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Curcumin induces cell cycle arrest and apoptosis of prostate cancer cells by regulating the expression of IκBα, c-Jun and androgen receptor

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Curcumin possesses chemopreventive properties against several types of cancer, but the molecular mechanisms by which it induces apoptosis of cancer cells and inhibits cancer cell proliferation are not clearly understood. To evaluate the antitumor activity of curcumin for prostate cancer, we used an androgen dependent LNCaP prostate cancer cell line and an androgen independent PC-3 prostate cancer cell line as experimental models. We treated these cells with curcumin and then evaluated the effects of curcumin on cell cycle profiling and apoptosis, as well as the activation of NF-κB and c-jun in these cells. The results showed that the ratios of apoptosis in LNCaP and PC-3 cells were significantly elevated in a dose dependent manner after exposure to curcumin. In addition, curcumin induces the G2/M cell cycle arrest of LNCaP and PC-3 cells in a dose dependent manner. Mechanistically, we found that curcumin upregulated the protein level of NF-kB inhibitor IκBα and downregulated protein levels of c-Jun and AR. These data suggest that curcumin is a promising agent for the treatment of both androgen-dependent and androgen-independent prostate cancer.

1. Introduction

Prostate cancer is the most common cancer and the leading cause of cancer death among men in the United States and Europe (Boyle and Ferlay 2004; Jemal et al. 2008). Recently, the occurrence of prostate cancer has rapidly increased in China as well (Ostrander and Stanford 2000). Current therapies including radical prostatectomy, chemotherapy, local radiotherapy, or hormone therapy have shown success for androgen-dependent and localized prostate cancer, but they are of limited efficacy against androgen-independent and metastatic prostate cancer. Androgen reduction therapy is largely effective against androgen-dependent tumors, although androgen-independent clones often emerge after hormone therapy. Experimental evidence also demonstrates clonal outgrowth of androgen-independent prostate cancer cells from androgen-dependent tumors (Craft et al. 1999). In addition, these tumors tend to be highly resistant to conventional cytotoxic agents such as cisplatin. Therefore, novel treatment modalities are needed to treat androgen-independent tumors and prevent the progression of androgen-dependent prostate cancer to the hormone-refractory stage.

Curcumin (diferulolylmethane) is an active ingredient derived from the rhizome of the plant *Curcuma longa*. It exhibits antioxidant and anti-inflammatory activities and has been proposed as a potential drug for the treatment of cancer and chronic diseases (Berginc et al. 2012). Although curcumin possesses chemopreventive properties against several types of cancer, the molecular mechanisms by which it inhibits cell growth and induces apoptosis are not clearly understood.

Nuclear factor-κB (NF-κB) is a transcription factor that contributes to many aspects of tumorigenesis, including cell growth and proliferation, anti-apoptosis, angiogenesis, and metastasis (Wu et al. 2010; Yeh et al. 2010; Wang et al. 2012). Normally, NF-κB resides in the cytoplasm in an inactive form in association with the inhibitory protein IκBα. Upon stimulation of cells with mitogens, antigens, or cytokines, IκBα is phosphorylated and degraded, allowing NF-κB to translocate to the nucleus where it binds to the κB motifs in the promoter region of the responsive genes and drive their transcription (Karin 1999).

C-Jun is an important member of the activator-protein 1 (AP-1) complex. Activation of c-Jun, primarily through phosphorylation by the c-Jun amino terminal kinase (JNK), leads to the regulation of survival, proliferation and apoptosis of a variety of cancer cells (Vogt 2001; Papachristou et al. 2003). Whether c-Jun promotes or inhibits apoptosis appears to depend on the cell type and the stimulating signal.

To evaluate the antitumor activity of curcumin for prostate cancer, in this study we used the androgen dependent LNCaP prostate cancer cell line and the androgen independent PC-3 prostate cancer cell line as experimental models. We treated these cells with curcumin and then evaluated the effects of curcumin on the apoptosis and the activation of NF-κB and JNK signaling in these cells.

