The Effect of Curcumin on Proliferation and Apoptosis in LNCaP Prostate Cancer Cells

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OBJECTIVE To observe the effect of curcumin on proliferation and apoptosis in the prostate cancer LNCaP cell line.

METHODS The AXSYMTM system luciferase method was used to examine the effect of various concentratious of curcumin on the content of prostate specific antigen (PSA) in prostate cancer LNCaP cells. A pGL3-PSA luciferase expression vector, containing 640 bp DNA of the PSA gene 5′-promoter region was constructed and transfected into the LNCaP cells with lipofectin. By measuring luciferase activity, the effect of 10 μmol/L, 20 μmol/L, 30 and 40 μmol/L curcumin on the promoter was studied. Effects on cell growth and apoptosis were analyzed by microscopy, the MTT colorimetric assay and flow cytometry. Western-blotting was used to measure expression of the androgen receptor (AR) in the LNCaP cells treated with different concentrations of curcumin.

RESULTS The results showed that the expression of PSA was inhibited as curcumin reduced the activity of luciferase. Curcumin also caused a sigificant concentration-dependent decrease in AR expession measured by Western-blotting. Cell growth was inhibited and apoptosis was induced.

CONCLUSION By inhibiting AR expression, curcumin reduced the function of the PSA promoter and inhibited PSA protein expression. Curcumin decreased the cellular proliferation and induced apoptosis in LNCaP cells in a concention-dependent manner.

KEYWORDS: curcumin, androgen receptor, prostrate specific antigen, apoptosis, LNCaP cells.

Curcumin is a natural, non-toxic yellow compound isolated from roots of the *Curcuma longa Linn* plant. It has been widely used in many Asian countries as a spice, to color cheese and butter, as a cosmetic and in some medicinal preparations. Curcumin (diferuloylmethane), a phenolic compound, possesses antioxidant, free radical scavenger and anti-inflammatory properties. [1] Epidemiological and animal model studies have revealed that curcumin can inhibit carcinogenesis. Natural curcuminoid, curcumin, I, II and III isolated from turmeric (*Curcuma longa*) have been compared for their cytotoxic, tumor reducing and antioxidant activities. Curcumin III was found to be more active than the other 2 substances as a cytotoxic agent for inhibiting Ehrlich ascites tumor cells in mice (ILS 74.1%). Synthetic curcumin I and III had activity similar to natural curcumins.[2]

Prostate cancer is the most common noncutaneous cancer and the second leading cause of cancer deaths in American men. The androgen receptor (AR) is required both for the development of the normal prostate gland and prostate cancer. [3] In the early stages of prostate cancer, almost all cancer cells are androgen-dependent and highly

sensitive to anti-androgens. However, prostate cancer usually recurs after a few years of androgen ablative treatment, and most cancer cells become androgen-independent, rendering anti-androgen therapy useless. Reports suggest that mutations in the AR ligand-binding domain, AR coregulators, or receptor phosphorylation may enable the AR to respond to non-androgen agonists.[4] Furthermore, the activation of the AR by these factors during androgen ablation therapy may facilitate androgen-independent prostate cancer growth. As androgen-independent prostate tumors are incurable, the prevention of such aberrant AR activation is an attractive therapeutic target.

Prostate-specific antigen (PSA) is a key androgen-regulated gene, and is a sensitive and selective marker for prostate cancer screening and assessment. Consequently, PSA is used as an indicator of disease progression and response for prostate cancer therapies.[5] In the present study, we have evaluated the effects of curcumin on cell growth. Here we have used the androgen-dependent LNCaP human prostate cancer cell line as a cell model to study the potential mechanism of curcumin effects on cellular proliferation and apoptosis.

MATERIALS AND METHODS

Cell culture

The androgen-dependent cell line LNCaP was obtained from the Charles Young Laboratory of America and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 10 mM glutamine, and 5 ml of PSN antibiotic complex (Invitrogen, Carlsbad, CA) at 37℃ in a 5% CO_2 incubator. Cell cultures grown to 80~90% confluence were exposed to 10, 20, 30 and 40 μmol/L curcumin for 24 h followed by microscopic examination to determine the effect of curcumin on proliferation of the LNCaP cells.

