Curcumin Potentiates the Antitumor Effects of Bacillus Calmette-Guerin against Bladder Cancer through the Downregulation of NF-κB and Upregulation of TRAIL Receptors

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Abstract

Although Bacillus Calmette-Guerin (BCG) intravesical therapy is a standard treatment for bladder cancer, eventual failure of response is a major problem. Treatments that can augment BCG therapy are urgently needed. We investigated whether curcumin, a component of *Curcuma longa* (also called turmeric), has potential to improve the current therapy using *in vitro* and *in vivo* MBT-2 murine tumor models. We found that curcumin potentiated BCG-induced apoptosis of human bladder cancer cells. BCG stimulated the release of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) from peripheral mononuclear neutrophils in a dose- and time-dependent manner, whereas curcumin enhanced the upregulation of TRAIL receptors. Electrophoretic mobility shift assay revealed that curcumin also suppressed the BCG-induced activation of the cell survival transcription factor NF-κB. In a syngeneic bladder cancer model, curcumin alone reduced the bladder tumor volume, but a significantly greater reduction was observed when BCG and curcumin were used in combination ($P < 0.0001$ versus control; $P < 0.003$ versus BCG alone). This was accompanied by a significant decrease in the proliferation marker Ki-67 ($P < 0.01$ versus control; $P < 0.01$ versus BCG alone) and microvessel density (CD31; $P < 0.01$ versus control; $P < 0.01$ versus BCG alone), decreased NF-κB in tumor tissue compared with the control, induced apoptosis, and decreased cyclin D1, vascular endothelial growth factor, cyclooxygenase-2, c-myc, and Bcl-2 expression in the tumor tissue. Upregulation of TRAIL receptor by the combination was also observed in tumor tissues. Overall, our results suggest that curcumin potentiates the antitumor effect of BCG through the inhibition of NF-κB and induction of TRAIL receptors in bladder cancer cells. [Cancer Res 2009;69(23):8958–66]

Introduction

Bladder cancer is a very common cancer in the United States (with an estimated 68,810 new cases and 14,100 deaths per year) and a significant cause of morbidity and mortality throughout the world (1). The mainstay of therapy for the majority of patients is complete transurethral resection of tumor followed by intravesical instillation of antitumor agents. Intravesical instillation of Bacillus Calmette-Guerin (BCG) was first used for bladder cancer therapy in 1974 (2); to date, it is the most successful adjuvant agent for treating noninvasive bladder cancer (3). Nonetheless, treatment failure is common, so intense researches into methods of augmenting this response are under way.

It has long been believed that the mechanism of action of intravesical BCG instillation was due to nonspecific inflammation in the bladder (4). It is generally accepted that the majority of activity shown by BCG is due to stimulation of the local immune system and recruitment of polymorphonuclear neutrophil granulocytes (PMN) and proinflammatory cytokines such as interleukin-12 (5). Under inflammatory conditions, PMN may promote tumor growth and progression, whereas, in the context of therapeutic interventions, they can exert important antitumor functions. Although the exact mechanisms of PMN-mediated activation of tumor immunity are poorly defined, it has been suggested that release of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) from PMNs is a key step in the antitumor effect of BCG therapy (6). It has been shown that patients who have a response from BCG therapy have higher urinary TRAIL levels, and this TRAIL is able to induce apoptosis in bladder cancer cells *in vitro*. Furthermore, flow cytometry of fresh urine suggests that this TRAIL comes from PMNs in the urine of patients (6).

Curcumin (diferuloylmethane) is a major constituent of the yellow spice turmeric derived from the rhizomes of *Curcuma longa*. It is safe and nontoxic and has demonstrable antitumor, anti-inflammatory, apoptotic, and antioxidant properties. We have shown previously that curcumin inhibits tumor metastasis, invasion, and angiogenesis (7–9). These data suggest that curcumin regulates multiple signaling pathways and possesses several therapeutic benefits. In this study, our purpose is to show that curcumin also upregulates DR5 in bladder cancer cells and in tumor tissue and this leads to potentiation of the effect of BCG through production of TRAIL.

Materials and Methods

Reagents. Curcumin with purity >95% was obtained from Synthite Chemicals. BCG, a lyophilized preparation containing 1×10^8 to 8×10^8 colony-forming units (CFU), was resuspended in PBS. Polyclonal antibodies against p65 and cyclin D1 and monoclonal antibodies against vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX-2), c-myc, and Bcl-2 were obtained from Santa Cruz Biotechnology. Antibodies against cleaved caspase-3 and cleaved caspase-8 were purchased from Cell Signaling. The liquid DAB+ substrate chromogen system-horseradish peroxidase used for immunocytochemistry was obtained from DakoCytomation. Penicillin, streptomycin, MEM, and fetal bovine serum were obtained from Invitrogen.

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Tris, glycine, NaCl, SDS, bovine serum albumin, and monoclonal antibody against β-actin were obtained from Sigma. Phycoerythrin-conjugated mouse monoclonal anti-human and anti-mouse DR5 and phycoerythrin-conjugated mouse IgG1 isotype control and hamster control were purchased from eBioscience. The ELISA kit for detecting soluble human TRAIL/Apo-2L was purchased from R&D Systems.

Cell lines and culture conditions. The MBT-2 cell line was a gift from Dr. Timothy Ratliff (4). Human bladder cancer cell line 253J-BV was generously provided by Dr. Colin P.N. Dinney (Department of Urology, The University of Texas M. D. Anderson Cancer Center). The human bladder carcinoma cell line KU-7 was obtained from the American Type Culture Collection. The cell line RT4V6 was generated by *in vivo* recycling of RT4. The cells were cultured in MEM supplemented with 10% fetal bovine serum, vitamins, sodium pyruvate, L-glutamine, nonessential amino acids, and penicillin-streptomycin.

Animals. C3H female mice were obtained from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research and Development Center. 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.

Neutrophil stimulation with BCG. Unstimulated PMNs (3×10^6/mL) and PMNs stimulated with cycloheximide (10 μg/mL, 1 h) were incubated in the presence or absence of BCG and were centrifuged and the supernatant was collected. Dose and time response of BCG to release TRAIL were analyzed by ELISA for TRAIL/Apo-2L.

