

Curcumin Decreases Specificity Protein Expression in Bladder Cancer Cells

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Abstract

Curcumin is the active component of tumeric, and this polyphenolic compound has been extensively investigated as an anticancer drug that modulates multiple pathways and genes. In this study, 10 to 25 $\mu\text{mol/L}$ curcumin inhibited 253JB-V and KU7 bladder cancer cell growth, and this was accompanied by induction of apoptosis and decreased expression of the proapoptotic protein survivin and the angiogenic proteins vascular endothelial growth factor (VEGF) and VEGF receptor 1 (VEGFR1). Because expression of survivin, VEGF, and VEGFR1 are dependent on specificity protein (Sp) transcription factors, we also investigated the effects of curcumin on Sp protein expression as an underlying mechanism for the apoptotic and antiangiogenic activity of this compound. The results show that curcumin induced proteasome-dependent down-regulation of Sp1, Sp3, and Sp4 in 253JB-V and KU7 cells. Moreover, using RNA interference with small inhibitory RNAs for Sp1, Sp3, and Sp4, we observed that curcumin-dependent inhibition of nuclear factor κB (NF- κB)-dependent genes, such as *bcl-2*, *survivin*, and *cyclin D1*, was also due, in part, to loss of Sp proteins. Curcumin also decreased bladder tumor growth in athymic nude mice bearing KU7 cells as xenografts and this was accompanied by decreased Sp1, Sp3, and Sp4 protein levels in tumors. These results show for the first time that one of the underlying mechanisms of action of curcumin as a cancer chemotherapeutic agent is due, in part, to decreased expression of Sp transcription factors in bladder cancer cells. [Cancer Res 2008;68(13):5345–54]

Introduction

Phytochemicals, microbial metabolites, and other natural products and their synthetic analogues have been extensively used for drug development and treatment of various diseases, including cancer (1, 2). Curcumin (diferuloylmethane) is a polyphenolic natural product and the active component of tumeric (*Curcuma* species), which is used in cooking and in traditional medicines (3–5). Curcumin has been extensively investigated as an anticancer drug in various cancer cells and laboratory animal models. Curcumin has also been evaluated in humans, and one of the major problems associated with clinical applications of curcumin

is the low bioavailability (6–10). The effects of curcumin in various tumor models is highly variable and dependent on both tumor type and cell context. In many studies, curcumin inhibits cancer cell proliferation, induces apoptosis, and inhibits angiogenesis (11–20). Mechanisms associated with these effects are variable and may involve direct effects on mitochondria, activation of endoplasmic reticulum (ER) stress, and modulation of kinase pathways including the inhibition of nuclear factor- κB (NF- κB).

Recent studies reported that curcumin decreased survival of RT4V6 and KU7 bladder cancer cells and this was accompanied by increased DNA fragmentation and other parameters associated with apoptosis (17). In addition, there was also evidence that curcumin potentiated the effects of other drugs and cytokines in bladder cancer cells and this has been observed in other studies (16, 17, 21, 22). Curcumin alone had minimal effects on NF- κB in RT4V6 or KU7 cells; however, in cells treated with agents, such as gemcitabine, tumor necrosis factor, and cigarette smoke, that induce NF- κB , cotreatment with curcumin inhibited NF- κB activation. It was concluded that suppression of induced NF- κB by curcumin may play a role in sensitizing bladder cancer cells and other cancer cell lines to various chemotherapeutic agents (17).

Betulinic acid is a triterpenoid natural product that inhibits growth of multiple cancer cell lines, and this compound induces apoptosis and inhibits angiogenesis (23). Studies in this laboratory showed that one of the underlying mechanisms of action of this compound in prostate cancer cells was the targeted degradation of specificity protein 1 (Sp1), Sp3, and Sp4 and Sp-dependent genes involved in cell growth, survival, and angiogenesis (24). We also observed similar effects for the analgesic tolfenamic acid (25, 26), and RNA interference studies with small inhibitory RNAs (siRNA) for Sp1, Sp3, and Sp4 confirmed the role of these proteins in regulating expression of *vascular endothelial growth factor* (VEGF), *VEGF receptor 1* (VEGFR1), *VEGFR2*, and *survivin* and repression of *p27* by Sp3 (26–30).

Curcumin induced apoptosis and inhibited KU7 and 253JB-V bladder cancer cell proliferation, and we hypothesized that curcumin may also affect Sp protein expression in these cells. Curcumin decreased Sp1, Sp3, and Sp4 protein expression in the bladder cancer cell lines; similar results were observed in tumors in a xenograft study. These results show that the curcumin-induced growth-inhibitory, proapoptotic, and angiogenic responses in bladder cancer cells and tumors are due, in part, to decreased expression of Sp1, Sp3, and Sp4. Moreover, curcumin-induced down-regulation of Sp proteins also plays a role in the effects of curcumin as an inhibitor of NF- κB -dependent genes.

Materials and Methods

Cell lines, antibodies, chemicals, and other materials. KU-7 human bladder cancer cells were obtained from the American Type Culture

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Collection and 253JB-V cells were provided by Dr. A. Kamat (M.D. Anderson Cancer Center, Houston, TX) and maintained essentially as described (17). With the exception of cleaved poly(ADP)ribose polymerase (PARP; Cell Signaling Technology), NF- κ B-p65 (Abcam, Inc.), and β -actin antibodies (Sigma-Aldrich), all remaining antibodies were purchased from Santa Cruz Biotechnology. Curcumin (98% pure) was purchased from Indofine Chemical Company, Inc. Lipofectamine and Lipofectamine 2000 were purchased from Invitrogen. β -Galactosidase reagent was obtained from Tropix.

Cell proliferation assays. Bladder cancer cells (3×10^4 per well) were plated using DMEM:Ham's F-12 medium containing 2.5% charcoal-stripped fetal bovine serum (FBS) in 12-well plates and left to attach for 24 h. Cells were then treated with either vehicle (DMSO) or the indicated concentrations of curcumin. Fresh medium and test compounds were added every 48 h, and cells were then counted at the indicated times using a Coulter Z1 particle counter. Each experiment was done in triplicate and results are expressed as means \pm SE for each determination.

Terminal deoxyribonucleotide transferase-mediated nick-end labeling assay. 253JB-V and KU7 cells (7×10^4) were seeded in four-chambered glass slides and left overnight to attach. After curcumin treatment for 12 h, the *in situ* cell death detection POD kit was used for the terminal deoxyribonucleotide transferase-mediated nick-end labeling (TUNEL) assay according to the instructions in the protocol manual for fixed cells. The percentage of apoptotic cells was calculated by counting the stained cells in eight fields, each containing 50 cells. The total number of apoptotic cells was plotted as a percentage in both cell lines.

Transfection and luciferase assays. Bladder cancer cells (1×10^5 per well) were plated in 12-well plates in DMEM:Ham's F-12 medium supplemented with 2.5% charcoal-stripped FBS. After 16 h, various amounts of DNA (i.e., 0.4 μ g pGL3/pGL2-Luc; 0.04 μ g β -galactosidase; and 0.4 μ g pSp1For4-Luc, pSp3For5-Luc, pVEGF-Luc, pNF κ B-Luc, and pSurvivin-Luc) were transfected using Lipofectamine reagent according to the manufacturer's protocol and luciferase activity (normalized to β -galactosidase) was determined essentially as described (24–29).