Detection of apoptosis

Thiazolyl blue (MTT) assay

LNCaP cells (5×10^4) were seeded in each well of 12-well plates. After 36~48 h, the medium was changed to phenol red-free RPMI 1640 medium with 8% FBS or charcoal-stripped FBS (CS-FBS) for another 2, 4 and 6 days, with different treatments. For cell counting, cells were trypsinized, neutralized with medium, and counted in hemocytometers. The MTT assay is a quantitative colorimetric assay for mammalian cell survival and proliferation.

Flow cytometry (FCM)

Curcumin-treated cells and control cells were collected, washed with phosphate buffered saline (PBS, dibasic sodium phosphate 9.1 mmol/L, monobasic sodium phosphate 1.7 mmol/L, and NaCl 150 mmol/L. pH was adjusted to 7.4 with NaOH), and resuspended in a final volume of 100 μl of iced-PBS. One ml of 70% (v/v) ethanol in PBS was added to the resuspended cells with vigorous mixing. Fixed cells were incubated with primary Ab (anti-p210bcr/abl, 1:400 dilution) for 30 min and then with FITC-labeled secondary IgG antibody before flow cytometric analysis. Flow cytometry measurements were made on a FACSCalibur instument, and the data analyzed with WinBryte software (Becton Dickinson).

PSA assay and measurement of luciferase activity

To observe the effect of curcumin on the prostate specific antigen (PSA), we used the AXSYMTM system-chemical luciferase method to examine the change of PSA in the culture of the curcumin-treated and transfected cells.

Plasmid constructs

A 640 bp fragment of the PSA promoter was generated using the polymerase chain reaction (PCR). The plasmid pPSA-EGFP was used as a template and a pair of specific primers were synthesized which contained digestion sites for Sac I and Kpn I. The sequence of the upstream primer was 5'-GGG GTA CCA TTG GAA TTC CAC ATT GTT TTG C-3' and the sequence of the downstream primer was 5'-GGG AGC TCA AGC TTG GGG CTG GGG AG-3'. The PCR product was digested with Sac I and Kpn I retriction enzymes, agrose gel purified, and ligated into the vector pGL-3Basic pre-cut with Sac I and Kpn I enzymes. The 640 bp DNA fragment was inserted upstream into the luciferase gene. The recombinant plasmid was transfected into *E.coli* DH-5α, then isolated and purified. After the plasmid was identified by double digestion and electrophoresis, it was sent to Boya Co. to determine the sequence. The recombinant was named pGL3-PSA.

Transient transfection assay

The day before transfection, LNCaP cells were seeded at a density of 4×10^5/well in 12-well plates. After 18~24 h, when the cells reached 90~95% confluency, the recombinent pGL3-PSA was transfected into the LNCaP cells by Lipofectamine TM2000 according to the manufacturer's instructions. The plasmid (0.4~1.6)

μg and 1~4 μl Lipofectamin were diluted into 100 μl of medium without serum and the 2 substances combinded and layed at room temperature for 20 min. The complexes were added onto the cells cultured with 0.8 ml serum-free medium. The pRL-TK was used as an internal control. The ratio of pRL-TK and experimental vector was 1:25. After 24 h of incubation with various levels of curcumin, the cells were harvested.

Harvesting of cells and measurement of luciferase activity

A dual-luciferase reporter assay system was used for this procedure. The transfected cells were harvested and the firefly and renilla luciferase activities measured concurrently for 12 s using a luminometer (LUMAT LB 9507, Berthold, Germany). Firefly luciferase activities were normalized by measuring renilla luciferase activities of the same lysate. The assays were conducted using quadruplet transfection replicates and at least 3 independent values were analyzed to confirm the reproducibility.

