Curcumin inhibits cellular condensation and alters microfilament organization during chondrogenic differentiation of limb bud mesenchymal cells

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Abbreviations: ECM, extracellular matrix; FAK, focal adhesion kinase; MMP, matrix metalloproteinase; PNA, peanut agglutinin

Abstract

Curcumin is a well known natural polyphenol product isolated from the rhizome of the plant *Curcuma longa*, anti-inflammatory agent for arthritis by inhibiting synthesis of inflammatory prostaglandins. However, the mechanisms by which curcumin regulates the functions of chondroprogenitor, such as proliferation, pre-cartilage condensation, cytoskeletal organization or overall chondrogenic behavior, are largely unknown. In the present report, we investigated the effects and signaling mechanism of curcumin on the regulation of chondrogenesis. Treating chick limb bud mesenchymal cells with curcumin suppressed chondrogenesis by stimulating apoptotic cell death. It also inhibited reorganization of the actin cytoskeleton into a cortical pattern concomitant with rounding of chondrogenic competent cells and down-regulation of integrin $\beta 1$ and focal adhesion kinase (FAK) phosphorylation. Curcumin suppressed the phosphorylation of Akt leading to Akt inactivation. Activation of

Akt by introducing a myristoylated, constitutively active form of Akt reversed the inhibitory actions of curcumin during chondrogenesis. In summary, for the first time, we describe biological properties of curcumin during chondrogenic differentiation of chick limb bud mesenchymal cells. Curcumin suppressed chondrogenesis by stimulating apoptotic cell death and down-regulating integrin-mediated reorganization of actin cytoskeleton via modulation of Akt signaling.

Keywords: apoptosis; cell differentiation; chondrocytes; curcumin; cytoskeleton; microfilament; proto-oncogene proteins c-akt

Introduction

The progressive degeneration of articular cartilage, indicating a disruption of the balance between anabolic and catabolic activities of the chondrocytes, results in osteoarthritis, a painful, debilitating disease of the synovial joints. Several events are known to play significant roles for the initiation and progression of joint diseases, including dysregulation of pro-inflammatory cytokines, pro-inflammatory enzymes that mediate the production of prostaglandins and leukotrienes (e.g. lipo-oxygenase), together with the expressions of adhesion molecules and matrix metalloproteinases. Curcumin is the major pigment derived from the rhizome of turmeric *Curcuma longa* Linn and has been found to exert various biological effects, such as anti-inflammatory, anti-oxidant and anti-tumorigenic effects, in addition to tissue repair and wound healing (Khanna, 1999). Even though the exact mechanisms involved in anti-inflammatory action in the degeneration of articular cartilage to relieve the aches and pains associated with arthritis are not fully understood, many recent studies provide a better understanding of these mechanisms and new insight into the field of arthritis. However, the role of curcumin in the differentiation of chondrogenic progenitor cells has not been studied even though their characteristics much differ from articular chondrocytes.

During embryonic development, most bones form by endochondral ossification, in which bones are first laid down as cartilage precursors (Karsenty

and Wagner, 2002). This is a precise series of events that includes aggregation and differentiation of mesenchymal cells, proliferation, hypertrophy and death of chondrocytes (DeLise *et al.*, 2000). In particular, chondrogenesis is characterized by 2 dramatic changes in cellular condensation that are mediated by cell-cell and cell-matrix adhesion molecules (Sandell and Adler, 1999; DeLise *et al.*, 2000; Knudson and Knudson, 2001) and cell shape changes from fibroblastoid to round or polygonal morphologies (von der Mark and von der Mark, 1977).

Several studies have shown that curcumin modulates the activities of numerous targets, including growth factors, growth factor receptors, transcription factors, cytokines, enzymes and genes regulating apoptosis (Gertsch *et al.*, 2003; Balasubramanian and Eckert, 2006; Zheng and Chen, 2006). It also suppresses the proliferation of a number of transformed cell types *in vitro*, including breast (Simon *et al.*, 1998; Ramachandran and You, 1999), colon (Chen *et al.*, 1999) and oral epithelial cells

(Khafif *et al.*, 1998). The anti-proliferative effects of curcumin include blocks at specific cell cycle stages, particularly a G2/M block (Hanif *et al.*, 1997; Mehta *et al.*, 1997; Ramachandran and You, 1999; Holy, 2002). Curcumin has also been reported to inhibit apoptosis in several cell types (Yamamoto, 1995; Sikora *et al.*, 1997) suggesting cell type-specific effects of curcumin during cell death triggering.