Western blot assays. Bladder cancer cells were seeded in DMEM:Ham's F-12 medium containing 2.5% charcoal-stripped FBS. Twenty-four hours later, cells were treated with either vehicle (DMSO) or the indicated compounds for 24 h and Western blot analysis was determined as described (26–29). Nuclear and cytoplasmic extracts were separated using NE-PER nuclear and cytoplasmic extraction reagents (Pierce). The tumor tissues were also processed similarly and probed for Sp1, Sp3, and Sp4 proteins and β -actin served as loading control. Protein quantification used Image J software, and optical densities for each protein was normalized to β -actin in the iLamin-treated group.

Fluorescence-activated cell sorting assays. Both 253JB-V and KU7 bladder cancer cells were treated with either the vehicle (DMSO) or the indicated compounds for 24 h. Cells were analyzed on a FACSCalibur flow cytometer using CellQuest acquisition software (Becton Dickinson Immunocytometry Systems). Propidium iodide (PI) fluorescence was collected through a 585/42 nm band pass filter, and list mode data were acquired on a minimum of 20,000 single cells defined by a dot plot of PI width versus PI area. Data analysis was performed using Modfit LT software (Verity Software House, Topsham, ME).

siRNA interference assays. siRNAs for Sp1, Sp3, and Sp4 were prepared by Dharmacon RNA Technologies. The iRNA complexes used in this study are indicated as follows:

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LMN 5'-CUG GAC UUC CAG AAG AAC ATT
Sp1 SMARTpool L-026959-00-0005, Human Sp1, NM_138473ss
Sp3 5'-GCG GCA GGU GGA GCC UUC ACU TT
Sp4 5'-GCA GUG ACA CAU UAG UGA GCT T
p65 (REL1096) 5'-GATTGAGGAGAAACGTAAATT
p50 (REL 1911) 5'-GTCACCTAACGTATGCAATT
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The two bladder cancer cell lines, 253JB-V and KU7, were seeded (1×10^5 per well) in 12-well plates in DMEM:Ham's F-12 medium supplemented with 2.5% charcoal-stripped FBS without antibiotic and left to attach for 1 d.

The triple Sp siRNA knockdown (iSp1, iSp3, iSp4 complex) along with iLamin as control was performed using Lipofectamine 2000 transfection reagent as per manufacturer's instructions. siRNA for p65 and p50 and NC were purchased from Integrated DNA Technologies.

Electrophoretic mobility shift assay. Cells were rinsed in cold PBS buffer and harvested in reporter lysis buffer (Promega). After 15-min incubation on ice and 10-min centrifugation at $16,000 \times g$, 4°C , the pellet was resuspended in reporter lysis buffer supplemented with 0.5 mol/L KCl and incubated on ice for 30 min. The supernatant containing nuclear proteins was collected after centrifugation for 10 min at $16,000 \times g$, 4°C and quantified for protein concentrations by Bradford method. The GC-rich probe was prepared by annealing the two complementary polynucleotides: 5'-CTGTCGGCCCCCGCCCTCT-3' and 5'-AGAGGGGCGGGGCGGACGAG-3'. The NF- κ B sense strand probe was 5'-AGTTGAGGGGACTTCC-CAGGC-3'. The annealed probe was 5'-end-labeled using T4 polynucleotide kinase (Invitrogen) and [γ - ^{32}P]ATP (Perkin-Elmer). The labeled probe was purified with the Chroma Spin TE-10 column (BD Biosciences). The electrophoretic mobility shift assay reaction was carried out in the reporter lysis buffer (Promega) supplemented with 0.1 mol/L KCl. Each reaction contained 2 μ g nuclear protein, 500 to 1,000 ng of poly(dI-dC) (Roche Molecular Biochemicals) with or without unlabeled competitor oligonucleotides, and 10 fmol of labeled probe; the mixture was incubated for 15 min on ice. Protein-DNA complexes were resolved by 5% native PAGE at 160 V at room temperature for 1.5 h and visualized using a Storm A60 PhosphorImager system (Molecular Dynamics).

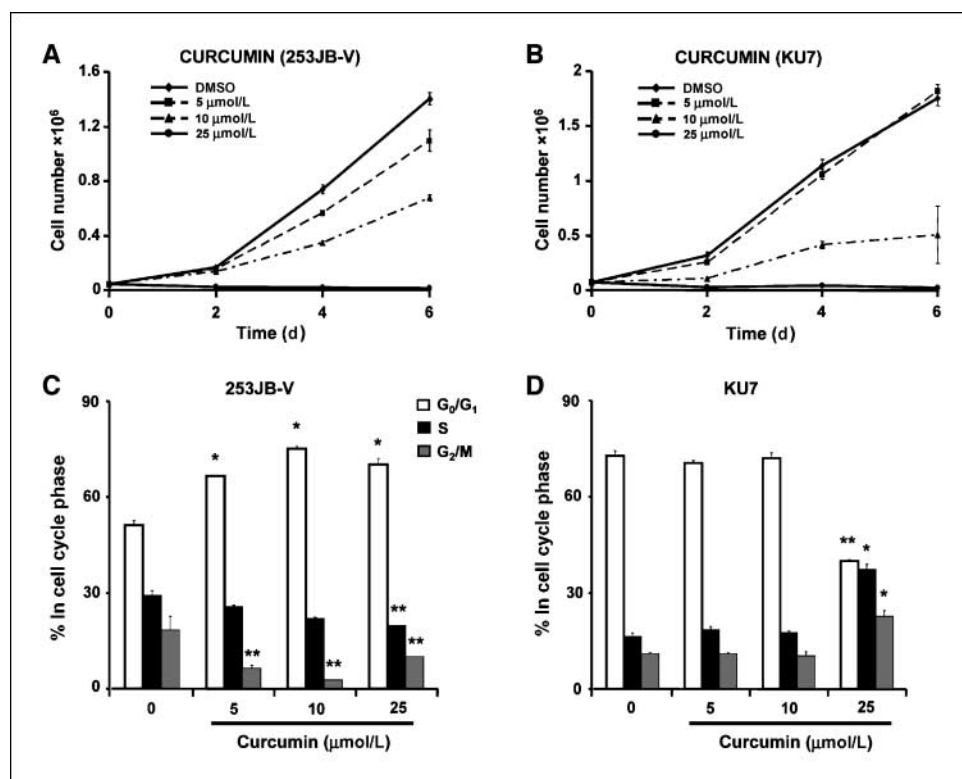
Xenograft study. Female athymic nude mice, age 4 to 6 weeks, were purchased from Harlan. KU7 cells (1×10^6) in 1:1 ratio of Matrigel (BD Biosciences) were injected into the either side of the flank area of nude mice. Seven days after the tumor cell inoculation, mice were divided into two groups of 10 animals each. The first group received 50 μ L vehicle (corn oil) by i.p. injection, and the second group of animals received 50 mg/kg/d injection of curcumin in corn oil every 2nd day for 18 d (9 doses) by i.p. injection. The mice were weighed, and tumor areas were measured throughout the study. After 20 d, the animals were sacrificed; final body and tumor weights were determined and plotted.

