Original Paper

Pathophysiology of Haemostasis and Thrombosis

Pathophysiol Haemost Thromb
DOI: 10.1159/000321375

Received: May 26, 2010
Accepted after revision: August 24, 2010
Published online: November 12, 2010

Curcumin Facilitates Fibrinolysis and Cellular Migration during Wound Healing by Modulating Urokinase Plasminogen Activator Expression

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Key Words
Curcumin · Urokinase plasminogen activator · Wound healing · Fibrinolysis · Cell migration

Abstract
Urokinase plasminogen activator (uPA) plays a vital role in the early phases of wound healing by aiding fibrin dissolution and promoting the migration, proliferation, and adhesion of various cells to the wound bed. The efficacy of botanicals in healing wounds is an area of active research. Among these, curcumin, a yellow pigment abundant in turmeric rhizome, has been the center of extensive studies. This study focused on the effect of curcumin on uPA expression and its consequence on fibrin dissolution and cellular migration. Treatment of human fibroblast cells with curcumin caused an upregulation of uPA mRNA and protein. Activation of JNK and p38 MAPK signal pathways was necessary for the upregulation of uPA. Curcumin treatment resulted in an increase in fibrinolytic activity and cell migration towards the wound area. The involvement of uPA in fibrinolysis and cell migration was confirmed by zymography and siRNA studies, respectively.

Introduction

Normal wound healing processes comprise 3 distinct but overlapping phases: inflammatory, proliferation, and remodeling. An immediate response to injury is the formation of a provisional, fibrin-containing matrix. The early stage of the inflammatory phase involves the rapid deposition and polymerization of fibrin. Replacement of the fibrin matrix by granulation tissue is necessary for normal healing to occur. The dissolution of fibrin is brought about by the serine protease, plasmin. Plasmin is generated from zymogen plasminogen by the action of either cell-derived urokinase-type (uPA) or tissue-type (tPA) plasminogen activators. uPA system is a multifunctional system involved in wound healing, tissue remodeling, immune response, and cancer by affecting cell migration, adhesion, and proliferation [1]. The system includes the serine protease uPA, its receptor uPAR, and 2 specific inhibitors, i.e. type 1 and type 2 plasminogen activator inhibitors (PAI-1 and PAI-2). Besides fibrin dissolution, the uPA system also plays a prominent role in the proliferation phase of wound repair by promoting the migration and adhesion of cells to the wound bed [2–4]. An important mechanism by which uPA promotes cell migration is the generation of plasmin which is capable of digesting basement membrane and extracellular ma-

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trix proteins [5]. uPA is produced by different cell types such as fibroblasts, keratinocytes, endothelial cells, and macrophages, all of which play prominent roles in wound healing.

The process of wound healing is accelerated by many natural products, including plant products that are rich in active components such as alkaloids, flavonoids, and anthroquinones [6–8]. Curcumin (diferuloylmethane), a major component of food flavor and turmeric *(Curcuma longa)* rhizomes is one such bioactive component and is well documented for its pharmacological activities such as antioxidant, anti-inflammatory, antimicrobial, anticancer, and wound healing effects [9]. Studies on wound healing have shown that curcumin is effective in healing cutaneous wounds [10], diabetic wounds [11], gamma-irradiated wounds [12] and gastric ulcers [13]. Although extensive studies have been carried out on curcumin and wound healing, the effect of curcumin on the expression of uPA and its functional consequence during wound healing has not yet been elucidated. This study investigated the effect of curcumin on the gene expression of uPA, the molecular mechanisms underlying the regulation, and the functional significance of the regulation.

Materials and Methods

Cell Culture and Reagents

Human fibroblast TIG 3-20 cells (HSRBB Cell Bank, Osaka, Japan) were cultured in Modified Eagle's Medium with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C, 5% CO_2, and 95% humidity. Semiconfluent cells between passages 3 and 5 were used for the study. Curcumin was purchased from Sigma, Japan. DMSO was used as the solvent for dissolving all compounds tested. The DMSO concentration (0.25%), kept constant in all experiments, did not cause cytotoxicity in TIG 3-20 cells.

Cytotoxicity Assay

Fibroblast cells were treated with different concentrations of curcumin for 24 h, after which the supernatants were collected to determine the amount of lactate dehydrogenase released as a result of cytotoxicity using a CytoTox 96® Non-Radioactive Kit (Promega Corp., Madison, Wisc., USA). The assay employs the principle of the calorimetric measurement of lactate dehydrogenase, a cytosolic enzyme released into the medium upon cell lysis.