2. Investigations and results

2.1. *Curcumin induces apoptosis and G2/M arrest in prostate cancer cells*

Flow cytometry analysis showed that curcumin induced the apoptosis of LNCaP and PC-3 cells (Table). Compared to controls, the ratio of apoptosis in LNCaP and PC-3 cells was significantly

Table: Apoptosis of LNCaP and PC-3 cells exposed to curcumin

Cell lines	Apoptosis rate	t	P value
LNCaP			
0 μmol/L	2.8 ± 0.7	–	–
10 μmol/L	6.0 ± 1.4$^{\Delta}$	−3.445	0.026
25 μmol/L	9.8 ± 1.2*	−8.983	0.001
50 μmol/L	18.2 ± 3.0*	−8.773	0.001
75 μmol/L	23.9 ± 1.5*	−20.229	0.000
100 μmol/L	42.4 ± 4.0*	−16.899	0.000
PC-3			
0 μmol/L	2.7 ± 1.0	–	–
10 μmol/L	6.3 ± 0.9$^{\Delta}$	−4.646	0.010
25 μmol/L	7.5 ± 2.3$^{\Delta}$	−3.292	0.030
50 μmol/L	12.7 ± 3.0*	−5.442	0.006
75 μmol/L	18.1 ± 2.5*	−9.843	0.001
100 μmol/L	27.5 ± 2.6*	−15.235	0.000

$^{\Delta}P < 0.05$, $^{*}P < 0.01$ compared to controls (0 μmol/L).

increased after exposure to curcumin ($p < 0.05$), and this effect was concentration dependent.

Next we analyzed the cell cycle profiling of LNCaP and PC-3 cells after curcumin treatment. The results showed that the rate of DNA content in the G2/M fraction of LNCaP and PC-3 cells was significantly increased following treatment with curcumin compared to the control, but the rate of DNA content in the G0/G1 fraction was not significantly different (Figs. 1, 2). These results demonstrate that curcumin induces apoptosis and G2/M arrest in prostate cancer cells.

2.2. *Curcumin induces the accumulation of IκBα in PC-3 cells*

To understand the molecular mechanisms by which curcumin induces apoptosis and G2/M arrest in prostate cancer cells, we first examined the activation of NF-κB signaling in cells treated with curcumin. The expression of IκBα in LNCaP cells was significantly higher than in PC-3 cells ($p < 0.01$, Fig. 2A) . This is consistent with the known fact that PC-3 cells express higher constitutive levels of NF-κB than LNCaP cells, indicating that the activation of NF-κB in PC-3 cells is due to the low level of IκBα.

Next we examined the effect of curcumin on IκBα level in prostate cancer cells. We found that the IκBα level was significantly increased after exposure to curcumin, and this effect was concentration dependent (Fig. 2B). However, the IκBα level was not significantly changed in LNCaP cells after 4 h of curcumin treatment (Fig. 2C). These data indicate that curcumin induces the accumulation of IκBα in androgen independent prostate cancer cells specially.

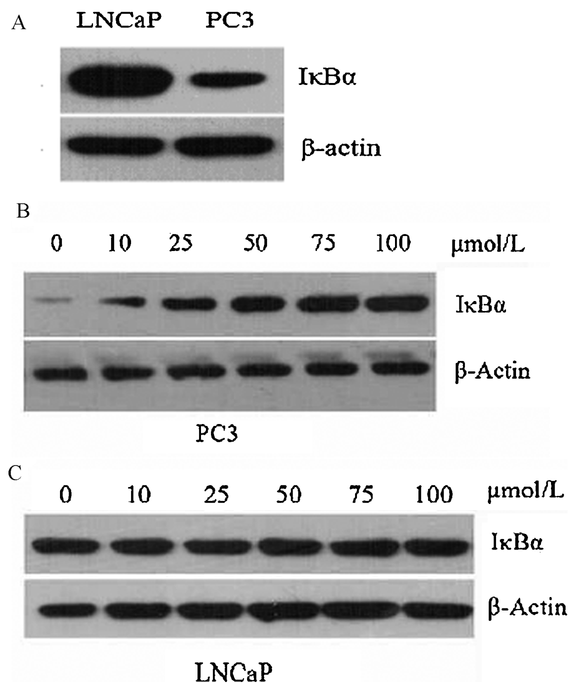

Fig. 2: Curcumin increases IκBα protein level in PC3 cells but has no such effect in LNCaP cells. A. Endogenous IκBα protein level in LNCaP and PC-3 cells. B. IκBα protein level in PC-3 cells was increased following the increase of curcumin concentration. C. IκBα protein level in LNCaP cells did not show a significant increase following the increase of curcumin concentration. Shown were representative blots from three independent experiments with similar results. β-actin served as loading control

