Sensitivity of Bladder Cancer Cells to Curcumin and its Derivatives Depends on the Extracellular Matrix

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Abstract. *Because the response of cancer cells to chemotherapeutic agents depends upon the supporting extracellular matrix (ECM), the response in vivo may not be reproduced in 2-dimensional cell culture. The dose-response to curcumin and two derivatives by bladder cancer cells grown on both normal (SISgel) and cancer-derived ECM (Matrigel) and on plastic were contrasted. Cells grown on Matrigel were resistant to curcumins, but cells growing on SISgel, which mimic cancer cells suppressed by normal ECM, were nearly as sensitive as cells grown on plastic. SV40-immortalized urothelial cells, which are models for premalignant cells, were the most sensitive, but even aggressive cell lines were nearly as sensitive when grown on SISgel as on plastic. Curcumin response depends highly on the supporting ECM, and cells grown on plastic poorly models cells growing on natural ECM. Curcumin could prove an effective chemopreventive for bladder cancer recurrence when administered intravesically post-therapy.*

Bladder cancer is the fifth most common cancer with 60,000 new cases per year in the US and 336,000 worldwide, resulting in 13,000 deaths per year in the US and 132,000 worldwide (1, 11, 20). The clinical problem with bladder cancer is the high recurrence rate, and although 75-85% of bladder cancers are initially superficial and noninvasive, as many as 70% of these patients will experience a recurrence within 5 years, and of those 20-30% will experience progression to invasive disease (1). The survival statistics of invasive cancer are 50% at 2 years, but the 5-year survival with metastatic bladder cancer is only 6% (1). Although immunotherapy with attenuated tuberculosis bacteria (BCG) shows efficacy, it nonetheless has appreciable toxicity and adverse reactions are common (1). Other, less toxic alternatives for preventing recurrences would be desirable.

The spice curcumin has significant antiviral, anti-inflammatory and anti-cancer activities (17). Curcumin has been shown to induce apoptosis in bladder cancer cells cultured on plastic (19) and to prevent implantation of bladder cancer cells *in vivo* when administered intravesically (16). In a small clinical trial of oral curcumin, doses of 8 g per day showed no toxicity, and improvement in 1 of 2 patients was noted in spite of not finding detectable levels of curcumin or metabolites in urine (3). However, most agents are less active against cells in tissues than against cancer cells growing on plastic (7, 12, 18), and the degree to which results obtained with cells cultured on plastic can be extrapolated clinically is not clear. *In vivo*, cancer cells must escape the regulatory controls of normal matrix and the evidence is strong that cancer cells suppressed by normal matrix are responsible for recurrences and delayed metastasis (6). In order to determine whether curcumin would be effective against cancer cells growing on an extracellular matrix and to develop a more realistic pre-clinical model of curcumin's anticancer activity, we investigated the dose-response of curcumin and two derivatives against several bladder cell lines growing on both a cancer-modulated extracellular matrix as well as a normal extracellular matrix. We tested two cell lines that are non-tumorigenic (HUC and UROtsa) as well as four cancer cell lines that vary in the degree of aggressiveness from papillomas (RT4) to cells that are derived from undifferentiated and invasive carcinomas (J82, TCC-Sup). Growth on Matrigel, a cancer-modulated basement membrane preparation, provided a tumormimetic model in which the cells recapitulated the phenotype reported in the patient from which the tumor cells were derived (8). Cells from invasive tumors invaded the Matrigel whereas those derived from papillary tumors grew papillary structures (8). In contrast, the malignant phenotype was suppressed when

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the cells were grown on a gel produced from small intestine submucosa (SIS), a normal extracellular matrix (8). The findings of this study suggest that intravesical curcumin might be an effective therapy in prevention of bladder cancer recurrence.