Reactive Oxygen Species Assay

The antioxidant effect of curcumin on TIG 3-20 cells was analyzed by studying the effect of curcumin on the generation of intracellular reactive oxygen species (ROS) using dichlorofluorescein diacetate (DCFH-DA) [10]. Following the incubation of fibroblast cells with various doses of curcumin for 24 h, 10 μM DCFH-DA was added to the cells. Nonfluorescent DCFH-DA is converted to fluorescent DCF in proportion to the amount of ROS generated in the cells. The fluorescent signal was measured using an FP-6200 spectrofluorometer (Jasco Engineering Co., Tokyo, Japan) at excitation and emission wavelengths of 485 and 530 nm, respectively.

Quantification of mRNA Expression

Cells were treated with different doses of curcumin for 24 h. Total RNA was isolated using RNAiso (Takara, Tokyo, Japan) as per the manufacturer's protocol. Reverse transcriptase polymerase chain reaction (RT-PCR) was carried out as per standard procedures using the ReverTra Ace enzyme (Toyobo, Tokyo, Japan) for first-strand cDNA synthesis and GoTaq Green Master Mix (Promega, USA) for PCR amplification with primers specific for uPA or 18S rRNA. Sequences targeting the coding region of the genes were selected for primer designing. The primer sequences were as follows: uPA (125 bp) forward 5′-TCACCACCAAAATGCTGTGT-3′ and reverse 5′-CCAGCTCACAATTCCAGTCA-3′; 18S rRNA (76bp) forward 5′-TGCATGGCCGTTCTTAGTTG-3′ and reverse 5′-AGTTAGCATGCCAGAGTCTCGTT-3′. The PCR-amplified products were run on 1% agarose gel and visualized by ethidium bromide staining. The expression intensities of optimized bands were quantified with a Luminescent Image Analyzer LAS-3000 (Fuji, Tokyo, Japan). In addition, to obtain quantitative data, real-time RT-PCR of the cDNAs was performed using Power SYBR® Green PCR Master Mix (Applied Biosystems, Japan) in an ABI 7000 real-time PCR system (Applied Biosystems). The real-time PCR results were analyzed and expressed as the fold increase relative to the control (untreated) group.

Western Blotting

Total protein extracts from cells were obtained by lysing cells in a lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, and 1 mM EDTA) containing protease inhibitors (10 μg/ml each of leupeptin, aprotinin, and pepstatin). Ten micrograms of proteins were resolved over 10% SDS-PAGE gels, electroblotted onto nitrocellulose membrane using Trans-Blot SD, semidry transfer cells (Bio-Rad Laboratories, Hercules, Calif., USA), and immunoblotted with the appropriate antibodies. Antibodies anti-uPA and anti-β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). Antibodies for the total and phosphorylated forms of p38 MAPK, JNK, ERK, and PI-3K were purchased from Cell Signaling Technology (Tokyo, Japan). Expression of proteins was detected by chemiluminescence using an ECL Plus Western Blotting Detection System (Amersham Life Science, Inc., Little Chalfont, UK). The intensities of the bands were quantified with Luminescent Image Analyzer LAS-3000 (Fuji). To ensure equal protein loading, membranes were stripped and reprobed with anti-β-actin antibodies using the protocol detailed above.

Signal Pathway Inhibition Studies

PI-3K, p38 MAPK, ERK, and JNK signal pathways were selectively inhibited using specific pharmacological inhibitors LY294002 (50 μM), SB203580 (10 μM), PD98059 (30 μM), and SP600125 (10 μM), respectively. Cells were pretreated with the respective inhibitors for 2 h before incubation with 20 μM curcumin for 24 h. Total RNA was isolated as described above and subjected to RT-PCR to detect uPA expression.

Fibrin Autography and Zymography

Following the 24-hour treatment of TIG 3-20 cells with different doses of curcumin, gross fibrinolytic activity in the conditioned medium of cells was assayed employing fibrin gel plates containing 6 mg/ml bovine fibrinogen (Miles, Kankakee, Ill., USA) and 1 U/ml bovine thrombin (Mochida Pharmaceutical, Tokyo, Japan). Thirty microliters of sample was loaded on a fibrin plate and incubated at 37°C for 16 h. Fibrinolytic activity was estimated by the lysis area obtained.

