Original Paper

Curcumin Treatment Suppresses CCR7 Expression and the Differentiation and Migration of Human Circulating Fibrocytes

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Key Words

Human Circulating Fibrocytes • Curcumin • CCR7/CCL21 pathway • Migration • Activation

Abstract

Background/Aim: Recent studies have demonstrated that circulating fibrocytes contribute to the formation and development of fibrosis. Curcumin, a polyphenolic compound isolated from turmeric, has been shown to have anti-fibrotic effects in various organs. We and others have demonstrated that curcumin beneficially affects the development of fibrosis. However the effect of curcumin on circulating fibrocytes has not been reported. **Methods:** Human circulating fibrocytes were isolated from leukocyte concentrates of healthy human donors and identified based on the expression of CD34, CD45, collagen I (COLI), and chemokine receptor CCR7 (CCR7) via flow cytometry. Cell Counting Kit-8 was used to evaluate cell viability. The effect of curcumin on the differentiation and migration of human circulating fibrocytes was evaluated by immunofluorescence staining, flow cytometry and a transwell migration assay. Transforming growth factor (TGF)- β 1 secretion was examined by ELISA. **Results:** Curcumin treatment (72 h; 20 μ M) significantly decreased the expression of COL I, α -SMA and CCR7, as well as TGF- β 1 secretion, in human circulating fibrocytes. The inhibitory effect of curcumin on the differentiation and migration of human circulating fibrocytes is likely via regulating the CCR7/CCL21 signaling pathway, in particular by reducing CCR7 expression. These observed effects may be beneficial in resolving fibrosis by suppressing TGF- β 1 secretion. **Conclusion:** Our results suggest that curcumin has the potential to suppress the differentiation and migration of circulating fibrocytes, which would provide new explanation for curcumin's application in the development of fibrosis in various organs.

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Introduction

Fibrosis is a pathological status characterized by excessive and persistent accumulation of fibrous connective tissue in an organ in response to pathological factors such as injury and inflammation [1, 2]. Scarring and the development of excess fibrous connective are two major symptoms of the fibrosis, and can obliterate the architecture and function of the underlying organ or tissue [3]. The symptoms of fibrosis may vary considerably depending on the location and extent of the condition as well as the symptoms of the underlying cause. This condition causes high morbidity and mortality globally [4], and limited therapeutic options are available for controlling the development of fibrosis thus far. It is widely acknowledged that (myo) fibroblasts are the main sources of extracellular matrix (ECM) production [1]. Discovered by Bucala [5] in 1994, blood-borne circulating fibrocytes have been reported to be involved in pulmonary [6], renal [7, 8], liver [7, 9, 10] and cardiac [7, 11] fibrosis. Derived from bone marrow, circulating fibrocytes exist in the peripheral circulation and migrate with the peripheral blood to wound chambers in response to chemokines [12]. Activated circulating fibrocytes not only secrete ECM, which is essential for the process of fibrosis, but also lead to pathological fibrosis by differentiating to (myo) fibroblasts [13]. This prompted us to investigate whether targeting circulating fibrocytes could open up a new avenue for resolving fibrosis.

Curcumin, a polyphenolic compound isolated from turmeric (Zingiberaceae), has been shown to exert a variety of biological effects such as anti-hypolipidemic, anti-hypoglycemic, anti-tumor, anti-oxidant and anti-inflammatory effects [14]. Increasing evidence suggests that curcumin has antifibrotic effects on cardiac fibrosis in spontaneously hypertensive rats via PPAR- γ activation [15]; on liver fibrosis in carbon tetrachloride treated rats via inhibition of transforming growth factor 1 and connective tissue growth factor expression [16], suppressing cannabinoid receptor type-1 [17], as well as ameliorating intrahepatic angiogenesis [18]; on renal fibrosis in unilateral ureteral obstruction (UUO) rat via inhibiting of nuclear factor kappa-B and activator protein-1 [19] and in 5/6 nephrectomized rat via modulating Nrf2-Keap1 pathway [20]; on pulmonary fibrosis in viral-induced acute respiratory distress syndrome mice through the alteration of inflammation and myofibroblast differentiation [21] and in bleomycin treated mice via overexpression of cathepsins K and L [22].