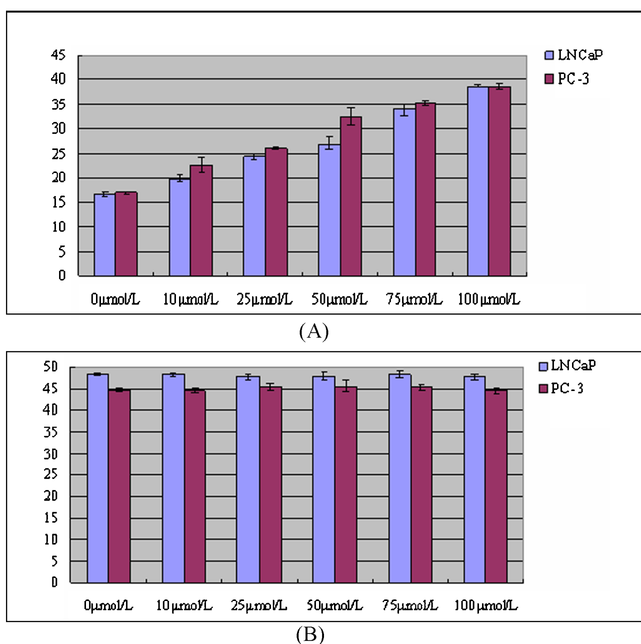

Fig. 1: Curcumin induces G2/M arrest of LNCaP and PC-3 cells. LNCaP and PC-3 cells were treated with curcumin and cell cycle was analyzed by flow cytometry. A. The rates of DNA content in the G2/M fraction of LNCaP and PC-3 cells were increased following the increase of curcumin concentration. B. The rates of DNA content in the G0/G1 fraction of LNCaP and PC-3 cells did not show a significant increase following the increase of curcumin concentration

2.3. *Curcumin reduces c-Jun level in LNCaP and PC-3 cells*

Since c-Jun is implicated in the regulation of cell cycle and apoptosis, we further evaluated the changes in c-Jun level in prostate cancer cell lines upon exposure to curcumin. c-Jun level in LNCaP and PC-3 cells was decreased in a concentration-dependent manner in response to 4 h of curcumin treatment (Fig. 3A, B).

2.4. *Curcumin decreases AR expression in LNCaP and PC-3 cells*

AR plays a crucial anti-apoptotic role in prostate cancer. Thus, we investigated the effect of curcumin on AR level in LNCaP and PC-3 cells. We observed that curcumin treatment decreased AR expression in both LNCaP and PC-3 cells, but the decreases were more dramatic in androgen dependent LNCaP cells (Fig. 4 A, B).

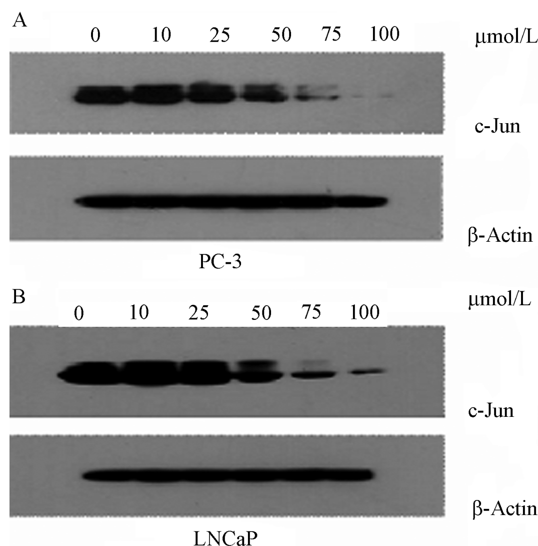

Fig. 3: Curcumin decreases c-jun protein level in PC3 and LNCaP cells. A. c-jun protein level in PC-3 cells was decreased following the increase of curcumin concentration. B. c-jun protein level in LNCaP cells was decreased following the increase of curcumin concentration. Shown were representative blots from three independent experiments with similar results. β-actin served as loading control

