RESEARCH COMMUNICATION

Antifibrotic Effect of Curcumin in TGF-β1-Induced Myofibroblasts from Human Oral Mucosa

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Abstract

Background: Myofibroblasts play an important role in the development of oral submucous fibrosis (OSF). In the current study, we investigate the effect of curcumin on growth and apoptosis of myofibroblasts derived from human oral mucosa. **Methods**: Myofibroblasts were generated by incubating fibroblasts, obtained from human oral mucosa, with transforming growth factor-β1 (TGF-β1). MTT, PI staining, and FACS assays were used to investigate curcumin's effect on proliferation and cell cycle of fibroblasts and myofibroblasts. Annexin V/PI binding and FACS assays were used to examine apoptosis of myofibroblasts, Western blotting to determine the levels of Bcl-2 and Bax, and enzyme-linked immunosorbant assay was employed to examine the levels of collagen type I and III in the supernatants of myofibroblasts. **Results**: Curcumin inhibits proliferation of fibroblasts and myofibroblasts; it also disturbs the cell cycle, induces apoptosis and decreases the generation of collagen type I and III in myofibroblasts, which are more sensitive to its effects than fibroblasts. Curcumin induces apoptosis in myofibroblasts by down-regulating the Bcl-2/ Bax ratio. **Conclusion**: Our results demonstrate the antifibrotic effect of curcumin *in vitro*. It may therefore be a candidate for the treatment of OSF.

Keywords: Curcumin - oral submucous fibrosis - myofibroblast - apoptosis - cell cycle

Introduction

Oral submucous fibrosis (OSF) is a chronic inflammatory disease, and has been defined by WHO as one of the precancerous conditions (International Agency for Research on Cancer, 2005). The main clinical symptoms include the stiffness of oral mucosa, restriction of mouth opening, atrophy of tongue papillae, blisters, and intolerance to hot and spicy food. Malignant transformation rate in OSF is high, reaching 7-13% (Tilakaratne et al., 2006). The disease exhibits characteristic histological features of excessive collagen deposition in the lamina propria, following the epithelium becomes atrophic (Singh et al., 2010).

OSF is closely associated with betel quid (BQ) chewing, a habit common in South and Southeast Asia, some parts of Africa, and among immigrants from these regions (Boucher and Mannan, 2002). An imbalance in collagen synthesis and degradation in oral mucosa is generally believed to be the main cause of OSF;The disease is considered a type of collagen metabolism disorder (Rajalalitha and Vali, 2005).

Myofibroblasts are typically activated fibroblasts, although they can also be derived from other cell types, including epithelial and endothelial cells via epithelial/endothelial mesenchymal transition (EMT/EndMT) process, as well as from the circulating fibroblast-like cells called fibrocytes, originating from bone marrow (Hinz et al., 2007). Myofibroblasts can be identified by certain features associated with the cytoskeleton, particularly by the expression of α-smooth muscle actin. They can synthesize collagen, the main component of extracellular matrix (ECM), and generate a variety of fibrogenic factors, such as TGF-β1 and TNF-α. A growing number of studies suggest that myofibroblasts are the major effector cells in OSF (Angadi et al., 2011).

Many drugs as well as surgical intervention and physical therapy have been used in OSF's therapy but a definitive, effective treatment is still elusive (Fedorowicz et al., 2008; Jiang and Hu, 2009; Kerr et al., 2011). Curcumin, 1,7-bis (4-hydroxy-3-methoxyphenol)-1,6-heptadiene-3,5-dione, is the primary active substance isolated from *Curcuma Longa* L. rhizome. It is inexpensive, widely available and has almost no side effects; it has been long used as a spice and pigment in food processing industry. Curcumin has some important biological properties such as anti-inflammatory, antioxidant and antitumoractivity (Goel et al., 2008). Recently, many studies have reported curcumin's role in the prevention and reduction of fibrosis caused by harmful factors (Venkatesan et al., 2007; Osawa, 2007).

In our study, we incubate TGF-β1-induced human oral mucosal myofibroblasts with curcumin to explore its possible application in the treatment of OSF. We

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report curcumin's effect on the proliferation, cell cycle, apoptosis, the expression levels of Bcl-2 and Bax, and extracellular collagen synthesis.