Thus, we speculated that curcumin has anti-fibrotic effects in various organs by targeting circulating fibrocytes. In order to test the hypothesis, we established methods for the culture and identification of human circulating fibrocytes, and evaluated the effects of curcumin on their viability, differentiation, and migration, as well as the possible mechanism of these effects.

Materials and Methods

Cell culture

Human circulating fibrocytes were isolated from leukocyte concentrates from healthy human donors (Beijing Red Cross Blood Center, China; the experiment has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and approved by the Medical Ethics committee of the Beijing University of Chinese Medicine) by centrifugation over Ficoll-Plaque PLUS (GE Healthcare, USA) according to the manufacturer's protocol. The leukocyte concentrate was diluted with phosphate-buffered saline (PBS; Macgene, China) at a volume ratio of 1:1. Subsequently, this diluted blood sample was layered on Percoll-Paque PLUS in a centrifuge tube. After being centrifuged at 400 $\times$ g for 30 minutes at room temperature, the lymphocyte layer was carefully transferred into a clean centrifuge tube containing at least 3 volumes of PBS. Cells were washed twice at 100 $\times$ g for 10 minutes. Following culture on T25 plastic dishes at a density of 0.75 $\times$ 10⁶ cells/cm² for 3 days in Dulbecco's Modified Eagle Medium (DMEM; Gibco, USA) with 20% fetal bovine serum (Gibco), L-glutamine, penicillin and streptomycin (Thermo, USA), non-adherent cells were removed. New media were applied to the adherent cells and cells remained in culture for the following experiments.

Immunofluorescence

Cells were seeded into 35 mm Petri dishes for 5 days and immunostained with phycoerythrin (PE)-conjugated mouse anti-human CD34 monoclonal antibody (1/50; BD Biosciences, USA), PE-conjugated rat anti-human C-C chemokine receptor type 7 (CCR7) monoclonal antibody (1/50; BD Biosciences, USA) or PE-Cy5-conjugated mouse anti-human CD45 monoclonal antibody (1/50; BD Biosciences) or PE-conjugated rat anti-human CCR7 monoclonal antibody (1/50; BD Biosciences) for 30 minutes at 4°C. After cells were washed three times, they were fixed and permeabilized by the addition of BD cytofix/cytoperm (BD Biosciences) for 20 minutes at 4°C. After washing and blocking, the cells were incubated for 30 minutes with mouse anti-human collagen I (COL I) primary antibody (1/200; Abcam). After the cells were washed three times with BD Perm/Wash™ buffer, fluorescein isothiocyanate (FITC)-labeled rabbit anti-mouse IgG H&L secondary antibody (1/200; Abcam, UK) against rabbit anti-mouse collagen I was added and incubated for 30 min, and the cells were then stained with 4,6-diamidinophenyl-2-indole (DAPI) (Roche, Germany), and viewed on a laser scanning microscope (OLYMPUS, Japan) using FV-ASW ver.3.1a software. Cells were identified by double immunocytochemistry with either CD34 and COL I, CD45 and COL I, CCR7 and COL I, or CD45 and CCR7.

Flow cytometry analysis

For flow cytometry analysis, 0.75×10^6 cells were collected and centrifuged. Cells were stained for CD34 (as described in the immunofluorescence section, same below), CD45, COL I, CCR7 or α -smooth muscle actin (α -SMA, rabbit anti-human primary antibody, 1/100; Millipore, USA; allophycocyanin (APC)-labeled goat anti-rabbit IgG H&L secondary antibody, 1/100; Southern Biotech, USA). After immunostaining, cells were washed twice and analyzed by flow cytometry (BD FACS Cantoll, USA) using BD FASDiva software and FlowJo 7.6.1 software. Negative controls used for non-specific background staining included: PE-labeled mouse IgG1 isotype control (BD Biosciences, USA), PE-Cy5-labelled mouse IgG1 isotype control (BD Biosciences, USA), FITC-labeled purified mouse IgG1 κ isotype control (BD Biosciences, USA), PE-labeled rat IgG2a κ isotype control (BD Biosciences), APC-labeled rabbit IgG isotype control (Epitomics, USA).

