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FAST TRACK

Inhibition of Cell Survival Signal Protein Kinase B/Akt by Curcumin in Human Prostate Cancer Cells

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Abstract Although curcumin has been shown to inhibit prostate tumor growth in animal models, its mechanism of action is not clear. To better understand the anti-cancer effects of curcumin, we investigated the effects of curcumin on cell survival factor Akt in human prostate cancer cell lines, LNCaP, PC-3, and DU-145. Our results demonstrated differential activation of Akt. Akt was constitutively activated in LNCaP and PC-3 cells. Curcumin inhibited completely Akt activation in both LNCaP and PC-3 cells. The presence of 10% serum decreased the inhibitory effect of curcumin in PC-3 cells whereas complete inhibition was observed in 0.5% serum. Very little or no activation of Akt was observed in serum starved DU-145 cells (0.5% serum). The presence of 10% serum activated Akt in DU-145 cells and was not inhibited by curcumin. Results suggest that one of the mechanisms of curcumin inhibition of prostate cancer may be via inhibition of Akt. To our knowledge this is the first report on the curcumin inhibition of Akt activation in LNCaP and PC-3 but not in DU-145 cells. J. Cell. Biochem. 89: 1–5, 2003.

Key words: curcumin; Akt; prostate cancer; LNCaP; PC-3; DU-145; cell survival; apoptosis

Prostate carcinoma is the most commonly diagnosed cancer and second leading cause of cancer-related deaths among men in the United States [Russell et al., 1998; Koeneman et al., 1999; Greenlee et al., 2000]. Most deaths from prostate cancer are caused by metastases mainly to bone and lymph nodes that resist conventional androgen-deprivation therapy [Kreis, 1995; Russell et al., 1998]. The most difficult problem in the treatment of prostate cancer is that initially it can be treated by androgen-deprivation, but androgen-treated patients experience relapse of the cancer that is largely androgen-independent [Russell et al., 1998; Koeneman et al., 1999]. The induction of prostate cancer in humans has been viewed as a multistage complex process that involves multiple stages of initiation, promotion, neoplastic transformation, and progression from androgen-dependent to androgen-independent metastasizing carcinoma [Russell et al., 1998]. The occurrence of prostate cancer is high in the Scandinavian countries, with the highest incidence and mortality rates in African American males, the later being twofold higher than in Caucasian American males. There is lower incidence of prostate carcinoma in Asian men than their Western counterparts [Denis et al., 1999]. These differences in cancer incidence have been attributed largely to diet, race, and geographical location [Denis et al., 1999]. Curcumin, a dietary ingredient used in India for centuries, has recently gained much attention for the prevention of colon and breast cancer [Kelloff et al., 1999] and may have clinical application in the prevention of prostate cancer. Curcumin (diferuloymethane), a major yellow pigment in turmeric obtained from rhizomes of the plant Curcumina longa Linn, is commonly used as a coloring agent in foods, drugs, and cosmetics [Srimal and Dhawan, 1973; Satoskar et al., 1986; Rao et al., 1993]. Turmeric has been used to treat inflammatory and other diseases, and pharmacological safety of curcumin is demonstrated by its consumption for centuries in Asian countries [Srimal and Dhawan, 1973; Satoskar et al., 1986].

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Several recent observations have shown that curcumin has anti-oxidant, anti-inflammatory, and anti-carcinogenic activity. Curcumin suppresses tumor initiation and promotion in animal models, and is a potent inhibitor of cycloxygenase-2 [Plummer et al., 1999], lipoxygenase, ornithine decarboxylase [Lu et al., 1993; Huang et al., 1997], c-Jun/AP-1 [Huang et al., 1991], NF-κB [Singh and Aggarwal, 1995; Plummer et al., 1999], c-Jun N-terminal kinase [Chen and Tan, 1998], and protein kinase C [Liu et al., 1993]. Curcumin inhibited EGF receptor activity in various tumors including prostate carcinoma [Dorai et al., 2000], marked decrease in cell proliferation and microvessel density, and increase in apoptosis in the tumors caused by LNCaP cells injected subcutaneously into nude mice [Dorai et al., 2001]. By virtue of its multiple effects, curcumin has potential clinical application in the prevention of prostate cancer. However, despite its anti-carcinogenic activity, very little is known about the effects and molecular mechanisms of curcumin action in prostate cancer. Cell proliferation and apoptosis (programmed cell death) play a critical role in tumor growth and progression. Protein kinase B (PKB/Akt), a serine-threonine protein kinase encoded by the c-Akt protooncogene, has been implicated in tumor growth and progression as a transformation stimulated cell survival factor because it stimulates cell proliferation and cell cycle progression, and suppresses apoptosis [Datta et al., 1999]. In this study, we investigated the effects of curcumin on Akt in different human prostate cancer cell lines. Our results demonstrated differential activation of Akt and inhibition by curcurmin in LNCaP, PC-3, and DU-145 cells.

