Lung (2019) 197:783–791
https://doi.org/10.1007/s00408-019-00272-x

OCCUPATIONAL LUNG DISEASE

The Role of CTGF in Inflammatory Responses Induced by Silica Particles in Human Bronchial Epithelial Cells

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Received: 12 June 2019 / Accepted: 4 September 2019 / Published online: 13 September 2019

Abstract

Background Prolonged exposure to crystalline silica leads to persistent pulmonary inflammation and progressive fibrosis. Connective tissue growth factor (CTGF) has emerged as a potent proinflammatory and profibrotic regulator to participate in a variety of chronic inflammatory diseases. However, the role of CTGF in silica-induced pulmonary inflammation remains poorly understood.

Methods To explore the effect of CTGF on inflammatory responses caused by silica particles, human bronchial epithelial cells (16HBE) were transfected with CTGF siRNA and exposed to silica particles at concentrations of 0, 12.5, 25, 50, 100 μg/ml for 48 h. Intracellular CTGF mRNA and protein expressions were determined by RT-PCR and Western blotting, respectively. The levels of inflammatory cytokines including IL-8, TNF-α, IL-6, IL-1β, IL-17A and TGF-β_1 were measured by ELISA kits.

Results Silica particles induce significantly elevated intracellular CTGF mRNA expression in 16HBE cells in a dose-dependent manner when compared with blank control group ($P<0.05$). The secretions of IL-8, TNF-α, IL-6 and IL-17A were also significantly increased by silica particles ($P<0.05$). After exposure to 25 or 50 μg/ml silica particles, the expression of intracellular CTGF mRNA was significantly inhibited in 16HBE cells when transfected with CTGF siRNA ($P<0.05$). The secreted levels of IL-8, TNF-α, IL-6 and IL-17A induced by silica particles were also significantly lower from CTGF siRNA-transfected cells than that from normal 16HBE cells ($P<0.05$).

Conclusion Inhibition of CTGF gene attenuates silica-induced inflammatory responses in bronchial epithelial cells, suggesting that CTGF could be a pivotal regulator in the development of silica-induced inflammation.

Keywords Silica particles · CTGF · Inflammatory cytokines · Bronchial epithelial cells

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Introduction

Crystalline silica dust is one of the major occupational hazards in a variety of industries including mining, construction, glass manufacturing and ceramic processing. Long-term inhalation of respirable crystalline silica dust can lead to several chronic pulmonary diseases, including silicosis, chronic obstructive pulmonary disease and lung cancer [1–3]. Silicosis is an occupational pulmonary disease, which is characterized by persistent pulmonary inflammatory responses that result in irreversible and incurable lung fibrosis [4]. Epidemiological studies have reported significantly increased expressions of TNF-α, IL-1β, IL-8 and IL-6 in plasma and bronchoalveolar lavage fluid from silicosis patients in comparison with unexposed controls [5–7]. A growing number of in vivo and in vitro studies have revealed that silica particles trigger pulmonary inflammatory cells infiltration, secretion of chemokines and cytokines including TNF-α, IL-1β, MCP-1 and TGF-β [8–11]. However, the crucial cellular and molecular mechanisms of silica-induced inflammation are still insufficiently understood.

Connective tissue growth factor (CTGF), also known as CCN2, is a cysteine-rich secretory protein that belongs to the immediate early gene CCN family (Cef10/cyr61, CTGF and Nov) [12, 13]. CTGF participates in the biological processes of adhesion, migration, proliferation and extracellular matrices (ECMs) formation, and is associated with the development of pathological fibrosis [14–16]. Overexpression of CTGF protein is observed in lung, kidney, and heart fibrosis, atherosclerosis and tumor tissues, whereas its expression is at low level or even undetectable in normal adult tissue or cells [17–21]. Additionally, emerging studies have established that CTGF plays a potential role in chronic inflammatory diseases including atherosclerosis, rheumatoid arthritis and inflammatory kidney diseases [22]. CTGF upregulates the expressions of TNF-α, IL-6, MCP-1 and IL-8 through a TGF-β-dependent pathway in cardiomyocytes [23]. Furthermore, CTGF can also induce higher levels of MCP-1 and IL-6 in renal tubular epithelial cells via MAPK/NF-κB signaling pathway [24].

A previous study has shown that CTGF protein is highly expressed in the serum of silicosis patients [25]. The transcriptional level of CTGF in fibroblasts is significantly elevated when co-cultured with silica-stimulated macrophages/monocytes and T cells [26]. However, the exact role of CTGF in the early inflammatory responses caused by silica particles remains unclear. Given that the bronchial epithelial surface is one of the largest primary barriers to inhaled environmental exposure and the initial deposition site of respirable silica particles, here we selected human bronchial epithelial cells (16HBE cells) as the target cells to investigate whether CTGF participates in the process of silica-induced inflammatory responses.

