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ORIGINAL PAPER

Suppression of TGF-β1/SMAD pathway and extracellular matrix production in primary keloid fibroblasts by curcuminoids: its potential therapeutic use in the chemoprevention of keloid

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Abstract Keloid is a fibrotic disease characterized by abnormal accumulation of extracellular matrix (ECM) in the dermis. It is a late spreading skin overgrowth and may be considered a plastic surgeon's nightmare. In nature, curcuminoid is composed of curcumin, demethoxycurcumin (DMC) and bisdemethoxycurcumin (bDMC). Curcuminoids have been found to inhibit fibrosis. However, their role in the synthesis of ECM in the keloid fibroblasts (KFs) has remained unclear. In this series of studies, a total of seven primary KFs cultures were used as the KFs model for investigating the inhibitory effect of curcuminoids on the expression of ECM and TGF-β1. A sensitive and reproducible HPLC method was developed to provide a quantitative analysis on the cellular uptake of curcuminoids onto the KF cells. The level of ECM in the primary KFs was elevated. The elevation of ECM and TGF-β1/p-SMAD-2 level was substantially blocked by the cellular uptake of curcumin in a dose-dependent manner in all the seven primary KFs. The results have led to the conclusion that the excessive production of ECM in the KF cells could be blocked and/or rapidly decreased by curcumin.

Keywords Keloid · Curcuminoids · Extracellular matrix (ECM) · Transforming growth factor-β1 (TGF-β1)

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Introduction

Turmeric (curcuminoid), a herb from the rhizome of the plant *Curcuma longa*, could be one of the most widely used spices in the world. It has been used extensively in Asian countries and also in traditional medicine. Interest in this herb has grown in recent years based on its putative beneficial pharmacological effects, which include antioxidant [26], anti-inflammatory [4] and cancer chemopreventive actions [13, 17]. It has been discovered that curcumin is also a potent scavenger of various reactive oxygen species (ROS), including superoxide anions [20] and hydroxyl radicals [16]. Moreover, there have been indications that curcuminoids may help in the prevention and treatment of patients with Alzheimer's disease by reducing oxidative damage and plaque burden in these patients and suppressing specific inflammatory factors [15, 22]. In addition to the actions of inhibiting cell proliferation and increasing apoptosis, other mechanisms have also been proposed to rationalize the anti-carcinogenic effect of curcuminoids, such as the anti-inflammatory and antioxidant activities, the induction of phase-II detoxification enzymes, the inhibition of cyclooxygenase 2 (COX-2), the effect on AP-1 and NFκB transcription factors, the effect on protein kinases and others [1, 27].

Keloids have been reported to exhibit a high rate of accumulation in collagen and proteoglycan by fibroblasts [18, 28], which is typical in the early phase of a wound but not in the normal skin or scars [23]. Collagen synthesis is very important for wound healing; it is synthesized and secreted in a soluble form by the fibroblast and deposited extracellularly. All of these steps have to progress in a coordinated manner and require the presence of various cytokines, growth factors and other bioactive molecules [24]. The biological molecules of nitric oxide (NO) have been reportedly synthesized in rodent wounds [3, 21]. Recently, this research group reported that the NO formed by iNOS produced the regulation of collagen synthesis by acting on the formation of keloid and enhancing the synthesis of fibrotic factors (TGF-β1, HSP47 and TIMP-1) in the keloid fibroblast (KF) [6–8].

To more clearly define the potential effect of curcuminoids on the inhibition of ECM synthesis in keloids, the KF isolated from the patients was used as a model system in this series of studies. In this article, the effect of curcuminoids on the TGF-β1/SMAD pathway and production of ECM in the KF are reported.

