Curcumin potentiates the apoptotic effects of chemotherapeutic agents and cytokines through down-regulation of nuclear factor-κB and nuclear factor-κB–regulated gene products in IFN-α–sensitive and IFN-α–resistant human bladder cancer cells

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Abstract
Bladder cancer mortality varies between the countries; whereas being highest in Western countries, it is lowest in Eastern countries, such as India. Cigarette smoking is one of the major risk factors for bladder cancer in affluent nations, such as United States. Localized early-stage bladder cancer is treated with resection and intravesical cytokine therapy, whereas metastatic cancer is typically treated with various combinations of systemic chemotherapy. Whether curcumin, a yellow curry pigment commonly consumed in countries, such as India, has any role in prevention or treatment of bladder cancer was investigated. We found that curcumin inhibited the proliferation, induced cell cycle arrest, and DNA fragmentation in both IFN-α–sensitive (RT4V6) and IFN-α–resistant (KU-7) bladder cancer cells. Curcumin also potentiated the apoptotic effects of the chemotherapeutic agents (gemcitabine and paclitaxel) and of cytokines (tumor necrosis factor (TNF) and TNF-related apoptosis-inducing ligand). This effect of curcumin was independent of sensitivity and resistance to IFN-α, commonly used for treatment of bladder cancer. Whether the effects of curcumin are mediated through modulation of the nuclear factor-κB (NF-κB) pathway known to mediate antiapoptosis was investigated. Both gemcitabine and TNF activated NF-κB in bladder cancer cells and curcumin suppressed this activation. Similarly, cigarette smoke, a major risk factor for bladder cancer, also activated NF-κB and curcumin suppressed it. Cigarette smoke–induced expression of the NF-κB–regulated gene products cyclooxygenase-2 and vascular endothelial growth factor, linked with proliferation and angiogenesis, respectively, was also down-regulated by curcumin. [Mol Cancer Ther 2007;6(3):1022–30]

Introduction
Bladder cancer is diagnosed in more than 61,000 people annually in the United States, and 14,000 people die of this disease each year (1). The treatment of early-stage bladder cancer involves complete transurethral resection of tumor followed by instillation of intravesical agents, such as Bacillus Calmette-Guerin (believed to act via induction of cytotoxic cytokines and IFN-α; ref. 2). Despite initial responses, these tumors can eventually recur and can progress to more invasive stages requiring surgical removal of the bladder. Most of the deaths from bladder cancer are due to advanced unresectable disease, which is resistant to chemotherapy (3, 4). For more than a decade, the standard treatment for advanced bladder cancer has been the combination of methotrexate, vinblastine, doxorubicin (Adriamycin), and cisplatin. This regimen is consistently reported to produce median survival durations ranging from 13 to 15 months (5). In spite of considerable effort to escalate the doses of the components of methotrexate, vinblastine, doxorubicin (Adriamycin), and cisplatin, there has been no observed improvement in survival with this regimen. Recently, paclitaxel, gemcitabine, and ifosfamide have been recognized to be quite active against invasive transitional cell carcinoma, and many novel combination regimens have been reported (6–9). Unfortunately, although some of these newer regimens are less toxic than methotrexate, vinblastine, doxorubicin (Adriamycin), and cisplatin, there is still no compelling evidence that survival rates are improving. Indeed, there is a growing conviction that the cytotoxic paradigm will not provide the means to qualitatively change the outcome for patients with metastatic bladder cancer.

Bladder cancer mortality rates vary in different countries. The highest rates are noted in European countries, such as Denmark, the United Kingdom, Belgium, and Italy,
whereas the lowest rates are noted in Asian countries, such as India, Japan, China, and Singapore (10). Populations in Southeast Asia have 4- to 10-fold lower incidences of and fewer deaths of bladder cancer than do populations in the United States (11). However, Asian individuals who migrate to the United States have an increased risk of bladder cancer equal to that in Americans in one generation (12).