Materials and Methods

Cell Culture

The 16HBE cells were obtained from American Type Culture Collection and maintained in DMEM (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, USA), 100 μg/ml penicillin (Sigma-Aldrich) and 100 μg/ml streptomycin (Sigma-Aldrich) at 37 °C and 5% CO_2.

Silica Particles Preparation and Exposure

Silica particles were provided by National Institute of Occupational Health and Poison Control, Chinese Center for Disease Control and Prevention (Beijing, China). The percentage of free SiO_2 (crystallize silica) was higher than 97%, and the particle diameter was less than 5 μm (diameter < 1 μm: 57%, 1–2 μm: 34%, 2–3 μm: 4.5%, 3–4 μm: 3.0%, 4–5 μm: 1.5%).

The silica particles were weighted, autoclaved at 121 °C for 20 min and then dried. The silica particle suspensions were freshly prepared in serum-free DMEM medium and sonicated for 15 min to ensure the homogeneity of the suspensions before experiment. The cells were immediately exposed to the silica particle suspensions at concentrations of 0, 12.5, 25, 50, 100 μg/ml for 24 h, 48 h and 72 h. The concentration of 0 μg/ml was considered as blank control group. All of the supernatants and cells were harvested for subsequent assays.

Cell Viability Assays

Cell viability was detected by Cell Counting Kit-8 (CCK-8) assay kit (Dojindo, Kumamoto, Japan). The cells were seeded at 3×10^4 cells/well in 96 well plates for 12 h and then treated with silica particles for 24 h, 48 h and 72 h, respectively. After being washed by 1 × phosphate buffered saline twice, each well was added 100 μl DMEM and 10 μl CCK-8 solution and then incubated at 37 °C for another 2 h. The optical density (OD) of the supernatant in each well was measured at 450 nm with a micro plate reader (BioTek Instrument Inc, USA). Cell viability (%) was calculated by the following equation: cell viability (%) = $(OD_{treatment}/OD_{control}) \times 100$.

Knockdown of CTGF Gene with Small Interfering RNA (siRNA)

The 16HBE cells were seeded at 5×10^5 cells/well in 6 well plates. After 24 h incubation at 37 °C, the cells were transfected with 1 μg CTGF siRNA and 4 μl siRNA-MATE (CTGF siRNA transfection reagent kit, Shanghai GenePharma Co., Ltd, China) or with transfection reagent alone for 12 h according to the manufacturer's recommendations. The expression of CTGF mRNA in 16HBE cells transfected with CTGF siRNA was reduced by more than 50% when compared with normal cells. Then the CTGF siRNA-transfected cells were treated with 25 and 50 μg/ml silica particles for 48 h.

Real-Time Quantitative PCR (RT-PCR)

The 16HBE cells (5×10^5 cells/well) in 6 well plates were harvested after being treated with silica particles as previously described. Total RNA was extracted from the treated cells by using Trizol reagents (Invitrogen, Carlsbad, CA, USA) and reverse transcribed to cDNA with PrimeScript ™ RT reagent kit (Takara Bio Inc, Japan) according to the manufacturer's instructions. Quantitative PCR was performed by using a SYBR® Premix Ex Taq™ Kit (Takara Bio Inc, Japan) and run on CFX96TM detection system (Bio-Rad Laboratories, Hercules, CA, USA). GAPDH was used as an internal control for each run. The primer sequences were as follows: CTGF: forward 5′-CAAGGGCCTCTTCTG TGACT-3′, reverse 5′-ACGTGCACTGGTACTTGCAG-3′; GAPDH: forward 5′-TCAAGAAGGTGGTGAAGCAG-3′, reverse 5′-AGGTGGAGGAGTGGGTGTCG-3′. The expression level of CTGF mRNA was standardized to GAPDH.