Materials and methods

Subjects

A total of seven keloid scar specimens were obtained from seven Taiwanese patients (four women and three men with age in the range of 21–76 years and a mean age of 45.0 years). Skin tissues were excised from the chest of three patients, the ear lobe of three patients and the shoulder of one patient (all had the disease for 1–6 months). Only the typical and clinically clear-cut cases, which had extended beyond the original boundary of the wound, were included in this study. None of the patients had received any previous treatment other than pressure therapy. Seven site-matched normal scar skin specimens (two women and five men, mean age 43.9 years; range 26–66 years) were obtained at the time of other unrelated operations. Prior written informed consent was obtained from the patients and all procedures received the approval of the ethics board at Kaohsiung Veterans General Hospital and Taipei Medical University in adherence to the Helsinki Principles [6].

Cell culture

Dermis from the scar skin tissues was minced and incubated in a solution of collagenase type I (0.5 mg/mL) and trypsin (0.2 mg/mL) at 37°C for 6 h. Cells were pelleted and grown at 37°C in the tissue culture flasks containing Dulbecco's Modified Eagle Medium (Gibco/BRL, Gaithersburg, MD, USA), which was supplemented with fetal bovine serum (HyClone, USA) for the primary culture and subsequent cultures, respectively, at 20 and 10% (v/v), and 5 mg/mL of L-glutamine in an atmosphere of 5% CO_2. The KF cells in the primary cultures were trypsinized with 0.05% of trypsin and 0.53 mM of EDTA (Life Technologies, Inc., Carlsbad, CA, USA) and passaged. All the experiments were performed using the third to fifth passage cells. During the experiments, all of the cultures were analyzed by flow cytometry (FACS Calibur, BD) and MTT test, and the results were shown to have no evidence of crisis or injury.

Preparation of extracting buffer

The extracting buffer was prepared from a combination of ethyl acetate and isopropanol [at the ratio of 9:1 (v/v)]. It was stored at room temperature until use (stable for at least 1 month).

Recovery of curcuminoids from KF cells by cell-liquid extraction method

The cell pellets were each re-suspended in the RIPA buffer (20 mM of Tris/HCl at pH 8.0, 137 mM of NaCl, 10% of glycerol, 5 mM of EDTA, 1 mM of phenylmethylsulfonyl fluoride, 1.5 mg of leupeptin and protease inhibitor cocktail) and the cell-liquid extraction was carried out. The cell extracts were each cleared by centrifugation (at 18,000 rpm for 15 min). The amount of total cellular protein in each of the extracts was determined using the Bradford method (Bio-Rad, USA). The cell extracts (100 μg) were each acidified by 6 N HCl, at 1:1 (v/v), vortexed for 30 s, and the extracting buffer (500 μl) was then added. The mixtures were re-vortexed and shaken (in an Orbital shaker) at 100 rpm, for 15 min. After centrifugation (at 18,000 rpm for 20 min), the upper organic layers were filtered by membrane filters (0.22 μm) and transferred to a clean injection sample vials ($\sim$100 μl) for quantitative analysis by high-performance liquid chromatographic (HPLC) method reported earlier [9].

HPLC analysis of curcuminoids

In nature, curcumin exists as curcuminoids. For this series of studies, curcuminoids were purchased from Sigma (Cat. No. C7727, Sigma, USA). Curcuminoids consist of curcumin, the primary constituent, in combination with two minor demethoxylated analogs (demethoxycurcumin and bisdemethoxycurcumin). The curcuminoids were separated and assayed by an HPLC instrument, which was a Hitachi L-2000 series high-performance liquid chromatograph

system (Hitachi, Japan) and consisted of a solvent delivery system (L-2130 model) equipped with a fluorescence detector (L-2480 model) and a reversed-phase column of LiChrospher Si60 (5 μm; Merck) packed in spherical particles (3 μm) maintained at the constant temperature of 37°C by a column oven (Hitachi L-2350). The combination of acetonitrile and 2% acetic acid (pH 2.5) at a ratio of 53:47 was used as the mobile phase and flowed through the column (at a flow rate of 0.8 ml/min), while the samples were each injected (at an injection volume of 20 μl) by an L-2130 model autosampler system (L-2130 model). Experiments were performed in triplicate and the data were expressed as the mean (±SEM).