Western blotting analysis

Cells (both floating and attached) were trypsinized, broken in lysing buffer (1×PBS pH 7.4; 1% Nonidet P-40; 0.5% sodium deoxycholate; 0.1% SDS; protease inhibitor cocktail tablets [Roche], frozen and thawed 3 times, and centrifuged (13000 *g*, 20 min at 4℃) to remove insoluble material. The supernatant was recovered and stored at -20℃ pending analysis. The protein content was determined using the Bio-Rad DC protein assay according to manufacturer's instructions. Protein extracts (50 μg) were heated (95℃, 3 min), resolved using 10% SDS-Polyacrylamide gel electrophoresis and electro-transferred to nitrocellulose membranes (15 V, 30 min) using a semi-dry transfer (Bio-Rad, Mississauga, ON, Canada). The membranes were then blocked (2 h, RT) with PBS containing 5% milk powder +0.05% Tween 20, then incubated with anti-AR (1:1000), anti-β-actin (overnight, 4℃), and subsequently with horse radish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (1:2000; RT, 45 min). Peroxidase activity was visualized with the ECL kit (Amersham, Arlington Heights, IL), according to the manufacturer's instructions.

Statistical analysis

All experiments were repeated at least 3 times. Data were subjected to one-way ANOVA or student t test (PRISM software version 4.0; GraphPad, San Diego, CA). Differences between experimental groups were determined by the Tukey's test.

RESULTS

Results of microscopy, MTT colorimetric assay and flow cytometric analysis

Curcumin was added to the LNCaP cells at levels of 10, 20, 30 and 40 μmol/L and the effects on proliferation examined by microscopy (Fig.1), the MTT colorimetric assay (Fig.2) and flow cytometry (Fig.3). We found that curcumin decreased proliferation of the prostate cancer cells in a concentration-dependent manner.

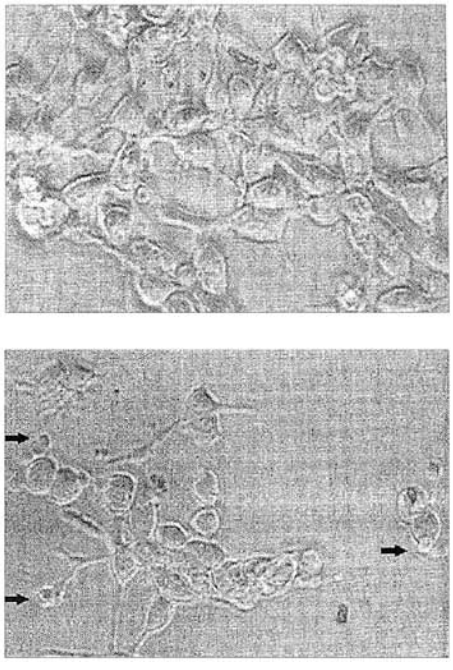

Fig.1. A: Curcumin-nontreated control. B: 40 μmol/L curcumin treatment (arrows point to typical apoptotic bodies).

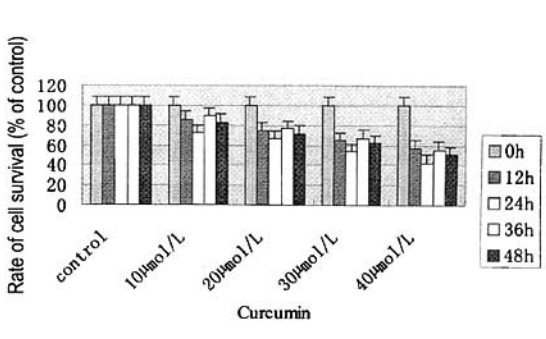

Fig.2. Rate of cell survival by the MTT colorimetric assay.