Fibrin zymography was carried out according to a standard method. Supernatants from treated cells were subjected to electrophoresis on 10% SDS-PAGE gels in nonreduced conditions. Standards for uPA and tPA were run alongside the proteins during SDS-PAGE. Following electrophoresis, gels were washed twice for 1 h each with 500 ml of 10 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.2% v/v Triton X-100 at room temperature with gentle agitation. After a brief wash with distilled water, the gels were overlaid on fibrin agar plates. After incubation of the plates in a moist chamber at 37°C, the fibrinolytic activity of enzymes was visualized as lytic bands against a black background.

In vitro Wound Healing Assay

Fibroblasts were grown in 6-well plates at a density of approximately 4×10^4/well and a small linear wound was created in the confluent monolayer by gently scraping with sterile cell scraper as per standard methods [14]. Cells were extensively rinsed with medium to remove cellular debris before treatment with 20 μM curcumin. Twenty-four hours later, images of the cells were obtained using a digital camera (Nikon, Japan) connected to the inverted microscope (TMS-F; Nikon) and analyzed using image analysis software (Image J 1.32e, National Institutes of Health, Bethesda, Md., USA). The extent of the wound healing was determined by the distance traversed by cells migrating into the denuded area. The data shown is representative of 3 independent experiments. In order to test the involvement of uPA in cell migration, wound healing assay was performed in uPA knocked down fibroblasts.

uPA Knockdown Studies

Knockdown of the uPA gene was facilitated by transient transfection of uPA siRNA. A lyophilized pool of 3 target-specific 20–25 nt siRNAs designed to knock down uPA gene expression (sc-36779) was purchased from Santa Cruz Biotechnology. Control siRNA consisting of a scrambled sequence that does not degrade any known cellular RNA (sc-37007, Santa Cruz Biotechnology) served as the negative control. The transient transfection of cells with siRNA was performed at 50–70% confluence using siRNA transfection reagent (sc-29528) and medium (sc-36868) from Santa Cruz Biotechnology following the manufacturer's protocol. Preliminary experiments conducted over a range of concentrations revealed a 60% inhibition of uPA expression at an siRNA concentration of 200 nM. Thereafter, all siRNA experiments were performed using 200 nM siRNA. The day following transfection, cells were provided with fresh normal growth medium. All assays were conducted 48 h after the addition of normal growth medium.

Statistical Analysis

All experiments were performed in triplicate. Data were expressed as means ± standard deviation, and the difference between the groups was analyzed using Student's t test. $p < 0.05$ was considered statistically significant.

Results

Cytotoxic and Antioxidant Profile of Curcumin

Preliminary experiments were performed to test the cytotoxic effect of curcumin on TIG 3-20 cells. Treatment of the fibroblasts with lower doses of curcumin (up to 40 μM) for a period of 24 h did not prove cytotoxic to the cells (fig. 1a). However, higher doses (50 μM and above) resulted in a reduction in viable cell numbers. Exposure of cells to 60 μM curcumin caused a 50% reduction in cell viability. Next, we tested the antioxidant property of curcumin on TIG 3-20 cells. Cells were treated with various doses of curcumin for 24 h and tested for ROS generation using DCFH-DA. ROS assay confirmed the antioxidant property of curcumin. Curcumin, at doses of 10, 20, and 30 μM, reduced ROS generation by 20, 50, and 70%, respectively (fig. 1b). Treatment of cells with higher doses of curcumin did not result in a further reduction in ROS generation, indicating that curcumin's antioxidant property reached a plateau at the dose of 30 μM.

Curcumin Upregulates uPA Expression

Cells treated with various doses of curcumin were analyzed for uPA gene expression. Both semiquantitative RT-PCR and quantitative real-time RT-PCR analyses showed that curcumin upregulated uPA mRNA in a dose-dependent manner (fig. 2a, b). The simultaneous analysis of PAI-1 expression revealed that curcumin did not alter the gene expression of PAI-1 (data not shown). Protein analysis by Western blotting showed that the uPA protein level was increased 2.8 fold and 4.0 fold by 10 and 20 μM curcumin, respectively (fig. 2c).