Materials and Methods

Cell culture. HUC, RT4, TCC-SUP, 5637, and J82 cells were obtained from American Type Culture Collection, Bethesda, MD, USA. UROtsa cells were obtained from Dr. Donald Sens (15). The HUC cell line was immortalized with SV-40 and is non-tumorigenic, RT4 cells were derived from a papilloma, 5637 cells were from an invasive grade II tumor, J82 cells were from an anaplastic tumor, and TCC-Sup cells were from a metastatic tumor. All tissue culture media and supplements were from Invitrogen (Rockville, MD, USA). Matrigel was obtained from Becton-Dickinson (Bedford, MA, USA). SIS gel was obtained from Cook Biotech (W. Lafayette, IN, USA) and prepared by mixing 5 ml of SISgel, 5 ml of 0.025 N HCl, and 1.25 ml 10x PBS, followed by adjustment of pH to 7.4 with 1 M NaOH. The three-dimensional cultures were conducted according to protocols published previously (8, 13). Briefly, 50 μl of ice-cold Matrigel or pH-adjusted SISgel was added to Costar 3610 white, clear bottom 96-well plates (Corning, Corning, NY, USA) and the liquid was allowed to gel at 37˚C. Cells were trypsinized with 1 ml 0.25% trypsin /1 mM EDTA, and 30,000 cells / 50 μl were added to each well of the 96 well plate. For cultures of cells on plastic, 2×10^6 cells were added to each T-75 flask.

Assay for curcumin activity. One day after seeding, cells were treated with various concentrations of curcumin, 4MC, or 4HC for 48 h in medium. Synthetic curcumin and analogues were provided by Professor Zhong-Li Liu of the National Laboratory of Applied Organic Chemistry, Lanzhou University, Lanzhou, China. The purities of Curcumin, 4HC and 4MC were 99.7%, 99.3% and 99%, respectively. A marker of cell proliferation using the substrate 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) cleaved to fluorescein by non-specific cellular esterases was used to assess the response to curcumin (9). Briefly, media was removed from the cells and 25BM CFDA-AM (Molecular Probes, Eugene, OR) in PBS was added for 2 hours at 37˚C. Plates were then read by a BIOCHEMI digital darkroom (UV Products, Upland, CA, USA) using 304 nm excitation, 430 nm broadband emission filters.

DAPI stain. The media was removed from the wells and cells were fixed with 50 μl of 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for 30 minutes at room temperature. The cells were then rinsed with PBS, incubated with 50 ng/ml DAPI in PBS for 30 minutes at room temperature and rinsed again with PBS. Cells were analyzed by fluorescence microscopy using a Nikon Eclipse inverted microscope TE2000-U. Images were collected using Lucia camera software. Apoptotic cells were identified as densely stained granular nuclear bodies (5).

Results

Chemosensitivity of bladder cell lines cultured on plastic, Matrigel, and SISgel. An example of the dose-response data showing sensitivity of urothelial cells to curcumin induced
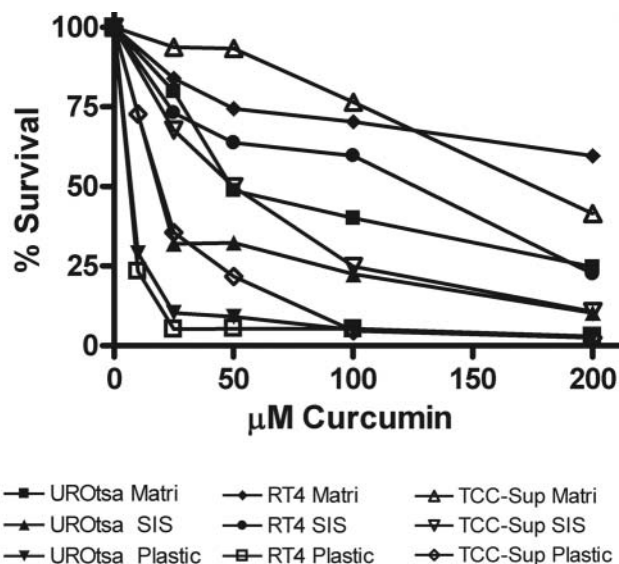