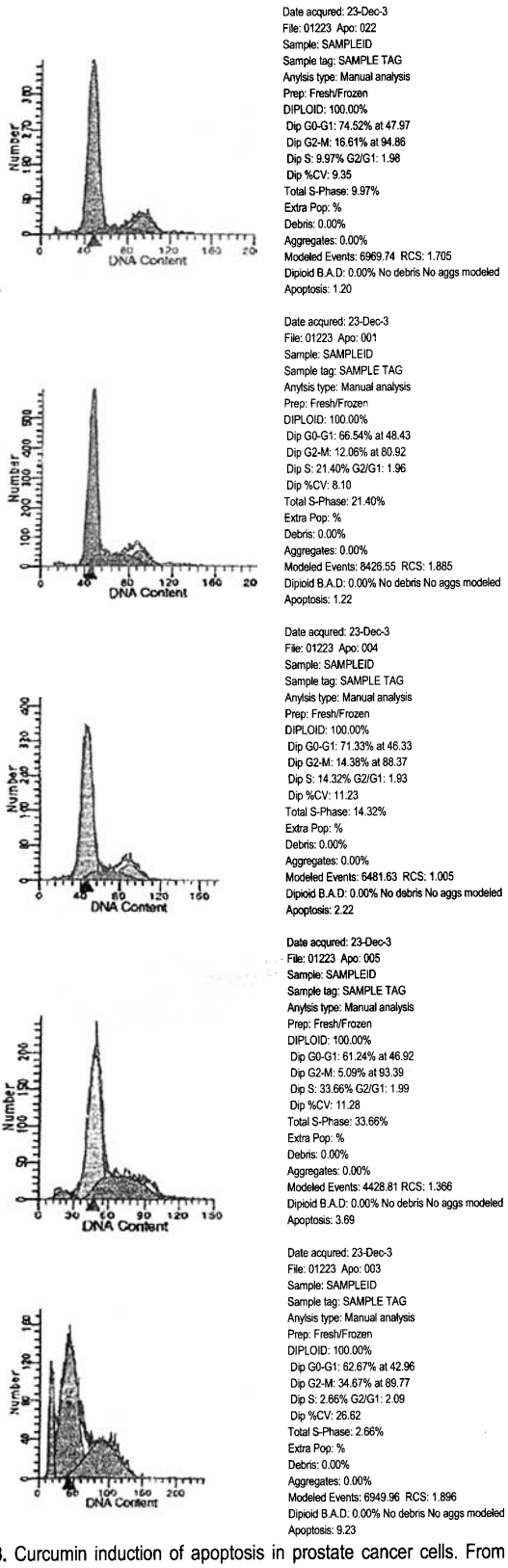

Fig.3. Curcumin induction of apoptosis in prostate cancer cells. From the top to the bottom : 0, 10, 20, 30 and 40 μmol/L curcumin treatment.

Effect of Curcumin on the prostate specific antigen (PSA)

The change of PSA in the cultures

THE AXSYMTM system luciferase method was used to examine changes in PSA in cultures of the curcumin treated and transfected cells. Curcumin depressed the expression of PSA in the cultures (Fig.4).

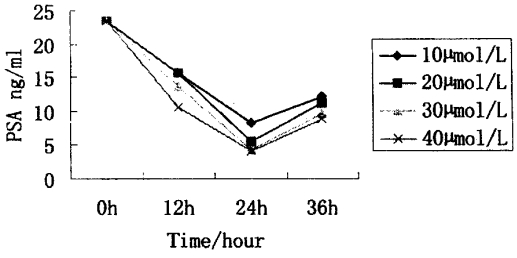

Fig.4. Mesurment of PSA by the AXSYMTM system after treatment with 10, 20, 30 and 40 μmol/L at 0, 12, 24 and 36 h.

The activity of luciferase

The effect of curcumin on the PSA promoter was studied by measuring the activity of luciferase. We found that curcumin inhibited the PSA promoter (Fig.5).

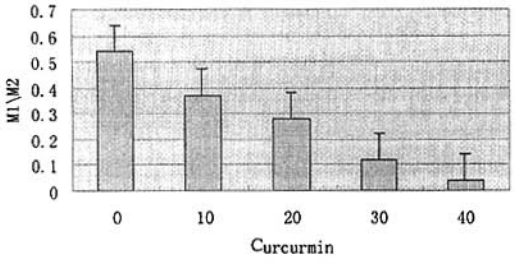

Fig.5. Measurement of lucifease activities in LNCaP cells treated with different concentrations of curcumin. From left to right: 0, 10, 20, 30 and 40 μmol/L curcumin treatment.