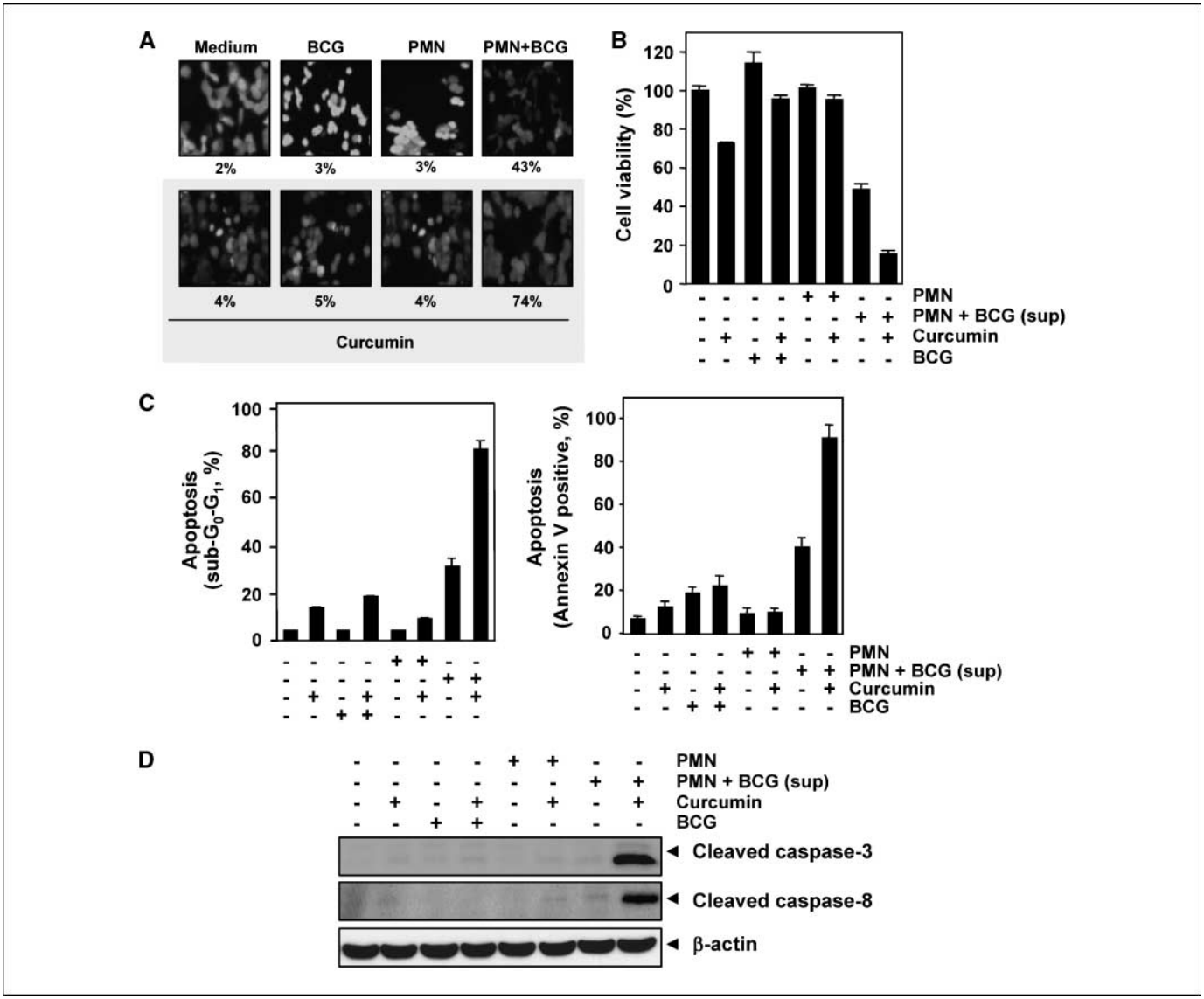

Figure 1. Curcumin potentiates the effects of BCG against human bladder cancer cells *in vitro*. *A,* PMNs (5×10^6/mL) were incubated with medium, BCG (10^6 CFU), cycloheximide (10 μg/mL), or combination for 1 h. After centrifugation, supernatants were collected and added to the bladder cancer cell line 253J-BV (1×10^6/mL). *B,* cells were also treated with curcumin (10 μmol/L), BCG (10^6 CFU), or the combination. After 24 h incubation, cells were stained with the appropriate assay reagents and cell viability was determined by Live/Dead Assay and MTT assay. *C,* cycloheximide-stimulated PMNs were incubated in the presence or absence of BCG (10^6 CFU). After centrifugation, supernatants were collected and added to the bladder cancer cell line 253J-BV. Cells were also treated with curcumin (10 μmol/L), BCG (10^6 CFU), or the combination. After 24 h incubation, cells were analyzed by apoptosis using propidium iodide (*left*) and Annexin V (*right*). *D,* supernatants from stimulated PMNs incubated in the presence or absence of BCG (10^6 CFU) were added to the bladder cancer cell line 253J-BV (1×10^6 per well). Cells were also treated with curcumin (10 μmol/L), BCG (10^6 CFU), or the combination and incubated at 37°C for 24 h. Whole-cell lysates were prepared and caspases was analyzed by Western blot.