Western blot

The cells, after harvesting, were immediately frozen to −70°C. The frozen cells were homogenized in a homogenizing buffer [prepared from the Tris buffer (20 mM at pH 8.0), NaCl (137 mM), glycerol (10%), EDTA (5 mM), phenylmethylsulfonyl fluoride (1 mM), leupeptin (1.5 mg) and protease inhibitor cocktail]. The cell lysate so produced was then cleared by centrifugation (at 13,000 rpm for 15 min). The amounts of protein in the lysate were determined using the Bradford method (Bio-Rad, USA). The levels of SMAD-2, phospho-SMAD-2 (both from US Biology, USA) and β-actin (Sigma, USA) in the total cellular extracts (60 μg) were analyzed by Western blot analysis, using the procedure described in literature [6]. The total proteins were separated, using the 10% SDS–polyacrylamide gel, transferred onto a nitrocellular membrane (Schleicher & Schnell, Keene, NH, USA) in Tris buffer (25 mM), which contained 0.192 M of glycine (pH 8.3) and 20% of methanol, and 30 volts were then applied overnight. The membranes were then each blocked with 5% non-fat milk in Tris–HCl (10 mM, pH 8.0), which contained 150 mM of NaCl and 0.05% of Tween-20, overnight, and then incubated with anti-SMAD-2 and anti-phospho-SMAD-2 for 1.5 h. The blots were further washed with Tris–HCl (pH 8.0), which contained 150 mM of NaCl and 0.05% of Tween-20, three times (10 min each), and incubated with the second antibody (IRDye Li-COR, USA) at 1/20000 dilution for 30 min. The antigen was then visualized by Odyssey infrared imaging system (Odyssey LI-COR, USA) and data analyzed by Odyssey 2.1 software (IRDye Li-COR, USA).

Determination of total soluble collagen concentration

The supernatant from the culture medium was obtained by filtering through a membrane filter (0.45 μm), and the total soluble collagens in the supernatant were determined by using the Sircol Collagen Assay kit (Sircol, USA). Experiments were performed in triplicate and the data were expressed as the mean (±SEM).

Enzyme-linked immunosorbent assay (ELISA)

The supernatant from the culture medium was obtained by filtering through a membrane filter (0.45 μm). The immunoassay of TGF-β1 was performed by ELISA using the manufacturer's protocol (R&D Systems, USA). Experiments were performed in triplicate and the data were expressed as the mean (±SEM).

Immunoassay

The concentrations of procollagen type I and fibronectin in the conditioned medium from the KF culture were measured using the Procollagen Type I C-peptide EIA kit and Fibronectin EIA kit (both from TakaRa, Japan). The supernatant from the culture medium was obtained by filtering through a membrane filter (0.45 μm). The immunoassay of procollagen type I and fibronectin was performed using the manufacturer's protocol. Experiments were performed in triplicate and the data were expressed as the mean (±SEM).

Statistical analysis

All data were reported as the means (±SEM) of three to four separate runs of the experiment. The ANOVA test was employed for statistical analysis, with significant differences determined as $P < 0.05$.

Results

Curcuminoids inhibited bleomycin-induced ECM expression in KF cells. The effect of curcuminoids on the expression of ECM (which includes total soluble collagens, pro-collagen I and fibronectin) in the primary KF cells has been studied. While the concentration of total soluble collagen was assayed by the Sircol Collagen Assay kit, the levels of pro-collagen I and fibronectin were measured by EIA analysis. The IC_{50} of curcuminoids in the primary KFs was determined to be 25–28 μM: the range of curcuminoids usage in this study without cytotoxicity effect in the primary fibroblasts (data not shown). Yamamoto et al. [30] showed that bleomycin 100 nM increases the levels of type collagen mRNA expression in skin fibroblast. In this study, the seven primary cultures of keloid fibroblasts and control fibroblasts were set up to study the roles played by curcuminoids in the expression and secretion of ECM.