3. Discussion

NF-κB family of transcription factors is constitutively activated in various human malignancies, including leukemias, lymphomas, and a number of solid tumors, and contributes to tumor progression by regulating the expression of genes involved in cell growth and proliferation, anti-apoptosis, angiogenesis, and metastasis (Wu et al. 2010; Yeh et al. 2010; Wang et al. 2012). IκBα negatively regulates the activation of NF-κB signaling by preventing NF-κB nuclear translocation and IκBα expression level is negatively correlated with NF-κB activity in the cells. In this study, we found that IκBα and AR levels in PC-3 cells were lower than those in LNCaP cells. This is consistent with an earlier study showing the inverse correlation between AR status and constitutive activation of NF-κB in prostate cancer cells (Papadopoulou et al. 2008). These data suggest either that AR inhibits NF-κB activity in prostate cancer cells or alternatively, that constitutive activation of NF-κB is correlated with AR loss and contributes to compensatory cellular changes allowing for cell survival and growth in the absence of AR activation. It is noteworthy that constitutive activation of NF-κB, known to induce potent anti-apoptotic effects, may play a role in the progression of prostate cancer and contribute to prostate cancer cell survival following androgen withdrawal. Therefore, we propose that constitutive activation of NF-κB is critically involved in the biological behavioral differences of prostate cancers, and the detection of NF-κB expression could discriminate between patients with androgen-dependent and androgen-independent prostate cancer. Although the precise mechanism by which AR help maintain IκBα level has not been elucidated, our study provides further evidence for the antagonistic effect of AR on NF-κB activity in prostate cancer cells.

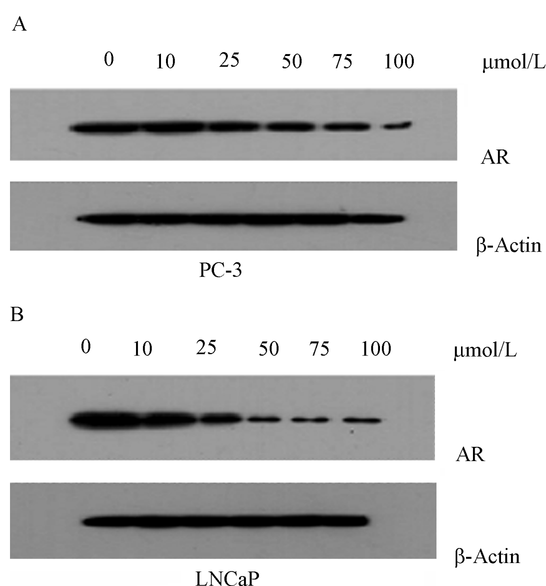

Fig. 4: Curcumin decreases AR protein level in PC3 and LNCaP cells. A. AR protein level in PC-3 cells was decreased following the increase of curcumin concentration. B. AR protein level in LNCaP cells was decreased following the increase of curcumin concentration. Shown were representative blots from three independent experiments with similar results. β-actin served as loading control

Because LNCaP cells are androgen dependent and differ from the androgen independent PC-3 cells in several aspects such as AR signaling and NF-κB function, we focused on the variation in the expression of IκBα in LNCaP and PC-3 cells after administration of curcumin. Inactivation of the NF-κB pathways by some anticancer agents has been reported to induce apoptosis and growth arrest at G2/M phase of cancer cells (Raffoul et al. 2006; Yao et al. 2006). Thus, it is tempting to speculate that the inhibition of NF-κB by curcumin in androgen-independent prostate cancer cells may promote the induction of cell cycle arrest and apoptosis we observed. However, because IκBα expression was not changed in LNCaP cells after the administration of curcumin, molecular targets of curcumin other than NF-κB may contribute to the induction of apoptosis in androgen-dependent prostate cancer cells.