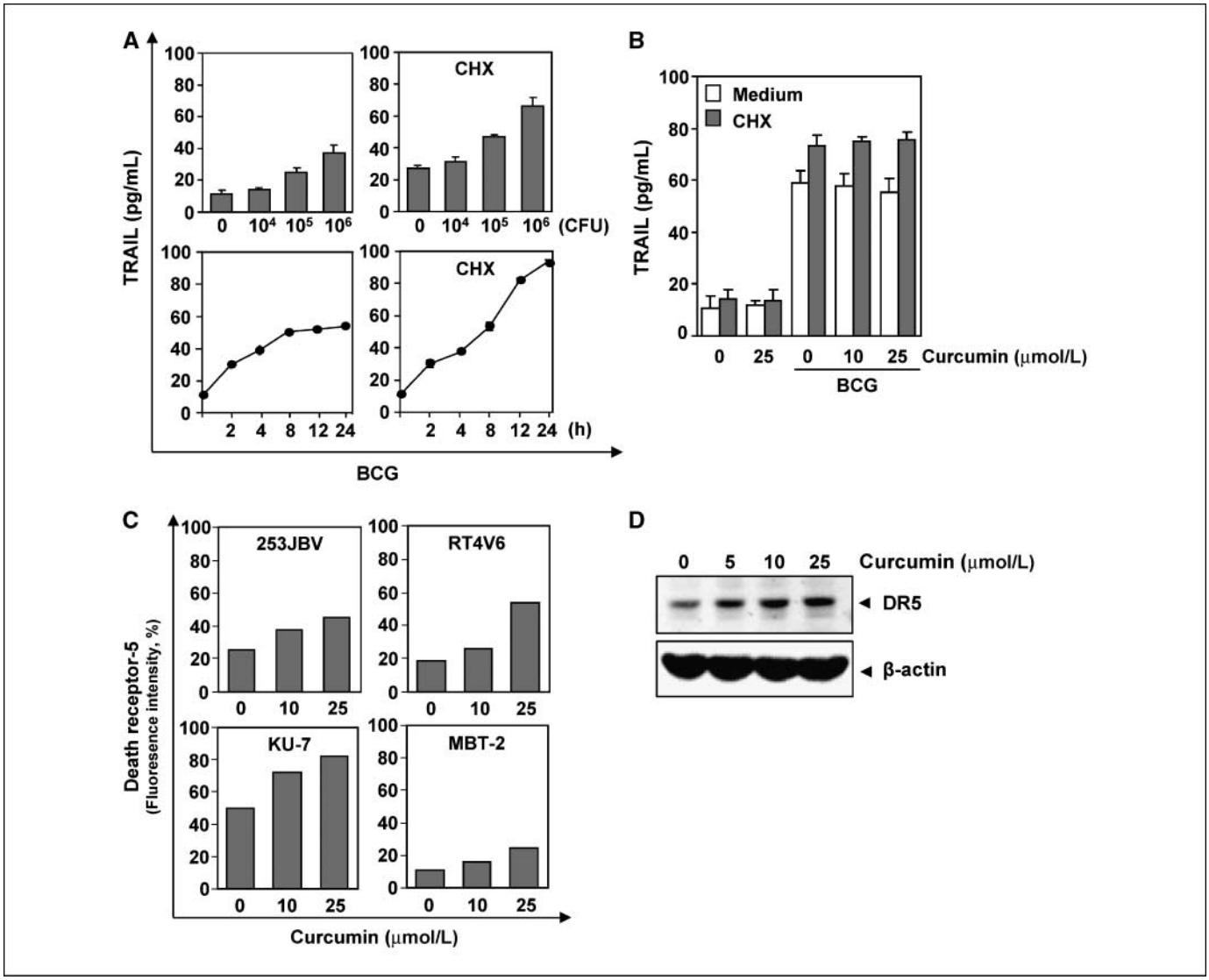

Figure 2. BCG induces PMNs to release TRAIL and DR5 expression. *A, top,* nonstimulated PMNs (5×10^6/mL; *left*) and PMNs (5×10^6/mL) stimulated with cycloheximide (*CHX*; 10 μg/mL, 1 h; *right*) were incubated with indicated concentrations of BCG for 4 h. The supernatants were collected and analyzed for the level of soluble TRAIL/Apo-2L by ELISA. *Bottom,* nonstimulated PMNs (*left*) and PMNs stimulated with cycloheximide (*right*) were incubated with BCG (10^6 CFU) for indicated times. The supernatants were collected and analyzed for the level of soluble TRAIL/Apo-2L by ELISA. *B,* nonstimulated PMNs and PMNs stimulated with cycloheximide were preincubated with curcumin for 4 h and then incubated with BCG (10^6 CFU) for 24 h. The supernatants were collected and analyzed for soluble TRAIL/Apo-2L level by ELISA. *C,* human bladder cancer cells (253J-BV, RT4V6, and KU-7) and murine bladder cancer cells (MBT-2) were treated with indicated concentrations of curcumin and incubated for 24 h. Cell surface expression of DR5 receptor was determined by flow cytometry as described. *D,* 253J-BV cells were treated with indicated concentrations of curcumin and incubated for 24 h. Whole-cell lysates were prepared and DR5 was analyzed by Western blot.

Analysis of cell surface DR5. Bladder cancer cell lines 253J-BV, RT4V6, and KU-7 (1×10^6/mL) were plated in six-well plates for 24 h. Different concentrations of curcumin were added and incubated for 24 h. Cells were trypsinized and washed with fluorescence-activated cell sorting buffer. This assay was done as described previously (10).

Western blot analysis. To determine the levels of protein expression, we prepared whole-cell extracts and analyzed by Western blot as described previously (11).

Preparation of nuclear extract from tumor samples and cell lines. Bladder cancer cells (1×10^6/mL) were treated with curcumin for 4 h and BCG for 1 h. For samples from mice, bladder tumor tissues (75-100 mg/mouse) from control and treated mice were minced. The preparation of nuclear extract from tissue samples was done as described previously (12).

Electrophoretic mobility shift assay. To evaluate the activation of NF-κB following radiation therapy, electrophoretic mobility shift assay (EMSA) was done as described previously (13).

Live/dead assay. This assay was done as described previously (14).

Cell cycle analysis. To determine the effect of curcumin on BCG-stimulated PMN on cell cycle analysis, we used propidium iodide/fluorescence-activated cell sorting analysis. PMNs (5×10^6/mL) were incubated in the absence or presence of BCG (10^6 CFU) in polypropylene tubes for 24 h, after which the supernatant was recovered by centrifugation. The neutrophil conditioned medium was then added to the human bladder tumor cell line 253J-BV in six-well plates for 24 h. Propidium iodide staining for DNA content analysis was done as described elsewhere.

MTT assay. The antiproliferative effect of curcumin on BCG-stimulated PMN against bladder cancer cell line 253J-BV was determined by MTT dye uptake method as described earlier (14).

Transplantation of MBT-2 and experimental protocol. MBT-2 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 were used for injections. Single-cell suspensions containing 2×10^5 MBT-2 cells in 0.1 mL HBSS were injected s.c. into both flanks of each mouse. Mice were randomized into the following treatment groups ($n = 9$): (*a*) untreated control (corn oil, 100 μL/d); (*b*) curcumin alone (1 g/kg), once daily, orally; (*c*) BCG (10^6 CFU), once weekly, intratumor injection; and (*d*) combination of curcumin (1 g/kg), once daily, orally, and BCG (10^6 CFU), once weekly, intratumorally. Therapy was continued for 4 weeks. Tumors were enumerated by inspection and palpation, and tumor dimensions were measured by Vernier caliper. Tumor volume was calculated using the formula: (shortest diameter)^2 × (longest diameter) / 2. On the 30th day, mice in all groups were sacrificed. Tumor volumes were compared among groups using unpaired Student's *t* test.