The signaling mechanisms of curcumin action are beginning to be elucidated. Curcumin is known to inhibit a number of kinase and enzyme activities, including PKC (Liu *et al.*, 1993), phosphorylase kinase (Reddy and Aggarwal, 1994), EGF receptor and erbB2, (Korutla and Kumar, 1994; Korutla *et al.*, 1995; Hong *et al.*, 1999). In spite of recent studies, large gaps exist in our knowledge of how curcumin functions during chondrogenesis. In particular, little information is available regarding the effects of curcumin on cytoskeletal organization or overall cell behavior.

In this study, we applied a high density (micro-

Figure 1. Curcumin suppresses chondrogenesis. (A) PNA and Alcian blue staining of chick limb bud mesenchymal cells grown in micro-mass cultures in the presence of varying concentrations of curcumin (upper panel) on day 3 and day 5 of cultures respectively. Quantification of chondrogenesis was determined by measuring the absorbance of bound Alcian blue at 600 nm (lower panel). (B) Changes in the levels of type II collagen in control and curcumin-treated cultures were determined by Western blotting on day 5 of culture. Results shown are representative of at least 4 independent experiments. HSP 70 was used as a loading control. *, statistically significant differences compared with control cells ($P < 0.01$).

mass) culture model system that allowed differentiation of chondrogenic progenitors into chondrocytes to clarify a mechanistic role for curcumin during chondrogenesis. For the first time, we found that curcumin suppressed chondrogenesis by stimulating apoptotic cell death, down-regulating integrin $\beta 1$ and lowering the phosphorylation level of FAK. This, in turn, lead to reorganization of the actin cytoskeleton via modulation of Akt signaling.

Results

Curcumin inhibits chondrogenesis by stimulating apoptotic death of chondrogenic competent cells

To determine any effects of curcumin on chondrogenesis, chondroblasts isolated from wing buds were cultured at a density of 2×10^7 cells/ml and stimulated with varying concentrations of curcumin. Precartilage condensation and chondrogenesis were assessed by peanut agglutinin (PNA) binding on day 3 and by Alcian blue staining for sulfated proteoglycans on day 5, respectively. At curcumin concentrations $> 20 \mu\text{M}$, PNA staining and Alcian blue uptake (Figure 1A) were significantly decreased. We therefore used $20 \mu\text{M}$ curcumin for all subsequent experiments. A significant decrease in type II collagen expression in curcumin-treated cultures, as compared with control cultures (Figure 1B), was consistent with this observation. These results demonstrate that curcumin inhibits sulfated proteoglycan accumulation and cartilage nodule formation during chondrogenesis.

Chondrogenic differentiation is known to be re-

gulated at 3 stages: cell proliferation, precartilage condensation and cartilage nodule formation (Solursh, 1989; Maini and Solursh, 1991). We first examined if curcumin-inhibited chondrogenesis was due to regulation of cell proliferation. During chondrogenic differentiation, the cell numbers increased in control cultures. Exposure of cells to curcumin suppressed the proliferation of chondrogenic competent cells as determined by direct cell counting (Figure 2A) and FACS analysis (Figure 2B). These results indicate that the negative regulatory action by curcumin results by modulating the proliferation and survival of chondrogenic competent cells.

Curcumin alters cytoskeletal reorganization in chick limb bud mesenchymal cells during chondrogenesis

Our laboratory previously showed that reorganization of the actin cytoskeleton to a cortical pattern concomitant with rounding of cells and reduced numbers of stress fibers occurs with time in culture (Jin *et al.*, 2007). We also demonstrated that microtubule polymerization is absolutely required for later stages of chondrogenic differentiation. Therefore, we investigated if suppressed chondrogenic differentiation by curcumin was also associated with the reorganization of the actin cytoskeleton. Actin stress fibers were markedly intensified by treatment with curcumin compared to the control (Figure 3A). This pointed to the actin cytoskeletal reorganization for curcumin-suppressed chondrogenesis.