Results

Figure 1A and B illustrates the concentration-dependent effects of 5, 10, and 25 μ mol/L curcumin on proliferation of 253JB-V and KU7 bladder cancer cells over a 6-day period with change of medium and treatment with DMSO (control) or curcumin every 48 h. Proliferation of both 253JB-V and KU7 cells was inhibited by curcumin. However, the pattern of inhibition was slightly different; 5 and 10 μ mol/L curcumin significantly inhibited 253JB-V cells after treatment for 4 and 6 days, whereas 10 μ mol/L (but not 5 μ mol/L) curcumin significantly inhibited KU7 cell proliferation after treatment for 2, 4, and 6 days. The highest concentration of curcumin (25 μ mol/L) was cytotoxic to both cell lines, and similar results were previously reported on the cytotoxicity of curcumin in KU7 and RT4V6 bladder cancer cells (17). The effects of curcumin on the distribution of 253JB-V and KU7 cells in G₀-G₁, S, and M phases of the cell cycle are illustrated in Fig. 1C and D, respectively. Curcumin (5–25 μ mol/L) increased the percentage of 253JB-V cells in G₀-G₁ and decreased the percentage in S and G₂-M phases, although the effects on G₂-M were concentration dependent and variable. In contrast, in KU7 cells, minimal effects were observed after treatment with 5 or 10 μ mol/L curcumin, whereas 25 μ mol/L curcumin decreased the percentage of cells in G₀-G₁ and increased the percentage in S and G₂-M phases (Fig. 1D). These results show that curcumin-induced changes in the distribution of 253JB-V and KU7 cells in different phases of the cycle were dependent on cell context.

The effects of curcumin on selected proteins involved in cell cycle progression are summarized in Fig. 2A. The cyclin-dependent

Figure 1. Curcumin inhibits bladder cancer cell growth and modulates the cell cycle. Inhibition of 253JB-V (A) and KU7 (B) cell growth. Cells were treated with DMSO (solvent control); 5, 10, or 25 $\mu\text{mol/L}$ curcumin; and the effects of cell growth were determined after treatment for 2, 4, or 6 d as described in Materials and Methods. Effects of curcumin on distribution of 253JB-V (C) and KU7 (D) cells in G_0 - G_1 , S, and G_2 -M phases. Cells were treated with DMSO (0) and 5, 10, or 25 $\mu\text{mol/L}$ curcumin for 24 h; the distribution of cells in G_0 - G_1 , S, and G_2 -M phases was determined by FACS analysis as described in Materials and Methods. Points and columns, mean for each data point in A-D; bars, SD. Significantly ($P < 0.05$) increased (*) or decreased (**) responses compared with DMSO (0, control) are indicated.



kinase inhibitors p21 and p27 were decreased in both cell lines by curcumin, but at different concentrations. p21 was decreased by 25 to 50 $\mu\text{mol/L}$ and 10 to 50 $\mu\text{mol/L}$ curcumin in 253JB-V and KU7 cells, respectively, whereas 25 to 50 $\mu\text{mol/L}$ curcumin decreased p27 expression in both cell lines. The role of these responses in mediating the distribution of bladder cancer cells in different phases of the cell cycle is unclear. Previous studies showed that curcumin induced apoptosis in bladder cancer cells (17) and, in pancreatic tumors, curcumin suppressed proliferation and inhibited angiogenesis (13, 16). Results in Fig. 2B show that curcumin induced PARP cleavage in 253JB-V and KU7 cells, and this was also accompanied by down-regulation of the antiapoptotic gene *survivin*. Decreased survivin and increased PARP cleavage were initially observed at curcumin concentrations of 10 and 25 $\mu\text{mol/L}$ in 253JB-V and KU7 cells, respectively, and maximal responses were observed in cells treated with 25 and 50 $\mu\text{mol/L}$ curcumin. In addition, curcumin also decreased expression of two angiogenic proteins, VEGF and VEGFR1, in both cell lines (Fig. 2B). The proapoptotic activity of curcumin was also confirmed in a TUNEL assay (Fig. 2C) where 40 $\mu\text{mol/L}$ curcumin induced increased TUNEL staining in 253JB-V and KU7 cells, whereas minimal effects were observed in the solvent (DMSO)-treated cells.

Results in Fig. 3A and B show that after treatment of 253JB-V and KU7 cells with curcumin (5–50 $\mu\text{mol/L}$) for 24 hours, there was a concentration-dependent decrease in the expression of Sp1, Sp3, and Sp4 proteins. Curcumin-induced down-regulation of Sp proteins was dependent on the concentration of curcumin, Sp protein (i.e., Sp1, Sp3, or Sp4), and cell context. However, in both 253JB-V and KU7 cells, decreased expression of all three proteins was observed after treatment with 10 $\mu\text{mol/L}$ and higher concentrations of curcumin for 24 hours. Because curcumin decreased expression of Sp1-, Sp3-, Sp4-, and Sp-dependent survivin, VEGF, and VEGFR1 proteins, we also investigated the

effects of curcumin on luciferase activity in 253JB-V and KU7 cells transfected with constructs containing GC-rich promoters that bind Sp proteins. Luciferase activity was decreased in 253JB-V and KU7 cells treated with 10 to 40 $\mu\text{mol/L}$ curcumin and transfected with GC-rich Sp1For4 and Sp3For5 constructs containing the –751 to –20 and –417 to –38 regions of the Sp1 and Sp3 gene promoters, respectively (refs. 31, 32; linked to the luciferase gene; Fig. 3C). In addition, curcumin (10–40 $\mu\text{mol/L}$) also decreased luciferase activity in 253JB-V and KU7 cells transfected with GC-rich pVEGF and pSurvivin constructs that contain the –2018 to +50 and –269 to +49 GC-rich regions of the VEGF and survivin gene promoters, respectively (Fig. 3D). Thus, like tolfenamic acid and betulinic acid, curcumin-induced down-regulation of Sp1, Sp3, and Sp4 not only decreased expression of Sp-regulated proteins, such as VEGF and survivin, but also decreased transactivation in 253JB-V and KU7 cells transfected with pVEGF and pSurvivin constructs containing GC-rich promoter inserts.

Previous studies show that tolfenamic and betulinic acids induce proteasome-dependent degradation of Sp1, Sp3, and Sp4 in pancreatic and prostate cancer cells (24, 25), and Fig. 4A and B summarize the effects of the proteasome inhibitor MG132 on curcumin-induced down-regulation of these proteins. In KU7 cells, MG132 inhibited curcumin-induced down-regulation of Sp1, Sp3, and Sp4 proteins, and similar effects were observed in 253JB-V cells. However, in the latter bladder cancer cell line, MG132 alone also decreased Sp protein expression and this response was most pronounced for Sp1. Other proteasome inhibitors such as lactacystin and gliotoxin gave similar results and, in combination with curcumin, high cytotoxicity was observed in 253JB-V cells (data not shown). Nevertheless, it was apparent that MG132 plus curcumin blocked Sp protein down-regulation in both cell lines, suggesting that curcumin, like betulinic and tolfenamic acids (24–26), induced proteasome-dependent down-regulation of Sp1,

Sp3, and Sp4 proteins in 253JB-V and KU7 cells. Figure 4C summarizes the effects of MG132 on curcumin-dependent decreased luciferase activity in 253JB-V and KU7 cells transfected with pSp1For4 and pSp3For5. In 253JB-V cells, MG132 only partially reversed down-regulation of activity by 25 $\mu\text{mol/L}$ curcumin, whereas in KU7 cells, MG132 completely inhibited the effects of curcumin on luciferase activity. The relative effectiveness of MG132 as an inhibitor of curcumin-dependent down-regulation of luciferase activity in cells transfected with pVEGF or pSurvivin also differed in 253JB-V and KU7 cells (Fig. 4D). MG132 reversed the effects of curcumin in KU7 cells (80–100%) but was less efficient in 253JB-V cells. This may be due, in part, to the cytotoxicity of MG132 alone and in combination with curcumin in 253JB-V cells. These results show that curcumin primarily induced proteasome-dependent degradation of Sp1, Sp3, and Sp4 in the bladder cancer cells, and similar effects have been observed for tolfenamic acid and betulinic acid in pancreatic and prostate cancer cells (24, 25).