Cigarette smoking is the major risk factor for bladder cancer in affluent nations; exposure to chemical carcinogens in the environment, particularly in the workplace, is also a contributing factor (10, 13). However, a definitive etiology of bladder cancer is still unknown. Considering the unknown etiologic factors for bladder cancer carcinogenesis and the fact that patients with bladder cancer face the threat of both metastasis and uncontrolled local recurrence after treatment, additional efforts to define alternative interventions for the prevention of bladder cancer progression and metastasis are urgently needed.

Nuclear factor-κB (NF-κB) is a transcription factor present in the cytoplasm as an inactive heterotrimer consisting of p50, p65, and IκBα subunits. On activation, IκBα undergoes phosphorylation- and ubiquitination-dependent degradation leading to nuclear translocation and binding to a specific consensus sequence in the DNA, which results in gene transcription. The kinase that phosphorylates IκBα is termed IκB kinase, which is composed of IκB kinase α, IκB kinase β, and IκB kinase γ (also called NF-κB essential modulator). NF-κB regulates the expression of genes involved in antiapoptosis (e.g., bcl-2 and bcl-xl), proliferation [e.g., cyclooxygenase-2 (COX-2) and cyclin D1], and metastasis [e.g., vascular endothelial growth factor (VEGF); refs. 14, 15].

Curcumin (diferuloylmethane), a yellow curry pigment derived from turmeric (Curcuma longa), is a pharmacologically safe agent that has been shown to suppress NF-κB activation and NF-κB gene products (16, 17). We did the study described herein to determine whether treatment with curcumin alone or in combination with chemotherapeutic agents or cytokines has a role in modulating the proliferation and apoptosis of bladder cancer cells by regulating the NF-κB pathway. Our results showed that curcumin alone can suppress the proliferation of these cells and further enhance the apoptotic effect of chemotherapeutic agents and cytokines through the down-regulation of NF-κB and NF-κB–regulated gene products.

Materials and Methods

Reagents

Curcumin (purity, >98%) was obtained from Sabinsa Corp. (Piscataway, NJ). Bacteria-derived human recombinant human tumor necrosis factor (TNF) that was purified to homogeneity with a specific activity of 5 × 10^7 units/mg was provided by Genentech (San Francisco, CA). TNF-related apoptosis-inducing ligand (TRAIL) was purchased from R&D Systems (Minneapolis, MN). Cigarette smoke condensate prepared as described previously (18) was supplied by C. Gary Gairola (University of Kentucky, Lexington, KY). Penicillin, streptomycin, RPMI 1640, and fetal bovine serum were obtained from Invitrogen (Grand Island, NY). Paclitaxel and an anti–β-actin antibody were obtained from Sigma-Aldrich (St. Louis, MO). Gemcitabine was purchased from MD Anderson Pharmacy (manufactured by Eli Lilly, Indianapolis, IN). An antibody against poly(ADP-ribose) polymerase (PARP) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody against VEGF was obtained from NeoMarkers (Fremont, CA). An anti–COX-2 antibody was obtained from BD Biosciences (San Diego, CA).

Cell Lines and Culture Conditions

The human bladder carcinoma cell line KU-7 was obtained from the American Type Culture Collection (Manassas, VA). The cell line RT4V6 was generated in Dr. Kumat’s laboratory by in vivo recycling of RT4 (ATCC, Manassas, VA). The cells were cultured as monolayers in modified Eagle’s MEM supplemented with 10% fetal bovine serum, vitamins, sodium pyruvate, l-glutamine, nonessential amino acids, and penicillin-streptomycin.

Electrophoretic Mobility Shift Assay

To assess NF-κB activation, nuclear extracts were prepared, and electrophoretic mobility shift assay (EMSA) was done as described previously (19). The dried gels were visualized, and the radioactive bands were quantitated using the Storm820 and ImageQuant software (Amer sham, Piscataway, NJ).

Western Blot Analysis

To determine the levels of protein expression, whole-cell extracts were prepared (20) and fractionated using SDS-PAGE. After electrophoresis, the proteins were electro-transferred onto nitrocellulose membranes, blotted with each antibody, and detected using an enhanced chemiluminescence regent (Amer sham). The bands obtained were quantitated using the NIH Image software program (NIH, Bethesda, MD).