Western Blotting

The harvested cells were lysed in 100 μl RIPA buffer containing protease inhibitor tablets (Pierce, Cat number: A32965). The protein concentration of each sample was determined by Bicinchoninic Acid (BCA) protein assay kit (Beyotime, China). Equivalent amounts of protein were heated to 95 °C in sample buffer for 5 min, loaded and ran in SDS–polyacrylamide gels, and then transferred electrophoretically to PVDF membranes (Millipore, Billerica, MA, USA) (100 V, 1 h).The membranes were blocked with 5% fat-free dry milk in Tris-buffered saline with Tween 20 (TBST) for 1 h at room temperature and then incubated with primary antibodies (CTGF: 1:1500, Abcam, Cat number: ab94939; or GAPDH: 1:3000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4 °C. After being washed with TBST three times, the membranes were incubated with secondary antibodies (a horseradish peroxidase conjugated goat anti-mouse IgG antibody, Biosharp, HeFei, China) at 1:3000 dilutions for 2 h at room temperature. The proteins were detected using SuperSignalTM West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA). The radioactive bands were quantitated with a Universal Hood II (Bio-Rad Laboratories, Hercules, CA, USA). The optical density of the immunoblots was analyzed with Image Lab™ Software Version 5.2.1.

Enzyme-Linked Immunosorbent Assay (ELISA)

The collected supernatants were centrifuged at $10{,}000 \times g$ for 10 min at 4 °C to eliminate the influence of remaining silica particles before measurement. The levels of cytokines (IL-8, TNF-α, IL-6, IL-1β, IL-17A and TGF-β_1) were determined by ELISA kits (Human, Neobioscience, Shanghai, China) according to the manufacturer's instructions.

Statistical Analysis

All results are represented statistically as means ± SD. The significant differences of mean values among groups were assessed by Student's t-test or one-way analysis of variance (ANOVA) followed by Dunnett's t-test using SPSS statistical package 11.5 (SPSS Inc., Chicago, IL, USA). *P*-values < 0.05 were considered to be statistically significant.

Results

Effect of Silica Particles on the Viability of 16HBE Cells

The viabilities of 16HBE cells affected by silica particles for 24 h, 48 h and 72 h are shown in Fig. 1. Compared with blank control group, the cell viability significantly decreased with exposure to 100 μg/ml silica particles for 24 h ($P < 0.05$). At 48 h time point, the cell viability was significantly reduced when silica particles' concentrations were increased from 12.5 μg/ml to 100 μg/ml ($P < 0.05$). After prolonged treatment with silica particles to 72 h, the cell viability was significantly lower than blank control group at concentrations of 50 μg/ml and 100 μg/ml ($P < 0.05$).

Intracellular CTGF Expression in 16HBE Cells After Exposure to Silica Particles

In Fig. 2a, compared with blank control group, intracellular CTGF mRNA expression in 16HBE cells significantly increased in a dose-dependent manner at 24 h and 48 h when silica particles' concentrations were equal to or higher than 12.5 μg/ml ($P < 0.05$). However, the intracellular CTGF

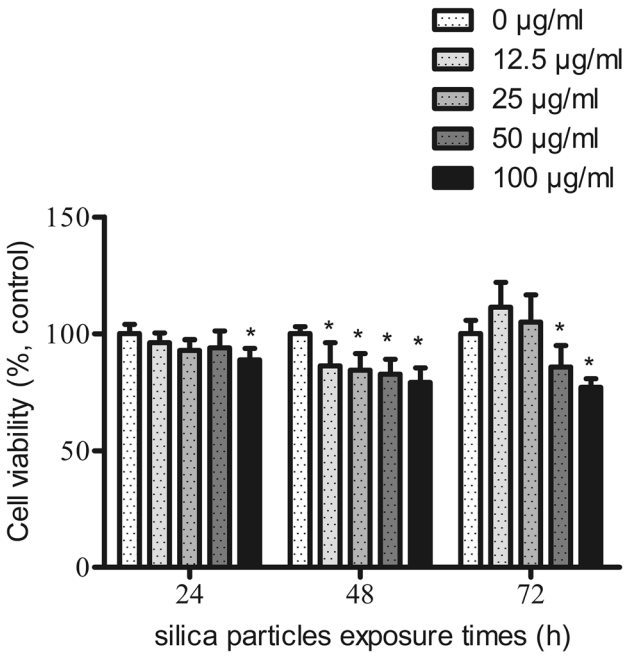

Fig. 1 The viability of 16HBE cells with exposure to silica particles. Values represent means ± SD ($n = 6$). *$P < 0.05$ compared with blank control group (0 μg/ml) at each time point

mRNA expression significantly declined in 16HBE cells with exposure to 100 μg/ml silica particles at 72 h ($P < 0.05$). According to the changed levels of cell viability and intracellular CTGF mRNA expression induced by silica particles, we finally chose 48 h as the time point to do the following toxicological experiments.

As shown in Fig. 2b and c, the intracellular CTGF protein expression slightly increased in 16HBE cells with exposure to silica particles at concentrations of 12.5 and 25 μg/ml, but there was no significant difference when compared with blank control group ($P > 0.05$).