Cell viability assay

Human circulating fibrocytes were seeded at 5×10^4 viable cells in a 96 well tissue culture plate in complete media (20% FBS in DMEM) for 3 days. The media was removed and replaced with new complete media in the presence or absence of various concentrations of curcumin (Sigma, USA; 1, 3, 10 and $20 \mu\text{M}$). Cells were cultured for 24, 48, or 72 hours before the addition of serum free media with Cell Counting Kit-8 (CCK-8; DOJINDO, Japan) for an additional 3 hours. The optical density (OD) at 450nm was recorded using a microplate reader.

ELISA for transforming growth factor beta 1 (TGF- β 1)

After the cells were cultured for 10 days, the media were removed and replaced with new complete media containing various concentrations of curcumin (0,1,3,10 and $20 \mu\text{M}$). The blank control group was set. The experiment was conducted according to the guidelines of the ELISA kit (eBioscience, USA). In brief, following the exposure of cells to curcumin for 72 hours, the culture supernatants were collected and pre-diluted with the assay buffer. The pretreated samples were added into wells and incubated for 2 hours. Then, after the addition of biotin-conjugate for 1 hour, the samples were incubated with TMB substrate solution for 1 hour. The reaction was then halted by the addition of the stop solution and the plate was read in a plate reader at 450 nm.

Transwell migration assay

Twenty-four-well chemotaxis chambers (Corning, USA) were used in the chemotaxis assays. The chambers were balanced in serum-free medium for 1 hour before seeding the cells. CCL21 (PeproTech, USA) and various concentrations of curcumin were added in the bottom wells of the chamber to function as the chemotactic stimuli. Human circulating fibrocytes (pretreated with different concentrations of curcumin for 72 hours) were collected and suspended in $100 \mu\text{l}$ of 2% BSA in DMEM, and 1×10^6 viable cells were plated in the top wells of the chamber. Cells were allowed to migrate for 3 hours at 37°C in a 5% CO₂ incubator. After migration, the cells on the upper surface of the filter were wiped and the cells sticking to the lower surface were collected and counted using an Automated Cell Counter (Bio-Rad, USA). The chemotaxis

rate was calculated by comparing the number of recruited cells with the total number of cells added into the top wells.

Statistical analysis

Results are expressed as the mean $\pm$ S.E.M. The statistical significance of differences among three or more variables was evaluated using analysis of variance (ANOVA) by Graph-pad Prism software (version 6.1). *P* values < 0.05 were considered statistically significant.

Results

Identification of human circulating fibrocytes

The classical markers for human circulating fibrocytes are CD34, CD45, COL I and CCR7 [23]. Immunocytochemistry results showed that COL I, CD45 and CCR7 strongly coexpressed in human circulating fibrocytes, whereas CD34 expression was very weak (Fig. 1A). Flow cytometry results indicated that approximately 99.8% of the human circulating fibrocytes were COL I -positive, 77.8% were CD45-positive, and only 1.0% were CD34-positive, suggesting that human circulating fibrocytes were successfully isolated and purified in the current study (Fig. 1B).

The effect of curcumin on the viability of human circulating fibrocytes

Our results showed that curcumin at higher concentrations (10 μ M and 20 μ M) exerted inhibitory effects on the proliferation of human circulating fibrocytes. Furthermore, this inhibitory effect was associated with an increase in curcumin concentration. Interestingly, curcumin at low concentrations (1 μ M and 3 μ M at 24 hours or 48 hours) has the potential to improve cell viability (Fig 1C). However we did not see significant differences between the treatment and control groups.

Curcumin elevates CD34 expression in human circulating fibrocytes

The expression of CD34 proved that human circulating fibrocytes were derived from bone marrow [5] and the expression of CD45 confirmed their hematopoietic origin [24]. Flow cytometry results showed that the ratio of CD34⁺ or CD45⁺ cells in the curcumin treatment group exhibited a slight increase compared to that in the control group. Specifically, CD34 expression showed a marked response to treatment with curcumin at the higher doses of 10 μ M ($p < 0.05$) and 20 μ M ($p < 0.01$) (Fig 2A), whereas CD45 did not show significant differences among these groups (Fig. 2B).