Involvement of p38 MAPK and JNK Signal Pathways

Next, potential signaling pathways that could be involved in uPA regulation by curcumin were evaluated. Treatment of TIG 3-20 cells with 20 μM curcumin induced a significant increase in the phosphorylated forms of the p38 MAPK and JNK pathways (fig. 3a) but failed to affect ERK or PI-3K pathways (data not shown). To test the involvement of these signal pathways in the induction of uPA by curcumin, the signal pathways were inhibited using specific inhibitors. Both SB203580 (p38 MAPK inhibitor) and SP600125 (JNK inhibitor) abrogated the stimuli of curcumin on uPA expression (fig. 3b). Inhibition of the PI-3K or ERK pathways had no impact on curcumin's effect (data not shown). The data obtained demonstrate that activation of the p38 MAPK and JNK signal pathways was necessary for uPA upregulation by curcumin.

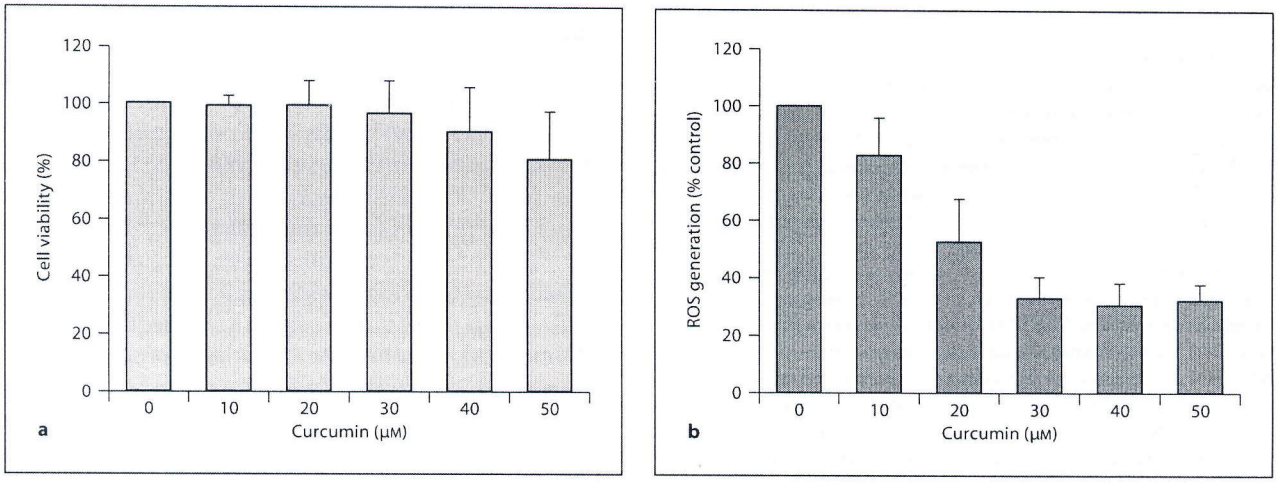

Fig. 1. Cytotoxic and antioxidant profile of curcumin on TIG 3-20 fibroblast cells. Fibroblast cells were treated with various doses of curcumin for 24 h and analyzed for cell viability (**a**) and ROS generation (**b**) as described in Materials and Methods. Values are the means ± SD of 6 independent experiments. * $p < 0.05$; ** $p < 0.01$ (statistical significance in curcumin-treated groups compared with controls).