MATERIALS AND METHODS

Materials

Curcumin, Dulbecco's phosphate-buffered saline (PBS), and trypsin-EDTA were purchased from Sigma Chemical Co. (St. Louis, MO). RPMI 1640 medium and fetal bovine serum (FBS) were obtained from Cellgro Mediatech (Herndon, VA) and Atlas Biologicals (Fort Collins, CO) respectively. Recombinant human FGF-2 and PDGF-BB were obtained from R & D Systems, Inc. (Minneapolis, MN). Phospho-Akt (Serine-473) and Akt antibodies were obtained from New England Biolabs (Beverly, MA). Stock solution of curcumin was made in DMSO. All other chemicals were of analytical grade.

Cell Culture

Human prostate cancer cell lines, LNCaP, PC-3, and DU-145 were obtained from American Type Culture Collection (Rockville, MD). Cells were routinely cultured in RPMI 1640 medium supplemented with 10% FBS and maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Cell Lysate Preparation

After cells reached confluency, cells were trypsinized and seeded in P-100 culture dishes at a density of 1×10^6 cells/dish. Cells were made quiescent in RPMI 1640 medium containing 1–0.5% FBS for 24 h. The required amounts of concentrated stock solutions of growth factors, serum, and curcumin were added directly to the medium and cells incubated for specified time period. Cells were rinsed with PBS and total cell lysates were prepared by adding 0.5 ml of boiling lysis buffer containing 1% SDS, 1 mM AEBSF, 10 mM Tris-HCl, pH 7.4, 1 mM sodium orthovanadate, and 20 mM NaF to each dish. Cell mixture was transferred to microcentrifuge tubes, boiled for 5 min, passed several times through a 25-gauge needle, and centrifuged for 15 min at 14,000*g* in a microcentrifuge. The supernatants were removed and stored at −80°C until used. Proteins in cell lysates were measured with the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA).

Western Blotting (Immunoblotting)

Cell lysates were subjected to SDS–PAGE (10% gel) and proteins were electrophoretically transferred to polyvinylidene fluoride (PVDF)/Immobilin-P membrane (Millipore Corp., Medford, MA) as described previously [Chaudhary and Hruska, 2001]. The membranes were incubated in blocking buffer (Tris-buffered saline-0.1% Tween-20 (TBST) containing 5% Carnation milk (anti-phospho-Akt and anti-Akt antibodies) at room temperature for 2 h, then incubated with appropriate antibodies according to the protocols of the manufacturers (New England BioLabs) and washed with TBST. Antibody binding was detected by enhanced chemiluminescence (ECL) Western blotting detection systems as directed by the manufacturer (Amersham Pharmacia Biotech, Arlington Heights, IL). Data shown are representative

of at least two individual experiments with similar results.

RESULTS

Effect of Curcumin on Akt Activation in LNCaP Cells

LNCaP cells have a mutated non-functional PTEN (phosphatase and tensin homologue deleted from chromosome 10 **[ten]**). Normal PTEN decreases Akt activation by virtue of its phospholipid phosphatase activity [Teng et al., 1997; Tsugawa et al., 2002]. Thus, Akt is constitutively activated in LNCaP cells. As shown in Figure 1, Akt was found to be constitutively activated in the presence of low serum in LNCaP cells. Treatment with curcumin completely inhibited the activation of Akt at 1, 2, and 3 h but had no effect on total levels of Akt. Since curcumin was inhibitory at 1 h, we designed all later experiments to determine the effects of curcumin at 1 h treatment in PC-3 and DU-145 cells.