The treatment of keloids and hypertrophic scars has been difficult and a recent study showed that bleomycin has

been useful in the treatment of these lesions [5, 29]. To each of the fibroblast cultures, 100 nM of bleomycin was added with varying doses of curcuminoids (0, 25, 50, 100 nM) for 24 h. The results summarized in Fig. 1 show that curcuminoids are observed to inhibit the expression of total soluble collagen, pro-collagen I and fibronectin production and secretion, induced by the bleomycin in the KF cells in a dose-dependent manner. Statistical analysis of the results indicated that the expression of the total soluble collagen, pro-collagen I and fibronectin in the primary KF cells was elevated, induced by the bleomycin, to a level significantly greater than the controls (treated with 0 nM of bleomycin at $P < 0.05$). For the treatment with bleomycin at 100 nM, the synthesis of total soluble collagen was increased twofold as compared to threefold for pro-collagen I and twofold for fibronectin. Interestingly, the addition of curcuminoids was observed to produce a reduction of 20–50% in total soluble collagen, 25–62.5% in pro-collagen I and 25–30% in fibronectin versus the controls (which were treated with 100 nM of bleomycin, but not curcuminoids) (Fig. 1).

Curcuminoids inhibited the bleomycin-induced elevation of TGF-β1 expression in KF cells. To explore the roles played by curcuminoids on the synthesis of TGF-β1 induced by bleomycin, in the primary KFs, ELISA analysis was carried out to determine the levels of TGF-β1 expression in each of the seven primary KF cells (four separate runs of experiment were conducted for each of the KF cells). Seven primary cultures of the keloid fibroblast (KF) cells were set up to determine the roles played by curcuminoids in the synthesis of TGF-β1, induced by 100 nM of bleomycin, with or without the coadministration of curcuminoids at varying doses (0, 25, 50, 100 nM) for a duration of 24 h. The results summarized in Fig. 2a indicate that curcuminoids showed an inhibitory action on the elevating effect of bleomycin on the expression of TGF-β1 in the KF cells. It is interesting to note that the inhibitory action of curcuminoids shows a dependency on the dose of curcuminoids.

Quantitative analysis of the results in Fig. 2a reveals that the expression of TGF-β1 in the primary KF cells is elevated by the addition of bleomycin, which is significantly ($P < 0.05$) higher than in the controls (treated with 0 nM of bleomycin). The synthesis of TGF-β1 was substantially reduced by 57% (for the treatment with 25 nM of curcuminoids) and by 73 and 89%, respectively (for the treatment with 50 and 100 nM of curcuminoids).

Curcuminoids inhibited the bleomycin-activated TGF-β1/SMAD-2 signal pathway in KF cells. To explore the roles that curcuminoids have played in the phosphorylation of the SMAD-2 signal pathway, following the induction by bleomycin, the primary KF cell cultures were treated with 100 nM of bleomycin in combination with varying doses