Expression of the androgen recepter

Curcumin treated and transfected cells were collected and Western-blotting performed to assess the expression of the AR. We found that curcumin significantly inhibited the expression of AR in the prostate cancer cells in a concentration-dependent manner (Fig.6).

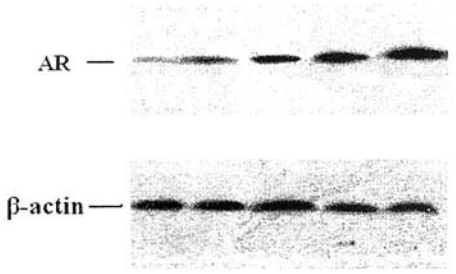

Fig.6. Western-blotting measurement of AR expession in LNCaP cells treated with different concentrations of curcumin. From right to left: 0, 10, 20, 30 and 40 μmol/L curcumin.

DISCUSSION

Prostate cancer is the most frequently diagnosed visceral cancer in the United States and the second leading cause of cancer deaths in men. Unfortunately, there are no proven primary prevention strategies for prostate cancer and no curative treatments for advanced-stage cancers. [5] Current available treatments often have troubling side effects such as urinary incontinence and erectile dysfunction. Androgen ablation therapy results in an initial response but destroys androgen-dependent cells without affecting the continuous growth of the androgen-independent cells. In contrast, the androgen-independent cells survive, resulting in the relapse of the disease and ultimate death of the patient. Androgens act via the intracellular androgen receptor (AR) and is known that AR activated by a ligand can stimulate or repress androgen-regulated genes.

Prostate cancer has an intimate relationship with the PSA in that it is expressed specifically in prostatic epidermic cells, which are regulated by androgen. The PSA promoter contains the ARE sequence AGA ACA GCA AGT GTC [6] at position -170 which is a responsive element for activated AR. Although the mechanism of ligand-independent activation of AR has not been as yet clarified, cross-talk between the AR and alternative signal-transduction pathways, such as protein kinases, growth factors, or cytokines, has been shown.[7]

Cancer control efforts have focused on detecting early-stage prostate cancer with screening tests and then aggressively treating the cancer with surgery or radiation. The most effective screening test is the PSA assay, which in combination with digital rectal examination substantially enhances the cancer detection rate. The American Cancer Society and the American Urologic Association recommend annual cancer screening with PSA testing and digital rectal examination for men with life expectancies greater than 10 years.[8,9]

Curcumin is a natural, non-toxic compound from a plant, *Curcuma longa Linn.* It has been traditionally used as a seasoning spice in Indian cuisine, and medicinally because of its anti-inflammatory and antioxidant properties for the treatment of inflammatory conditions and other diseases. Studies using an animal model also indicated that dietary administration of curcumin significantly inhibited the incidence of colon adenocarcinomas and suppressed the colon tumor volume compared to the control group.[10]

Curcumin appears to be a potent inhibitor of cell growth or proliferation in a variety of tumor cells. To investigate the effects of curcumin on prostate cancer and induce apoptosis of prostate cancer cells, we used 10, 20, 30 and 40 μmol/L curcumin, and examined its effect on proliferation by electron microscopy, the MTT colorimetric assay and flow cytometry. The results showed that curcumin decreased the proliferation of LNCaP cells with inhibition increasing as the level of curcumin increased. The optimal effect was 40 μmol/L at 24 h. Curcumin induced apoptosis of the LNCaP cells, with the maximal effect at a concentration of 40 μmol/L resulting in an apoptotic rate of 9.23%.