Secretions of Inflammatory Cytokines Induced by Silica Particles from 16HBE Cells

The levels of inflammatory cytokines released from 16HBE cells with exposure to silica particles for 48 h are shown in Fig. 3. The secretions of IL-8, TNF-α, IL-6 and IL-17A induced by silica particles gradually increased in a dose-dependent manner. Compared with blank control group, the secreted level of IL-8 was significantly higher from 16HBE cells with exposure to 25 and 50 μg/ml silica particles ($P < 0.05$). The expressions of TNF-α and IL-6 were much higher when silica particles' concentrations

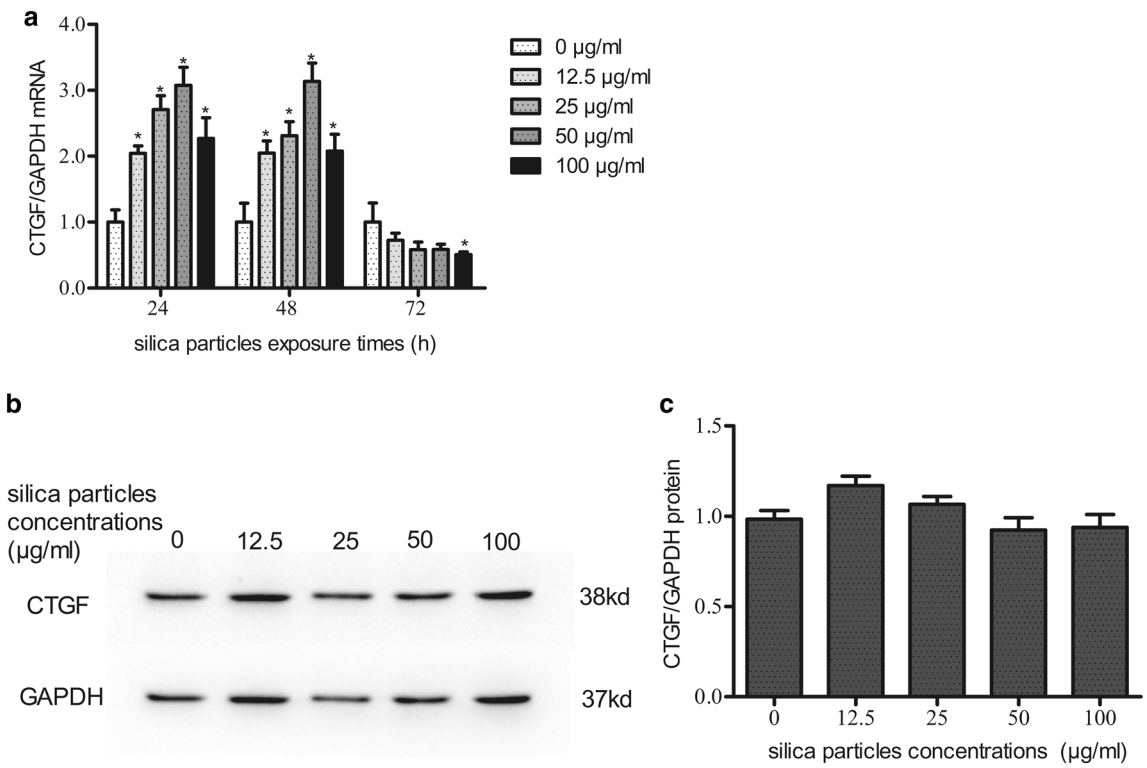

Fig. 2 Intracellular CTGF expression in 16HBE cells with exposure to silica particles. **a** The changed level of intracellular CTGF mRNA at different time points. **b** Intracellular CTGF protein expression analyzed by Western blotting at 48 h. **c** Relatively quantitative analysis of intracellular CTGF protein expression at 48 h. Data were expressed as means ± SD ($n = 3$). *$P < 0.05$ compared with blank control group (0 μg/ml) at each time point