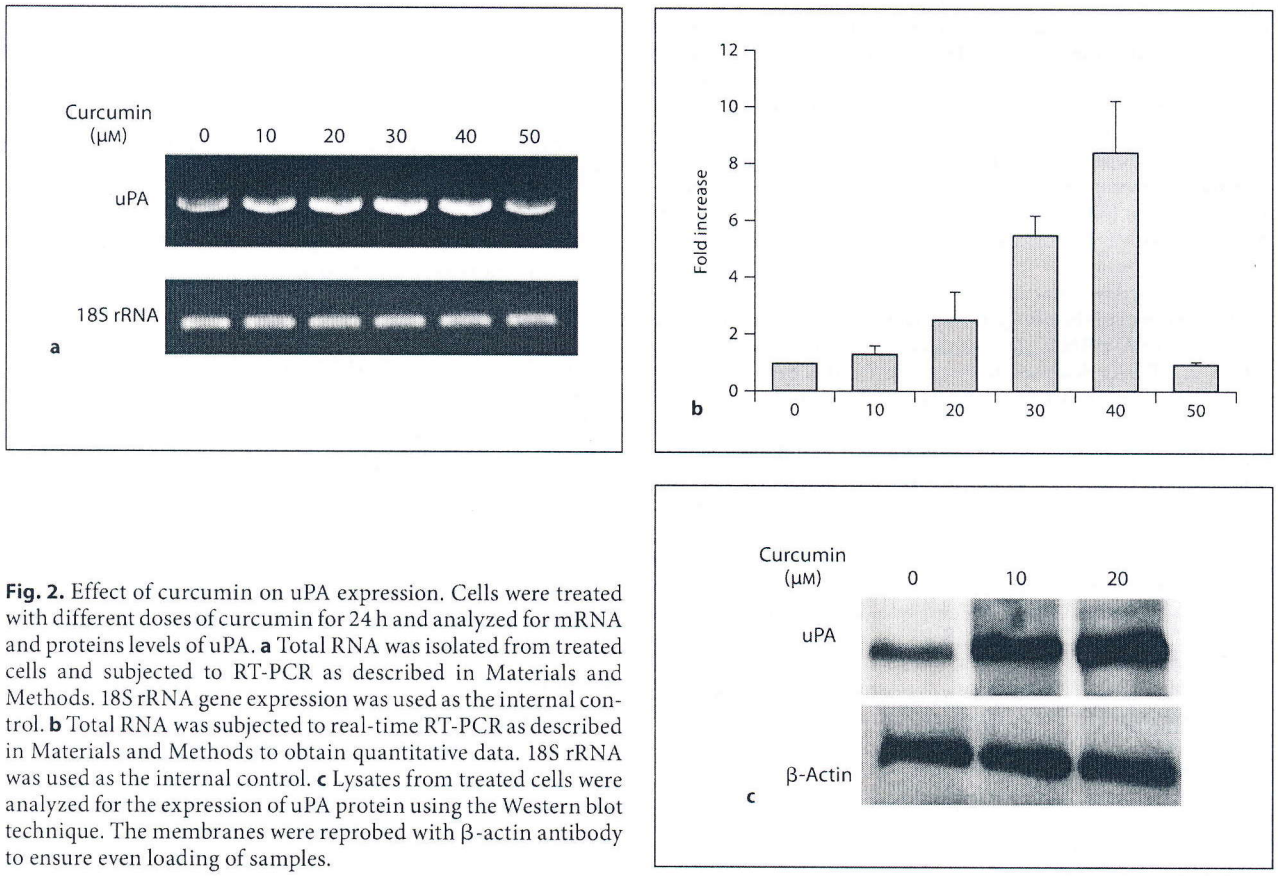

Fig. 2. Effect of curcumin on uPA expression. Cells were treated with different doses of curcumin for 24 h and analyzed for mRNA and proteins levels of uPA. **a** Total RNA was isolated from treated cells and subjected to RT-PCR as described in Materials and Methods. 18S rRNA gene expression was used as the internal control. **b** Total RNA was subjected to real-time RT-PCR as described in Materials and Methods to obtain quantitative data. 18S rRNA was used as the internal control. **c** Lysates from treated cells were analyzed for the expression of uPA protein using the Western blot technique. The membranes were reprobed with β-actin antibody to ensure even loading of samples.