Ki-67 immunohistochemistry. Formalin-fixed, paraffin-embedded sections were stained with anti–Ki-67 (rabbit monoclonal clone SP6; Thermo Scientific) antibody as described previously (15). Results were expressed as percentage ± SE of Ki-67–positive cells per ×40 magnification field. A total of ten ×40 fields was examined and counted from each group.

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 (Pharmingen) as described previously (15). The density of vessels was determined by counting the number of vessels per high-power field (×100) in four areas of each tumor section. Results were expressed as the mean ± SE number of vessels per high-power field. A total of 20 high-power fields was examined and counted from three tumors of each of the treatment groups.

Quantification of apoptosis in tumor sections. Apoptotic cells in tumor samples were quantified by fluorescent terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling using a commercial kit (Promega) according to the manufacturer's instructions. Immunofluorescence microscopy was done using a Zeiss Plan-Neofluar lens on an epifluorescence microscope and captured image using a cooled CCD camera. DNA fragmentation was detected by localized green fluorescence within the nucleus of apoptotic cells. For total terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling expression, apoptotic events were quantified manually (16).

Immunohistochemical analysis for VEGF, COX-2, p65, cyclin D1, and DR5 in tumor tissues. The nuclear localization of p65 and the expression of VEGF, COX-2, cyclin D1, and DR5 were evaluated using an immunohistochemical method described previously (17).

Statistics. All experiments were done as at least three independent assays. The values were compared using one-way ANOVA and Dunnett's multiple comparison post-test.

Results

The goal of this study is to determine whether curcumin potentiates the activity of BCG against bladder cancer. For this, we used *in vitro* and *in vivo* model systems to investigate the effect of curcumin on BCG activity. Because BCG mediates its effects through activation of PMN, freshly isolated human PMNs were used for these studies.

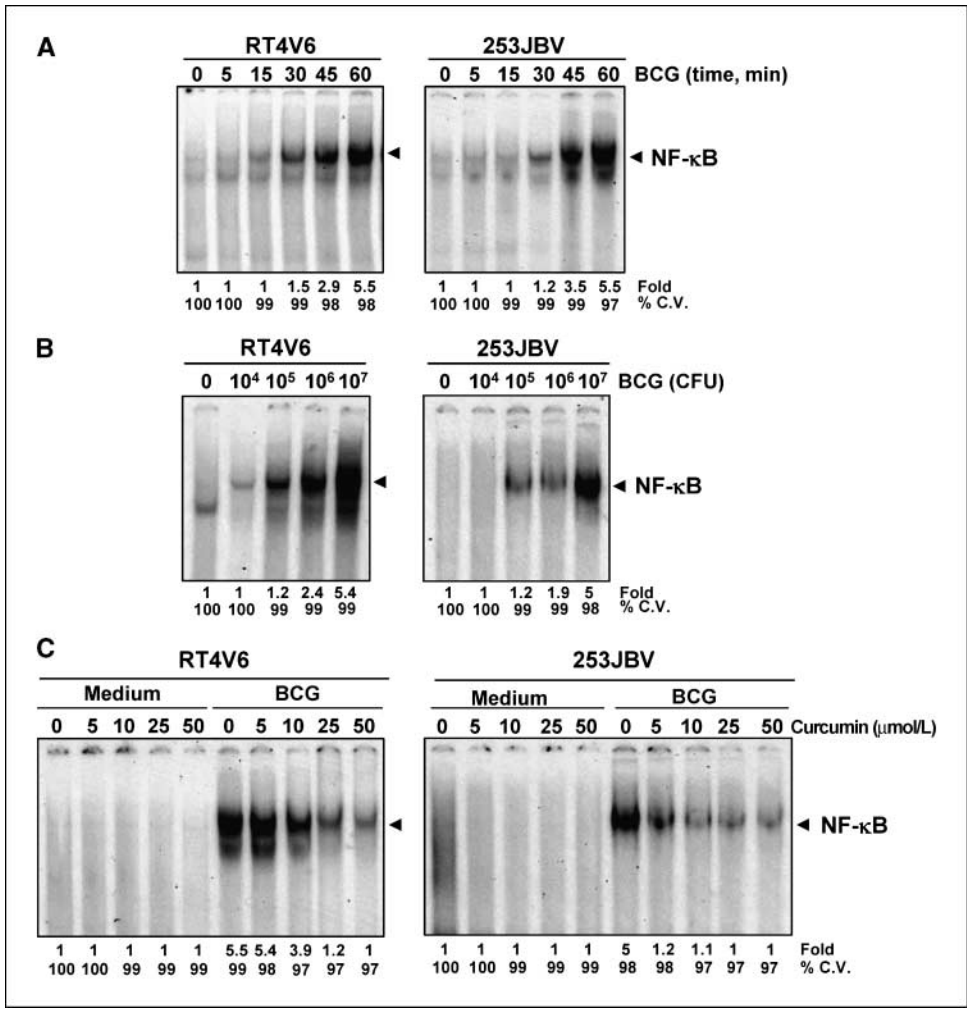

Figure 3. BCG induces NF-κB activation in human bladder cancer cells; this activation is abrogated by exposure to curcumin. *A,* human bladder cancer cells (RT4V6 and 253J-BV) were exposed to BCG (10^7 CFU) for indicated times. Nuclear extracts were prepared and assayed for NF-κB activation using EMSA. Cell viability (*% C.V.*) was measured by trypan blue assay. *B,* cells were incubated with indicated concentration of BCG for 1 h. Nuclear extracts were prepared and assayed for NF-κB by EMSA. *C,* cells were incubated with curcumin at different concentrations for 4 h and then treated with BCG (10^7 CFU) for 1 h. Nuclear extracts were prepared and assayed for NF-κB by EMSA.

