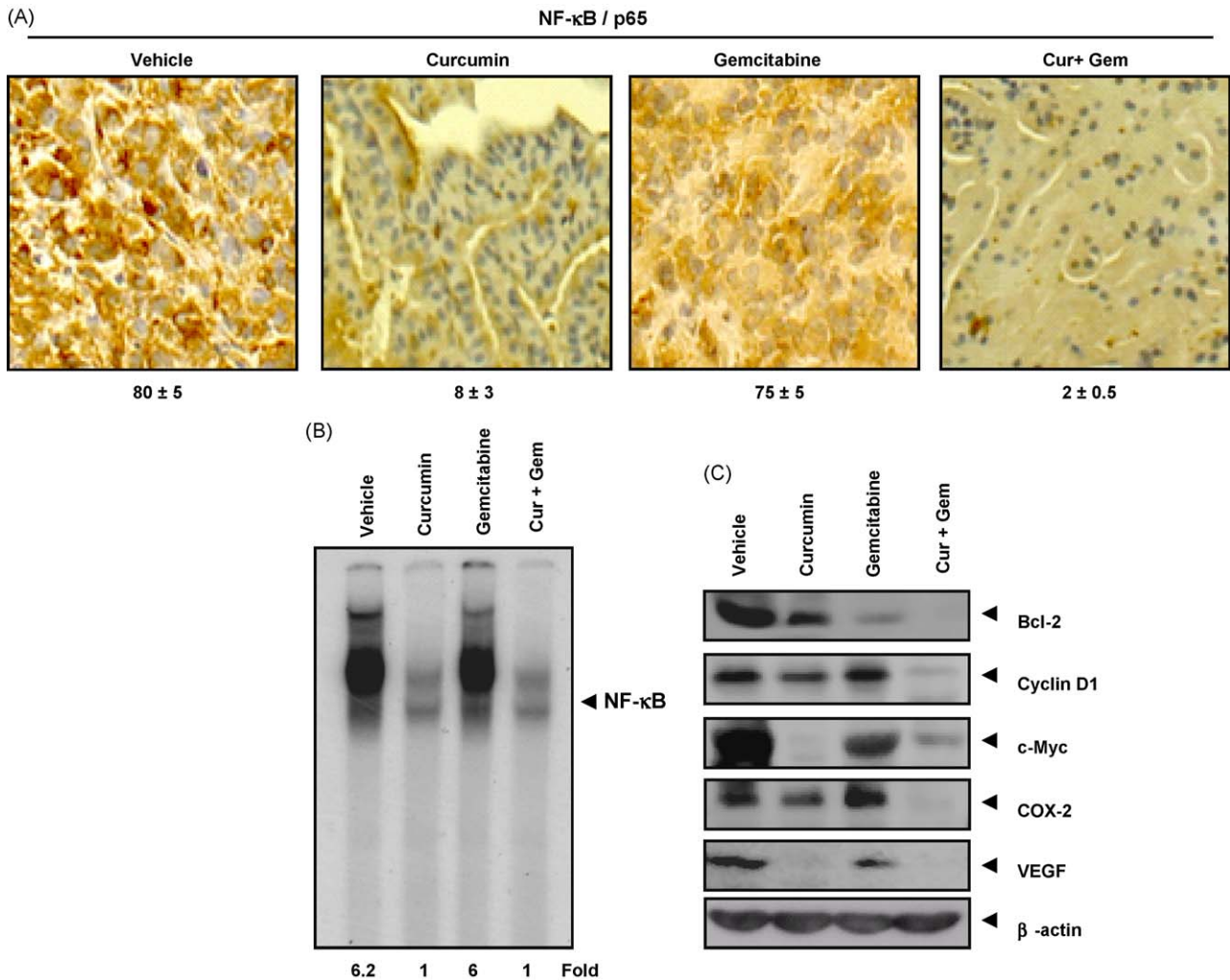
Fig. 6. Curcumin inhibits NF-κB and NF-κB-regulated gene products in tumor tissue. (A) Immunohistochemical analysis of nuclear p65. The percentage inhibition nuclear p65 by curcumin alone and in combination with gemcitabine is indicated. (B) Detection of NF-κB by DNA binding in orthotopic tumor tissue samples. (C) Western blot showing that curcumin inhibits the expression of NF-κB-dependent gene products cyclin D1, COX-2, VEGF, c-myc, and Bcl-2 in human bladder tumors.

alone or in combination with other agents in bladder cancer therapy [5]. Our laboratory has reported that curcumin can also potentiate the antitumor activity of gemcitabine in an orthotopic model of pancreatic cancer through the suppression of proliferation, angiogenesis, and inhibition of NF-κB-regulated gene products [24]. Curcumin is in clinical trial at M. D. Anderson for the treatment of pancreatic cancer [8] and multiple myeloma [7]. It has been found to be highly safe and well tolerated even at very high doses [25]. Considering that the median age for diagnosis of bladder cancer is 73 years an age at which ability to tolerate toxic therapy is low; curcumin should prove highly beneficial. Because the recurrence rate in bladder cancer, even with the best treatment available, is very high, whether curcumin could prevent recurrence should be evaluated. Overall, we propose that based on our present study, curcumin should be explored further for therapy of human bladder cancer.

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