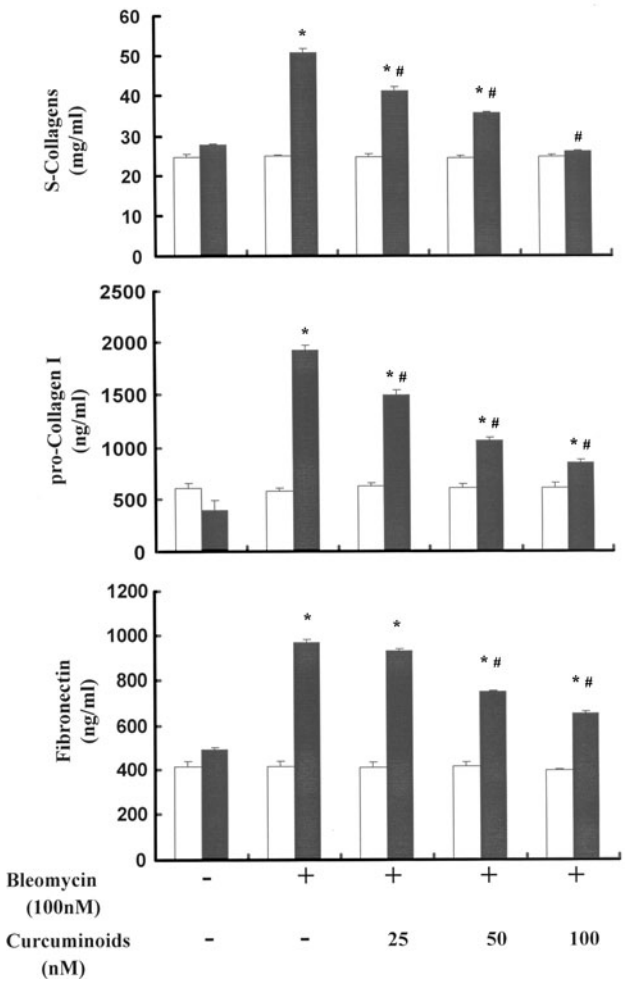

Fig. 1 The promoting effect of bleomycin on the synthesis of total s-collagens, pro-collagen I and fibronectin in the KF cells, as well as the inhibition of the bleomycin-enhanced synthesis of total s-collagens, pro-collagen I and fibronectin as a function of curcuminoids concentration. The amount of total s-collagens was determined by Sircol Collagen assay kit, while EIA analysis was used to determine the levels of pro-collagen I and fibronectin expression in the seven primary KF cells. The same studies were also performed on the normal scar skin fibroblast ($n = 7$; *open bars*) in comparison to the primary KF cultures ($n = 7$; *solid bars*). Each of the *bars* represents the expression of total s-collagens, pro-collagen and fibronectin in the KF cells and the normal scar fibroblast. All data are the mean (±SEM) of four separate experiments. ANOVA with post hoc was employed for statistical analysis, with significant differences determined at $P < 0.05$

(0, 25, 50, 100 nM) of curcuminoids. The results compared in Fig. 2b indicate that the addition of 100 nM bleomycin induces the production of SMAD-2 and phoph-SMAD-2 (p-SMAD-2) in the primary KF cell (versus the controls), while the production of SMAD-2 is observed to be reduced by the co-treatment of curcuminoids (versus the controls treated with 0 nM of curcuminoids). The phosphorylation of the SMAD-2 and protein expression was repressed by curcuminoids treatment in KFs.