Live/Dead Assay

To measure apoptosis, the Live/Dead assay (Molecular Probes, Carlsbad, CA), which measures intracellular esterase activity and plasma membrane integrity, was used. This assay uses calcein, a polyanionic dye that is retained within the live cells and provides green fluorescence. It also uses the ethidium monomer dye (red fluorescence), which can enter the cells only through damaged membranes and bind to nucleic acids but is excluded by the intact plasma membranes of live cells. Briefly, cells were incubated with curcumin and/or chemotherapeutic agents (paclitaxel and gemcitabine) and cytokines (TNF and TRAIL) at 37°C for 24 h. Thereafter, cells were stained with the Live/Dead reagent (5 µmol/L ethidium homodimer and 5 µmol/L calcein-AM) and then incubated at 37°C for 30 min. Cells were then analyzed under a fluorescence microscope (Labophot-2).

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. To identify apoptosis, we used an Annexin V antibody, which was conjugated with the FITC fluorescence dye. Briefly, cells were incubated with curcumin and/or chemotherapeutic agents (paclitaxel and gemcitabine) and cytokines (TNF and TRAIL) at 37°C for 24 h and subjected to Annexin V staining. The cells were washed in PBS, resuspended in 100 μL of binding buffer containing a FITC-conjugated anti–Annexin V antibody, and then analyzed with a flow cytometer (FACSCalibur, BD Biosciences).

**Immunoblotting Analysis for PARP Cleavage**

Apoptosis was assessed by determining the proteolytic cleavage of PARP (21). Briefly, cells (2 × 10^6/mL) were

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**Figure 1.** Curcumin suppresses proliferation of bladder cancer cells. A, RT4V6 and KU-7 cells (5,000 cells/0.1 mL) were incubated with the indicated concentrations of curcumin at 37°C for 24 h, and cell proliferation was assayed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent. Results are representative of three independent experiments. Columns, cell proliferation; bars, SD (between the triplicates). B, curcumin induces cell cycle arrest in bladder cancer cells. RT4V6 and KU-7 cells (1 × 10^6) were incubated with the indicated concentrations of curcumin for 24 h, and cell cycle analysis was done as described in Materials and Methods. Results are representative of three independent experiments. C, curcumin induces DNA fragmentation in bladder cancer cells. RT4V6 and KU-7 cells (1 × 10^6) were incubated with the indicated concentrations of curcumin for 48 h, and DNA fragmentation was analyzed as described in Materials and Methods. Columns, DNA fragmentation; bars, SD (between the triplicates).
treated with indicated concentrations of curcumin and/or gemcitabine for indicated periods. The cells were then washed and extracted by incubation for 30 min on ice in 0.05 mL of a buffer consisting of 20 mmol/L HEPES (pH 7.4), 2 mmol/L EDTA, 250 mmol/L NaCl, 0.1% NP40, 2 μg/mL leupeptin, 2 μg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, 0.5 μg/mL benzamidine, 1 mmol/L DTT, and 1 mmol/L sodium vanadate. The lysate was centrifuged, and the supernatant was collected. Cell extract proteins (30 μg) were resolved on 7.5% SDS-PAGE gels, electrotransferred onto a nitrocellulose membrane, blotted with a mouse anti-PARP antibody, and then visualized using an enhanced chemiluminescence reagent. Apoptosis was represented by cleavage of 116-kDa PARP into an 85-kDa product.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay**

The antiproliferative effect of curcumin on bladder cancer cells was determined by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye uptake in bladder cancer cells (22). Briefly, cells were incubated in triplicate in 96-well plates in the presence or absence of indicated concentrations of curcumin in a final volume of 0.1 mL at 37°C for 24 h. Afterward, 0.025 mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (5 mg/mL in PBS) was added to each well. After 2 h of incubation at 37°C, 0.1 mL of lysis buffer (20% SDS, 50% dimethylformamide) was added, incubation was continued overnight at 37°C, and the absorbance at 590 nm was read using a 96-well multiscanner.