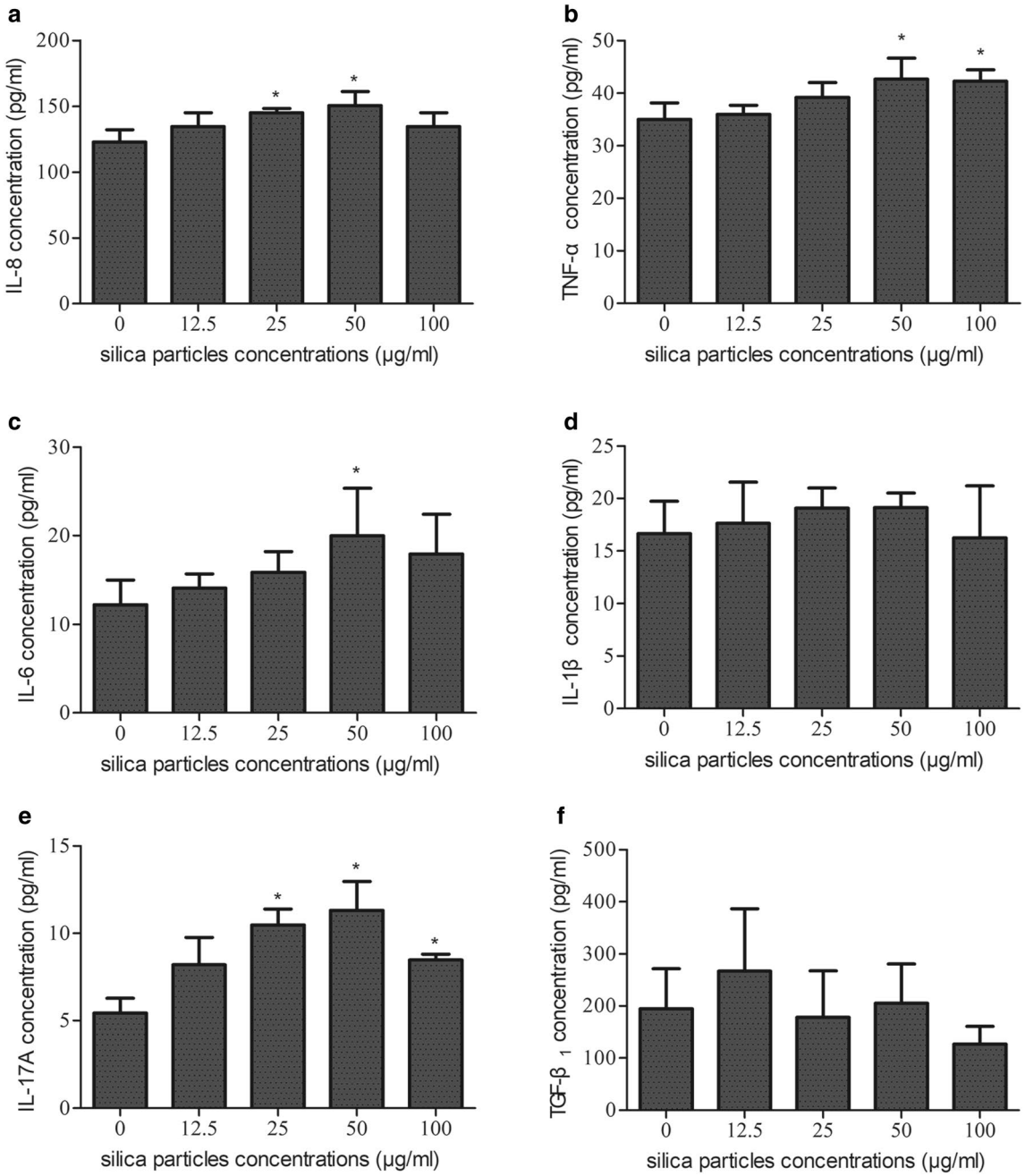

Fig. 3 Secretions of inflammatory cytokines induced by silica particles from 16HBE cells at 48 h. The secreted levels of IL-8 (**a**), TNF-α (**b**), IL-6 (**c**) IL-1β (**d**), IL-17A (**e**) and TGF-β_1 (**f**) were determined by ELISA kits. Data are presented as means ± SD ($n = 6$). *$P < 0.05$ compared with blank control group (0 μg/ml)

were equal to or higher than 50 μg/ml ($P < 0.05$). In addition, IL-17A expression significantly increased by 25, 50 and 100 μg/ml silica particles ($P < 0.05$). However, compared with blank control group, no significant differences were observed in IL-1β and TGF-β_1 secretions induced by silica particles at any concentrations ($P > 0.05$).

Effects of CTGF siRNA on the Intracellular CTGF Expression Induced by Silica Particles in 16HBE Cells

As shown in Fig. 4a, intracellular CTGF mRNA expression in 16HBE cells transfected with CTGF siRNA was significantly lower than that in normal cells with exposure to silica particles at concentrations of 25 and 50 μg/ml ($P < 0.05$).