Curcumin potentiates the apoptotic and antiproliferative effects of BCG in bladder cancer cells. We first investigated the effect of curcumin on PMN-BCG–induced apoptosis by intracellular esterase activity method that examined plasma membrane integrity. These results showed that whereas BCG and PMN alone had little effect on apoptosis, together they induced significant apoptosis and curcumin enhanced the apoptotic effects of PMN-BCG from 43% to 74% against bladder cancer cells (Fig. 1*A*).

We also investigated the effect of curcumin on cell viability affected by PMN-BCG by the MTT method that examines the mitochondrial activity. Results showed that curcumin significantly enhanced the antiproliferative effects of BCG (Fig. 1*B*). When examined by sub-G_0-G_1 fraction by fluorescence-activated cell sorting, curcumin enhanced apoptosis by BCG from 30% to ~80% (Fig. 1*C, left*).

When apoptosis was examined by Annexin V method, curcumin enhanced apoptosis from 40% to >80% (Fig. 1*C, right*). We also examined apoptosis by activation of caspase-8 and caspase-3. Highly significant apoptosis was produced only when curcumin was used in combination with BCG (Fig. 1*D*). All these results together suggest that curcumin can potentiate the apoptotic effect of BCG against bladder cancer cells.

Stimulation of PMN with BCG results in TRAIL/Apo-2L expression. How BCG, a standard treatment for bladder cancer, induces apoptosis is not known. Induction of TRAIL by PMN, a potent apoptosis-inducing cytokine, has been described as one of the mechanisms (6, 18). Whether BCG induces apoptosis in our system through induction of TRAIL was examined. Unstimulated or stimulated PMNs were treated with BCG for 4 h and supernatants were analyzed for soluble TRAIL/Apo-2L using ELISA. We found that there was an increase in TRAIL/Apo-2L in all groups of neutrophils stimulated with BCG and that the soluble TRAIL increased with BCG dose (Fig. 2*A, top*).

To further examine the response of neutrophils to BCG stimulation, we analyzed the response for different periods and different doses of BCG. Soluble TRAIL/Apo-2L increased with BCG stimulation throughout the 24 h of exposure in a time-dependent manner (Fig. 2*A, bottom*).

Curcumin does not modulate the production of TRAIL/Apo-2L by BCG-stimulated PMN. Next, we also examined what is the effect of curcumin on BCG-stimulated production of TRAIL in PMNs. Unstimulated and stimulated PMNs were pretreated with curcumin for 4 h, and then incubated with BCG for 24 h. The supernatants were analyzed for TRAIL. As shown in Fig. 2*A*, BCG increased the level of TRAIL in PMNs in a dose- and time-dependent manner. To exclude the role of *de novo* protein synthesis, we also examined the effect of protein synthesis inhibitor cycloheximide on production of TRAIL (18, 19). We have found that presence of cycloheximide did not inhibit the production of TRAIL, indicating that protein synthesis is not involved. TRAIL production, however, was slightly enhanced.

Whether curcumin modulate BCG-stimulated soluble TRAIL production from PMNs was also examined. We found that curcumin had no effect on BCG-induced production of TRAIL from PMNs (Fig. 2*B*).

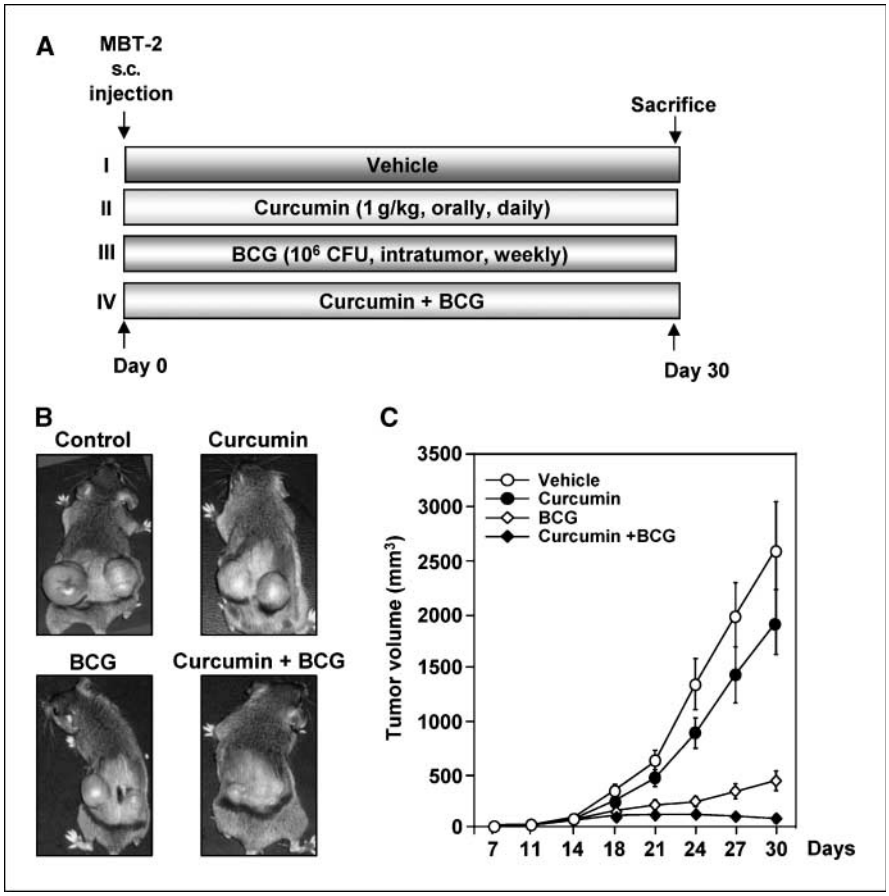

Figure 4. Curcumin potentiates the antitumor effects of BCG against MBT-2–induced murine tumors. *A,* schematic representation of experiment protocol described in Materials and Methods. Animals were divided into four groups ($n = 9$). Group I (vehicle) was treated with corn oil (100 μL, orally/daily), group II was treated with curcumin alone (1 g/kg, orally/daily), group III was treated with BCG alone (10^6 CFU, intratumor/weekly), and group IV was treated with the combination of curcumin (1 g/kg, orally/daily) and BCG (10^6 CFU, intratumor/weekly). *B,* representative photographs of animals from each group on the last day of study showing marked response in mouse treated with combination therapy. *C,* tumor volumes measured at indicated time intervals ($n = 9$); as expected, BCG reduced tumor volumes, but combination therapy was even more effective.