Previous studies in bladder and other cancer cell lines report that the broad spectrum of anticancer activities of curcumin have been associated, in part, with decreased NF- κ B-dependent activity (3, 4). Results illustrated in Fig. 5A show that 10 to 40 $\mu\text{mol/L}$ curcumin decreased luciferase activity in 253JB-V cells transfected with pNF κ B, a construct containing five tandem NF- κ B response elements that regulate a luciferase reporter gene. Thus, curcumin

decreases NF- κ B-dependent transactivation in the 253JB-V cell line. Because curcumin also decreases expression of Sp1, Sp3, and Sp4 in bladder cancer cells, we investigated the effects of Sp protein knockdown on luciferase activity in cells transfected with pNF κ B-luc (Fig. 5B). 253JB-V and KU7 cells were transfected with iSp, which is a cocktail of siRNAs for Sp1 (iSp1), Sp3 (iSp3), and Sp4 (iSp4; ref. 33). The results showed that transfection with iSp significantly inhibited NF- κ B-dependent activity and this was not directly related to a decrease in p65 or p50 protein levels after transfection with iSp (Fig. 5B). There was a minimal but not significant decrease in these proteins over replicated experiments, suggesting that decreased NF- κ B-dependent transactivation by iSp was not dependent on decreased expression of p65 or p50. We also investigated the effects of iSp1, iSp3, and iSp4 on p65 and p50 protein expression in 253JB-V and KU7 cells (Supplement 1). In the former cell line, iSp3 and iSp4 (but not iSp1) slightly decreased p65 protein levels, whereas none of the siRNAs affected p65 or p50 expression in KU7 cells and p50 expression in 253JB-V cells. Figure 5C shows that curcumin decreased expression of cyclin D1 and the antiapoptotic protein bcl-2 in 253JB-V and KU7 cells, and both of these genes are also regulated by NF- κ B and Sp transcription factors (34, 35). It is possible that curcumin-induced down-regulation of Sp proteins may directly affect NF- κ B because both p65 and p50 are Sp-dependent genes in some cell lines

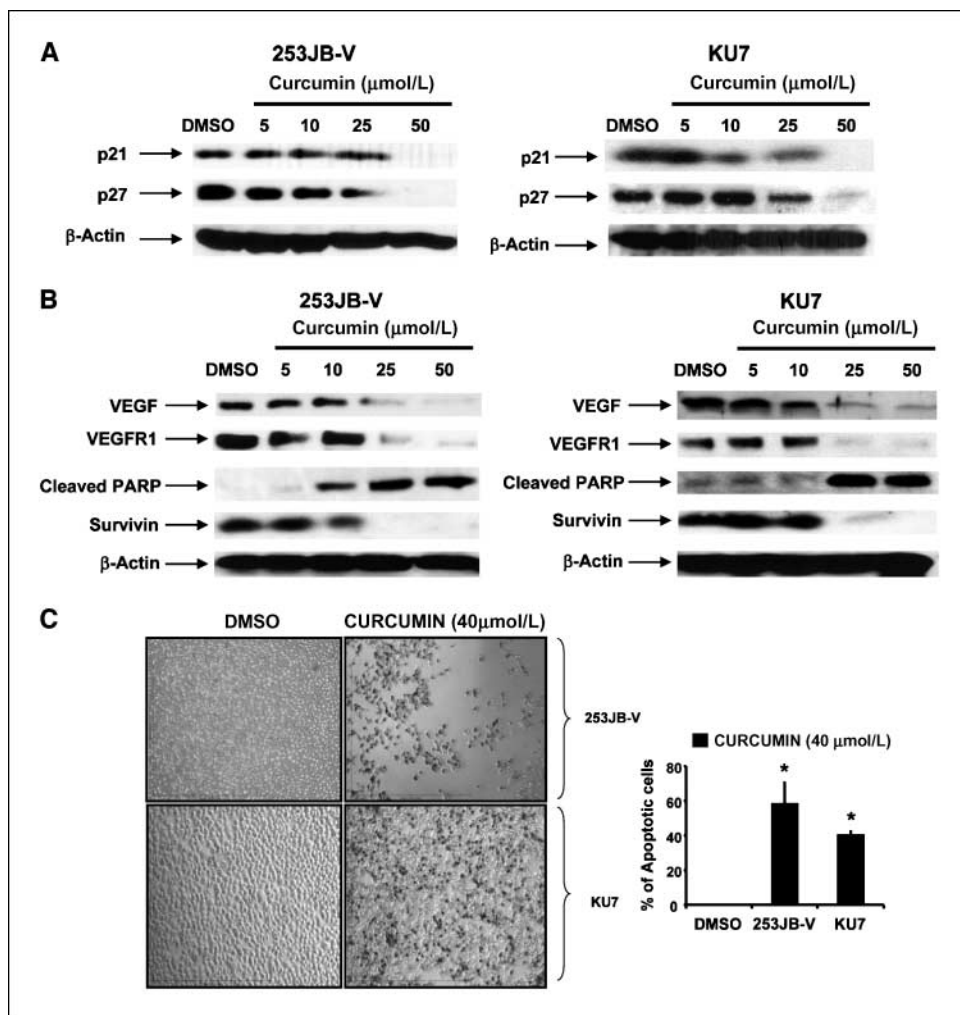
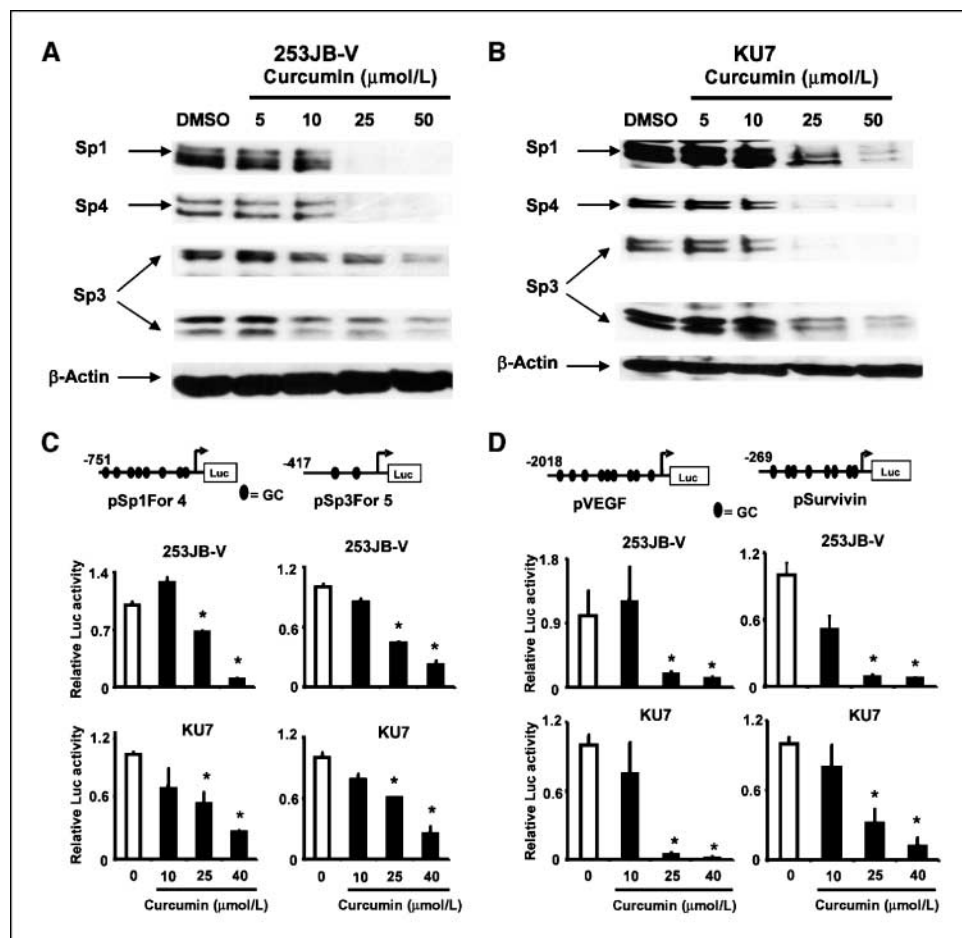