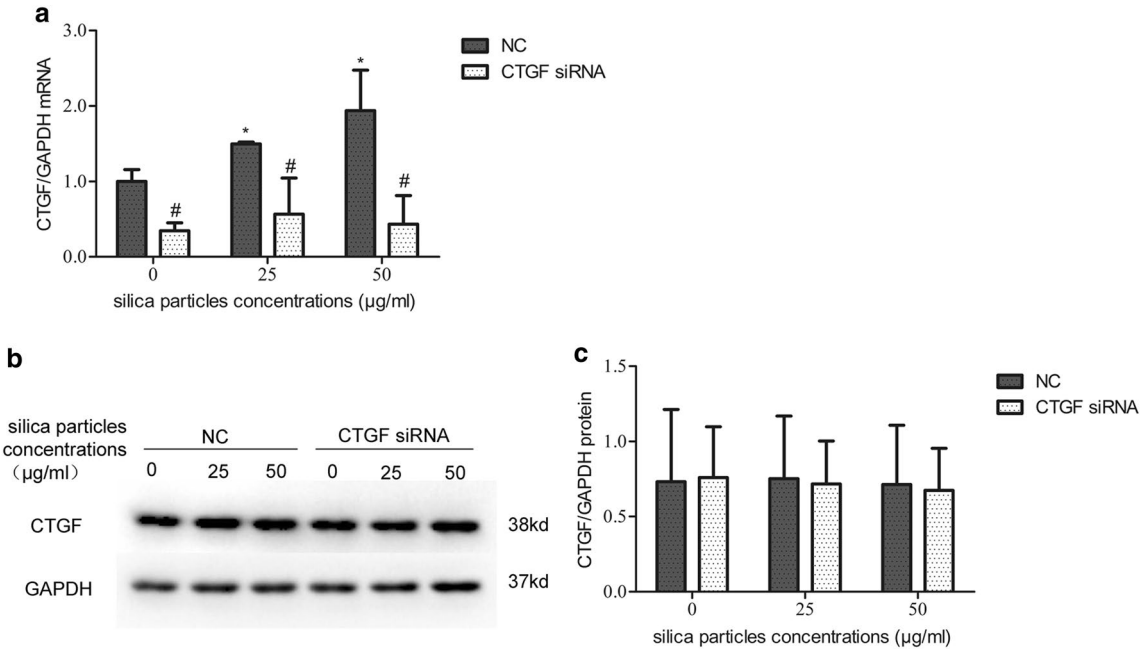

Fig. 4 Effects of CTGF siRNA on intracellular CTGF expression induced by silica particles in 16HBE cells at 48 h. **a** The changed level of intracellular CTGF mRNA expression. **b** Intracellular CTGF protein expression analyzed by Western blotting. **c** Relatively quantitative analysis of intracellular CTGF protein expression. Data were expressed as means ± SD ($n = 3$). *$P < 0.05$ compared with blank control group (0 μg/ml) in normal 16HBE cells. #$P < 0.05$ CTGF siRNA-transfected cells vs. normal 16HBE cells when exposed to the same concentration of silica particles

However, the level of intracellular CTGF protein was not changed in CTGF siRNA-transfected cells (Fig. 4b, c).

CTGF siRNA Attenuated Inflammatory Responses Induced by Silica Particles in 16HBE Cells

In Fig. 5, the secretions of IL-8, TNF-α, IL-6 and IL-17A from CTGF siRNA-transfected 16HBE cells were significantly lower than that from normal cells with exposure to 25 or 50 μg/ml silica particles ($P < 0.05$). However, there was no statistical difference in the expressions of IL-1β and TGF-β_1 between CTGF siRNA-transfected cells and normal 16HBE cells with exposure to 25 and 50 μg/ml silica particles ($P > 0.05$).

Discussion

In the present study, we found that exposure to silica particles significantly induced cytotoxicity and elevated expression of intracellular CTGF mRNA in a dose-dependent manner at 48 h, which was similar to the increased secretions of IL-8, TNF-α, IL-6 and IL-17A in 16HBE cells. This is largely in accordance with the wealth of literature on the inflammatory responses of silica particles on bronchial epithelial cells [27–29]. After CTGF siRNA interference, the expression of intracellular CTGF mRNA was significantly lower than that in normal 16HBE cells caused by silica particles. The levels of IL-8, TNF-α, IL-6 and IL-17A secreted from CTGF siRNA-transfected cells also significantly decreased when compared with normal 16HBE cells with exposure to silica particles. Our findings suggest that inhibition of CTGF gene attenuates silica-induced pulmonary inflammatory responses in bronchial epithelial cells.