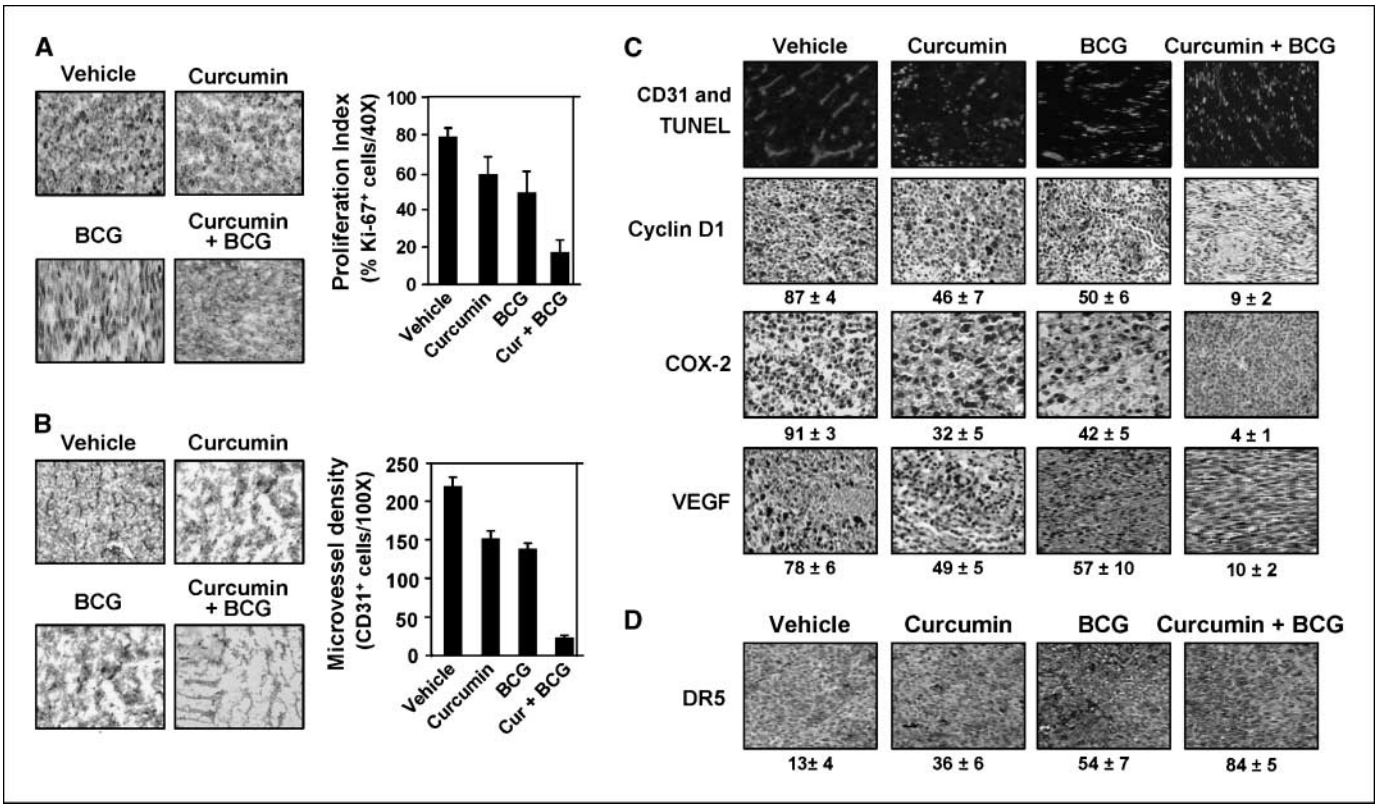

Figure 5. Combination of curcumin and BCG inhibits cell proliferation and expression of NF-κB–dependent gene products. *A, left,* immunohistochemical analysis of Ki-67–positive cells in bladder tumor indicated that curcumin alone and in combination with BCG suppressed cell proliferation; *right,* quantification of Ki-67 proliferation index as described in Materials and Methods. *B, left,* immunohistochemical analysis of tumors indicates the inhibition of microvessel density (CD31); *right,* quantification of $CD31^+$ for microvessel density as described in Materials and Methods. *Columns,* mean of triplicates; *bars,* SE. *C,* immunohistochemical analysis of murine tumors indicates induction of apoptosis (terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling and CD31) and NF-κB–dependent gene products in tumors treated with curcumin and BCG. Green fluorescence indicated apoptosis and red fluorescence indicated microvessel density ($CD31^+$ cells). Percentage, positive staining for the given biomarker. *D,* immunohistochemical analysis of DR5 showed the upregulation of DR5 by curcumin alone or in combination with BCG.

Curcumin upregulates TRAIL receptor (DR5) in various cancer cells. To assess the molecular mechanisms underlying the potentiation of BCG-induced apoptosis by curcumin in bladder cancer cells, we examined its effect on the expression of DR5, one of the major receptors for TRAIL, by fluorescence-activated cell sorting analysis. Increased fluorescent intensity of DR5 staining was observed in treated versus untreated cells, which increased with increasing concentration of curcumin (Fig. 2*C*). Curcumin induced the expression of DR5 protein in a dose-dependent manner on all bladder cancer cell lines. We also examined the effect of curcumin on induction of DR5 by Western blot analysis. These results also confirmed the induction of DR5 by curcumin (Fig. 2*D*).

Curcumin inhibits BCG-induced NF-κB activation in bladder cancer cells *in vitro*. Interaction of BCG mycobacteria with cells often accompanies the activation of the transcription factor NF-κB (20, 21). This NF-κB activation has been shown to suppress the apoptotic effect of TRAIL (22). Whether curcumin potentiates BCG-mediated apoptosis through downregulation of NF-κB was examined. Our results show that BCG can induce NF-κB in a time-dependent (Fig. 3*A*) and dose-dependent (Fig. 3*B*) manner in two different bladder cancer cell lines. Furthermore, we found that curcumin downregulated BCG-induced NF-κB activation in a dose-dependent manner in both cell lines (Fig. 3*C*).

