Silica promotes the transdifferentiation of rat circulating fibrocytes *in vitro*

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Abstract. To investigate the effects of silica on circulating fibrocytes (cFbs), the present study established a primary culture model of rat alveolar macrophages and cFbs *in vitro*. Macrophages were treated with free silica, and their supernatant was used to stimulate cFbs. The mRNA expression levels of collagen I, collagen III and α-smooth muscle actin (SMA) in cFbs were analyzed by reverse transcription-quantitative polymerase chain reaction. The intracellular and extracellular protein expression levels of collagen I, collagen III and α-SMA were detected by ELISA and immunofluorescence staining. The results indicated that in the cell model, the free silica effectively increased the protein and mRNA expression levels of collagen-I, collagen-III and α-SMA. The free silica significantly promoted the transdifferentiation of cFbs into myofibroblasts in a dose-and time-dependent manner.

Introduction

Silicosis, which is among the longest known occupational diseases, is caused by the inhalation of silica particles, usually at low levels but over long time periods, which eventually causes irreversible lung fibrosis. Regardless of its source, purity and age, crystalline silica induces various pathological reactions in the lung and causes irreversible fibrosis and damage (1,2). Fibrocytes are a cell type arising from monocyte precursors expressing surface markers for leukocytes and mesenchymal cells, which are capable of differentiating into myofibroblasts (3-5). They were described in 1994 as a circulating, bone marrow-derived cells with the ability to adopt a mesenchymal phenotype (4). Fibrocytes are initially present in injured organs and have the inflammatory features of macrophages; they have been shown to participate in granuloma formation, antigen presentation and various fibrotic disorders (6). Fibrocytes represent a minor component of the circulating pool of leukocytes and express a characteristic pattern of markers, including collagen I and collagen III (4). α smooth muscle actin (α-SMA) expressed on the surface of fibrocytes is a surface marker of myofibroblasts (7,8). A previous study demonstrated that peripheral blood fibrocytes migrate to skin wound chambers in humans as well as in mice (9). Fibrocytes have been identified at sites of active fibrosis and in fibrotic pathologies, including hypertrophic scars, asthma and idiopathic pulmonary fibrosis (IPF) (10-12). Fibrocytes contribute to fibrogenesis by directly producing collagen, hematopoietic growth factors, inflammatory cytokines and chemokines (13).

It is well known that fibroblasts are responsible for repair and remodeling within the lung. Circulating fibrocytes (cFbs) in the blood are involved in tissue damage repair and were shown to be able to transform into fibrocytes *in vitro* (5). cFbs have been described as a potential source of increased extracellular matrix and myofibroblasts, markers of fibrotic pathologies, and to contribute to the pathogenesis of pulmonary fibrosis (14). Without treatment, continued fibrosis leads to loss of lung function and ultimately, sufferers succumb to the disease. cFbs locate to the damaged area of lung tissue via the circulatory system through the action of chemotactic factors and differentiate into active fibrocytes prior to repairing and rebuilding the lung tissue (15,16). cFbs may function as precursors of myofibroblasts in proliferative vitreoretinopathy membranes (8). cFbs have an important role in fibrosis damage, including pneumoconiosis (17).

The effects of crystalline silica (SiO_2) on cFbs and on the development of silicosis have yet to be determined. cFbs may be recruited to injured lungs as an integral component of the pathogenesis of pulmonary fibrosis. The aim of the present study was to evaluate the effects of free SiO_2 on the differentiation of cFbs *in vitro*, specifically with regard to the effects of free SiO_2 on the expression levels of collagen I, collagen III and α-SMA. Sprague Dawley (SD) rat cFbs and alveolar macrophages (AMs) were separated and cultured in order to establish a primary rat culture model of AMs and cFbs *in vitro*. AMs were treated with free SiO_2 at 0, 20, 40, 60, 80, 100 and 120 μg/ml, the cell culture supernatant was collected and then used to stimulate cFbs. cFbs stimulated by the supernatant were then identified by immunohistochemical analysis. The mRNA expression levels of collagen I, collagen III and α-SMA in the cFbs were analyzed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The extracellular protein

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expression levels of collagen I, collagen III and α-SMA were detected by ELISA. The results of the present study further clarified the role of cFbs in silicosis.

Materials and methods

Animals. Specific pathogen-free male SD rats were purchased from the animal center of Henan province (Henan, China). The rats (age, 8-13 weeks; weight, 120-150 g) were housed in polycarbonate isolator cages with autoclaved bedding, and provided with *ad libitum* access to autoclaved reverse-osmosis water and standard rat food. Animals were maintained in accordance with the guidelines of the Chinese Association of Laboratory Animal Care, all experiments and surgical procedures complied with the relevant provisions of the Experimental Animal Ethics Committee of Zhengzhou University (Zhengzhou, China).