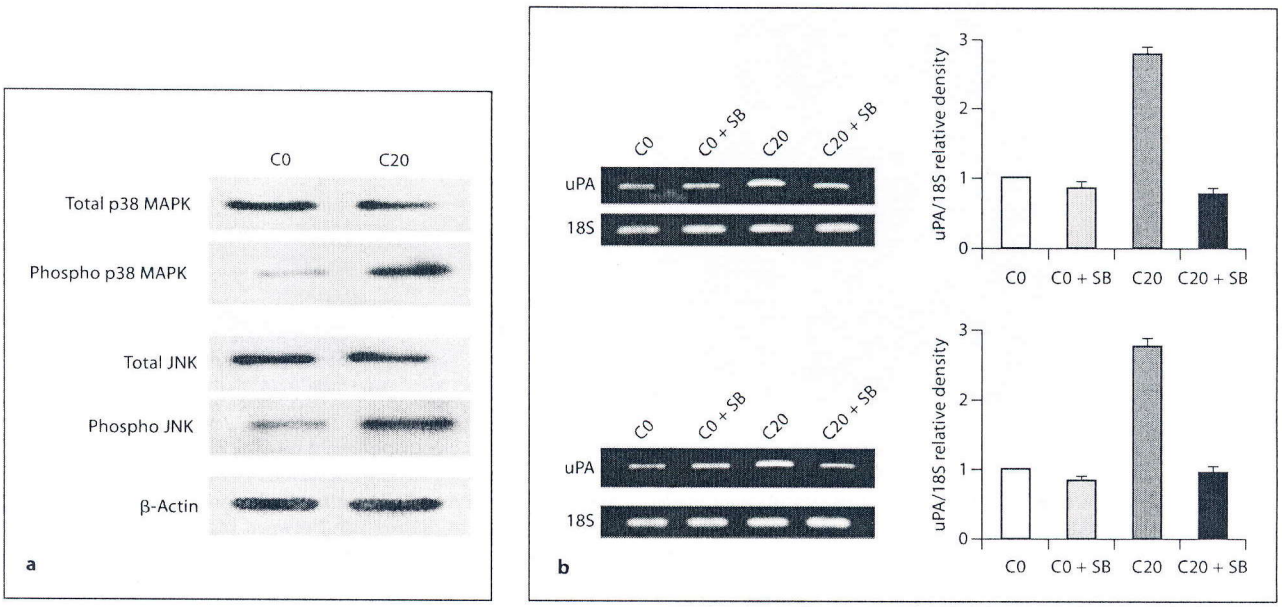

Fig. 3. Involvement of p38 MAPK and JNK pathways in the up-regulation of uPA by curcumin. **a** Lysates from cells treated with (C20) or without (C0) 20 μM curcumin for 24 h were analyzed for expressions of the total and phosphorylated forms of p38 MAPK and JNK proteins using the Western blot technique. The membranes were reprobed with β-actin antibody to ensure even loading of samples. **b** Cells were pretreated with SB 203580 (p38 MAPK inhibitor) or SP600125 (JNK inhibitor) for 2 h before incubation with (C20) or without (C0) 20 μM curcumin for 24 h. Total RNA was isolated and subjected to RT-PCR to detect uPA expression. 18S rRNA gene expression was used as internal control.

Curcumin Enhances uPA-Dependent Fibrinolytic Activity

Since dissolution of the fibrin clot is an important process in the initial phase of wound healing, we studied the effect of curcumin on the fibrinolytic activity of conditioned medium from treated cells. Treatment of cells with curcumin caused a significant increase in fibrinolysis (fig. 4a). Fibrinolysis increased in a dose-dependent manner up to a dose of 40 μM. Cells treated with 50 μM curcumin demonstrated a very low fibrinolytic activity. This data was concurrent with the cytotoxicity profile of curcumin, where it was observed that 50 μM curcumin demonstrated cytotoxic properties. Fibrinolysis can occur as a result of the action of either of the 2 plasminogen activators, uPA or tPA. Since TIG 3-20 fibroblasts express undetectable amounts of tPA, it is reasonable that curcumin-enhanced fibrinolytic activity could be a result of uPA activity. Besides, zymography results revealed a dose-dependent increase in lysis area corresponding to a uPA standard marker (fig. 4b), thus attributing curcumin-enhanced fibrinolytic activity to increased uPA activity.

Curcumin Promotes Cell Migration towards the Wounded Region in a uPA-Dependent Manner

The effect of curcumin on cellular migration in a wound environment was tested using a standard in vitro wound healing assay. Treatment of cells with 20 μM curcumin resulted in a significant increase in the number of cells that migrated into the wound area (fig. 5a). In order to study the influence of uPA in curcumin-induced cell migration towards the wound, uPA expression was transiently knocked down by transfecting uPA siRNA into TIG 3-20 cells, and the cells were subjected to a wound healing assay. Cells transfected with control siRNA served as control. Contrary to figure 5a, curcumin failed to increase the migratory rate of the cells towards the wound when uPA was silenced (fig. 5b). These findings demonstrate that curcumin promotes cellular migration towards the wound in a uPA-dependent manner.