Materials and Methods

Chemicals and Reagents

Curcumin, 3- (4, 5-dimethylthiazol-2-yl) -2, 5-diphenyl tetrazolium bromide (MTT) and propidium iodide (PI) were purchased from Sigma (St. Louis, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and other cell culture biologicals were purchased from Gibco (NY, USA). Recombinant human TGF-β1 was obtained from Peprotech (Rocky Hill, NJ). α-SMA polyclonal antibody was purchased from Proteinteck (Chicago, USA), and Bcl-2 polyclonal antibody was obtained from Abcam (MA, USA).Vimentin monoclonal antibody, cytokeratin (CK)14 monoclonal antibody, and Bax monoclonal antibody were purchased from Santa Cruz Biotechnology (CA, USA). Annexin V-FITC/PI kit was from BD Biosciences (CA, USA). Human Collagen type I and III ELISA kits were obtained from Zhongshan Goldenbridge (Beijing, China). All other reagents were of analytical grade.

Cell culture

Fibroblasts were obtained from the fragment of the oral mucosa of three patients, aged 1-3 years, undergoing the cheiloplasty. Informed consent, as required by the local ethics committee, was obtained from their parents.

The tissues were washed three times with phosphate-buffered saline (PBS), cut into pieces of 1-2 mm^3, and placed in tissue culture dishes. The explants were incubated in DMEM containing 20% FBS at 37°C in a humidified atmosphere of 5% carbon dioxide in air. When outgrowth was observed in the cultures, the medium was replaced twice a week until the cells reached 70-80% confluence. The cells were then detached by brief treatment with 0.1% trypsin and re-cultured in DMEM containing 10% FBS until confluent monolayers were again obtained. Cells between the fourth and sixth passages were used in the experiments.

Immunocytochemistry

We used immunocytochemical staining to identify the phenotype of the primary cultured cells. The third passage cells were fixed with 4% paraformaldehyde, incubated with primary antibody against vimentin and CK 14 (1:100) overnight at 4°C, and then incubated with corresponding secondary antibody for 30min. Visualization was performed using freshly prepared diamino benzidine tetrahydrochloride (DAB).

The cells collected between the fourth and sixth passage were pretreated with 10% FBS-DMEM containing 10ng/ml TGF-β1 for 48h.The expression of α-SMA was detected by immunocytochemical staining, as described above.

MTT assay

Fibroblasts and myofibroblasts (approximately 5×10^3 cells /well) were seeded in 96-well culture plates. After12

h, the culture medium was changed and then various amounts of curcumin (final concentrations of 0, 2.5, 5, 10, 20 and 40μmol/L) were added. Only dimethyl sulfoxide (DMSO) was added to control wells. Cells were further incubated for12 or 24 h. Next, MTT was added to each well and the plates were incubated for 4h at 37°C in the dark. The culture medium was decanted. To estimate cell viability, the formazan generated by viable cells was dissolved in DMSO and read against reagent blank (DMSO), at a wavelength of 540 nm, usinga microplate reader. The inhibition of cell growth was calculated according to the equation: growth inhibitory rate = [1-Ab_{540} treated/Ab_{540} control]× 100%.

Flow cytometric analysis of cell cycle distribution and apoptosis

Fibroblasts and myofibroblasts (approximately 1×10^5 cells/well) were incubated in 12-well plates with 0, 2.5, 5, 10, 20 or 40μmol/L of curcumin for 12h. After the treatment, the cells were fixed, permeabilized, and stained with PI in the dark. Cellular DNA content was measured using a FACS Calibur flow cytometer (Beckman Coulter, CA, USA) to evaluate cell cycle distribution. A total of 10,000 events were determined for each sample.

We performed double staining with Annexin V-FITC and PI to detect apoptotic myofibroblasts. After treatment with 0, 10, 20 or 40μmol/L of curcumin for 24h, the cells were collected and washed with PBS. The cells were stained with Annexin V-FITC/PI in the dark, according to the instructions provided by the manufacturer. Stained cells were analyzed using the flow cytometer. Early apoptotic cells were defined as positive for Annexin V-FITC but negative for PI staining.