Curcumin blocks the expression of COL I and α -SMA in human circulating fibrocytes

We next evaluated the effect of curcumin on the transdifferentiation of human circulating fibrocytes. COL I is present in scar tissue [25], which is main symptom of fibrosis development. Fibrocytes can be activated into fibroblast, which are main producer of collagen, especially COL. Our study has revealed that COL I is strongly expressed in human circulating fibrocytes, suggesting that human circulating fibrocytes could transdifferentiate into fibroblasts and induce a fibrogenic response. Furthermore, we observed a curcumin concentration-dependent reduction in COL I levels at 72 hours (Fig. 2 C)

Previous studies have shown that bone-marrow-derived circulating fibrocytes can differentiate into α -SMA⁺ myofibroblasts [26, 27], and make crucial contributions to liver fibrosis [27]. We observed that α -SMA was strongly expressed on human circulating fibrocytes cultured for 12 days, and that there was a dose-dependent decrease in α -SMA levels after 72-hour treatment with curcumin. Compared with the control group (94.8%), curcumin significantly reduced the numbers of α -SMA⁺ human circulating fibrocytes at concentrations of 10 and 20 μ M ($p < 0.01$; Fig. 2D).

Fig. 1. Expression levels of CD34, CD45, CCR7 and COL I in human circulating fibrocytes and the effect of curcumin on the viability of human circulating fibrocytes. (A) Representative confocal images showed CD34, CD45, CCR7 and COL I coexpression in human circulating fibrocytes. Cells were costained for (a) COL I and CD34; (b) COL I and CD45; (c) COL I and CCR7; (d) CD45 and CCR7. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; blue). Cells were examined at 60× magnification. Results shown are representative of triple determinations (n=3). (B) Flow cytometry results show CD34, CD45 and COL I coexpression in human circulating fibrocytes. Results shown are representative of triple determinations (n=3). (C) CCK-8 results show that curcumin regulates the viability of human circulating fibrocytes. Data are representative of three separate experiments. * $p < 0.05$ and ** $p < 0.01$ indicate significant differences between control and curcumin treatment.

Curcumin suppresses the secretion of TGF- β 1 in human circulating fibrocytes

As the acknowledged fibrogenic and growth-regulating cytokine, TGF- β 1 was able to regulate multiple signaling pathways involved in fibrogenesis [28]. ELISA results showed a concentration-dependent decrease in TGF- β 1 levels in cell culture media after 72-hour treatment with curcumin. This effect was not significant at low concentration of curcumin, but became prominent at concentrations ranging from 3 to 20 μ M ($p < 0.01$; Fig 3).

Curcumin blocks the migration of human circulating fibrocytes via the CCR7/CCR21 pathway

Expressed in various lymphoid tissues and activates B and T lymphocytes, the chemokine receptor CCR7 and its ligand CCL21 play a major role in immune responses [29]. CCL21/CCR7 signaling has been demonstrated to be the primary pathway inducing the recruitment of human circulating fibrocytes into the kidney, and is thereby involved in renal fibrogenesis [8, 30]. Given that CCR7 was found to be strongly expressed in human circulating fibrocytes in our previous study, we investigated the effect of curcumin on CCR7 levels by flow cytometry. The results showed that curcumin inhibited CCR7 expression in human circulating fibrocytes. This inhibitory effect of curcumin was initiated at doses of 1 μ M, and was marked at doses of 3–20 μ M ($p < 0.01$; Fig 4A and 4B).

Fig. 2. Effects of curcumin on differentiation of the expression of CD34, CD45, COL I and α -SMA expression in human circulating fibrocytes. (A-B) Flow cytometry results show that curcumin up-regulates CD34 and CD45 expression in human circulating fibrocytes. Human circulating fibrocytes were treated with curcumin (1, 3, 10 and 20 μ M; 72 hours). The bar diagram represents data obtained from three separate experiments, with the bars representing mean $\pm$ SEM. (A) CD34 levels in human circulating fibrocytes stained with PE. Cells were cultured for 4 days before treatment with curcumin. (B) CD45 levels in human circulating fibrocytes stained with PE-Cy5. Cells were cultured for 6 days before treating with curcumin. (C-D) Representative flow cytometry results showed that curcumin down-regulates COL I expression and α -SMA expression in human circulating fibrocytes. (C) Human circulating fibrocytes were treated with curcumin (1, 3, 10 and 20 μ M; 72 hours) and stained with FITC. Cells were cultured for 9 days before treating with curcumin. (D) Human circulating fibrocytes were treated with curcumin (1, 3, 10 and 20 μ M; 72 hours) and stained with APC. Cells were cultured for 12 days before treatment with curcumin. * p <0.05 and ** p <0.01 indicate significant differences between control and curcumin treatment.

