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Research Article

Curcumin induces differential expression of cytoprotective enzymes but similar apoptotic responses in fibroblasts and myofibroblasts

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ABSTRACT

Excessive extracellular matrix (ECM) deposition and tissue contraction after injury can lead to esthetic and functional problems. Fibroblasts and myofibroblasts activated by transforming growth factor (TGF)-β1 play a key role in these processes. The persistence of (myo)fibroblasts and their excessive ECM production and continuous wound contraction have been linked to pathological scarring. The identification of compounds reducing myofibroblast survival and function may thus offer promising therapeutic strategies to optimize impaired wound healing. The plant-derived polyphenol curcumin has shown promising results as a wound healing therapeutic in vivo; however, the exact mechanism is still unclear. In vitro, curcumin induces apoptosis in various cell types via a reactive oxygen species (ROS)-dependent mechanism. Here we treated human dermal fibroblasts with TGF-β1 to induce myofibroblast differentiation, and compared the responses of fibroblasts and myofibroblasts to 25 μM curcumin. Curcumin induced caspase-independent apoptosis in both fibroblasts and myofibroblasts in a ROS-dependent manner. Oxidative stress leads to the induction of several antioxidant systems to regain cellular homeostasis. We detected stress-induced induction of heme oxygenase (HO)-1 in fibroblasts but not in myofibroblasts following curcumin exposure. Instead, myofibroblasts expressed higher levels of heat shock protein (HSP)72 compared to fibroblasts in response to curcumin, suggesting that TGF-β1 treatment alters the stress-responses of the cells. However, we did not detect any differences in curcumin toxicity between the two populations. The differential stress responses in fibroblasts and myofibroblasts may open new therapeutic approaches to reduce myofibroblasts and scarring.

Abbreviations: αSMA, alpha-smooth muscle actin; ECM, extracellular matrix; HO-1, heme oxygenase-1; HSP, heat shock protein; NAC, N-acetyl cysteine; ROS, reactive oxygen species; TGF-β1, transforming growth factor β1

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Introduction

Wound healing is a well-regulated yet complex process. Deregulated wound healing may result in tissue contraction, excessive deposition of extracellular matrix (ECM), and abundant scarring. This process is also known as fibrosis and can occur in many different tissues leading to impaired tissue and organ function [30]. A specialized cell, the myofibroblast, plays a pivotal role in fibrotic processes [63]. After skin injury, dermal fibroblasts differentiate into myofibroblasts that produce large amounts of ECM. They also express alpha-smooth muscle actin (αSMA) which enables the cells to contract the wound. After tissue regeneration and regained tissue homeostasis, myofibroblasts undergo apoptosis, leaving a rather acellular scar. However, in hypertrophic scars, myofibroblasts persist even after the wound has closed, resulting in bulky, contracted scars [17,36]. Fibroblast-myofibroblast differentiation is regulated by chemical and mechanical signals, of which transforming growth factor (TGF)-β1 is the key factor [63].

The topical application of curcumin, a plant-derived polyphenol, improves cutaneous wound healing of both normal and chronic diabetic wounds by improving the formation and quality of the granulation tissue [53,52]. In addition, curcumin possesses beneficial anti-inflammatory and antioxidant properties [1,40].

We and others have demonstrated that curcumin induces apoptosis in various cell lines in a cell- and concentration-dependent manner [51,50,20]. This apoptotic response is mediated via reactive oxygen species (ROS) [20], and can be prevented by various antioxidants such as glutathione precursor N-acetyl cysteine (NAC) [34,54,50], vitamin E [33], and bilirubin [50]. For instance, NAC blocks curcumin-induced apoptosis in gingival and dermal fibroblasts as well as in leukemia cells [2,3].

Redox regulation is critical for cell survival as low levels of ROS serve as second messengers, whereas high levels lead to oxidative stress and subsequent damage of DNA, membranes, and proteins. To cope with oxidative stress, the cells contain various cytoprotective systems. Heme oxygenase-1 (HO-1) is an inducible enzyme that responds to a wide range of cellular stresses, including inflammation and oxidative stress [30]. In fact, curcumin is also an HO-1 inducer in various cell types [20]. HO-1 breaks down heme and thereby generates carbon monoxide, biliverdin and free iron [31]. Biliverdin is quickly converted by biliverdin reductase into the powerful antioxidant bilirubin [5]. The antioxidant and anti-inflammatory effects of HO-1 have been demonstrated both in vitro and in vivo, and are thought to be mediated via the generated effector molecules [19]. We have previously demonstrated that pharmacologic induction of HO-1 inhibits curcumin-induced apoptosis in dermal fibroblasts [50].