The role of c-Jun in apoptotic response appears to be cell type and stimulus dependent. In this study, we found that enhanced apoptosis of LNCaP and PC-3 cells after curcumin treatment was associated with a decreased level of c-Jun. These data suggest that curcumin has profound effects on apoptosis and cell cycle arrest of prostate cancer cells by targeting survival pathways including the JNK pathways. This is consistent with previous reports that JNK inhibitor promoted CD95-mediated apoptosis of prostate cancer cells (Park et al. 2010). Thus it appears that c-Jun is required for the survival of prostate cancer cells, and curcumin induces the downregulation of c-Jun, leading to increased apoptosis of LNCaP and PC-3 cells. Interestingly, a recent study showed that resveratrol inhibited the transcriptional activities of both NF-κB and c-jun (Kim et al. 2011). This is consistent with our data that curcumin increases the level of IκBα but decreases the level of c-jun.

Moreover, we observed that curcumin decreased AR expression levels in LNCaP and PC-3 cells. The transcriptional program activated by AR plays a critical role in the regulation of normal and malignant prostate tissue. AR is able to retain activity even in the androgen independent prostate cancer cells through aberrant mechanisms of activation (Brinkmann and Trapman 2000; Ntais et al. 2003). Since AR is a main driver of prostate cancer cell proliferation and progression, the inhibition of AR is an important approach for prostate cancer therapy. Although further studies are needed to elucidate the mechanism by which curcumin downregulates AR expression in prostate cancer, our data clearly indicate that curcumin could act as a natural, efficient and specific AR antagonist for prostate cancer.

In summary, in this study we demonstrate that curcumin induces the cell cycle arrest and apoptosis of prostate cancer cells in a dose dependent manner, and this is associated with the down-regulation of c-Jun and AR and upregulation of NF-kB inhibitor IκBα. These data suggest that curcumin is a promising agent for the treatment of both androgen-dependent and androgen-independent prostate cancer.

4. Experimental

4.1. Cell lines

LNCaP cell line (Cell Center of Preclinical Medicine, Chinese Union Medical College, Beijing, China) was cultured in RPMI 1640 medium (Life Technologies, Rockville, MD, USA) supplemented with 10% fetal calf serum (Hyclone, Logan, UT, USA), 1% penicillin/streptomycin, 1 ug/ml hydrocortisone, and 100 nM testosterone. PC-3 cell line (The Cell Bank of Chinese Academy of Sciences, Shanghai, China) was cultured in DMEM medium (Life Technologies, Rockville, MD, USA) supplemented with 10% fetal calf serum (Hyclone, Logan, UT, USA) and 1% penicillin/streptomycin. All cells were cultured at 37 °C in a humidified atmosphere consisting of 5% CO_2 and 95% air.

4.2. Flow cytometric analysis

LNCaP and PC-3 cells were treated with 0, 10, 25, 50, 75 and 100 μmol/L curcumin for 24 h, washed, and then fixed with 70% ethanol overnight at −20 °C. The cells were washed with PBS and then suspended in staining buffer (10 mg/ml propidium iodide, 0.5% Tween-20 and 0.1% RNase in PBS). The cell suspension was then analyzed using a FACS vantage flow cytometer and the results were analyzed by cell quest acquisition and analysis programs (Becton Dickinson, San Jose, CA, USA). Gating was set to exclude cell debris, cell doublets, and cell clumps.

4.3. Western blot analysis

LNCaP and PC-3 cells (5×10^7) were treated with various concentrations of curcumin (0, 10, 25, 50, 75 and 100 μmol/L) for 5 h. The cell lysate was collected and 30 mg of protein were resolved on 9% sodium dodecyl sulfate-polyacrylamide gels. Resolved samples were electrotransferred to nitrocellulose membranes, which were blocked with 5% nonfat milk protein for 1 h and then incubated with anti-IκBα, anti-c-Jun and anti-AR polyclonal antibodies for 1 h at room temperature. The membranes were washed and incubated with HRP-conjugated secondary antibodies and finally detected by chemiluminescence (Supersignal West Pico Chemniluinescent Substrate; Pierce Biotechnology, Rockford, CA, USA). Each blot was stripped and reprobed with a β-actin antibody as an internal control. Densitometric analysis was performed using Quantity One version 4.4.1 Basic (Bio-Rad, Hercules, CA, USA).

4.4. Statistical analysis

The data were expressed as mean ± SD. Statistical analyses were performed using the two-tailed Student's t test for paired values and one-way ANOVA for multiple values when appropriate. Statistical significance was considered to be $p < 0.05$. All statistical analyses were performed using SPSS software for Windows 12.0 (SPSS, Chicago, IL, USA).

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