Effects of Curcumin, Serum, and PDGF-BB on Akt Activation in PC-3 Cells

Since serum and PDGF-BB stimulate Akt [Chaudhary and Hruska, 2001], we examined their effects on the activation of Akt in PC-3 cells. Results demonstrated that PC-3 cells also expressed constitutively activated Akt (Fig. 2). Treatment with 10% serum or PDGF-BB had no further stimulatory action on Akt as compared to 0.5% serum. Curcumin treatment of PC-3 cells completely inhibited Akt activation without affecting total Akt. Even though serum did not increase the constitutively active Akt, the inhibitory effect of curcumin was attenuated in the presence of 10% serum but not PDGF-BB.

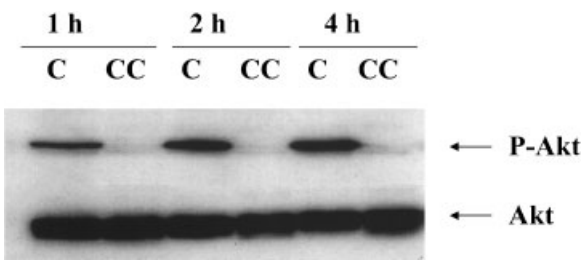

Fig. 1. Effect of curcumin on Akt activation in LNCaP cells. Cells were made quiescent in RPMI-1640 medium containing 1% FBS for 24 h. Thereafter curcumin (35 μM) was added and cells were incubated for 1, 2, and 3 h. Total Cell lysates were prepared and samples (60 μg protein) subjected to SDS–PAGE followed by transfer of proteins to PVDF membranes. Membranes were immunoblotted with anti-phospho-Akt and anti-Akt antibodies and proteins were detected by ECL kit as described in Materials and Methods. C, control; CC, curcumin.

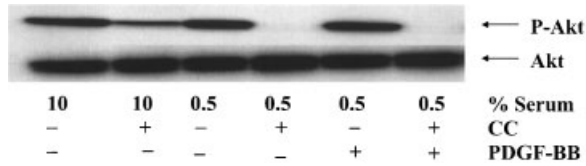

Fig. 2. Effects of curcumin, serum, and PDGF-BB on Akt activation in PC-3 cells. Cells were made quiescent in RPMI-1640 medium containing 0.5% FBS for 24 h. Cells were pretreated with curcumin (35 μM) for 30 min and then serum (10%) or PDGF-BB (30 ng/ml) were added to the specified samples and cells were incubated for additional 1 h. Thereafter, cells lysates were prepared, samples (60 μg protein) subjected to SDS–PAGE, and immunoblotted as described in the Materials and Methods.

Effects of Curcumin, Serum, PDGF-BB, and FGF-2 on Akt Activation in DU-145 Cells

We next examined the effects of curcumin and growth factors on the activation of Akt in DU-145 cells. Results showed very little or no activation of Akt in serum starved DU-145 cells (Fig. 3). However, 10% serum activated Akt in DU-145 cells but the activation was much lower than the activation observed in LNCaP and PC-3 cells even in the presence of low serum. Curcumin did not inhibit Akt activation by 10% serum. FGF-2 and PDGF-BB had very little or no effect on the activation of Akt.

DISCUSSION

In the present study we investigated the effects of curcumin on the Akt activation as

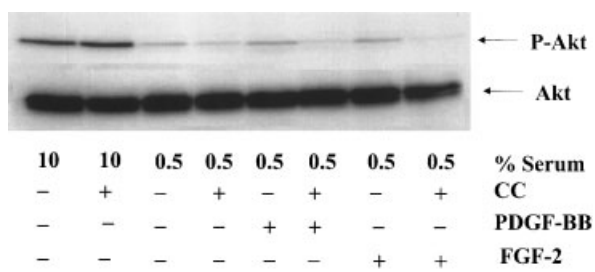

Fig. 3. Effects of curcumin, serum, PDGF-BB, and FGF-2 on Akt activation in DU-145 cells. Cells were made quiescent in RPMI-1640 medium containing 0.5% FBS for 24 h. Cells were pretreated with curcumin (14 μM) for 30 min and then serum (10%), PDGF-BB (30 ng/ml) or FGF-2 (30 ng/ml) were added to the specified samples and cells were incubated for additional 1 h. Thereafter, cells lysates were prepared, samples (60 μg protein) subjected to SDS–PAGE, and immunoblotted as described in the Materials and Methods.