Curcumin potentiates the antitumor effects of BCG in murine tumors in C3H mice. Based on the studies described above, we investigated the effect of curcumin on the antitumor effects of BCG in mice. BCG requires an immunocompetent *in vivo* model to show efficacy, presumably because it requires functioning immune competent cells such as PMNs. Therefore, we investigated the effects of curcumin on the antitumor activity of BCG in a syngeneic mouse model. We examined the effects of curcumin alone, BCG alone, and the combination of the two *in vivo* in C3H mice implanted with MBT-2 bladder cancer cells (Fig. 4*A*). On day 0, MBT-2 cells were injected s.c. into both flanks of mice. Curcumin therapy was started on day 1 and BCG on day 3. At indicated time intervals, tumor volume was measured by Vernier calipers. The tumor volume was reduced in curcumin- and BCG-treated groups and in those given the combination. BCG alone reduced tumor growth; growth was further reduced by the combination treatment (Fig. 4*B*). All animals were sacrificed on the 30th day. The final tumor volumes on day 30 after the start of treatment showed significant decrease in the curcumin + BCG group (67 mm^3) compared with control (2598 mm^3; $P < 0.0001$ versus control) or with BCG alone (419 mm^3; $P < 0.003$ versus BCG; Fig. 4*C*). The tumor volumes in curcumin alone group was not statistically significant compared with control group ($P = 0.197$).

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 the expression of Ki-67, a biomarker for growth of tumors,

and CD31, a biomarker for microvessel density in tumor tissues, by immunohistochemistry. Results in Fig. 5*A* showed that both curcumin and BCG, when compared with the untreated control, decreased the expression of Ki-67 to a similar extent, but the maximum decrease was noted when the two agents were used in combination ($P < 0.01$ versus control; $P < 0.01$ versus BCG alone). Curcumin and BCG were equally effective in decreasing CD31 expression, and the two in combination were maximally effective (Fig. 5*B*; $P < 0.01$ versus control; $P < 0.01$ versus BCG alone).

Curcumin potentiates BCG-induced apoptosis in the tumor tissue. As shown in Fig. 5*C*, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining indicated that whereas treatment with curcumin and BCG alone did induce apoptosis, combination therapy had the greatest effect. We used fluorescent staining with CD31 to indicate that the combination therapy not only increased apoptosis (green) but also resulted in lower microvessel density (red) in the same region of the tumor tissue, indicating that curcumin potentiated the apoptotic effects of BCG as well as reducing angiogenesis.

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 immunohistochemistry. As shown in Fig. 5*C*, curcumin and BCG alone reduced the expression of cyclin D1 to 53% and 58% of vehicle control, respectively. The two agents together, however, reduced the expression by 90% ($P < 0.01$ versus control; $P < 0.01$ versus BCG alone) in the tumor tissue.

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 immunohistochemistry. As shown in Fig. 5*C*, curcumin and BCG alone reduced the expression of COX-2 to 35% and 46% of vehicle control, respectively. The two agents together, however, reduced the expression by 96% ($P < 0.01$ versus control; $P < 0.01$ versus BCG alone) in the tumor tissue.

Curcumin decreases VEGF expression in the tumor tissue. Whether curcumin can affect the VEGF expression, a marker for angiogenesis, in the tumor tissue was examined by immunohistochemistry. As shown in Fig. 5*C*, curcumin and BCG alone reduced the expression of VEGF to 63% and 73% of vehicle control, respectively. The two agents together, however, reduced the expression by 87% ($P < 0.01$ versus control; $P < 0.01$ versus BCG alone) in the tumor tissue.

Curcumin increases DR5 expression in the tumor tissue. To test our hypothesis in the animal model, we assessed whether curcumin upregulates DR5 expression in tumor tissues as studied by immunohistochemistry. As shown in Fig. 5*D*, curcumin and BCG together increases the upregulation of DR5 ($P < 0.01$ versus control; $P < 0.01$ versus BCG alone).

Curcumin inhibits NF-κB and downregulates the NF-κB–regulated gene products in tumor tissues. NF-κB expression has been linked with survival, proliferation, and angiogenesis. Here, we examined the effects of therapy on its expression using immunohistochemistry. As shown in Fig. 6*A*, curcumin alone reduced the expression of NF-κB by 90%, whereas BCG alone had no significant effect. Two agents together reduced the expression by 94% in the tumor tissue.

We also analyzed the effect of curcumin and BCG on NF-κB in tumor tissues by EMSA. Curcumin completely downregulated the NF-κB DNA-binding activity in tumor tissue, whereas and BCG had minimal effect (Fig. 6*B*). We tested the effects of curcumin, BCG alone, and the combination on the expression of genes involved in tumor cell survival (Bcl-2, Bcl-xL, and survivin), proliferation (c-myc), inflammation (COX-2), and angiogenesis (VEGF). Western blot results indicated that curcumin alone downregulated the expression of all these proteins whether alone or with BCG (Fig. 6*C*). These data are in agreement with the immunohistochemical analysis (Fig. 5*C*).

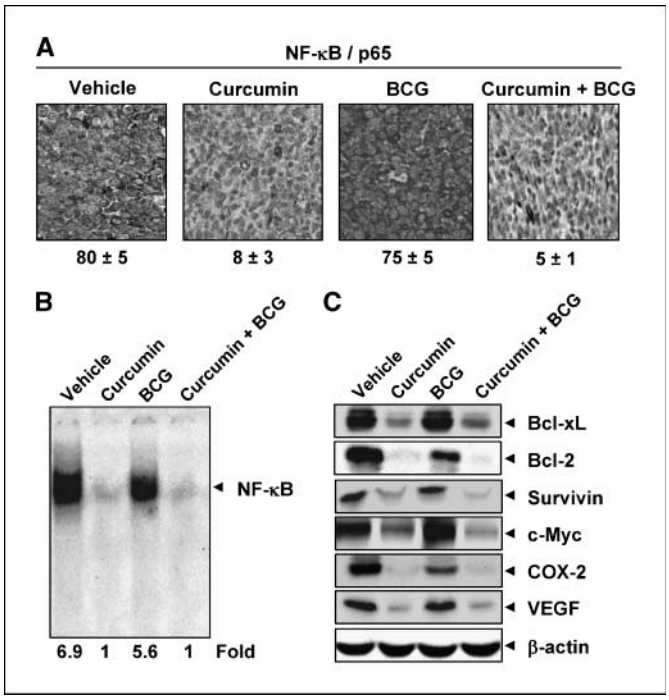

Figure 6. Curcumin inhibits NF-κB and NF-κB–regulated gene products in tumor tissue. *A,* immunohistochemical analysis of nuclear p65. The percentage inhibition of nuclear p65 by curcumin alone and in combination with BCG is indicated. *B,* EMSA analysis revealed that curcumin inhibited NF-κB activation in nuclear extracts from animal tissue. *C,* Western blot showing that curcumin inhibits the expression of NF-κB–dependent gene products Bcl-xL, VEGF, c-myc, survivin, COX-2, and Bcl-2 in tumor tissue.