All experiments were repeated at least 3 times.

Western blotting analyses for Bcl-2 and Bax

After treatment with 0, 10, 20 or 40μmol/L of curcumin for 24h, myofibroblasts were harvested and washed with ice-cold PBS. Whole-cell protein extracts were prepared. SDS-PAGE with a 10% resolving gel was used to separate the proteins (30μg/lane). The separated proteins were transferred onto PVDF membranes. After blocking in 5% fat-free milk for 1h at room temperature, membranes were incubated with primary antibodies (antibodies against Bcl-2 and Bax, 1: 1000), followed by appropriate secondary antibodies. Immunoreactive proteins were visualized using an enhanced chemiluminescent detection system (Amersham Biosciences; Piscataway, NJ, USA), then the membranes were exposed to X-ray film. After scanning the films, the intensities were determined densitometrically using QuantityOne image software (Bio-Rad).

Enzyme-linked immunosorbant assay (ELISA) analyses for collagen type I and III

Myofibroblasts (approximately 5×10^4 cells/well) were incubated in 24-well plates with 0, 2.5, 5, 10, 20 or 40μmol/L of curcumin for 12, 24 and 48h, and then the supernatants were collected. The expression levels of collagen type I and III in the supernatants were measured usinga commercially available ELISA kit, following the manufacturer's instructions.
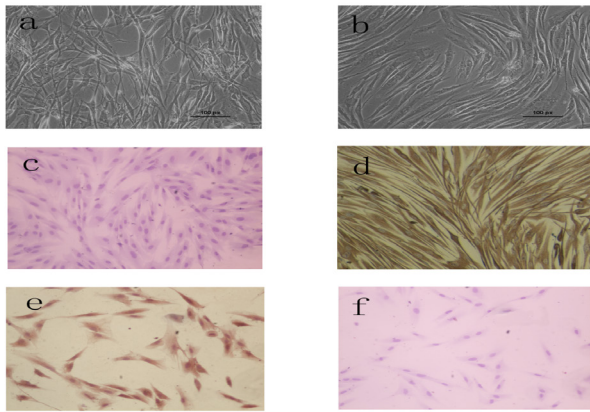
Figure 1. The Morphology of The Cells Cultured from Oral Mucosa. Cells are spindle-shaped (a) After treatment with 10ng/ml TGF-β1 for 48h, cells become wider (b) The third passage cells express vimentin (c) but not CK 14 (d). After pretreatment with TGF-β1, over 95% of the cells express α-SMA (e) in comparison with control (f) Original magnification 100×
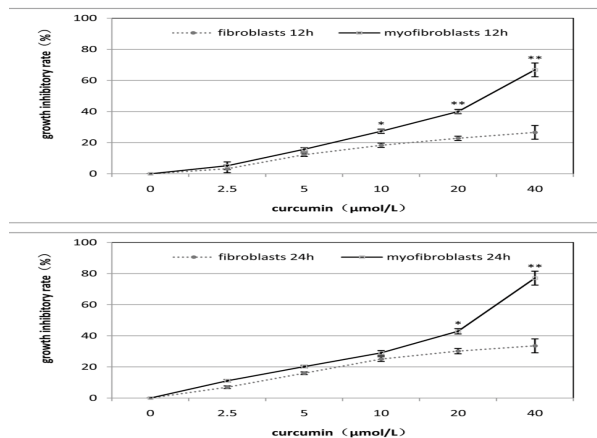

Figure 2. Curcumin Inhibits Fibroblasts and Myofibroblasts Proliferation. Fibroblasts and myofibroblasts were treated with different concentrations of curcumin (2.5, 5, 10, 20 or 40μmol/L) for 12 h (a) and 24 h (b) and were subjected to MTT assay. Curcumin inhibits fibroblasts and myofibroblasts proliferation in a dose-dependent manner; the effect is stronger for myofibroblasts. The results are expressed in terms of the growth inhibition rate. Each point represents the mean±SEM of three independent experiments. *P<0.05, **P<0.01 relative to fibroblasts

Statistical analysis

Data are presented as the mean±standard error of the mean (SEM) of three or more independent experiments. The results were analyzed by one-way analysis of variance (ANOVA) and post hoc Tukey's test. Statistical significance was set at p <0.05.