Besides classical antioxidant enzymes and endogenous antioxidants, heat shock proteins (HSPs) comprise another class of cytoprotective molecules that are induced by cellular stresses [6]. The 70-kDa HSP proteins are a major family in humans with the inducible HSP72 (HSP70) and the constitutive HSP73 (HSC70) as prominent members with chaperone functions in folding, transport, and degradation of proteins [13]. In addition, the cytoprotective effects of HSP70 proteins may be explained, in part, by inhibiting key apoptotic effectors [49]. Interestingly, HSP72 inhibits the TGF-β1 pathway by inducing TGF-β1 receptor degradation, suggesting a possible role of HSPs in anti-fibrotic therapies [49].

We have previously demonstrated that high doses of curcumin induce caspase-independent apoptosis in dermal fibroblasts as well as decrease fibroblast-mediated collagen gel contraction [3], and this may thus provide a therapeutic approach to reduce hypertrophic scarring. In this study, we compared the responses of human dermal fibroblasts and TGF-β1-induced myofibroblasts following curcumin exposure with respect to apoptosis, functional markers, and stress protein expression. The aim was to gain more insight into the effects of curcumin and its potential use as anti-scarring therapeutic agent.

Methods and materials

Reagents

Curcumin (Fluka Chemika) 2 mM stock solution was freshly prepared by dissolving in 100% ethanol. N-acetyl cysteine (NAC; Sigma-Aldrich) was dissolved in PBS to 1 M and filter-sterilized. Recombinant human Transforming Growth Factor-β1 (Millipore) 50 ng/μl stock solution was prepared according to the manufacturer's instructions. Stock solutions were diluted in culture medium immediately before experiments.

Fibroblast culture

Fibroblasts derived from human foreskin were cultured in Dulbecco's modified Eagle's medium (DMEM; 4.5 g/L glucose) containing 10% fetal calf serum, and 2% penicillin/streptomycin at 37 °C in a 5% CO_2 atmosphere. Medium was refreshed every 2–3 days and when the cells reached 90% confluence, they were subcultured in a 1:3 dilution using trypsin-EDTA. Fibroblasts were differentiated into myofibroblasts by seeding fibroblasts at 20–30% confluence and treating these cells for 72 h with 10 ng/ml TGF-β1. Thereafter, further treatment of myofibroblasts was always performed in the presence of TGF-β1. Experiments were carried out between passage 8–16. All materials for cell culturing were from Invitrogen.

Immunocytochemistry

Fibroblasts were seeded at 30% confluence on glass chamber slides (Nunc) and cultured in the presence or absence of 10 ng/ml TGF-β1 for three days followed by additional culturing for 48 h in the presence or absence of 25 μM curcumin. Myofibroblasts were continuously exposed to 10 ng/ml TGF-β1. After culturing cells were fixed with 4% para-formaldehyde for 20 min before permeabilization with 0.1% Triton X-100 in PBS. Unspecific epitopes were blocked for 30 min with blocking buffer (PBS, 2% bovine serum albumin, 2% goat serum, 0.1% Triton X-100, 0.05% Tween-20) supplemented with 100 mM glycine to quench residual formaldehyde. Primary antibodies against αSMA (1:2500, monoclonal anti-αSMA clone 1A1; Sigma-Aldrich), and HO-1 (1:200, SPA-895, Stressgen) were diluted in blocking buffer and incubated 2 h. As negative control the primary antibody was omitted. After washing with PBS-T (PBS, 0.05% Tween-20) cells were incubated for 45 min with the appropriate fluorescently labeled secondary antibody (AlexaFluor-596 or AlexaFluor-488 1:200; Invitrogen) diluted in blocking buffer. After washing with PBS-T and PBS cells were stained with DAPI for nuclear visualization. Slides were mounted