Discussion

Although BCG treatment has been improved incrementally through dose modifications, changes in schedule, and symptomatic treatment of side effects (23), there has not been substantial improvement largely because of poor understanding of its mechanism of action. Here, we present a mechanism-based rationale for combination therapy of BCG with curcumin. Our results suggest that the effects of BCG therapy mediated via release of TRAIL from PMNs can be enhanced by curcumin. The latter enhances BCG-induced apoptosis through upregulation of death receptors, suppression of NF-κB activation, and inhibition of angiogenesis, all of which translates into decreased tumor volumes.

Recent advances have shed light on the potential mode of action of BCG via release of TRAIL from PMNs in the bladder and subsequent apoptosis of bladder cancer cells (6). However, not all tumors respond to this treatment, so agents that can enhance response to BCG are required. We found that PMNs exposed to BCG do indeed release soluble TRAIL into the supernatant. When the supernatant is added to bladder cancer cell lines *in vitro*, it resulted in decreased cell proliferation and apoptosis. The addition of curcumin increased the number of cells undergoing apoptosis,

an event that was accompanied by marked activation of caspases. Caspase activation plays pivotal roles in both mitochondria and death receptor–mediated pathways. The activation of caspase-8 has been shown to play an important role in both mitochondria and death receptor–mediated pathways and is mediated by the increased expression of a death receptor and/or ligation of a receptor with its ligand (24). We found that curcumin treatment enhanced the BCG-induced activation of both caspase-8 and caspase-3.

We found that curcumin upregulated DR5 expression in bladder cancer cell lines. The induced expression of DR5 correlated with curcumin-induced apoptosis. Recently, these death receptors have attracted much more attention because their ligand preferentially induces apoptosis in transformed cells showing potential as a tumor-selective apoptosis-inducing cytokine for cancer treatment (25). DR5 can be regulated by several factors including p53, NF-κB, c-Jun NH_2-terminal kinase, and CAAT/enhancer-binding protein homologous protein (26–29).

We also explored whether BCG itself can activate NF-κB in tumor cells because this is one avenue whereby certain tumor cells escape apoptosis. We were able to show activation of NF-κB by BCG and curcumin suppressed this activation, showing yet another reason why the combination might be relevant in the clinical situation. Inhibition of NF-κB activation by curcumin has been thought to enhance apoptosis induction and mediate anti-inflammatory effects (30, 31).

We found that curcumin upregulated DR5 expression and downregulated NF-κB–regulated cancer cell survival proteins (e.g., Bcl-2, Bcl-xL, and survivin) in both *in vitro* and *in vivo*. These observations are in contradictions to the report that TRAIL receptors including DR5 are transcriptionally regulated by NF-κB (32). Although curcumin can inhibit NF-κB activation, the upregulation of DR5 by curcumin has been shown to be mediated through activation of CAAT/enhancer-binding protein homologous protein in human renal cancer cells (33). Our results are in agreement with a report that curcumin suppressed NF-κB activation but upregulated DR5 expression at the same time, which led to the sensitization of cancer cells against TRAIL (34, 35). The constitutive activation of NF-κB has been implicated in TRAIL resistance and this resistance can be reversed by suppression of NF-κB in human pancreatic cancer cells (36).

In an immunocompetent mouse model, BCG reduced tumor volume, and its effects were enhanced by coadministration of curcumin. Immunohistochemical analysis of murine tumors indicates that the inhibition of cell proliferation, induction of apoptosis, upregulation of DR5, and decreased VEGF and microvessel density were most pronounced in the tumors of mice treated with combination BCG plus curcumin. Curcumin inhibits the expression of NF-κB–dependent gene products cyclin D1, COX-2, and VEGF in tumors.

Our results indicate that BCG treatment generates conflicting signals by activating both proapoptotic (release of TRAIL) and antiapoptotic signaling. It seems that the proapoptotic signaling (TRAIL) can overcome the activation of antiapoptotic signaling, leading to the induction of apoptosis and enhancement of TRAIL-induced apoptosis. Recently, it was shown that myc interferes with the TRAIL-dependent NF-κB–mediated transcriptional activation of prosurvival genes such as Mcl-1 and cellular inhibitor of apoptosis protein-2 (37). TRAIL functions as a DR5 ligand and rapidly induces apoptosis in a wide variety of cancer cells but is not cytotoxic in normal cells *in vitro* and *in vivo* (25). Therefore, TRAIL is considered to be a tumor-selective, apoptosis-inducing cytokine and is being evaluated in clinical trials as a new candidate for cancer treatment. Overexpression of Bcl-2 inhibits TRAIL-induced apoptosis, although it has been proposed that Bcl-2 provides only a partial protection at lower doses of TRAIL and not at higher concentrations (38). We found that curcumin inhibited Bcl-2 levels both *in vitro* and *in vivo*. Mechanisms of TRAIL resistance and ways to overcome these by targeted agents that either neutralize apoptotic blockades or suppress prosurvival signals also triggered by TRAIL are the subject of intense drug development, including molecules such as inhibitors of inhibitor of apoptosis proteins, Bcl-2 family members, and modulators of NF-κB and epidermal growth factor receptor signaling (38). Although combined treatment with standard chemotherapeutics or targeted agents may be an option to increase the efficacy of TRAIL therapy, the ideal combination would be one that does not carry any added toxicity.

It should be noted that BCG is the most effective therapy for bladder cancer and is able to treat even unresected tumors in the human patient (e.g., carcinoma *in situ*). To date, no therapy has shown added benefit over BCG alone (other than a randomized trial of BCG plus chemotherapy, with its ensuing problems). Thus, the finding that the combination of curcumin and BCG was more effective than BCG alone is remarkable. In conclusion, we provide a novel mechanistic-based rationale for combining curcumin, which is nontoxic and safe for oral ingestion, with BCG for bladder cancer therapy. We have shown that this is effective in the murine model and propose that this combination be tested in clinical trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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