Results

Cell morphology and immunocytochemistry

The cultured cells derived from the oral mucosa have spindle-shape morphology (Figure 1a). After pretreatment with 10ng/ml TGF-β1 for 48h, they become wider (Figure 1b). The phenotypes of the cells were identified by immunocytochemistry. The third passage cells are positive for vimentin (Figure 1c) and negative for CK 14 (Figure 1d). After pretreatment with TGF-β1, over 95% of the cells expressed α-SMA (Figure 1e and f), confirming the
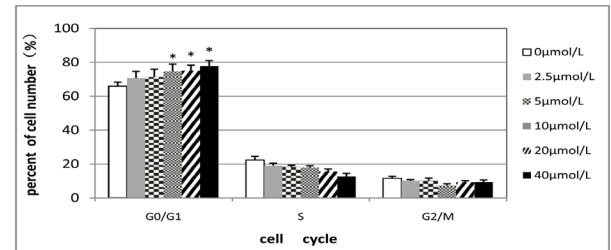

Figure 3. Cell Cycle Distribution of Curcumin-treated Myofibroblasts. Results represent the mean±SEM of three independent experiments. *P<0.05 relative to untreated cells
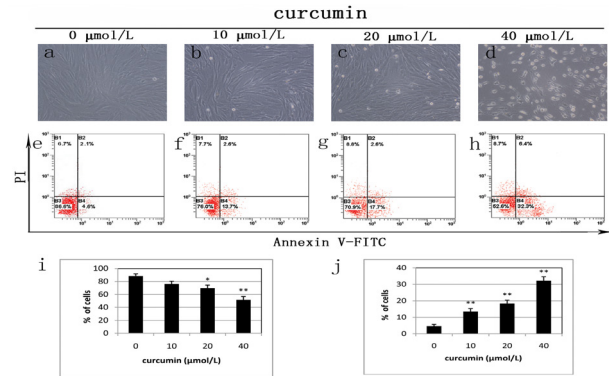

Figure 4. Curcumin Induces Myofibroblast Apoptosis. The morphology of myofibroblasts (a–d). Myofibroblasts were not treated (a) or treated with 10 (b), 20 (c) or 40μmol/L (d) of curcumin for 24 h. Images were taken using a light microscope and a digital camera. Original magnification 100×. FACS analysis of myofibroblasts after curcumin treatment (e–h). Myofibroblasts were treated for24h with the indicated doses of curcumin. Afterwards, all cells were collected and stained with Annexin V-FITC and PI. Staining intensity was determined for 10,000 cells using flow cytometry and subsequent quadrant analysis. The mean ±SEM are shown for the living (i) and early apoptotic (j) cell fractions from 3 independent experiments. *P < 0.05, **P < 0.01 compared to untreated

transformation into myofibroblasts.

Curcumin inhibits cell proliferation in fibroblasts and myofibroblasts

MTT assay revealed that curcumin treatment significantly decreases the proliferation of fibroblasts and myofibroblasts, in a dose-dependent manner. This effect is more pronounced in myofibroblasts; the growth inhibitory rate for myofibroblasts incubated with curcumin was double of that for the similarly treated fibroblasts (Figure 2).

Curcumin induces cell cycle arrest in myofibroblasts

Cell cycle analysis shows that curcumin treatment results in a dose-dependent increase in the proportion of myofibroblast cells in G0/G1 phase (Figure 3). The cell cycle of fibroblasts didn't change significantly (data not shown). An approximate 20% elevation in the G0/G1 phase population was seen in myofibroblasts after treatment with 40μmol/L curcumin. These data show that curcumin causes a G0/G1 cell cycle arrest in myofibroblasts.