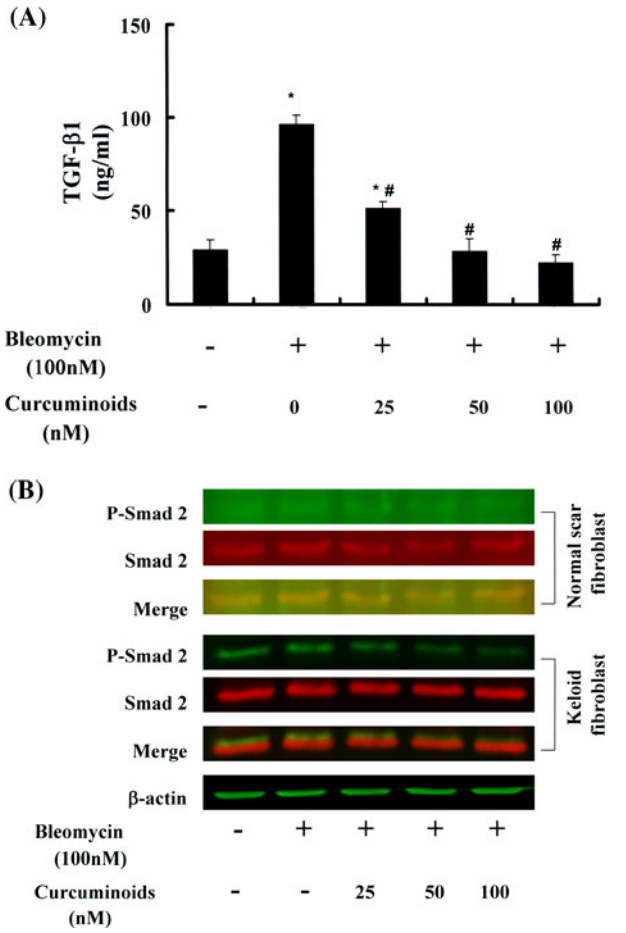

Fig. 2 The inhibition of TGF-β1 expression and the phosphorylation of SMAD2, induced by the bleomycin in the KF cells as a function of curcuminoids concentration. The KF cells were treated with bleomycin (100 nM) alone or in combination with varying concentrations of curcuminoids (0, 25, 50, 100 nM). **a** The synthesis of TGF-β1 in the KF cells and the expression of TGF-β1 were analyzed quantitatively by ELISA technique. The *bars* each represent the quantity of TGF-β1 expression in the KF cells. ($^{*}P < 0.05$, compared with the negative control treated with 0 nM of bleomycin; $^{\#}P < 0.05$, compared with the positive control treated with 100 nM of bleomycin and 0 nM of curcuminoids). All data represent the mean (±SEM) of three separate experiments. **b** The Western blot analysis of the p-SMAD 2 and SMAD 2 expression in the KF and normal scar fibroblast cells, which had been treated with bleomycin (100 nM) alone or in combination with various concentrations of curcuminoids

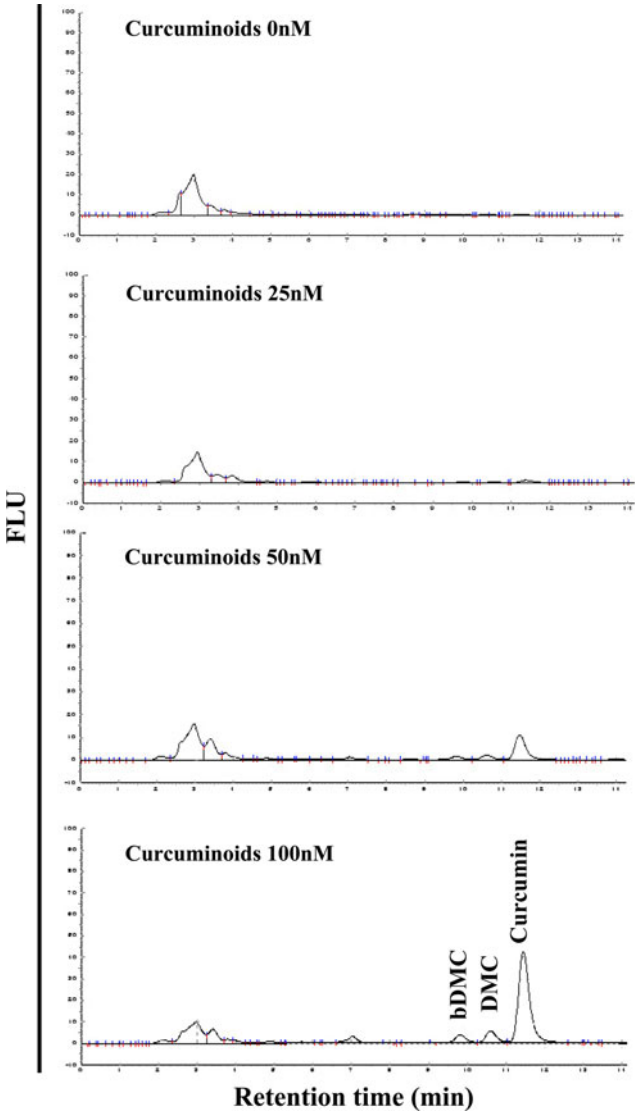

Fig. 3 The HPLC chromatogram for the curcuminoids recovered from the seven primary KF cells having been exposed to a varying concentration of curcuminoids (0, 25, 50 or 100 nM) for 24 h

