Curcumin, a nutritional supplement with antineoplastic activity, enhances leiomyoma cell apoptosis and decreases fibronectin expression

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Objective: To determine if curcumin has an antiproliferative effect on leiomyoma cells via apoptosis induction and whether curcumin impacts extracellular matrix (ECM) production by assessing the fibronectin expression in leiomyoma cells treated with curcumin.

Design: Tissue culture study of immortalized human leiomyoma and patient-matched myometrial cells treated with curcumin.

Setting: University hospital.

Patient(s): Immortalized leiomyoma and myometrial cells from patients with symptomatic leiomyomata.

Intervention(s): Tissue culture, followed by proliferation studies, RNA, and protein analysis.

Main Outcome Measure(s): Cell proliferation, alteration in apoptotic signaling pathways.

Result(s): Curcumin demonstrated an antiproliferative effect on leiomyoma cell lines (IC50 = 20 μM). Importantly, no statistically significant inhibition of growth was observed when patient-matched myometrial cells were exposed to equivalent concentrations of curcumin. Curcumin stimulated caspase-3 and caspase-9 expression while inhibiting extracellular signal-regulated kinase 1 (ERK 1), ERK 2, and nuclear factor kappa B (NF-κB), suggesting regulation of leiomyocyte apoptosis. Finally, curcumin inhibited expression of fibronectin in leiomyoma cells.

Conclusion(s): Our findings demonstrate that curcumin inhibited uterine leiomyoma cell proliferation via regulation of the apoptotic pathway, and inhibited production of the ECM component fibronectin. Curcumin provides a novel direction for leiomyoma therapies. (Fertil Steril 2009;91:2177–84. ©2009 by American Society for Reproductive Medicine.)

Key Words: Leiomyoma, myometrium, curcumin, apoptosis, MAP kinase, NF-κB, fibronectin

Uterine leiomyomas cause significant morbidity, including menorrhagia, pelvic pain, pelvic pressure, infertility, miscarriage, and bladder dysfunction (1–9). The significant symptoms and the progressive nature of the disease frequently require hysterectomy (10), which is associated with surgical morbidity and precludes the opportunity for future childbirth. To avoid the risks of surgical intervention, many novel therapies are currently under investigation. An optimal therapy for these tumors would be a medication that has demonstrated efficacy and proven safety.

We have previously reported that leiomyomas are a disease of excessive and dysregulated extracellular matrix (ECM) production (11–12). We have further hypothesized that leiomyomas may develop from a disorder in wound healing, similar to that seen in keloid formation (11, 13). The bulk of leiomyoma tumors are made up of ECM. Any effective therapy therefore must regulate both leiomyocyte proliferation and ECM production.

Curcumin has been used for thousands of years as a food additive, in doses as high as 100 mg/day, and as a medicine (14). Curcumin is the active component found in dried ground rhizomes of Curcuma longa. As a dietary spice, curcumin has a safety profile well superior to those currently used for cancer therapy. Furthermore, curcumin both enhances wound healing (15, 16) and inhibits fibrosis (17–19).

With regard to regulation of cell proliferation, curcumin acts through many signaling pathways to regulate cell growth of various cancers (20). For example, curcumin suppresses activation of nuclear factor kappa B (NF-κB), activator protein 1 (AP-1), and signal transducer and activator of
transcription 3 (STAT3) and STAT5, and modulates early growth response protein 1 (Egr-1), peroxisome proliferator-activated receptor gamma (PPAR-γ), β-catenin, and nuclear erythroid 2 p45-related factor 2 (Nrf2) (21). It also down-regulates B-cell lymphoma 2 (Bcl-2), Bcl-XL, cyclooxygenase 2 (COX-2), matrix metalloproteinase 9 (MMP9), tumor necrosis factor (TNF), and cyclin D1 (22). Through these pathways, curcumin inhibits cell proliferation and induces apoptosis in a broad array of abnormal cells.

Curcumin can regulate cell proliferation and fibrosis in various cancers. This compound could therefore have activity in uterine leiomyomas. In this study, we hypothesized that curcumin will have antiproliferative effect on leiomyoma cells via an apoptotic pathway and that curcumin could regulate abnormal extracellular matrix production by leiomyocytes.

MATERIALS AND METHODS

Tissue Culture

Immortalized leiomyoma and patient-matched myometrial cells (23) were plated in 96-well plates and allowed to grow to 50% confluency before being exposed to concentrations of curcumin ranging from 5 to 40 μM. The plates were collected every 24 hours for up to 96 hours. The proliferation of the cells was measured using sulforhodamine-B method (Sigma-Aldrich, St. Louis, MO) as described previously elsewhere (24). The development of the cell lines was done under an institutional review board–approved protocol.