Curcumin induces cell apoptosis in myofibroblasts

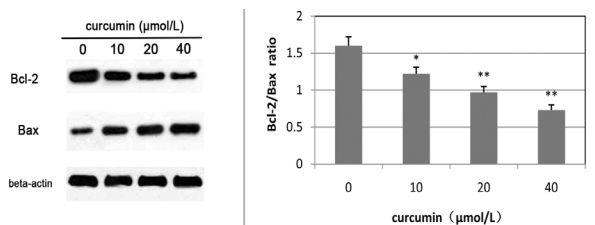We investigated the effect of different doses of

Figure 5. Western Blot Analysis of Bcl-2 and Bax Expression in Curcumin-treated Myofibroblasts. Curcumin treatment decreased Bcl-2 expression and increased Bax expression in a dose-dependent manner. Blots representative of three independent experiments are shown in the left panel. Bar graphs (right panel) depict Bcl-2/Bax ratio analysis of the Western blots. *P <0.05, **P < 0.01 compared to untreated
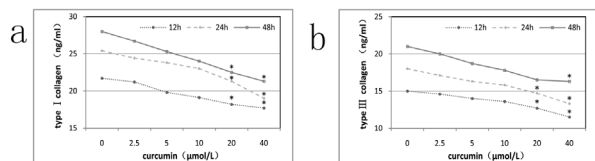

Figure 6. ELISA Analysis of TypeI (a) and III (b) Collagen in the Supernatants of Myofibroblasts Incubated with 0, 2.5, 5, 10, 20 or 40μmol/L of Curcumin for12 h, 24 h and 48 h. Curcumin decrease sthe expression levels of type I and type III collagen in a dose-time-dependent manner.*P < 0.05compared to untreated

curcumin on myofibroblasts morphology. Treatment with 10, 20 and 40μmol/L of curcumin for 24 h causes an increase in the number of floating and rounded cells (Figure 4a, b, c and d), showing that curcumin increases death rate in myofibroblasts. We next investigated whether the curcumin-induced cell death was a result of myofibroblasts necrosis or apoptosis. We show that the treatment with 40μmol/L of curcumin for 24h results in increased Annexin-V-FITC staining. The percentage of living cells decreased significantly from 88% in the untreated group to 60% after curcumin treatment (P < 0.01) (Figure 4i). Moreover, 10-40μmol/L of curcumin caused a significant increase in the percentage of early apoptotic cells (Figure 4j). Only a very small percentage stained positive for PI, indicating that most cells were not necrotic (data not shown). Thus, we demonstrate that curcumin induces myofibroblasts apoptosis in a dose-independent manner.

Curcumin decreases the Bcl-2/Bax ratio in myofibroblasts

The expression levels of Bcl-2 and Bax were analyzed by Western blotting. As illustrated in Figure 5a, curcumin treatment diminished Bcl-2 expression and increased Bax expression. The Bcl-2/Bax ratio was significantly decreased in curcumin-treated myofibroblasts compared to untreated cells (P<0.01) (Figure 5b).

Curcumin decreases the generation of type I and type III collagen in myofibroblasts

Using ELISA, we investigated the content of type I and III collagen in the supernatants of myofibroblasts incubated with curcumin. The expression levels of both type I and type III collagen decreased in a dose-time-dependent manner after curcumin treatment (Figure 6).

Discussion

Myofibroblasts, typically considered to be activated fibroblasts, play an important role in morphogenesis, oncogenesis, inflammation, wound healing and fibrosis in most organs and tissues (Watsky et al., 2010). Myofibroblast persistence is a key feature of fibrotic diseases including OSF, scleroderma, and hepatic, pancreatic, and pulmonary fibrosis(Gabbiani, 2003; Angadi et al., 2011).

Myofibroblasts can be detected in the OSF-affected tissues; this phenomenon is related to the severity of OSF (Angadi et al., 2011). Myofibroblasts not only synthesize collagen, but also produce numerous inflammatory mediators, chemokines, and growth factors (Powell et al., 1999), intensifying and prolonging the inflammation in OSF by activating the inflammatory corpuscles. This self-excitation of inflammation increases the expression of fibrogenic cytokines such as TGF-β1, and enhances fibrosis. The possibility of inhibiting proliferation and inducing apoptosis in myofibroblasts offers a new, promising therapy line in the treatment of OSF.