Fig. 3. Curcumin down-regulates TGF- β 1 secretion from human circulating fibrocytes by ELISA. Data are expressed as the mean $\pm$ SEM and representative of three separate experiments. ** p <0.01 indicate significant differences between control and curcumin treatment.

Next, we evaluated the effect of curcumin on the migration of circulating fibrocytes in response to CCL21. As shown in Figure 4C, CCL21 induced the migration of human circulating fibrocytes in a dose-dependent manner. Compared with the control group, cells migration was overactivated in response to CCL21 treatment (1-200 ng/ml; p <0.05 or p <0.01). However, curcumin did not induce the migration of human circulating fibrocytes in the absence of CCL21.

Given that curcumin blocks CCR7 expression in human circulating fibrocytes, we further assessed the effect of curcumin on cell migration via the CCL21/CCR7 pathway. As shown in

Fig. 4. Effects of curcumin on CCR7 expression and the migration of human circulating fibrocytes. (A) Curcumin down-regulates CCR7 levels in human circulating fibrocytes, as evidenced by flow cytometry. Human circulating fibrocytes were treated with curcumin (1, 3, 10 and 20 μM; 72 hours) and stained with PE. Cells were cultured for 6 days before treating with curcumin. The bar diagram represents data obtained from three separate experiments, with the bars representing mean ± SEM. (B) Representative histogram plot from three different flow cytometry assays. (C) CCL21 up-regulates the migration of human circulating fibrocytes cultured for 9 days. The left fourth column represents the control group. (D) Curcumin down-regulates the migration of human circulating fibrocytes. Cells were cultured for 6 days before treating with curcumin. Cells were pretreated with various concentrations of curcumin for 72 hours in the upper wells. Data are from three separate experiments. The left third column represents the control group. * $p < 0.05$ and ** $p < 0.01$ indicate significant differences between the curcumin treatment and control groups. ("CUR" means "curcumin").

Figure 4D, compared with the control group, curcumin pretreatment for 72 hours at doses of 3–20 μM led to a remarkable suppression of cell migration (3 μM: $p < 0.05$; 10–20 μM: $p < 0.01$). In addition, there was a non-significant difference between curcumin and CCL21 levels in the bottom chambers.

Discussion

In the last two decades, since the discovery of circulating fibrocytes in 1994[5], our knowledge of the pathogenesis of fibrosis has expanded dramatically. Existing in the peripheral circulation, circulating fibrocytes can be recruited into multiple organs in response to chemokines, and actively contribute to fibrogenesis. It has been proved that circulating fibrocytes play a key role in pulmonary [6, 31, 32], renal [7, 8], skin [7, 33], hepatic [7, 9] and cardiac [7, 11] fibrosis. Consequently, it can be speculated that circulating fibrocytes could be one of the common targets for anti-fibrotic therapy in various organs.

As bone marrow-derived mesenchymal progenitors, circulating fibrocytes are a special population of peripheral leukocyte subsets with hallmarks of fibrotic pathologies, co-expressing hematopoietic stem cell antigens and markers of the monocyte lineage as well as fibroblasts[2]. Fibrocytes are markedly positive for specific molecular markers, including leukocyte (CD45), monocyte (CD11a, CD11b, CD13), and bone marrow stem cell

markers (CD34), chemokine receptors (CXCR4, CCR7, CCR2, CXCR6), major histocompatibility complex (MHC I, MHC II), and mesenchymal cell makers (collagen I, pro-collagen I, collagen III, vimentin, prolyl4-hydroxylase and α -SMA) [2, 12, 34]. The combination of collagen production and hematogenous origin features as a good standard to identify circulating fibrocytes in most settings, i.e. co-expression of CD34 or CD45 and COL I or pro-COL I [2].