Consistent with our results, CTGF stimulation also induces high levels of TNF-α, IL-6, MCP-1 and IL-8 in cardiomyocytes [23]. In addition, systemic administration of CTGF increases renal expressions of MCP-1, IL-6, IL-4 and IFN-γ that recruit immune cells including T lymphocytes and monocytes/macrophages to promote inflammation by activating NF-κB signaling pathway [24]. Although we did not evaluate NF-κB gene expression, a large body of evidence have demonstrated that NF-κB is a pivotal transcription factor activated by silica particles in macrophages, lung epithelial cells and other types of lung cells, which is critical for the productions of inflammatory mediators in silica-associated lung diseases [30–33]. Therefore, it may be suggested that intracellular CTGF mRNA could regulate the inflammatory responses caused by silica particles in bronchial epithelial cells through activating NF-κB signaling pathway. Partially supported by Wahab's study, addition of exogenous CTGF to mesangial cells could be internalized

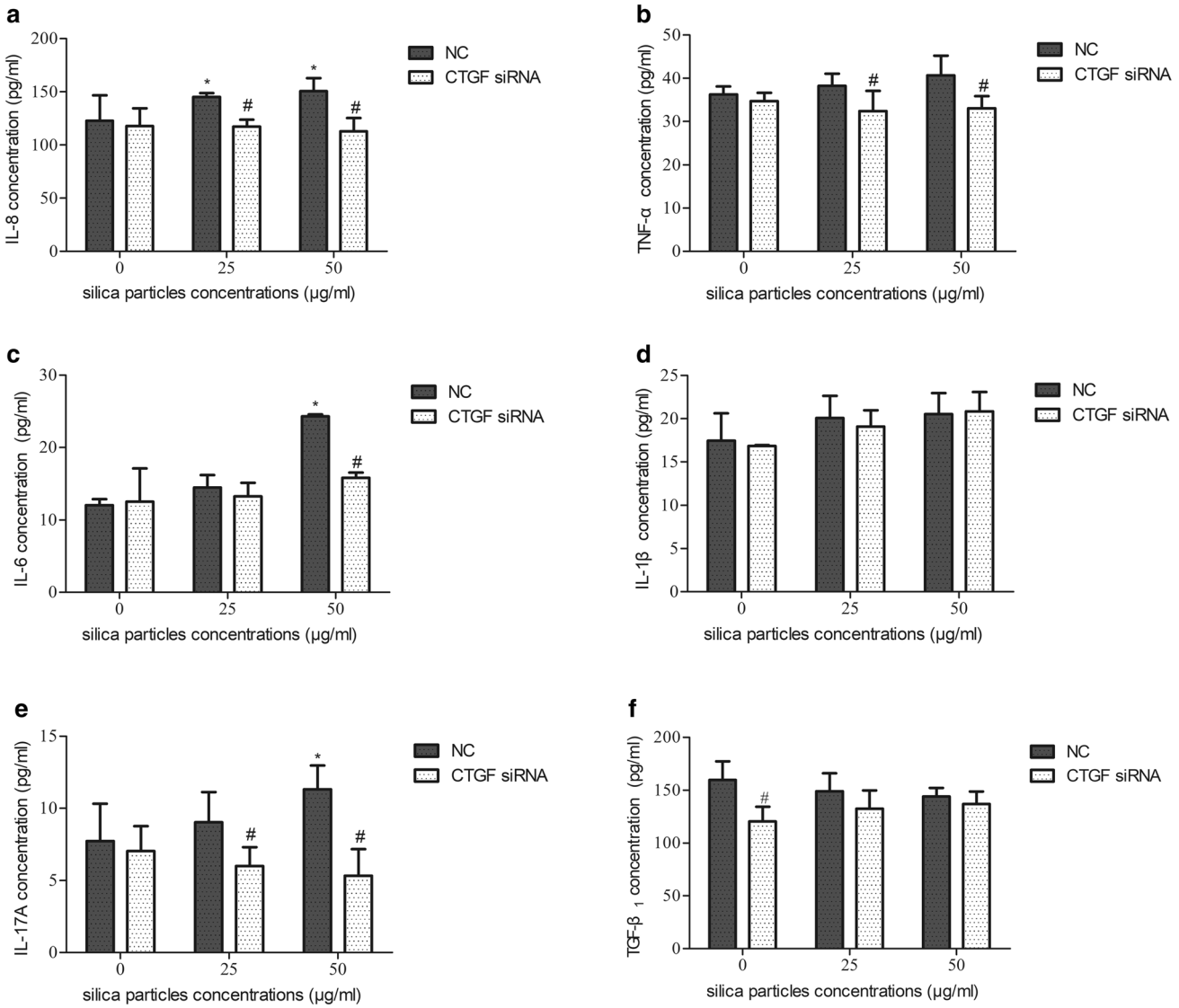

Fig. 5 CTGF siRNA attenuated inflammatory cytokines secretions induced by silica particles from 16HBE cells at 48 h. The secreted levels of IL-8 (**a**), TNF-α (**b**), IL-6 (**c**) IL-1β (**d**), IL-17A (**e**) and TGF-β_1 (**f**) were determined by ELISA kits. Data are presented as means ± SD ($n = 6$). $^{*}P < 0.05$ compared with blank control group (0 μg/ml) in normal 16HBE cells. $^{\#}P < 0.05$ CTGF siRNA-transfected cells vs. normal 16HBE cells when exposed to the same concentration of silica particles

and transported into the nucleus where it may directly affect the transcription of total ribosomal RNA [34]. It is further confirmed by Gao's study, showing that CTGF-induced IκBα phosphorylation and degradation is associated with the translocation of cytoplasmic NF-κB to nucleus [35]. CTGF could promote three activated NF-κB dimers to individually bind to DNA binding sites and subsequently trigger NF-κB transcriptional activity [35].

A recent epidemiological study revealed that the ratio of neutrophil to lymphocyte is significantly higher in patients with stage II and III silicosis in comparison with healthy controls, suggesting that neutrophils play a crucial role in the progression of early inflammation into terminal fibrosis [36]. IL-8 is a major inflammatory cytokine secreted by epithelial cells, which contributes to recruiting and activating neutrophils in the lesions of inflammatory reaction [37]. We found that silica particles induce significantly high level of IL-8 with a similar dose–response trend to CTGF mRNA expression, whereas inhibition of CTGF obviously reduces IL-8 secretion. To date, there is considerable evidence that CTGF treatment stimulates the expression of IL-8 gene transcription and production in various cells [23, 38, 39]. Thus, CTGF may mediate silica-induced inflammatory responses by regulating IL-8 expression in bronchial epithelial cells.