**Figure 2.** Curcumin potentiates apoptosis induced by chemotherapeutic agents and cytokines. RT4V6 cells (A) and KU-7 cells (B), 1 × 10^6, were incubated with 5 μmol/L curcumin alone or in combination with 5 nmol/L gemcitabine, 300 nmol/L paclitaxel, 1 nmol/L TNF, and 1 ng/mL TRAIL for 24 h. Cells were stained with the Live/Dead assay reagent for 30 min and then analyzed under a fluorescence microscope. RT4V6 cells (C) and KU-7 cells (D), 1 × 10^6, were incubated with 5 μmol/L curcumin alone or in combination with 5 nmol/L gemcitabine, 300 nmol/L paclitaxel, 1 nmol/L TNF, and 1 ng/mL TRAIL for 24 h. Cells were incubated with anti–Annexin V antibody conjugated with FITC and then analyzed with a flow cytometer for early apoptotic effects. Results are representative of three independent experiments.
Curcumin also potentiated the cytotoxic effects of paclitaxel, gemcitabine, and TRAIL.

Results

The focus of this study was to determine whether curcumin has a role in prevention or treatment of bladder cancer. Apoptosis, NF-κB, COX-2, and VEGF, which we used as targets, are relevant to both prevention and treatment of cancer (25–28). Throughout, we used two different bladder cancer cell lines: RT4V6, which is highly sensitive to IFN-α and TRAIL, and KU-7, which is resistant to IFN-α and TRAIL.

Curcumin Suppresses the Proliferation of Bladder Cancer Cells

Because NF-κB has been implicated in cell survival and proliferation (29, 30), we examined the effect of treatment with curcumin on the proliferation of RT4V6 and KU-7 cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Treatment with curcumin at a concentration as low as 5 μmol/L inhibited the growth of both cell types (Fig. 1A); at 25 μmol/L, the treatment significantly suppressed the growth of both cell types. We also examined whether curcumin has any effect on cell cycle by using flow cytometric analysis. The results showed a significant, dose-dependent increase in the percentage of cells in the S phase of cell cycle (Fig. 1B). These results clearly showed that curcumin induces arrest of these bladder cancer cells. In addition, we examined whether curcumin can induce DNA fragmentation in bladder cancer cells. We found that treatment with curcumin induced substantial DNA fragmentation in both RT4V6 and KU-7 cells in a dose-dependent manner (Fig. 1C). The results of Fig. 1C, which measures DNA fragmentation, correlates well with Fig. 1A, which measures mitochondrial activity. For instance, 10 μmol/L curcumin induced ~40% DNA fragmentation and 50% reduction in mitochondrial activity in RT4V6 cell line.

Curcumin Potentiates Apoptosis Induced by Chemotherapeutic Agents and Cytokines

Because we found that curcumin inhibited the proliferation of KU-7 and RT4V6 cells, we next sought to determine whether curcumin potentiates the apoptotic effects of chemotherapeutic agents and cytokines used against bladder cancer. The Live/Dead assay, which measures membrane stability, indicated that treatment with combination of curcumin and gemcitabine increased the percentage apoptosis in RT4V6 cells from 10% to 90% compared with cells treated with gemcitabine alone. Curcumin also potentiated the cytotoxic effects of paclitaxel, TNF, and TRAIL, to the same extent (Fig. 2A). We obtained similar results with KU-7 cells (Fig. 2B). To
further confirm the potentiation effect of curcumin, we used Annexin V staining, which detects an early-stage of apoptosis. These results also suggested the enhancement of apoptotic effects of TNF, TRAIL, paclitaxel, and gemcitabine in RT4V6 (Fig. 2C) and KU-7 cells (Fig. 2D). Thus, all these results together indicated that curcumin could potentiate the apoptotic effects of chemotherapeutic agents and cytokines against different bladder cancer cell lines.