Figure 1. *Effect of growth environment on curcumin-induced cytotoxicity. UROtsa, RT4, or TCC-Sup cells were cultured on plastic, SISgel, or Matrigel and treated with variable doses of curcumin for 48 hours then CFDA-AM was added and fluorescence was measured after a 2 hour incubation. Data are plotted as percent control of the CFDA-AM conversion to fluorescein of untreated cells.*

cytoxicity is shown in Figure 1; complete IC_{50} data are shown in Table I. As shown in Table I, treatment of cells on plastic with curcumin resulted in an IC_{50} of 4-21 μM, demonstrating that the sensitivity of all of the cell lines was roughly equivalent. All of the six urothelial cell lines tested were most sensitive to treatment with curcumin, 4MC, and 4HC when they were cultured on plastic. All cell lines were less sensitive when cultured on SISgel; treatment of cells cultured on SISgel with curcumin resulted in an IC_{50} between 10-87 μM. When cells were cultured on SISgel or plastic, as the aggressiveness of the tumor cells increased, they usually became more resistant to killing by curcumin and the analogs. Cells grown on Matrigel exhibited the highest resistance to curcumin, with IC_{50} values of 64 to >400 μM. The efficacy of curcumin was independent of aggressiveness for cells cultured on Matrigel. In comparing the efficacy of the curcumin derivatives, 4MC was much less active. The 4HC derivative was roughly as active as curcumin, but was significantly more effective than curcumin in killing HUC, RT4, 5637 and J82 cells cultured on Matrigel.

Curcumin induces apoptosis of bladder cell lines. To determine if curcumin and its derivatives killed cells by apoptosis in all matrices, J82 cells cultured on SISgel were treated with curcumin, 4MC, and 4HC. Only the cells that were treated with curcumin, 4MC, or 4HC exhibited

Table I. *Effects of curcumin, 4MC, and 4HC on the viability of urothelial cells cultured on Matrigel, SISgel, or plastic.*

	IC$_{50}$ (μM)		
	Matrigel	SISgel	Plastic
Curcumin			
UROTSA	64.2±1.1	10.3±1.4	3.9±1.2
HUC	>400	24.6±1.1	7.8±1.1
RT4	243.4±1.1	87.0±1.1	3.5±1.3
5637	234.4±1.1	38.2±1.1	10.5±1
J82	115.5±1	22.9±1.3	20.5±1.1
TCCSup	171.6±1	46.0±1.1	18.3±1.1
4MC			
UROTSA	237.1±1	9.1±1.4	1.2±2.6
HUC	>400	7.9±1.4	7.3±1
RT4	>400	134.4±1.1	20.8±1.2
5637	>400	41.5±1.1	10.5±1.8
J82	>400	30.4±1.2	21.2±1.7
TCCSup	>400	85.7±1.1	15.3±1.2
4HC			
UROTSA	84.3±1.1	8.3±1.4	6.9±1.1
HUC	171.2±1.1	2.8±2	1.6±1.4
RT4	102.5±1	63.6±1.1	10.6±1
5637	106.8±1.1	29.1±1.1	7.6±1
J82	155.3±1	22.4±1.2	12.8±1.1
TCCSup	164.7±1.1	46.5±1	21.4±1.2

Figure 2. *Nuclear fragmentation is observed when J82 cells are cultured on SISgel and treated with curcumin, 4MC, or 4 HC. J82 cells were cultured on SISgel and were left untreated for Control (A), or were treated with 100 μM of 4HC for 48 hours (B), then the cells were fixed and the nucleus was stained with DAPI as described in the Materials and Methods. Similar results were observed for 4MC and curcumin (data not shown).*

features typically observed in apoptotic cells (Figure 2A, B), such as nuclear fragmentation, while no signs of apoptosis were detected in the untreated control cells (2A).