Three structural elements are required for cell morphological changes, including an extracellular matrix (ECM) ligand, its cell surface receptor and

Figure 2. Curcumin induces apoptotic death of chondrogenic competent cells. Chick limb bud mesenchymal cells were cultured with or without $20 \mu\text{M}$ curcumin. (A) The number of viable cells was counted on the indicated days. (B) Percentages of apoptotic cells were quantified by flow cytometric analysis on days 2 and 3 of culture. Results are averages with standard deviations of 5 independent experiments. *, statistically significant differences compared with control cells ($P < 0.01$).

the cell cytoskeleton. Recent studies have shown that members of the integrin family, including the $\alpha 5 \beta 1$ fibronectin receptor, $\alpha 2 \beta 1$ and $\alpha 3 \beta 1$, and the vitronectin receptor $\alpha v \beta 3$ interact reversibly with both ECM proteins and cytoskeletal structures (Li *et al.*, 2004). Therefore, actin cytoskeleton reorganization could be regulated by the type of receptor-ligand interactions. First, possible curcumin-induced alterations of fibronectin and integrin $\beta 1$ expressions in chick wing bud mesenchymal cells were examined by immunoblotting (Figure 3B). The level of fibronectin remained unchanged after curcumin treatment. However, integrin $\beta 1$ expression in chick wing bud mesenchymal cells was completely abolished. Furthermore, the engagement of integrins to the ECM triggers the activation of protein tyrosine kinases (Felsenfeld *et al.*, 1999; Parsons *et al.*, 2002). The phosphorylation levels of focal adhesion kinase (FAK) were also decreased by curcumin (Figure 3B).

Involvement of Akt signaling in curcumin-suppressed chondrogenic differentiation of chick limb bud mesenchymal cells

Activation of Akt has been shown to block the actions of several pro-apoptotic proteins, such as Bad (Hayakawa *et al.*, 2000), and block cytochrome C release from the mitochondria through the regulation of Bcl-2 (Davies *et al.*, 1999). Previously, we demonstrated that Akt signaling positively regulates chondrogenic differentiation of chick limb bud mesenchymal cells via reorganization of the actin cytoskeleton to a cortical pattern with concomitant rounding of chondrogenic competent cells (Jin *et al.*, 2007). Therefore, we investigated if Akt signaling was associated with curcumin-suppressed chondrogenic differentiation. No obvious changes in Akt protein levels were observed. However, the phosphorylation level of Akt was decreased when cells were exposed to curcumin (Figure 4A).

To further verify Akt involvement in curcumin-suppressed chondrogenic differentiation, chondrogenic progenitors were transfected with vectors expressing either a myristoylated constitutively active form of Akt (myr-Akt), a dominant inhibitory mutant Akt (mutant-Akt) or an empty vector (mock). Compared to cultures treated with curcumin alone, activation of Akt after introducing myr-Akt showed recoveries of precartilaginous condensation (Figure 4B upper panel), chondrogenic differentiation (Figure 4B lower panel), apoptotic cell death (Figure 4C), expression levels of pFAK and integrin $\beta 1$ (Figure 4D), and rounding of cells (surrounded by dotted circle in Figure 4E). In contrast, inhibition of Akt by introducing mutant-Akt was associated with even

Figure 3. Curcumin suppresses reorganization of the actin cytoskeleton during chondrogenesis of chick limb bud mesenchymal cells. Chick limb bud mesenchymal cells were cultured with or without 20 μ M curcumin. (A) Cells were immunostained for F-actin using Alexa488-phalloidin on 4 days of culture. (B) Changes in the levels of pFAK³⁹⁷, FAK, paxillin and integrin $\beta 1$ in control and curcumin-treated cultures were determined by Western blotting on the indicated days. Results shown are representative of at least 4 independent experiments.

greater decreases of precartilaginous condensation, chondrogenic differentiation, apoptotic cell death, and expression levels of pFAK and integrin $\beta 1$ compared to cultures treated with curcumin alone. The formation of stress fiber was also intensively increased by inhibition of Akt compared to cultures treated with curcumin alone.

Discussion

Curcumin has been shown to inhibit cell growth and to have a number of biological effects, such as anti-metastatic, anti-inflammatory and anti-infectious (Kuo *et al.*, 1996; Jaruga *et al.*, 1998; Kawamori *et al.*, 1999). However, the essential cellular and molecular targets and a signaling mechanism for curcumin have not been completely defined. Here, we demonstrate several biological properties of curcumin by showing that curcumin suppressed chondrogenic differentiation of chick limb mesenchymal cells by stimulating apoptotic cell death and