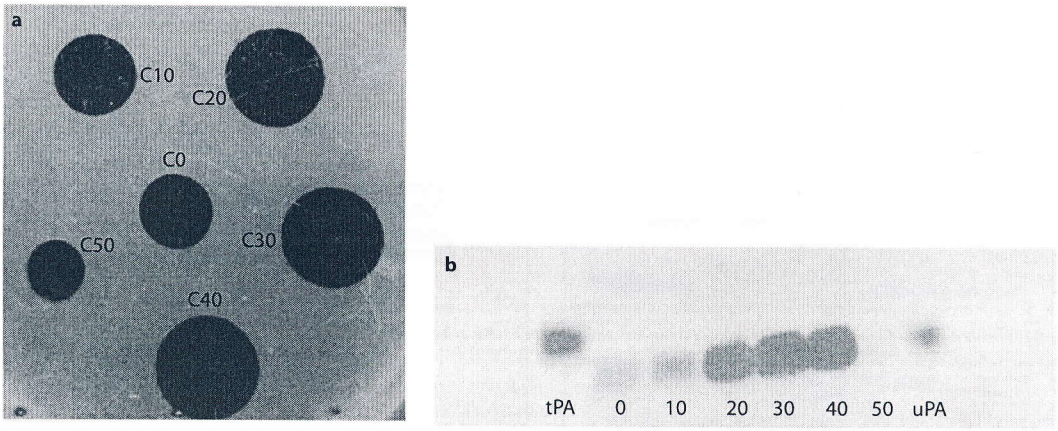

Fig. 4. Fibrin autography using conditioned medium from treated cells. TIG 3-20 cells were treated with different doses of curcumin (0–50 μg/ml) for 24 h. **a** Thirty microliters of conditioned medium from treated cells was loaded on a fibrin plate and incubated at 37°C for 16 h. Fibrinolytic activity was measured by the lysis area obtained. **b** Twelve microliters of conditioned medium from treated cells was electrophoresed on 10% SDS-PAGE and subjected to a zymography assay as described in Materials and Methods. Standards for uPA and tPA were run alongside the samples during electrophoresis to distinguish between lysis resulting from uPA and tPA activity. The figures are representative of 3 independent experiments.

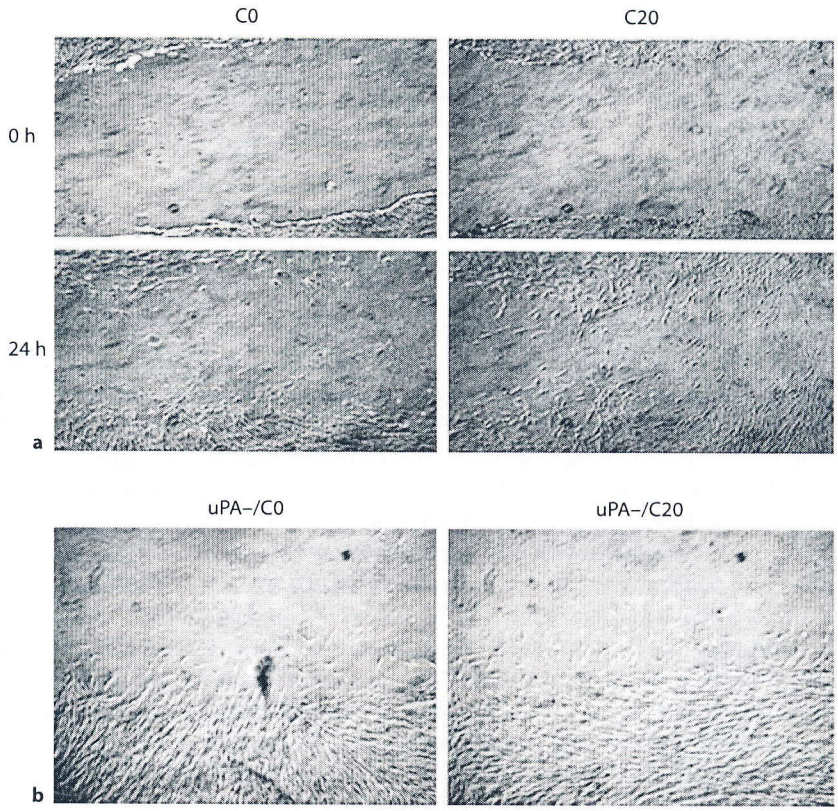

Fig. 5. In vitro wound healing assay. **a** Effect of curcumin on cellular migration towards the wound area. Semiconfluent TIG 3-20 cells were wounded by gentle scratching using a cell scraper. Cells were extensively washed with PBS to remove debris before incubation in serum-free medium with (C20) or without (C0) 20 μM curcumin for 24 h. The condition immediately after wounding is denoted by 0 h; 24 h denotes 24 h after wounding. **b** Cells were transfected with uPA siRNA to transiently knock down uPA expression. uPA– cells were subjected to a wound healing assay as in **a**. The figure denotes the condition of the wound area 24 h after wounding. The images are representative of 3 independent experiments.