Fluorochrome Inhibitor of Caspases (FLICA) Assay

Immortalized human leiomyoma and patient-matched myometrial cells were plated on chamber slides and allowed to grow to 60% confluency before treatment with 10 μM, 20 μM, and 40 μM curcumin. The cells were treated with fluorescent-labeled inhibitor, FAM-VAD-FMK that binds to activated caspases, according to the manufacturers’ protocol (Apoptosis detection kit; Immunochemistry Technologies, Bloomington, MN). Briefly, immortalized cells were grown to 60% confluence on glass chamber slides (Nalge Nunc International, Rochester, NY) before treatment with different concentrations of curcumin for 24 hours and 48 hours. At the end of treatment time, 30X fluorochrome inhibitor of caspases (FLICA) was added to the medium at a 1:30 ratio, and cells were incubated for 1 hour in the cell culture incubator. The cells were washed with 1X wash solution and fixed. Green fluorescence indicated caspase-positive cells under fluorescence microscopy (excitation 450 nm, emission 520 nm).

Western Blot

Protein was isolated using radioimmunoprecipitation assay (RIPA) lysis and extraction buffer (Pierce Biotechnology, Rockford, IL) containing 1X Halt protease inhibitor (Pierce Biotechnology). Aliquots of the proteins extracted from cultured cells treated with 10 μM, 20 μM, and 40 μM curcumin for 24 hours underwent electrophoreses on a sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The proteins were electrotransferred onto nitrocellulose membranes (Invitrogen, Carlsbad, CA). The methodology has been described previously elsewhere (25). For the detection of apoptosis-related proteins, blots were incubated with mouse monoclonal antibody against caspase-3 (sc-7272; dilution 1:200), caspase-9 (sc-17784; dilution 1:200), rabbit polyclonal antibody against extracellular signal-regulated kinase 1 and 2 (ERK1/ERK2) (sc-93; dilution 1:200), NF-κB p50 (sc-7178; dilution 1:200), NF-κB p65 (sc-109; dilution 1:200). All antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase (HRP)–conjugated secondary antibody (ImmunoPure; Pierce Biotechnology) in combination with the SuperSignal West Pico (Pierce Biotechnology) was used for detection of the proteins. As an internal standard between the samples, HRP labeled anti-human β-actin (sc-1616; Santa Cruz Biotechnology) was used.

Quantitative Reverse Transcriptase Polymerase Chain Reaction Analysis

We used the real-time reverse transcriptase polymerase chain reaction (RT-PCR) method to evaluate gene expression of fibronectin, as described previously elsewhere (25, 26). We used 18S ribosomal RNA gene as an internal control, and each sample was analyzed in triplicate. Bio-Rad iCycler software, version 3.1 (Bio-Rad Laboratories, Hercules, CA) was used for data analysis.

Statistical Analysis

The results are reported as mean ± standard error of the mean (SEM). All experiments were done in triplicate. For real-time RT-PCR data, relative quantification was done with normalization with 18S gene. Relative expression was calculated based on Pfaffl method (27). Wilcoxon-signed rank test was used for nonparametric statistical evaluation. In Western blots, the densities of protein bands were analyzed using Quantity-1 software (Bio-Rad Laboratories). The data is represented as the fold difference between control and treated samples after normalization against protein loading internal control β-actin.

RESULTS

To assess the influence of curcumin exposure on human immortalized leiomyoma and myometrial cell lines, we performed proliferation studies at curcumin concentrations between 5 and 40 μM (Fig. 1). We found that curcumin treatment resulted in a statistically significant reduction in leiomyoma cell concentrations at curcumin concentrations as low as 5 μM; however, at concentrations at or above 20 μM, cell concentrations of both cell lines was inhibited. These results demonstrate that curcumin can inhibit...
leiomyoma cell numbers at concentrations that have insignificant impact on patient-matched myometrial cell proliferation.