We also investigated whether curcumin mediates its apoptotic effects through activation of caspases. As shown in Fig. 3A, treatment of RT4V6 cells with curcumin alone induced caspase-3–mediated PARP cleavage in a time-dependent manner. At a dose at which neither curcumin nor gemcitabine alone had much effect on PARP cleavage, treatment with the two together was found to be very effective in inducing PARP cleavage (Fig. 3B). We obtained similar results on treatment with curcumin alone (Fig. 3C) and in combination with gemcitabine (Fig. 3D) in KU-7 cells.

**Gemcitabine Induces NF-κB Activation, but Curcumin Inhibits this Activation**

How curcumin potentiates the apoptotic effects of gemcitabine was investigated. Because chemotherapeutic agents have been shown to activate NF-κB (31, 32), and NF-κB is known to suppress apoptosis (33), we investigated the role of NF-κB pathway as a potential mechanism. We first investigated whether gemcitabine can activate NF-κB in bladder cancer cells. We found that gemcitabine induced NF-κB activation in both RT4V6 (Fig. 4A) and KU-7 (Fig. 4B) cells in a time-dependent manner. We also examined whether curcumin suppresses gemcitabine–induced NF-κB activation in RT4V6 and KU-7 cells. We found that curcumin inhibited gemcitabine–induced NF-κB activation in both cell types (Fig. 4C and D).

Because treatment with curcumin potentiates TNF–induced apoptosis of bladder cancer cells, we also sought to determine whether curcumin suppresses TNF–induced NF-κB activation in bladder cancer cells. We found that TNF activated NF-κB and curcumin inhibited TNF–induced NF-κB activation in both RT4V6 (Fig. 4E) and KU-7 cells (Fig. 4F).

**Cigarette Smoke Induces NF-κB Activation in Bladder Cancer Cells, but Curcumin Suppresses this Activation**

Cigarette smoking is a potential risk factor for bladder cancer (13). Therefore, we investigated whether cigarette smoke induces NF-κB activation in RT4V6 and KU-7 cells. Our results showed that exposure of bladder cancer cells to cigarette smoke activated NF-κB in both cell types in a time-dependent manner (Fig. 5A). Cigarette smoke–induced NF-κB activation in these cells was persistent.

We also investigated whether curcumin can suppress cigarette smoke–induced NF-κB activation in bladder cancer cells. We found that treatment with curcumin inhibited cigarette smoke–induced NF-κB activation in both RT4V6 and KU-7 cells in a dose-dependent manner (Fig. 5B).

**Curcumin Represses Cigarette Smoke–Induced Expression of NF-κB–Dependent Gene Products Involved in the Proliferation and Metastasis of Tumor Cells**

Additionally, we investigated whether curcumin can modulate NF-κB–regulated gene products involved in the proliferation and metastasis of tumor cells. NF-κB activation has been shown to regulate the expression of COX-2 and VEGF (14).

Western blot analysis using specific antibodies showed that cigarette smoke induced the expression of both COX-2 and VEGF in a time-dependent manner, whereas treatment with curcumin suppressed their expression in both RT4V6
This is the first study to show that curcumin alone can suppress the proliferation of both TRAIL/IFN-α-sensitive and TRAIL/IFN-α–resistant bladder cancer cells. Specifically, curcumin seems to induce cell cycle arrest and DNA fragmentation in these cells. Considering that TRAIL and IFN-α are implicated mechanistically in response of bladder tumors to Bacillus Calmette-Guerin, our results suggest that combination therapy with curcumin would be worth exploring. How curcumin overcomes resistance to IFN-α is unclear, but a previous study showed that this cytokine mediates its apoptotic effects through the induction of TRAIL (34). Similar to curcumin, genistein (derived from soy) and analogues of indole-3-carbinol (derived from cruciferous vegetables) have been shown to suppress the proliferation of bladder cancer cells (35, 36). Curcumin has been shown to have no effect on normal cells. For instance, treatment of normal lymphocytes, hepatocytes, human foreskin fibroblast cells, and rat skin fibroblasts with 50 μmol/L curcumin for 24 h did not affect cell proliferation (37, 38).