To quantitate the uptake of curcuminoids by the primary KF cells, a sensitive and reproducible HPLC method for curcuminoids uptake assay was developed earlier to achieve the quantitative and reproducible assay of curcuminoids. Using the HPLC system and conditions reported [9], as briefly outlined above in the "Methods" section, the major components of the curcuminoids, which consist of curcumin, demethoxycurcumin (DMC) and bisdemethoxycurcumin (bDMC), were observed to yield three well-defined specific peaks with retention times in the range of 9–13 min (Fig. 3). These peaks were not detectable in the HPLC chromatogram for the controls (with no curcuminoids treatment, Fig. 3). Furthermore, the peak area under the peak for curcumin generated from a series of six standard curcuminoid solutions (with concentration range of up to 150 nM) yielded a perfect linearity with the concentrations of curcuminoids (with a correlation coefficient of 0.99). Moreover, the same linearity was also achieved for the peak areas measured from the DMC and bDMC peaks (with a correlation coefficient above 0.99). The attainment of a correlation coefficient of >0.99 for all the standard curves confirmed the accuracy and precision of the HPLC method used for the assay of curcumin, DMC and bDMC in the curcuminoids (Table 1). Furthermore, statistical analysis indicated that this HPLC method has achieved a satisfactory day-to-day reproducibility (2.98%

Table 1 The quantitative assay of curcumin, demethoxycurcumin (DMC) and bisdemethoxycurcumin (bDMC) uptake by KFs

Dose (nM)	Culture medium (area)			Cells (area/mg total protein)		
	Curcumin	DMC	bDMC	Curcumin	DMC	bDMC
0	ND	ND	ND	ND	ND	ND
25	43,847 ± 1,134	42,354 ± 1,537	27,204 ± 1,107	8,639 ± 1,651	6,903 ± 989	4,138 ± 464
50	388,879 ± 5,362	211,499 ± 3,912	164,717 ± 2,755	127,096 ± 3,427	35,709 ± 2,579	22,821 + 1,313
100	9,818,069 ± 81,580	3,746,524 ± 13,837	2,000,983 ± 28,682	332,224 ± 9,240	62,462 ± 5,228	45,863 ± 6,018

ND non-detectable

for curcumin, 1.39% for DMC and 3.12% for bDMC). The satisfactory results on accuracy, precision and intra- and inter-day reproducibility combined together have made this HPLC method suitable for curcuminoid quantitative analysis and its major components taken up by the primary KF cells and also the amounts remaining in the culture medium, as reported by the results in the next section.

To study the uptake of curcuminoids by the primary KF cells after the 24-h treatment with curcuminoids, the amount of curcuminoids taken up by the KF cells and that remaining in the culture medium were first recovered by extraction and then analyzed by the sensitive HPLC method (outlined in the "Methods" section). The HPLC chromatograms indicate that the specific peaks for curcumin and its demethoxylated analogs (DMC and bDMC) all increase in parallel to the increase in the concentration of curcuminoids added to the KF cell cultures. The observations suggest that the amounts of curcumin, DMC and bDMC taken up by the KF cells increase in proportion to the dose of curcuminoids to which the KF cells were exposed for 24 h. The results summarized in Fig. 4 demonstrate that this is the case, in which the level of curcumin taken up by the KF cells (displayed as the percentage of the total curcuminoids recovered from the cell pellets and the culture medium) is elevated, in a dose-dependent manner, as the dose of curcuminoids increases in the range of 0–100 nM. On the other hand, the levels of DMC and bDMC are observed to increase at first and then decrease after reaching the peak levels at 25 nM of curcuminoids. The elevation of ECM and TGF-β1/p-SMAD-2 levels was substantially blocked by the cellular uptake of curcumin, but not DMC and bDMC in primary KFs.

In summary, the results of this series of studies lead to the conclusion that curcuminoids inhibit the bleomycin-induced ECM expression in the keloid fibroblast, and the inhibition results from the cellular uptake of curcumin.