Prior studies demonstrated that curcumin can induce apoptosis in various cell types (22), and leiomyoma development may result from disruption of apoptosis (28), which would result in an excess of leiomyocytes that continue to produce disrupted ECM. We evaluated the role of curcumin on the induction of apoptosis in human leiomyoma cells by assessing the expression of caspase-3 and caspase-9 with curcumin treatment (Figs. 2 and 3). Caspase-3 is frequently activated in the protease cascade that results in apoptosis in many mammalian tissues (29), and caspase-9 is a well-described initiator of apoptosis (30). Using the FLICA assay, we found a dramatic increase in total caspase activity in human leiomyoma cells when treated with curcumin (Fig. 2). Furthermore, leiomyoma cells demonstrated a concentration-dependent increase in caspase-3 expression with curcumin treatment (Fig. 3A), with an approximately 2.5-fold increased amount of caspase-3 in leiomyoma cells treated with 40 μM curcumin. Comparatively, 40 μM curcumin in myometrial cells increased caspase-3 only 1.19 ± 0.22-fold, suggesting that apoptosis involving caspase-3 was not induced by curcumin in myometrial cells at any of the curcumin concentrations tested. When we evaluated caspase-9, we similarly found little stimulation in myometrial cells (data not shown), while curcumin stimulated caspase-9 in leiomyoma cells in concentration-dependent manner (Fig. 3B). These findings demonstrated that curcumin selectively activated caspase activity in leiomyoma cells.

In further support of a proapoptotic mechanism for curcumin, we evaluated the impact of curcumin on the mitogen-activated protein (MAP) kinase pathway in human leiomyoma cells (Fig. 3C, D). Treatment of leiomyoma cells with curcumin resulted in a concentration-dependent decrease in ERK1 and ERK2 proteins that are regulated by MAP-kinase (Fig. 3C). At the 40 μM curcumin concentration, there was a 3.6-fold lower amount of total ERK1 and ERK2 protein (44 kd and 42 kd) in treated leiomyoma cells relative to untreated cells. As ERK activity is known to be involved in transcriptional regulation of activating transcription factor 3 (ATF3) gene through inhibition of TNF-α–mediated induction of ATF3 (31), we hypothesized that ATF3 activity would increase with curcumin treatment. We demonstrated a 1.45-fold increase in total ATF3 amount in leiomyoma cells at 10 μM curcumin concentration with no further statistically significant increase at higher concentrations (Fig. 3D).

The proapoptotic action of curcumin is also supported by its ability to block the activity of transcription factor NF-κB (32). Increased expression of this transcription factor is associated with increased survival of many tumor cells, while inhibition often results in decreased cellular proliferation and increased apoptosis. Use of curcumin inhibits TNF-α–induced NF-κB activation in human myelomonoblastic leukemia cells (32) and cytokine-mediated NF-κB activation in colonic epithelial cells (33). We therefore hypothesized that the NF-κB pathway would be altered in human leiomyoma cells. Indeed, treatment of leiomyoma cells with curcumin resulted in a decrease in both NF-κB p50 and p65 in a concentration-dependent fashion (Fig. 3E). On treatment with 10 μM curcumin, a 1.14-fold lower amount of NF-κB p50 was observed, which further decreased to 1.57-fold at the 40 μM curcumin concentration (Fig. 3F). We found that NF-κBp65 demonstrated a 4.7-fold decreased amount at the 40 μM curcumin concentration (Fig. 3F).

Leiomyomas are characterized by excessive ECM production. Fibronectin is an ECM protein that is highly expressed in leiomyomas as compared with myometrium (23). In this study, we demonstrated that curcumin down-regulated expression of fibronectin in leiomyomas as well as myometrium in a concentration-dependent manner. At 40 μM curcumin concentration, fourfold down-regulation of the fibronectin gene expression was observed in leiomyoma cells (Fig. 4).

**DISCUSSION**

Our results demonstrate that curcumin inhibited leiomyoma cell proliferation and stimulated proapoptotic genes at concentrations that do not significantly impact patient-matched normal myometrial cells. Leiomyoma cell proliferation was
inhibited by 38% at 5 μM curcumin, and there was less than 10% inhibition in myometrial cells at this concentration. Inhibition of cellular proliferation in presence of external compound can be caused either through cytostatic effect on cells (34) or induction of the apoptotic pathway.

The mechanism by which curcumin can induce apoptosis in a wide array of cells is varied, including activation of caspase-3 (35–37), the fas receptor/caspase 8 pathway (38), AP-1/NF-κB activity (39, 40), the ERK/MAP-kinase pathway (41), and the Akt dephosphorylation, Bcl-2, Bcl-XL pathway (42). Marin et al. (39) demonstrated that curcumin selectively induces apoptosis of melanoma cells but not normal melanocytes by inhibiting NF-κB activity and the expression of its downstream target genes. It has also been demonstrated that curcumin can inhibit proteasome activity in mice, potentially leading to induction of apoptosis through caspase-9 activation (43). As curcumin is known to cause apoptosis in a large number of cancer cells through various pathways (21, 41, 44), we were interested in understanding its effect on apoptotic pathways in benign tumors. In our studies, leiomyoma cell inhibition correlated with induction of caspase-3 and caspase-9, and a reduction in ERK1, ERK2, and NF-κB p50 and p65 subunits. Taken together, these results suggest that curcumin induces apoptosis in leiomyoma cells through the MAP kinase and NF-κB pathways.