Discussion

The high rate of recurrence of bladder cancer may be attributed to the several factors, including failure to detect all of the lesions with cystoscopy (4), or the possibility that the microenvironment may suppress cancer cells in the normal appearing regions of the bladder, where the scar microenvironment can promote them to emerge at a later time (6). In addition, earlier findings of abnormal biomarkers in histopathologically normal urothelium that was located up to 10 cm away from tumors (14) suggests that the phenotypic suppression seen *in vitro* (8) occurs *in vivo* as well. In that study, over 30% of the cases showed abnormalities in EGFR and Her2/neu expression, and 20% of cases showed abnormal ploidy. The main therapy for prevention of recurrence is BCG, but the toxicity is high and many patients are accordingly lost to follow-up or are forced to abandon the therapy (1, 10).

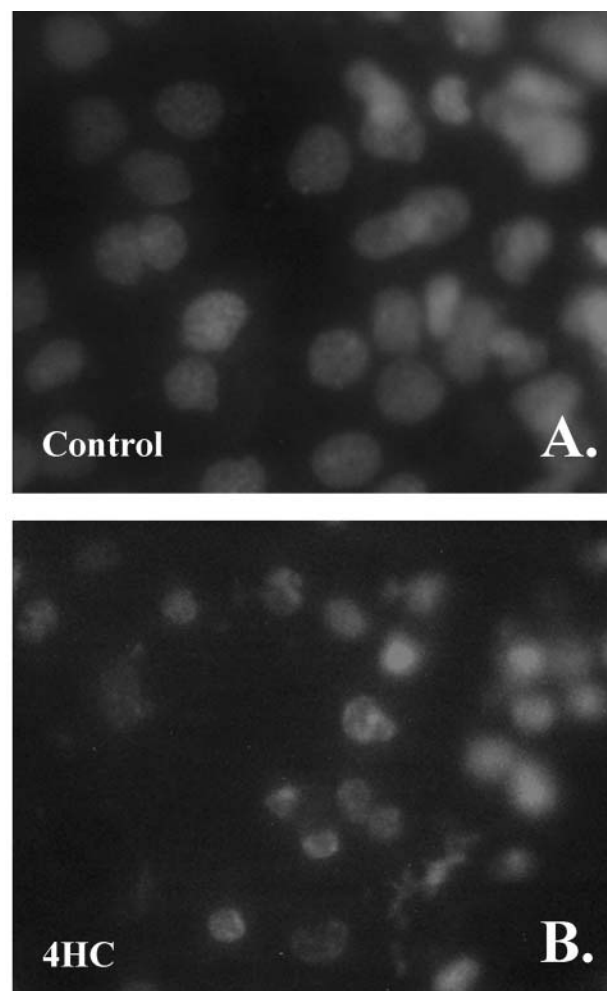Our findings here suggest that curcumin may provide an alternative or at least a supplement for prevention of recurrence. Although curcumin was effective against bladder cancer cells of all grades and immortalized bladder cells growing on plastic, it was not particularly effective against the aggressive lines growing on Matrigel, where they express their full malignant phenotype. Curcumin therefore is unlikely to be effective as a chemotherapeutic agent. However, curcumin was quite effective against suppressed cancer cells and immortalized urothelial cells growing on normal extracellular matrix. This suggests it would be effective in a chemotherapeutic mode against precisely those cells that evade BCG. That it was effective against SV40-immortalized urothelial cells is particularly encouraging because such cells likely represent a model for premalignant cells.

Curcumin is generally considered to be non-toxic (3, 17). It induces apoptosis in target cells (19), which we confirmed occurs as well in cells growing on either Matrigel or SISgel. Of the forms tested, curcumin and 4HC were slightly more effective than the MC derivative. Curcumin is poorly absorbed and none appears in urine even after large oral doses (3) and will therefore likely need to be administered intravesically. In a mouse model, intravesically administered curcumin showed little evidence of toxicity to the normal bladder but was effective in preventing implantation of tumor cells (16). We therefore suggest that curcumin administered intravesically could be tested as a chemopreventive for bladder cancer recurrence following transurethral resection or BCG therapy. Because bladder cancer is the most expensive cancer to manage from diagnosis to death of the patient from any cause (2), alternatives to current approaches are needed.

Acknowledgements

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