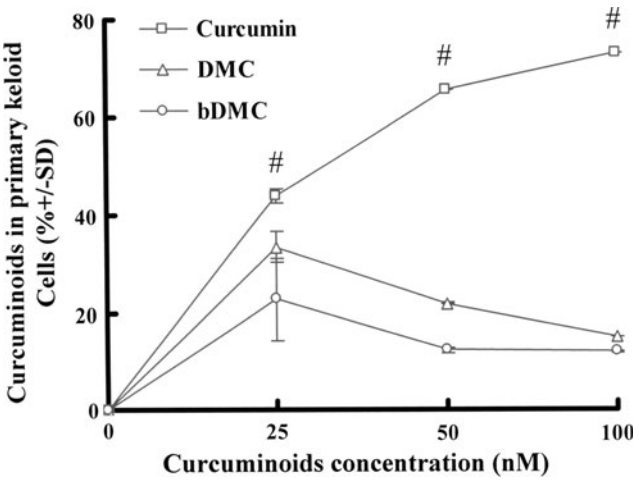

Fig. 4 The cellular uptake of curcumin and its related compounds *(DMC and bDMC) by the primary keloid fibroblast cells treated with a varying concentration of curcuminoids (0, 25, 50, 100 nM). Data shown are the mean (±SEM) of at least three independent experiments. $^{\#}P < 0.05$ versus DMC and bDMC

Discussion

There is no single effective therapeutic regimen for the treatment of keloids. Numerous treatment options have been described including occlusive dressings, compression therapy, intralesional steroid injections, imiquimod cream, laser and radiation therapy, cryosurgery, 5-fluorouracil, bleomycin and interferon therapy [5, 25]. The treatment of keloids and hypertrophic scars has been difficult, although a recent study showed that bleomycin was useful in the treatment of these lesions [29]. Curcuminoids have been used traditionally in Asia as a treatment for skin wounds, inflammation and tumors [2, 14, 18, 19]. Recent studies have also shown the inhibition of liver fibrosis lesions by curcuminoids [12]. However, no direct evidence has been provided on the effect of the curcuminoids in the expression of ECM and TGF-β1 in the KFs. In the present investigation, experimental evidence demonstrated that in the KF cells, the increased levels of ECM and TGF-β1, stimulated by bleomycin, was reduced by the coadministration of curcuminoids.

To further provide a validation of our hypothesis that bleomycin could stimulate the synthesis of ECM and TGF-β1 in the KF, KF cells were treated with 100 nM bleomycin (at a fixed level) and curcuminoids (at varying concentrations). A dose-dependent decrease was observed

in the production of ECM (which includes total s-collagens, pro-collagen I and fibronectin) and TGF-β1 in the KF cells (Fig. 1). Curcumin suppressed not only TGF-beta(1)-induced Smad2 phosphorylation in a dose- and time-dependent manner, but also the nuclear accumulation of receptor-regulated Smads (R-Smad), Smad2 and Smad3 in human proximal tubule cells (HK-2) [10]. In the present study, the role of curcuminoids in the bleomycin-enhanced TGF-β1/SMAD 2 pathway was investigated.

TGF-β1 is known to have a promoting effect on the extracellular matrix in various cells by stimulating the synthesis of matrix proteins. An important physiologic feature of TGF-β includes the de novo synthesis of extracellular matrix proteins and inhibition of the expression of matrix metalloproteinases [11]. At last, an increased expression of TGF-β isoforms has been documented in the fibrotic cells [31] and keloid [1]. In this study, we provided further evidence that the increased level of TGF-β1, stimulated by bleomycin, inhibited the production and transactivation of autocrine TGF-β1 in the KFs by the coadministration of curcuminoids (Fig. 2a). The production of SMAD 2 and phosphorylation were also inhibited by the addition of curcuminoids in the KFs (Fig. 2b).

These observations have led us to conclude that curcuminoids inhibited the TGF-β1/SMAD signal cascade and decreased the expression of ECM in the keloid fibroblasts. A better understanding of the underlying molecular mechanisms will greatly improve our current knowledge of keloid therapy, which in turn could also help to elucidate the pathology of keloid, and has the potential of leading us to the development of an effective and a specific drug for keloid fibroblast therapy.

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