Figure 2. Curcumin modulates expression of cell cycle, survival, and angiogenic proteins and induces apoptosis. **A**, decreased expression of p27 and p21 proteins. 253JB-V and KU7 cells were treated with DMSO and 5, 10, 25, or 50 $\mu\text{mol/L}$ curcumin for 24 h, and whole-cell lysates were analyzed by Western blot analysis as described in Materials and Methods. **B**, decreased expression of survivin, VEGF, and VEGFR1, and increased PARP cleavage. 253JB-V and KU7 cells were treated with DMSO and 5, 10, 25, or 50 $\mu\text{mol/L}$ curcumin for 24 h, and whole-cell lysates were analyzed by Western blot analysis as described in Materials and Methods. β -Actin served as a loading control. **C**, TUNEL assay. 253JB-V and KU7 cells were treated with DMSO and 40 $\mu\text{mol/L}$ curcumin for 12 h, and TUNEL staining was determined as described in Materials and Methods. Results of the Western blot (**A** and **B**) and TUNEL staining experiments were similar in replicate experiments in both cell lines. *, significant ($P < 0.05$) increases (TUNEL staining).

Figure 3. Effects of curcumin on Sp proteins and Sp-dependent transactivation. Curcumin decreases Sp proteins in 253JB-V (A) and KU7 (B) bladder cancer cells. Cells were treated with DMSO and 5, 10, 25, or 50 $\mu\text{mol/L}$ curcumin for 24 h and whole-cell lysates were analyzed by Western blot analysis as described in Materials and Methods. β -Actin served as a loading control and similar results were observed in duplicate experiments. Curcumin decreases luciferase activity in cells transfected with Sp1/Sp3 promoter (C) and Sp-regulated promoter (D) constructs. Bladder cancer cells were transfected with the indicated constructs, treated with DMSO or different concentrations of curcumin, and luciferase activity was determined as described in Materials and Methods. Columns, means for at least three separate determinations for each treatment group; bars, SE. *, significantly ($P < 0.05$) decreased activity.



(36, 37). However, treatment of 253JB-V and KU7 cells with DMSO and 10, 25, or 40 $\mu\text{mol/L}$ curcumin for 24 hours did not affect nuclear p65 expression, whereas nuclear p50 protein levels were decreased only at the higher (40 $\mu\text{mol/L}$) concentration (Fig. 5D) and 25 $\mu\text{mol/L}$ curcumin, which decreases Sp protein levels, did not affect p65 or p50 expression. Thus, curcumin-mediated inhibition of NF- κ B-dependent gene expression is not due to direct effects on p65 or p50.

A close inspection of many NF- κ B-regulated genes, such as *cyclin D1*, *bcl-2*, *survivin*, and *VEGF*, indicates that these genes also contain multiple GC-rich promoter sequences and are coregulated by Sp proteins in many cell lines (24–28, 34, 35, 37–40). Results in Fig. 6A show that in 253JB-V and KU7 cells transfected with iLamin (control) or the iSp cocktail (containing iSp1, iSp3, and iSp4), there was a significant decrease in levels of *bcl-2*, *cyclin D1*, *survivin*, and *VEGF* and this correlated with the effects of curcumin on these same proteins (Figs. 2B and 5C). In contrast, combined knockdown of p65 and p50 by RNA interference (Fig. 6B) did not affect expression of all these proteins. Only *VEGF* and *bcl-2* proteins were decreased by p65/p50 knockdown, suggesting that basal expression of *cyclin D1* and *survivin* were NF- κ B independent in the bladder cancer cells. Figure 6C illustrates gel mobility shift assays in which nuclear extracts from 253JB-V and KU7 cells bound a ^{32}P -labeled GC-rich oligonucleotide to form Sp-DNA retarded bands as previously characterized in other cell lines (27–29). Treatment with 25 or 40 $\mu\text{mol/L}$ curcumin decreased retarded band intensities and coinubation with unlabeled wild-type GC-rich or mutant

oligonucleotides decreased or did not affect retarded band intensities, respectively (Fig. 6C). Supershift experiments with Sp1 antibodies did not give a defined supershifted antibody-protein complex but a diffuse band. However, the Sp1 band intensity was decreased due to immunodepletion. We also determined the effects of curcumin on NF- κ B-DNA binding in a gel mobility shift assay using a consensus NF- κ B response element and extracts from cells, and the results showed that curcumin decreased retarded band intensities associated with the NF- κ B-DNA complex (Supplement 2). These data confirm that curcumin decreases Sp proteins and Sp-DNA complex formation and the NF- κ B-DNA retarded band and this correlated with decreased expression of NF- κ B-regulated proteins that are also coregulated by Sp transcription factors. These factors suggest that curcumin-dependent down-regulation of Sp proteins contributes to decreased expression of several NF- κ B-dependent proteins, which are coregulated by both Sp and NF- κ B transcription factors (24–28, 34, 35, 37–40).

We also investigated the antitumorigenic activity of curcumin in athymic nude mice bearing KU7 cells as xenografts. At a dose of 50 mg/kg/d, curcumin significantly decreased tumor volumes and tumor weights (Fig. 6D). Moreover, Western blot analysis of tumor lysates from three control and three treated mice show that Sp1, Sp3, and Sp4 protein expression is decreased in the latter group. These results complement the *in vitro* studies and show that curcumin-induced effects on Sp proteins also plays a role in the mechanism of action of this compound in bladder cancer cells.