CD34 belongs to a special class of transmembrane glycoproteins that are heavily glycosylated, and has the capacity to transfer hematopoietic cells from bone marrow into peripheral blood [35]. In our study, the expression of CD34 indicated the hematogenous stem origin of human circulating fibrocytes, consistent with previous studies [36]. We found that only 1.0% of the cells were CD34⁺ in the early period (less than 2.99% in a study by Liu et al [36]). It has been reported that the proportion of CD34⁺ cells in peripheral blood ranges from 0.01% to 0.1% by flow cytometry [37]. We believe that the level of CD34⁺ cells may vary due to differences in blood separation methods. The expression of CD45 indicates the pan-hematogenous origin of human circulating fibrocytes. The high level of COLI demonstrated that human circulating fibrocytes could transdifferentiate into fibroblasts, and thereby play a role in fibrogenesis. Flow cytometry results showed that 77.8% of cells were both CD45⁺ and COLI⁺, typical of human circulating fibrocytes.

In this study, we have discovered that curcumin treatment (72 hours, 20 μ M) significantly down-regulated COLI, α -SMA, and CCR7 levels, and blocked TGF- β 1 secretion, as well as the migration of human circulating fibrocytes. Curcumin remarkably improved the expression of CD34 on human circulating fibrocytes, but did not significantly increase CD45 levels.

CD34 and CD45 are hematopoietic markers, COLI is the hallmark of fibroblasts, and α -SMA is the classical marker of myofibroblasts. The modulation of curcumin on these surface phenotypes indicated that curcumin was able to maintain the hematopoietic features of cells and suppress the transdifferentiation of human circulating fibrocytes into fibroblasts or myofibroblasts.

TGF- β 1 can regulate the synthesis and degradation of ECM and induce the activity of other fibrogenic cytokines that contribute to the development of fibrosis [28]. It has been reported that TGF- β 1 can enhance the expression of COLI and α -SMA, and induce the differentiation of CD45⁺CD34⁺COLI⁺ fibrocytes to myofibroblasts by the activation of the Smad2/3 and SAPK/JNK signaling pathways [26]. In the present study, curcumin exhibited anti-fibrotic effects by inhibiting TGF- β 1 secretion in human circulating fibrocytes. Furthermore, the effects of curcumin on the expressions of CD45, CD34, COL I and α -SMA expressions may be mediated via regulating TGF- β 1 secretion, suggesting that curcumin could block the differentiation of human circulating fibrocytes by reducing the secretion of TGF- β 1.

Recent studies indicated that the CCL21 and CCR7 signaling pathways induce the trafficking of circulating fibrocytes in pulmonary [38] and renal fibrogenesis [39]. In a murine UUO model, a large number of circulating CD45⁺/COLI⁺ fibrocytes were shown to be able to infiltrate the renal interstitium via CCL21-positive HEV-like vessels [8]. Most fibrocytes were positive for CCR7, and the inhibition of CCL21/CCR7 signaling reduced the number of infiltrating fibrocytes, and blocked the pathogenesis of renal fibrosis in UUO-treated mice [19]. Here, flow cytometry results showed a dose-dependent inhibitory effect of curcumin on CCR7 expression, and revealed that pretreatment with curcumin for 72 hours effectively inhibited the migration of human circulating fibrocytes in response to CCL21. Taken together, these results suggested that curcumin may block the differentiation and migration of human circulating fibrocytes by down-regulating the level of CCR7 via the CCL21/CCR7 pathways.

In conclusion, our study revealed that curcumin can reduce the migration, and down-regulate the COLI, α -SMA and CCR7 levels, as well as the TGF- β 1 secretion in human circulating fibrocytes. The possible mechanism mediating these effects may be through regulating CCR7/CCL21 signaling pathway, mainly via reducing CCR7 expression. These observed effects of curcumin may contribute to resolve fibrosis via suppressing TGF- β 1 secretion. This also suggest that curcumin may exert its anti-fibrotic effects in various organs by targeting circulating fibrocytes.

Disclosure Statement

All authors declare that they have no any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work.

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