These effects may be explained by the following distinct mechanisms [23, 38]. First, CTGF exposure has a positive influence on IL-8 mRNA stability through inhibiting IL-8 mRNA degradation by p38 MAPK activation. Second, CTGF increases IL-8 promoter and promotes its transcription, which is dependent on CTGF-activated NF-κB and AP-1 binding to IL-8 DNA sites. Finally, IL-8 mRNA could also be regulated by CTGF via TGF-β and TrkA signaling pathway.

In addition, Kular et al. reviewed that inflammatory cytokines including TNF-α, IL-1β, TGF-β and IL-17A are capable of regulating CTGF expression, and reciprocally, CTGF gene could participate in the regulation of their expressions [22]. TNF-α alone or in combination with TGF-β exerts a positive role in CTGF expression [40, 41]. Besides, in the presence of IL-1β, CTGF treatment upregulates the levels of IL-8 and IL-6 [42]. Thus, although the expressions of IL-1β and TGF-β_1 were not altered by silica particles, their presence may have a synergistic effect on CTGF expression or promote the role of CTGF on inflammation. Furthermore, several studies have indicated that IL-17A directly elevates CTGF gene expression in a dose-dependent manner [43, 44], and the expression of CTGF mRNA is significantly reduced in IL-17A-deficient mice with bleomycin-induced skin fibrosis [45]. In contrast, Rodrigues-Díez R found that C-terminal module of CTGF induces human Th17 cells differentiation and upregulates IL-17A and IL-6 mRNA and protein [46], which is consistent with our results. Therefore, it is indicated that these inflammatory cytokines are capable of regulating CTGF expression, and conversely, CTGF gene could mediate the productions of inflammatory cytokines in silica-induced inflammation, which warrant further investigation.

In conclusion, we found evidence of enriched expression of CTGF mRNA in association with inflammatory cytokines induced by silica particles in human bronchial epithelial cells. Inhibition of CTGF gene attenuates the increased expressions of IL-8, TNF-α, IL-6 and IL-17A caused by silica particles. Therefore, this raises the possibility of CTGF being an important regulator in the development of the silica-induced inflammatory responses.

Acknowledgements This work was financially supported by grants from the National Natural Scientific Foundation of China (81402659), the China Scholarship Council Fund (201808420128), the Natural Scientific Foundation of Hubei Province (2014CFB811, 2016CFB520) and the Outstanding Project of Young and Middle-aged Team in Wuhan University of Science and Technology (2018TDZ03).

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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