indicated by level of phosphorylation in prostate cancer cells. Our results demonstrated for the first time that curcumin completely inhibited Akt activation in serum starved LNCaP and PC-3 cells. The presence of serum decreased inhibitory effect of curcumin. In contrast, no activation of Akt was observed in serum starved DU-145 cells. It is important to mention that Akt was expressed in all cell lines. Only the activation of Akt was altered in different prostate cancer cell lines which was determined using specific antibody which specifically recognized phosphorylated form indicating activation of Akt. Our results are consistent with the fact that LNCaP expresses non-functional PTEN product and PC-3 cells lack PTEN [Teng et al., 1997; Bastola et al., 2002; Grünwald et al., 2002]. Loss of PTEN leads to constitutive activation of the phosphatidylinositol 3′-kinase (PI3K)/serine-threonine kinase Akt signal transduction pathway [Datta et al., 1999]. Thus, PTEN plays a critical role in regulating cell signaling in prostate cancer cells [Tsugawa et al., 2002]. Akt is an important regulator of cell proliferation, cell survival, and significantly contributes to tumor growth and progression by promoting cell invasiveness and angiogenesis. Overexpression of Akt has been reported in a variety of human cancers including prostate cancer [Datta et al., 1999; Hill and Hemings, 2002] and cells expressing elevated levels of Akt are less sensitive to apoptotic stimuli [Hill and Hemings, 2002].

Akt enhances cell survival by phosphorylating key apoptotic regulator Bad, a pro-death member of the Bcl-2 family that initiates apoptosis by binding to Bcl-x_L on the outer mitochondrial membrane, causing the release of cytochrome c into the cytosol. Phosphorylated Bad has low affinity to Bcl-x_L and high affinity to the 14-3-3 proteins, thus inactivating its proapoptotic function [Datta et al., 1999]. Our results suggest that curcumin may increase cell death by inhibiting Akt and downstream modulating activity of proapoptotic factor Bad. Furthermore, Akt plays an important role in the stimulation of cell proliferation by inactivating cyclin-dependent kinase inhibitor $p21^{Cip1/WAF1}$ and decreasing the transcription of $p27^{kip1}$ [Hill and Hemings, 2002]. As Akt promotes cell survival and proliferation the conditions that are favorable for tumor growth and progression, use of specific inhibitors of its activity is a good therapeutic strategy for human cancers with elevated levels of Akt. Curcumin inhibition of Akt activation in LNCaP and PC-3 cells suggests that curcumin could be a potent anti-cancer dietary ingredient. Although curcumin may not be a specific inhibitor of Akt, it can prove to be a potential therapeutic agent for the prevention and possibly treatment of prostate cancer and other cancers because of its proven safety in human population.

Curcumin has been shown to stimulate apoptosis by downregulating anti-apoptotic factors NF-κB, Bcl-2, and Bcl-x_L, and activation of procaspase-3 and procaspase-8 in LNCaP and DU-145 cells [Mukhopadhyay et al., 2001]. Caspase-8 mediated cleavage of BID, a proapoptotic member of Bcl-2 family, causes release of cytochrome c from the mitochondria, an essential step in the apoptosis pathway [Datta et al., 1999; Mukhopadhyay et al., 2001]. Interestingly, we discovered that the presence of serum decreased the inhibitory effects of curcumin on Akt activation in PC-3 cells. At the moment it is not clear why serum attenuated curcumin response. However, it is possible that curcumin may bind to serum proteins resulting in the decreased availability of the curcumin to the cell. Another possibility could be that serum factor(s) might increase anti-apoptotic factors such as Bcl-2 and Bcl-x_L which have been shown to resist curcumin inhibitory effect [Anto et al., 2002].

In summary, we have demonstrated that curcumin inhibited cell survival signal Akt in LNCaP and PC-3 cells. Our results suggest that curcumin may induce programmed cell death by Akt/Bad signaling pathway and play a critical role in prostate cancer cell survival.

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