Curcumin has been used for centuries both as a medicinal and dietary spice. Given curcumin’s low side-effect profile, it has been evaluated as a therapeutic agent in a wide array of diseases with demonstrated efficacy (20). As a cancer therapy agent, curcumin has molecular function at the transcriptional level (NF-κB, STAT, AP-1, PPAR-γ, Egr-1, β-catenin, and Nrf-2 signaling pathways), and has demonstrated efficacy as an inducer of apoptosis in multiple human cancers including skin (45), leukemia (46), colon (47), liver (48), breast (49), lymphoma (50), neuroblastoma (51), pancreatic (52), lung (52), endometrial (53), and ovarian (54). In human leiomyomas, there is evidence of a disruption in apoptosis, resulting in tumor growth (28); and various potential leiomyoma therapies, including gonadotropin-releasing hormone (GnRH) agonists (55), CDB-2914 (56), cetorelix (57), asoprisnil (58), genestein/TKS050 (59), 2-methoxyestradiol (60), raloxifene (61), and ciglitizone (62), have demonstrated efficacy by regulating apoptosis in leiomyomas. Our results demonstrate that curcumin stimulated a metabolic profile compatible with the induction of apoptosis in human leiomyoma cells.

Although regulation of leiomyocyte proliferation is critical to controlling leiomyoma development, the bulk of the tumor is made up of excessive and disorganized extracellular matrix rather than cells. As a result, any effective therapy for leiomyomas must also regulate the production of extracellular matrix components. Previously, we demonstrated that fibronectin template is elevated in human leiomyoma cells compared with myometrial cells (23). In this study, we have found that curcumin at concentrations of 10 μM or greater inhibits the production of fibronectin template relative to untreated leiomyoma cells. These findings provide the groundwork to support evaluation of curcumin for global regulation of the disrupted extracellular matrix found in leiomyomas.

Fibronectin template and protein are elevated in leiomyomas compared with myometrium, both in tissue and in culture (23, 63). In smooth muscle cells, fibronectin is involved in cell
Expression of proapoptotic proteins in leiomyoma cells exposed to different concentrations of curcumin for 24 hours, as analyzed by Western blot analysis. (A) Caspase-3 expression was up-regulated in leiomyoma cells in a concentration-dependent manner, demonstrating a 2.5-fold increase at 40 μM curcumin concentration. (B) Caspase-9 expression in leiomyoma cells was elevated above baseline at all concentrations tested, demonstrating a 1.8-fold higher amount of protein at 40 μM curcumin concentration. The results for both caspase-3 and caspase-9 represent an average of two separate experiments. (C) Western blot of ERK1 and ERK2 in leiomyoma cells treated with various concentrations of curcumin. Both ERK1 and ERK2 demonstrated a concentration-dependent decreased expression in leiomyoma cells when exposed to curcumin. (D) ATF-3 protein increased in leiomyoma cells exposed to curcumin. Curcumin stimulated ATF-3 protein expression at 10 μM, with no additional ATF-3 protein production at higher curcumin concentrations. (E) NF-κB p50 and p65 proteins demonstrated decreasing total amount in leiomyoma cells with increasing curcumin concentrations, as seen by Western blot analysis. (F) Further analysis of the blots demonstrated that NF-κB p65 (blue bars) was down-regulated to 4.7-fold decreased amount at 40 μM compared with 1.57-fold decrease in total p50 protein amount (yellow bars). *P < .05 relative to untreated cells.

Our studies have demonstrated that curcumin can preferentially inhibit human leiomyoma cell proliferation at concentrations that do not significantly impact patient-matched myometrial cell proliferation. This impact likely occurs by regulating apoptosis, as confirmed by the curcumin-induced increases in caspase-3 and caspase-9 as well as reductions in ERK 1, ERK 2, and NF-κB p50 and p65 subunits. Finally, curcumin also inhibited fibronectin template production, which is otherwise increased in human leiomyoma cells in the absence of treatment relative to patient-matched myometrial cells (23). These studies provide the groundwork for the investigation of curcumin in clinical trials as a potential leiomyoma therapeutic agent.

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