PSA, produced by an androgen-regulated gene, is a sensitive and selective marker for prostate cancer screening and assessment and is used as an indicator of disease progression and response for prostate cancer therapies. To observe the effect of curcumin on PSA expression, we used the AXSYM™ system luciferase method to examine the levels of PSA in the cultures. The prostate tissue-specific expression vector pGL3-PSA luciferase expression vector containing 640 bp of the PSA gene 5'-promoter region was constructed. The PSA promoter lies upstream from the luciferase gene, so the expression of the luciferase is regulated by the PSA promoter. pGL3-PSA was transfected into LNCaP cells with Lipofectamine™2000. and the pRL-TK plasmids were cotransfected into the LNCaP cells. By measuring the activity of luciferase, the effect of curcumin on the promoter of PSA was studied. The result showed that curcumin inhibited the expression of PSA in the LNCaP cells. The greatest effect occurred with 40 μmol/L at 24 h. The PSA in the cells was 20% of the control group. The results showed that curcumin inhibited the promoter of the prostate specific antigen.

Androgen is essential for the development, differentiation and normal function of the prostate and AR is a ligand-mediated transcriptional factor that belongs to

the superfamily of nuclear receptors. Activated by androgen binding, the AR moves into the nucleus, binds to the specific DNA ARE and regulates the transcriptional activity of target genes. Androgens are believed to promote prostate carcinogenesis, via AR-mediated mechanisms. Western blots used in our study to assess the effects of curcumin on expression of AR showed that as the concentration of curcumin increased, AR expression decreased. Curcumin, a known non-toxic dietary ingredient, may have a therapeutic effect on prostate cancer cells through down-regulation of AR gene expression. The AR is important in the progression of prostate cancer to hormone independence. [10] The finding that curcumin can inhibit cell survival will help to elucidate the anti-tumorigenic mechanism of androgen-independent cells. In addition to its activity as a chemopreventive agent, the potential therapeutic role of curcumin in hormone refractory and advanced prostate cancer is worthy of further evaluation and clinical trials.

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REFERENCES

1 Dorai T, Gehani N, Katz AE. Therapeutic potential of curcumin in human prostate cancer. II. Curcumin inhibits tyrosine kinase activity of epidermal growth factor receptor and depletes the protein. Mol Urol. 2000;4:1–6.

2 Deeb D, Jiang H, Gao X, et al. Curcumin sensitizes prostate cancer cells to tumor necrosis factor –related apoptosis –inducing ligand/Apo2L by inhibiting nuclear factor {kappa}B through suppression of {kappa} {alpha} phosphorylation. Mol Cancer Ther. 2004;3:803–812.

3 Collado B, Sanchez MG, Diaz–Laviada I, et al. Vasoactive intestinal peptide (VIP) induces c–fos expression in LNCaP prostate cancer cells through a mechanism that involves Ca^{2+} signalling. Implications in angiogenesis and neuroendocrine differentiation. Biochim Biophys Acta. 2005;1744:224–233.

4 Montgomery BT, Young C Y, Bilhartz DL, et al. Hormonal regulation of prostate–specific antigen (PSA) glycoprotein in the human prostatic adenocarcinoma cell line, LNCaP. Prostate. 1992;21:63–73.

5 Holy J. Curcumin inhibits cell motility and alters microfilament organization and function in prostate cancer cells. Cell Motil Cytoskeleton. 2004;58:253–268.

6 Montgomery BT, Young CY, Bilhartz DL, et al. Hormonal regulation of prostate–specific antigen (PSA) glycoprotein in the human prostatic adenocarcinoma cell line, LNCaP. Prostate. 1992;21:63–73.

7 Weiss–Messer E, Merom O, Adi A, et al. Growth hormone (GH) receptors in prostate cancer: gene expression in human tissues and cell lines and characterization, GH signaling and androgen receptor regulation in LNCaP cells. Mol Cell Endocrinol. 2004;220:109–123.

8 Dorai T, Gehani N, Katz A. Therapeutic potential of curcuminin human prostate cancer –I. Curcumin induces apoptosis inboth androgen–depende androgen–independent prostatecancer cells. Prostate Cancer. 2000;3:84–93.

9 Taplin ME, Balk SP. Androgen receptor: a key molecule in the progression of prostate cancer to hormone independence. J Cell Biochem. 2004;91:483–490.

10 Sokoloski JA, Shyam K, Sartorelli AC, Induction of the differentiation of HL–60 promyelocytic leukemia cells by curcumin in combination with low levels of vitamin D3. Oncol Res. 1997;9:31–39.