Figure 4. Akt signaling mediates inhibitory actions of curcumin during chondrogenesis. Chick limb bud mesenchymal cells were cultured with or without 20 μ M curcumin. (A) Changes in the levels of Akt or pAkt in control and curcumin-treated cultures were determined by Western blotting on the indicated days. (B-E) Chondrogenic progenitors were electroporated with vectors expressing either a myristoylated constitutively active form of Akt (myr-Akt), a dominant inhibitory mutant Akt (mutant-Akt) or an empty vector (mock) using a square wave generator (BTX-830; Gentronics, San Diego, CA) with 20 msec, 200 square pulses. (B) Cells were stained with PNA or Alcian blue on day 3 or 5 of culture, respectively. (C) Percentages of apoptotic cells were quantified by flow cytometric analysis on day 3 of culture. (D) Changes in the levels of pFAK³⁹⁷, FAK, and integrin β 1 were determined by Western blotting. (E) Cells were immunostained for F-actin using Alexa488-phalloidin on 4 days of culture. Notice many rounding cells inside dotted circle in control- or curcumin/myr-Akt-treated culture.

altering actin cytoskeleton reorganization, possibly by down-regulating Akt signaling.

Curcumin induces apoptosis in a variety of cells, including prostate cancer cells (Dorai *et al.*, 2004, Guo *et al.*, 2006). Curcumin is known to induce the up-regulation of pro-apoptotic proteins of the Bcl-2 family and the down-regulation of anti-apoptotic proteins Bcl-2 and Bcl-xL in cancer cells, including U937 cells (Bae *et al.*, 2003), human renal Caki cells (Woo *et al.*, 2003) and DU145 cells (Mukhopadhyay *et al.*, 2001). Consistent with these observations, we also found that curcumin induced apoptosis of chondrogenic progenitors in chick limb buds.

Actin filaments are integral components of the plasma membrane-associated cytoskeleton and are thought to play important roles in the determination of cell polarity, shape and membrane mechanical properties (Swales *et al.*, 2004; Wawro *et al.*, 2007). Chondrogenesis is characterized by dramatic changes in cell shape from fibroblastoid to round or polygonal morphologies (von der Mark and von der Mark, 1977). Undifferentiated chondrogenic progenitor cells are characterized by pronounced fibrillar actin fibers, whereas chondrocytes display a primarily cortical organization of their actin filaments (Idowu *et al.*, 2000; Langelier *et al.*, 2000). Previously, we demonstrated that actin cytoskeleton reorganization is essential for chondrogenic differentiation of chick limb bud mesenchymal cells (Jin *et al.*, 2007). Here, we demonstrate an additional biological property of curcumin, showing that actin stress fibers were markedly intensified by curcumin treatment, which points to a negative regulatory role for curcumin in the reorganization of the actin cytoskeleton.

Although the precise mode of curcumin action is not yet elucidated, a few signaling pathways and molecular targets have been suggested, and the results often appear to be cell-type specific and dose-dependent (Bernt *et al.*, 2006; Lev-Ari *et al.*, 2006). Previously, our laboratory showed that chondrogenic differentiation of chick limb bud mesenchymal cells is regulated by complex protein kinase signaling cascades involving PKC, MAPKs and Akt (Choi *et al.*, 1995; Chang *et al.*, 1998; Oh *et al.*, 1999; Yoon *et al.*, 2000). Curcumin has been shown to inhibit tyrosine kinase activity in AU565 tumor cells (Narasimhan and Ammanamanchi, 2008). Pre-treatment of MCF-7 cells with curcumin resulted in an inhibition of TNF- α -induced JNK activation (Chen and Tan, 1998). In this study, treatment of chondrogenic progenitors with curcumin inhibited Akt phosphorylation, indicating that the Akt pathway contributed to curcumin-modulated chondrogenic differentiation of chick limb bud

mesenchymal cells.

The phosphorylation of Akt is routinely used as an indicator of Akt activation. Suppression of Akt activation promoted apoptotic cell death. It also hindered integrin-mediated signal transduction by the down-regulation of integrin β 1 and lowering the phosphorylation level of FAK, which in turn lead to reorganization of the actin cytoskeleton. However, exactly how curcumin exerts its inhibitory activity on chondrogenesis is still little understood.

It has been reported that a mixture of curcuminoids inhibited the expression of gelatinase B (matrix metalloproteinase-9, MMP-9) in endothelial cells (Mohan *et al.*, 2000). MMP-9 can be regulated via AP-1, PI3K/PKB and GSK-3 β (Troussard *et al.*, 2000; Kim *et al.*, 2001). The first report that curcumin has potential against arthritis was in 1980 (Deodhar *et al.*, 1980). Neutral matrix MMPs responsible for degradation of cartilage in arthritis were inhibited by curcumin (Onodera *et al.*, 2000). Previously, we also demonstrated that Akt signaling is responsible for MMP-2 activation (Jin *et al.*, 2007). Our previous studies indicated a possible linkage between MMP and integrin-mediated migration during curcumin-suppressed chondrogenic differentiation of chick limb bud mesenchymal cells. Our on-going research will test the possible involvements of MMPs in curcumin-suppressed chondrogenesis.