Discussion

Curcumin has been reported to promote wound healing in both in vivo and in vitro experimental models. Curcumin enhances wound healing by increasing granulation tissue formation, neovascularization, and faster reepithelialization [11, 15]. It also increases the synthesis of fibronectin and transforming growth factor (TGF)-β [11, 12, 16–18] and the maturation and cross-linking of collagen besides accelerating the rate of wound contracture and decreasing the wound healing time [17]. It is also argued that fibroblast apoptosis induced by high doses of curcumin could provide a novel therapeutic strategy to modulate pathological scar formation [16]. The present study reports that curcumin accelerates fibrinolysis and cell migration during wound healing by upregulating the serine protease uPA via the activation of the JNK and p38 MAPK signal pathways.

The importance of fibrin lysis in wound healing is evidenced in plasminogen-deficient mice models where insufficient plasmin generation resulted in impaired fibrinolysis and eventually impaired healing of wounds [19]. uPA is one of the earliest mediators of the fibrinolysis cascade, playing a critical role in local fibrin dissolution [20]. Plasmin generation is an important mechanism by which uPA affects cell migration. In addition to its proteolytic activity, uPA promotes cell migration by triggering intracellular signaling systems [6, 21]. The binding of uPA to uPAR can initiate vitronectin- or integrin-mediated cell adhesion and migration cascades [22, 23]. uPA can activate motogenic factors such as hepatocyte growth factor [24] and platelet-derived growth factor [25], while plasmin can activate basic fibroblast growth factor and TGF-β [26]. The release of growth factors into the wound bed greatly affects the recruitment, migration, and differentiation of various cell types necessary for effective wound repair.

uPA expression is subject to regulation by many different signals, including growth factors, hormones, genotoxic factors, and transcription factors [27]. Several transcription factors modulate uPA expression. The uPA gene promoter contains specific sequences that are recognized by NFκB, AP-1, HIF-1α, and SP-1 [28]. These transcription factors are subject to activation by members of the MAP kinase family as well as other signals. The JNK pathway has been shown to be involved in AP-1-mediated uPA induction by genotoxic stress [29] and TGF-β [30]. JNK pathway mediated the phosphorylation of SP-1 to activate the minimal promoter of the uPA gene [31]. Although the most studied route of the MAPK regulation of gene expression is the activation of transcription factors, it is also plausible that MAPKs can regulate gene expression through posttranscriptional mechanisms such as mRNA stability. Studies have provided evidence that p38 MAPK stabilizes uPA mRNA via the AU-rich elements in the 3′UTR [32]. It has been demonstrated that MAPK-activated protein kinase 2 is the downstream target in the p38 MAPK modulation of the αv integrin-induced stability of uPA mRNA [33]. The present study shows that regulation of uPA by curcumin requires the activation of both the JNK and p38 MAPK pathways. Whether curcumin regulates uPA at the transcriptional or posttranscriptional level is not known and requires further evaluation. Work in this regard is ongoing in our laboratory.

The MAP kinase family itself is activated by various extracellular stimuli, such as growth factors, cytokines, hormones, genotoxic agents, and oxidative stress. Curcumin has been shown to modulate MAPK signaling in different cell lines. It augmented aldose reductase expression in vascular smooth muscle cells and heme oxygenase-1 in breast epithelial cells and fibroblasts by activating the p38 MAPK pathway [34, 35]. It induced apoptosis by activating JNK in colon cancer cells [36] and p38 MAPK in primary human neutrophils [37]. On the other hand, it inhibited p38 MAPK in primary human intestinal microvascular endothelial cells [38] and JNK in Jurkat T cells [39]. Since the JNK and p38 signal cascades can be activated by the overexpression of at least a dozen MAPKK kinases [40], it is likely that signals upstream of the MAPK cascade could be primary targets of curcumin.

In summary, this study shows that JNK and p38 MAPK mediate the upregulation of uPA gene expression by curcumin and that the upregulation of uPA is instrumental in promoting fibrinolysis and cell migration, two mechanisms that are important for wound healing.

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