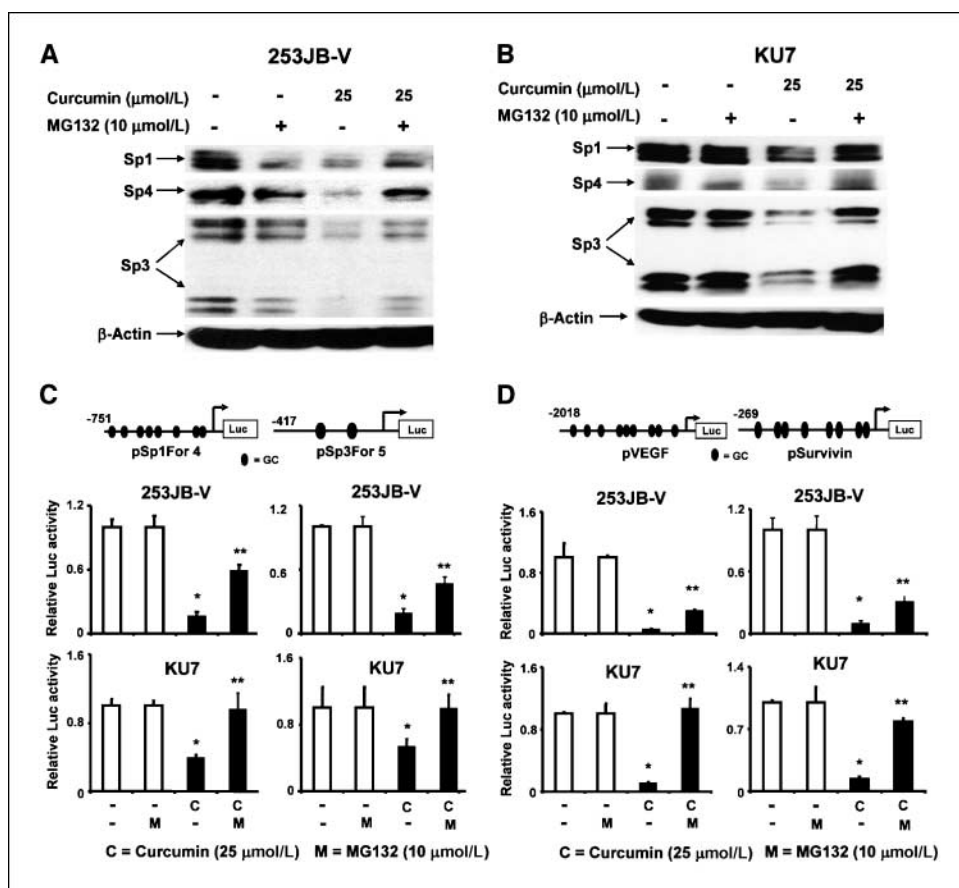


Figure 4. MG132 inhibition of curcumin-induced effects on Sp proteins and Sp-dependent transactivation. MG132 inhibits curcumin-dependent down-regulation of Sp proteins in 253JB-V (A) and KU7 (B) cells. Bladder cancer cells were treated with DMSO alone, 25 $\mu\text{mol/L}$ curcumin, or 10 $\mu\text{mol/L}$ MG132 in the presence or absence of 25 $\mu\text{mol/L}$ curcumin for 24 h, and whole-cell lysates were analyzed by Western blots as described in Materials and Methods. β -Actin served as a loading control, and similar results were observed in duplicate experiments. MG132 inhibits the effects of curcumin on luciferase activity in bladder cancer cells transfected with Sp1/Sp3 promoter (C) and Sp-regulated promoter (D) constructs. Cells were transfected with the indicated constructs and treated with DMSO and 10 $\mu\text{mol/L}$ MG132, 25 $\mu\text{mol/L}$ curcumin, or MG132 plus curcumin (combined), and luciferase activity was determined as described in Materials and Methods. Columns, means for at least three separate determinations for each treatment group; bars, SE. Significantly decreased activity after treatment with curcumin (*) and reversal of the effect after cotreatment with MG132 (**).

Discussion

Curcumin has been widely used in traditional medicines, and its chemoprotective and chemotherapeutic effects are due to many different activities that are also dependent on cell context (3–5). One of the most predominant effects of curcumin observed in many studies is associated with inhibition of constitutive or induced NF- κ B-dependent genes/proteins associated with cell survival, angiogenesis, and inflammation (3–5, 33, 41–43). However, other pathways contribute to the effects of curcumin, and these may be NF- κ B independent. For example, curcumin induces ER stress in human leukemia HL-60 cells, and this results in activation of ER stress-dependent proapoptotic pathways associated with cleavage of caspase-8, caspase-9, caspase-3, and caspase-4 (18). In addition, curcumin-induced apoptosis in human leukemia U937 cells was also linked to the direct effects of this compound on mitochondria function and subsequent activation of the intrinsic apoptosis pathway (44).

Recent studies in this laboratory have shown that compounds such as celecoxib, tolfenamic acid, and betulinic acid inhibit cancer cell and tumor growth and metastasis, and exhibit both antiangiogenic and proapoptotic activity through decreased expression of Sp proteins such as Sp1, Sp3, and Sp4 (25–27). The role of Sp proteins in mediating these responses was consistent with RNA interference studies showing that Sp proteins regulate angiogenic, growth-promoting, and prosurvival genes in cancer cells (28–30). Results in Fig. 1 illustrate that curcumin decreased proliferation of bladder cancer cells and induced apoptosis as evidenced by induction of PARP cleavage (Fig. 2B) and DNA

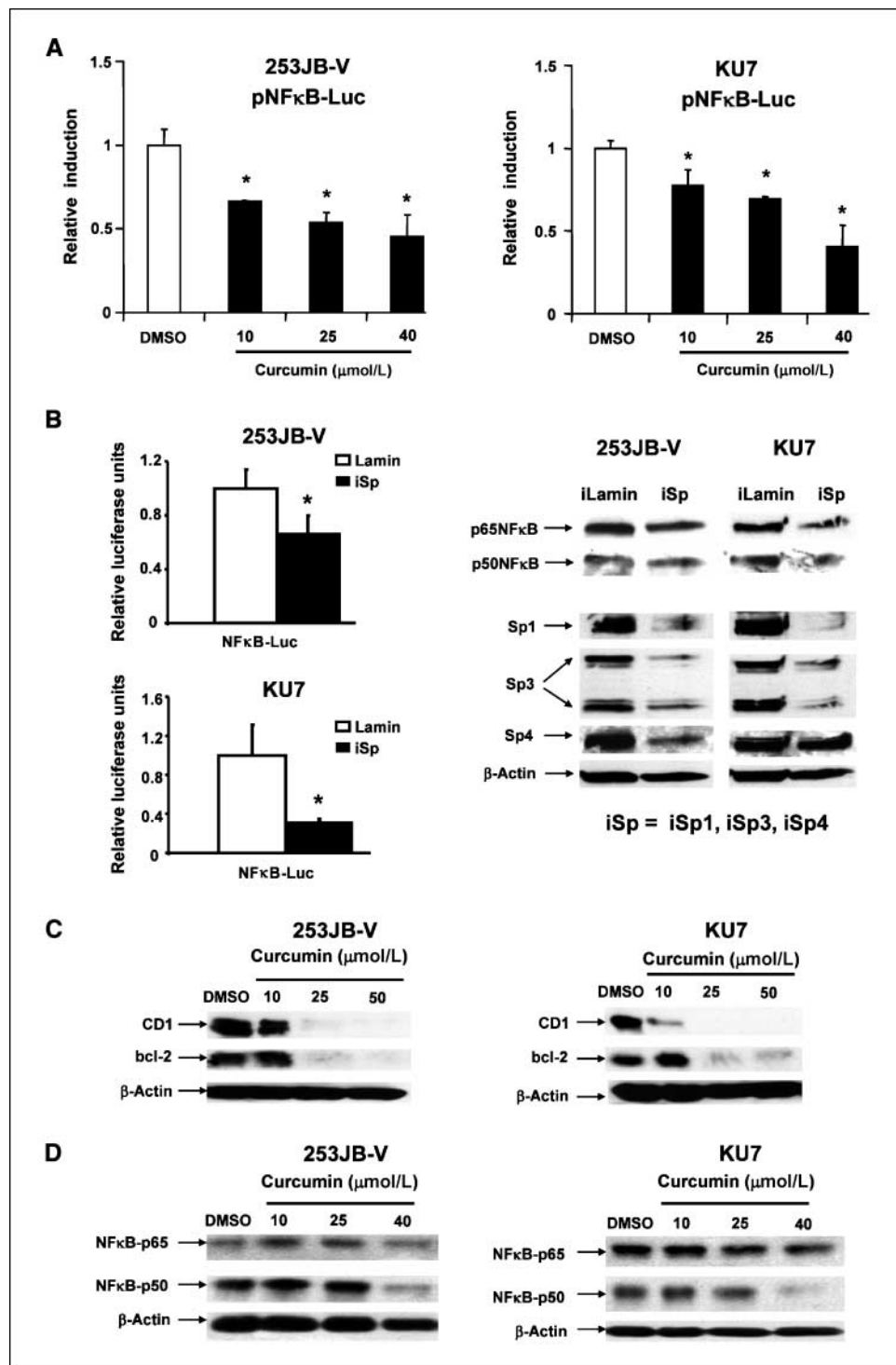
laddering (Fig. 2C) in 253JB-V and KU7 bladder cancer cells. These results are consistent with a report (17) showing that curcumin exhibited growth inhibitory and proapoptotic activity in KU7 and RT4V6 bladder cancer cells. In the prior study (17), 10 $\mu\text{mol/L}$ curcumin slightly inhibited G₀-G₁ to S phase progression in KU7 cells, whereas our results showed that 10 $\mu\text{mol/L}$ curcumin inhibited G₀-G₁ to S phase progression in 253JB-V but not in KU7 cells, which were essentially unaffected by 10 $\mu\text{mol/L}$ curcumin (Fig. 1C and D). Differences in the effects of curcumin on cell cycle progression in these cell lines may be due, in part, to the effects of curcumin on p21, p27, and cyclin D1 expression (Figs. 2A and 5C), and this is currently being investigated.