In summary, for the first time, this study describes biological properties of curcumin during chondrogenic differentiation of chick limb bud mesenchymal cells. Curcumin suppressed chondrogenesis by stimulating apoptotic cell death. It also down-regulated integrin β 1 and lowered the phosphorylation level of FAK, which in turn lead to a reorganization of the actin cytoskeleton via modulation of Akt signaling.

Methods

Cell culture and treatment

Mesenchymal cells were derived from the distal tips of wing buds from Hamburger-Hamilton (Hamburger and Hamilton, 1992) stage 22/23 embryos of fertilized white Leghorn chicken eggs. The cells were micromass-cultured as described previously (Jin *et al.*, 2007). Cells at 2×10^7 cells/ml were plated onto 35 or 60 mm dishes in 3 or 19 drops of 15 μ l each. The cells were incubated for 1 h at 37°C under 5% CO₂ to allow attachment, and were then maintained in Ham's F-12 medium containing 10% FBS, 100 IU/ml penicillin and 50 μ g/ml streptomycin (Gibco Invitrogen, Grand Island, NY). The cells were maintained in culture medium for the indicated time periods in the presence or absence of Curcumin (1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) purchased from

Sigma Chemical Company. Control cultures were exposed to solvent only.

Analysis of cell condensation and differentiation

Chondrogenic differentiation was measured by Alcian blue staining of sulfated cartilage glycosaminoglycans (GAG). To demonstrate the deposition of cartilage matrix proteoglycans, representative cultures were collected on day 5 of incubation and stained with 0.5% Alcian blue 8GX, pH 1.0 (Lev and Spicer, 1964; Hassell and Horigan, 1982). Alcian blue bound to sulfated GAG was extracted with 6 M guanidine-HCl, and quantified by measuring the absorbance of the extracts at 600 nm. Binding of peanut agglutinin (PNA) was used as a specific marker for precartilage condensation. Briefly, cultures were rinsed twice with 0.02 M PBS, pH 7.2, fixed in methanol:acetone (1:1) for 1 min, air-dried, and then incubated with 100 µg/ml biotinylated PNA (Sigma) for 1 h. Bound PNA was visualized using the VECTASTAIN ABC and DAB substrate solution kit (Vector laboratories Inc., Burlingame, CA).

Cell proliferation assay

Proliferation of mesenchymal cells was determined by direct counting of cells from micromass cultures. Control and treated cultures were maintained for the indicated number of days, detached with trypsin/EDTA solution and counted in triplicate using a hemacytometer.

Apoptosis assay

Apoptosis was analyzed by flow cytometry (FACS Calibur, Becton-Dickinson, France). To detect the extent of propidium iodide staining, cells were excited at 488 nm and emission was detected at 585 nm.

Western blot analysis

Total protein was prepared using RIPA buffer and Western blot analyses were performed. Thirty µg total protein was loaded and resolved by 10% SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane and probed with the following primary antibodies: (p)Akt, (Cell Signaling Technology Inc., Danvers, MA), type II collagen and FN (Sigma), integrin β1 (BD Science, Franklin Lakes, NJ), (p)FAK³⁹⁷ (Covance, Princeton, NJ). Membranes were washed and incubated with the appropriate HRP-conjugated secondary antibodies (Santa Cruz). Signals were detected using enhanced chemiluminescence (Pierce Biotechnology inc., Rockford, MN). Three independent experiments were performed for all Western blot analyses.

Actin staining

Cells were micromass cultured on cover slips and treated with various chemicals as indicated in the figure legends, washed 3 times with PBS, then fixed and permeabilized as described above. Each culture was then stained with Alexa488-phalloidin (Molecular Probes, Eugene, OR) prepared in PBS containing 1% (v/v) BSA for 1 h at room

temperature in a lightproof box. The cultures were then washed 3 times with water and mounted with Gel/Mount (Biomedica, Foster City, CA). The slides were examined using a confocal microscope (MRC 1024/ES, Bio-Rad Laboratory, CA).

Electroporation

Vectors expressing HA-wild-type Akt1 (wt-Akt) or dominant inhibitory mutant Akt1 (mutant-Akt) were electroporated into isolated mesenchymal cells using a square wave generator (BTX-830; Gentronics, San Diego, CA) with 20 msec, 200 square pulses.

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