We also found that treatment with curcumin potentiated the apoptotic effects of chemotherapeutic agents (gemcitabine and paclitaxel) and cytokines (TNF and TRAIL) against bladder cancer cells. Histone deacetylase inhibitors have been found to enhance the TRAIL-induced apoptosis of bladder cancer cells (39). We have shown previously that treatment with a proteasome inhibitor bortezomib enhanced gemcitabine-induced apoptosis of bladder cancer cells (24).

When investigated for the mechanism by which curcumin mediates its effects, we found that both cytokines and chemotherapeutic agents activated NF-κB and that curcumin inhibited this activation. The role of NF-κB in

(Fig. 6A) and KU-7 cells (Fig. 6B). These results support our hypothesis that treatment with curcumin blocks the expression of cigarette smoke–induced NF-κB–regulated gene products.

Discussion

The goal of this study was to determine whether curcumin has a role in the prevention and/or treatment of bladder cancer. We found that curcumin inhibited the proliferation and induced apoptosis of both IFN-α–sensitive and IFN-α–resistant bladder cancer cells. Curcumin also potentiated the apoptotic effects of gemcitabine, paclitaxel, TNF, and TRAIL. This effect of curcumin was found to be independent of sensitivity or resistance to IFN-α or TRAIL, both of which are believed to mediate the effects of Bacillus Calmette-Guerin, which is the current ‘gold standard’ treatment for localized, noninvasive bladder cancer. Furthermore, we found that both gemcitabine and TNF activated NF-κB in bladder cancer cells and that treatment with curcumin suppressed this activation. In addition, curcumin suppressed cigarette smoke–induced NF-κB activation. Finally, treatment with curcumin down-regulated the cigarette smoke–induced expression of the NF-κB–regulated gene products COX-2 and VEGF in bladder cancer cells.
suppression of apoptosis induced by cytokines and chemotherapeutic agents is well established (40). Thus, suppression of NF-κB by curcumin may sensitize bladder cancer cells to chemotherapeutic agents and cytokines. Cigarette smoking is one of the major risk factors for bladder cancer. We found that cigarette smoke can activate NF-κB in bladder cancer cells and that curcumin can suppress this activation. Because NF-κB activation has been shown to mediate cellular transformation, proliferation, invasion, angiogenesis, and metastasis (14), it is possible that suppression of cigarette smoke-induced NF-κB activation by curcumin may have implications in suppression of tumorigenesis. Previously, NF-κB has been targeted in bladder cancer using genistein (35). We also found that cigarette smoke–induced expression of NF-κB–regulated proteins, COX-2 and VEGF, which are required for cell proliferation and angiogenesis, respectively, is downregulated by curcumin. Whether all of these effects of curcumin are mediated by targeting NF-κB is not clear. Previously, we showed that the indole-3-carbinol analogue DIM can mediate its effects against bladder cancer by targeting peroxisome proliferator-activated receptor-γ (36). Curcumin is agonist of peroxisome proliferator-activated receptor-γ as well (41). Similarly, a previous study found that curcumin mediates its effects by modulating histone acetylation (42); others have shown that histone deacetylase inhibitors enhance TRAIL-induced apoptosis of bladder cancer cells as described above (39). Thus, curcumin may mediate its effects by targeting more than one cell signaling pathway (26). This is consistent with recent reports that multitargeted therapy has a better chance of success against cancer than monотargeted therapies (25, 28).

Phase I clinical trials in patients with resected bladder cancer have indicated that curcumin is pharmacologically safe even when consumed up to 12 g/d for 3 months and there is an indication of a histologic response to it (43). The present study suggests that curcumin can be used either alone for prevention of bladder cancer or in combination with chemotherapeutic agents and cytokines for treatment of the disease. This may account for the lower incidence of bladder cancer in countries where curcumin is consumed on a regular basis. Future animal studies are required to further validate these findings. Overall, our results support the use of curcumin either alone or in combination with existing therapy.

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References


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