Curcumin also decreased expression of the angiogenic proteins VEGF and VEGFR1 in both bladder cancer cell lines, and decreased expression of the antiapoptotic protein survivin was accompanied by induction of caspase-dependent PARP cleavage (Fig. 2B) and increased staining in the TUNEL assay (Fig. 2C). This evidence for activation of apoptosis by curcumin is consistent with previous studies on this compound in bladder and other cancer cell lines (3–5, 12–18). However, the coordinate decrease in expression of survivin, VEGF, and VEGFR1 in bladder cancer cells treated with curcumin was reminiscent of similar effects observed in prostate and pancreatic cancer cells treated with betulinic acid and tolfenamic acid, respectively (24–26). In those studies, it was shown that decreased expression of survivin, VEGF, and VEGFR1 was due to degradation of Sp1, Sp3, and Sp4, which are known to regulate basal expression of survivin, VEGF, VEGFR1, and VEGFR2 (27–30, 38–40). Results summarized in Fig. 3 illustrate that treatment of 253JB-V and KU7 cells with curcumin caused a

concentration-dependent decrease in Sp1, Sp3, and Sp4 protein expression. The Sp1 and Sp3 promoters contain GC-rich motifs that bind Sp proteins (31, 32), and curcumin inhibited transactivation in 253JB-V and KU7 cells transfected with constructs (pSp1For4 and pSp3For5) containing Sp1 and Sp3 gene promoter inserts (Fig. 3C). Sp proteins also regulate expression of survivin and VEGF (38–40), and curcumin decreased luciferase activity in bladder cancer cells transfected with pVEGF and pSurvivin.

Betulinic acid- and tolfenamic acid-induced down-regulation of Sp1, Sp3, and Sp4 in prostate and pancreatic cancer cells is due to activation of proteasomes, and these are blocked by the proteasome inhibitor MG132 (24–26). This same inhibitor inhibited curcumin-induced down-regulation of Sp1, Sp3, and Sp4 proteins in 253JB-V and KU7 cells (Fig. 4A and B), and similar effects were observed in cells transfected with pS1For4, pSp3For5, pVEGF, and pSurvivin and treated with curcumin plus MG132 (Fig. 4C and D).

Figure 5. Effects of curcumin and Sp knockdown on NF- κ B. *A*, curcumin decreases NF- κ B promoter activity in 253JB-V and KU7 cells. 253JB-V and KU7 cells were transfected with pNF- κ B-luc treated with DMSO and 10, 25, or 40 μ mol/L curcumin; luciferase activity was determined as described in Materials and Methods. *Columns*, means for three replicate determinations for each treatment group; *bars*, SE. *, significantly ($P < 0.05$) decreased activity. *B*, effects of iSp on NF- κ B. 253JB-V and KU7 cells were transfected with iSp (a cocktail of iSp1, iSp3, and iSp4) or iLamin, and transfected with pNF- κ B-Luc; luciferase activity was determined as described in Materials and Methods. *Columns*, means for three replicate determinations. *, significantly ($P < 0.05$) decreased activity. The efficiency of Sp knockdown and the effects of p65 and p50 protein levels were determined by Western blots as described in Materials and Methods. Effects of curcumin on bcl-2 and cyclin D1 (*C*) and p65 and p50 (*D*) proteins in 253JB-V and KU7 cells. Cells were treated with DMSO and different concentrations of curcumin, and whole cell and nuclear extracts were analyzed by Western blots as described in Materials and Methods.