The polyphenol curcumin has been shown to be an effective treatment for liver and lung fibrosis both in vivo and in vitro (Bruck et al., 2007; Venkatesan et al., 2007; Rivera-Espinoza and Muriel, 2009). It prevents fibrosis by blocking leukocyte influx, inhibiting the activation of inflammatory cells and subsequent release of pro-inflammatories. Curcumin also induces apoptosis of lung fibrosis and hepatic stellate cells, attenuating accumulation of ECM (Bruck et al., 2007; Venkatesan et al., 2007; Lin et al., 2009). However, up to date, there have been no reports on the effect of curcumin on myofibroblasts of human oral mucosa.

We obtained myofibroblasts by incubation of cultured human oral mucosal fibroblasts with TGF-β1. MTT and cell cycle analysis show that curcumin inhibits the proliferation of fibroblasts and myofibroblasts in a dose-dependent manner, and the effect is stronger in myofibroblasts. Curcumin also causes G0/G1 cell cycle arrest in myofibroblasts, confirming their higher sensitivity to that chemical.

Control of the cell cycle is accomplished via the coordinated interaction of cyclins with their respective cyclin-dependent kinases (CDKs) to form active complexes and drive cells into the next phase at the appropriate time (Elledge, 1996). Some studies show that curcumin affects the expressin of cyclinD1, CDK4, CDK6 and p53, p21, p16 which are related to the regulation of cell cycle (Chen and Xu, 2005; Choudhuri et al., 2005; Divya and Pillai, 2006; Puliyappadamba et al., 2010).

Disordering the cell cycle may result in genomic instability and apoptosis. Studies of human gingival fibroblasts, lung fibroblasts, hepatic stellate cells, synoviocytes and cell lines from various malignant tumors demonstrated that curcumin inhibits cell proliferation and induces apoptosis (Jiang et al., 1996; Atsumi et al., 2006; Chen et al., 2006; Divya and Pillai, 2006; Jackson et al., 2006; Lin et al., 2009; Montopoli et al., 2009; Smith et al., 2010; Wu et al., 2010; Liu et al., 2011). The mechanisms implicated in curcumin-induced apoptosis appear to be

multifactorial, including effect on the stability of p53, the release of cytochrome c from mitochondria, and the generation of ROS (Strimpakos and Sharma, 2008).

Bcl-2 family proteins are important regulators of apoptosis. The family comprises both anti-apoptotic (e.g., Bcl-2) and pro-apoptotic proteins (e.g., Bax) with opposing biological functions (Miyashita et al., 1994; Miyashita and Reed, 1995). We show that after curcumin treatment of myofibroblasts, Bcl-2 is down-regulated whereas Bax is up-regulated, and the Bcl-2/Bax ratio decreases. These changes might tip the balance between pro- and anti-apoptotic BCL-2 family members in mitochondrial outer membranes towards apoptosis, thus causing the leakage of cytochrome c into the cytosol. Cytosolic cytochrome c would then activate caspase-9 which trans-activates caspase-3, and apoptotic cell death would follow. We can postulate that curcumin, by decreasing Bcl-2/Bax ratio, can induce apoptosis of myofibroblasts through such mitochondrial pathway.

Type I and type III collagens are the main components of ECM (Utsunomiya et al., 2005). A reduction in the deposition of ECM is of great importance in the treatment of OSF. By inhibiting the proliferation and inducing apoptosis of myofibroblasts, curcumin effectively decreases the generation of type I and III collagens, confirming its potential therapeutic value in OSF treatment.

To summarize, we show that curcumin inhibits proliferation, disrupts the cell cycle, induces apoptosis, and decreases the expression levels of type I and III collagen; our results clearly demonstrate curcumin's antifibrotic potential in vitro. This effect remains to be investigated in animal models of OSF, the task already incorporated into our future research plans.

The FDA has approved curcumin as "generally regarded as safe"; It is widely used in food industry as a spice and coloring agent. The safety of curcumin, combined with its potential efficacy and low cost, makes it an ideal therapeutic drug for OSF, a disease prevalent mainly in the developing countries.

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