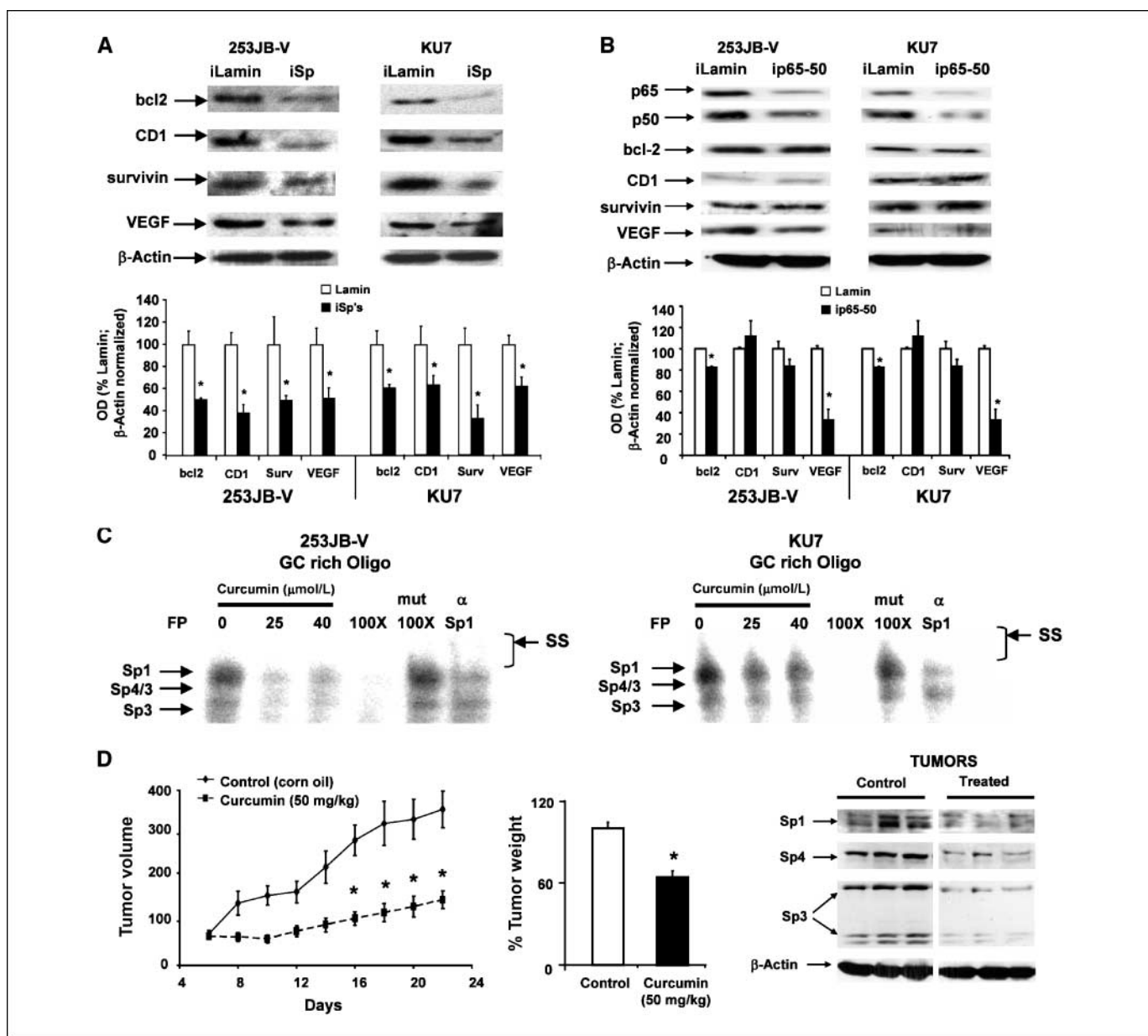


Figure 6. Role of Sp protein and NF- κ B on protein expression and the effects of curcumin on Sp-DNA binding and bladder tumor growth. Effects of iSp and iLamin (A) and small inhibitory RNAs for p65 (ip65) and p50 (ip50) [combined (B)] on genes regulated by Sp and NF- κ B. 253JB-V and KU7 cells were transfected with iSp (a cocktail of iSp1, iSp3, and iSp4) or iLamin, or ip65 plus ip50, and protein expression was determined by Western blots of whole cell lysates. β -Actin served as a loading control and for standardizing quantitative protein determinations where iLamin protein levels were set at 100%. Columns, means for three separate determinations; bars, SE. *, significantly ($P < 0.05$) decreased protein levels. C, gel mobility shift assay. 253JB-V and KU7 cells were treated with DMSO (0) and 25 or 40 μ mol/L curcumin for 24 h, and nuclear extracts were incubated with 32 P-labeled GC-rich oligonucleotide alone or in the presence of other factors. Retarded bands were analyzed by electrophoretic mobility shift assay as described in Materials and Methods. The identities of the retarded Sp-DNA bands and Sp1 antibody supershifted complex (SS) are indicated. D, curcumin inhibits bladder tumor growth. Athymic nude mice bearing KU7 cells as xenografts were treated with corn oil (control) or curcumin in corn oil (50 mg/kg/d), and tumor volumes (mm^3) and weights were determined as described in Materials and Methods. Western blot analysis of tumor lysates from three mice in the treated and control groups was also determined as described in Materials and Methods. *, significant ($P < 0.05$) decreases.

MG132 and other proteasome inhibitors such as gliotoxin and lactacystin were cytotoxic to 253JB-V cells, and MG132 alone decreased Sp1 protein in this cell line. However, despite these effects, our results show that curcumin-dependent down-regulation of Sp1, Sp3, and Sp4 proteins was partially reversed in 253JB-V cells cotreated with curcumin plus MG132. Moreover, similar results were observed in the transfection studies (Fig. 4C and D), suggesting that curcumin-dependent down-regulation of Sp1, Sp3, and Sp4 in KU7 and 253JB-V cells was primarily due to activation of

the proteasome pathway. These results suggest that some of the proapoptotic and antiangiogenic activities of curcumin in bladder cancer cells are due, in part, to degradation of Sp proteins and Sp-dependent survivin, VEGF, and VEGFR1.

In cancer cells and tumors, NF- κ B regulates expression of prosurvival and angiogenic genes, and the efficacy of several chemotherapeutic agents, including curcumin, has also been linked to their inhibition of NF- κ B-mediated responses (3–5, 11, 16, 17). Inhibition of NF- κ B by curcumin has been associated with

modulation of multiple pathways; however, the effects of curcumin-dependent down-regulation of Sp proteins on NF- κ B has not been determined. p65 and p50/105 are regulated by Sp1 in some cell lines (36, 45), and our results show that concentrations of curcumin that decreased NF- κ B-dependent activity (≤ 25 μ mol/L) did not affect nuclear p65 or p50 levels in KU7 or 253JB-V cells (Fig. 5D). Moreover, in KU7 and 253JB-V cells transfected with the iSp cocktail containing siRNAs for Sp1, Sp3, and Sp4, there was also only a minimal decrease in expression of p65 or p50 (Fig. 5B). Curcumin decreased expression of several NF- κ B-dependent genes, including *VEGF*, *survivin*, *bcl-2*, and *cyclin D1* (Figs. 2B and 5C; refs. 24, 26, 28, 29, 34, 35, 37–40), and similar results were observed in bladder cancer cells transfected with iSp (Fig. 6A). Because curcumin decreases both Sp proteins and NF- κ B-dependent transactivation in bladder cancer cells, the role of NF- κ B in mediating these responses was investigated by p65 and p50 knockdown (Fig. 6B). Only VEGF and *bcl-2* were decreased in both cell lines after transfection with siRNAs for p65 and p50 (combined). This suggests that curcumin-induced effects on *bcl-2*, *cyclin D1*, VEGF, and *survivin* expression are primarily due to Sp down-regulation; however, the contribution of Sp proteins and NF- κ B on regulation of these gene products are gene and cell context dependent although their promoters contain both GC-rich and NF- κ B elements (24, 26, 28, 29, 34, 35, 37–40).

We also examined the *in vivo* activity of curcumin (50 mg/kg/d) in athymic nude mice bearing KU7 cells as xenografts (Fig. 6D). Curcumin inhibited tumor growth and weight and this corresponded to parallel effects in 253JB-V and KU7 cells (Fig. 1A and B) where curcumin inhibited cell proliferation. Moreover, curcumin-induced effects on Sp protein expression in bladder cancer cells (Fig. 3A and B) were also observed in bladder tumors (Fig. 6D),

demonstrating that curcumin-dependent Sp protein degradation is an integral part of the anticancer activity of this compound in bladder cancer cells and tumors.

In summary, results of this study show for the first time that curcumin induces proteasome-dependent degradation of Sp1, Sp3, and Sp4 in bladder cancer cell lines, and this is accompanied by decreased expression of Sp-dependent survival and angiogenic proteins. The results suggest that at least some of the important anticancer activities of curcumin may be due, in part, to decreased expression of Sp proteins. A recent study (46) reported that curcumin decreased Sp1 protein expression in pancreatic cancer cells and this was related to increased microRNA-22, which directly targets Sp1 but not Sp3 or Sp4. Because curcumin induces proteasome-dependent degradation of all three Sp proteins in bladder cancer cells, the mechanism of curcumin action clearly differs in pancreatic and bladder cancer cells. Current studies are focused on the cancer cell context-dependent differences in the contributions and mechanisms of action of curcumin-dependent down-regulation of Sp proteins on the overall activity of this compound as an anticancer agent.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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