Reagents. The SiO_2 dust (purity, >99%; particle size, <5 μm) was obtained from The Center for Disease Control and Prevention (Beijing, China) and sterilized by hot air sterilization at 190˚C for 1.5 h. The stock solutions (100 mg/ml) of SiO_2 were suspended in Dulbecco's modified Eagle's medium (DMEM; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and diluted to the desired working concentrations for further experimentation.

Cell isolation and culture. The cFbs were prepared as previously described (4). Total peripheral blood lymphocytes were first isolated from 45 ml rat blood by centrifugation in Ficoll® Paque Plus (GE Healthcare Life Sciences, Little Chalfont, UK) according to the manufacturer's instructions. Following overnight culture in six-well plates in high-glucose DMEM supplemented with 20% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT, USA), non-adherent cells were removed by gentle aspiration. The experiments were conducted at 37˚C in a 5% CO_2 humidified atmosphere after six weeks of culture.

Rat AMs were collected from the SD rats by bronchoalveolar lavage. Briefly, the rats were anesthetized with 2% pentobarbital (Sigma-Aldrich, Shanghai, China) by intraperitoneal injection, and following tracheal exposure and cannulation, the airways of the rats were gently treated with 5 ml cold sterile D-Hank's solution. This procedure was repeated two additional times. The bronchoalveolar lavage fluid was collected and centrifuged at 800 x g, for 5 min at 4˚C to form pellets of AMs, which were re-suspended in DMEM supplemented with 10% FBS. The AMs were counted using a hemocytometer, and the cells were incubated at 37˚C for 2 h in an atmosphere containing 5% CO_2. The medium was then discarded and any non-adherent cells were removed by washing.

Fluorescence-activated cell sorting analysis. The cells were harvested after six weeks of culture with 0.25% trypsin (Beijing Solarbio Science & Technology Co., Ltd.) and pelleted by centrifugation for 7 min at 250 x g. The cells were then re-suspended in 0.5 ml phosphate-buffered saline (PBS), and phycoerythrin-conjugated anti-collagen I and fluorescein isothiocyanate-conjugated anti-CD45 antibodies (BD Biosciences, Franklin Lakes, NJ, USA) were added according to the manufacturer's instructions. Following incubation in a dark environment for 30 min at 4˚C, cell fluorescence was evaluated by flow cytometry (Accuri C6; BD Biosciences).

Cytotoxicity assay and cellular activity. The AMs were seeded into 96-well plates at a density of $3x10^5$ cells/well (250 μl media per well). After 24 h of incubation, the media was replaced with fresh media supplemented with SiO_2 at 0, 20, 40, 60, 80, 100 or 120 μg/ml. Each concentration was assayed in six replicates, and the control wells received no SiO_2 (0 μg/ml). An MTT assay was performed after 24 h of culture. cFbs from six wells in each group were seeded in 96-well plates at a density of $1.5x10^4$ cells/well (200 μl media per well). A total of 24 h after seeding, the cells were collected and counted under a light microscope (Eclipse TS100-F; Nikon, Tokyo, Japan) with trypan blue exclusion (Sigma-Aldrich). The MTT assay was performed once daily from the first day of culture, the cells from six wells of each day were assessed for ten consecutive days, and the cell numbers were used to produce a growth curve. The optical density (OD) was measured at 490 nm using a plate microreader (Tecan Infinite M200; Tecan, Wetzlar, Germany). The proliferation rate was determined using the following formula: Cell proliferation (%) = $OD_{experimental\ samples}/OD_{control}$ x 100% [n=6, mean ± standard deviation (SD)]. The cell growth curve was used to determine the activity levels of the cFbs, and to evaluate the inhibitory effects of SiO_2 on the growth of the macrophages in order to ascertain the maximum non-lethal concentration of SiO_2.

Cell treatment. A primary culture model of rat AMs and cFbs was established *in vitro*. After 24 h of culture, the AMs were treated with various concentrations of SiO_2 (0, 20, 40, 60, 80, 100 and 120 μg/ml). After 24 h of SiO_2 treatment, the cell culture supernatant and cells were collected for the subsequent experiments. After three days of cFbs culture, the cells were treated with supernatant for 24 h. The supernatant was collected from the AMs, which were stimulated with SiO_2 at various concentrations (0, 20, 40, 60 and 80 μg/ml), and each concentration was assayed in six replicates. The cell culture supernatant was collected from AMs and was then transferred to cFbs each day for 28 days consecutively, the cFbs were cultured with DMEM supplemented with 10% FBS (control), 0 μg/ml SiO_2-treated AM supernatant (0 μg/ml, 2 ml for $1x10^5$ cells) and 80 μg/ml SiO_2-treated AM supernatant (80 μg/ml, 2 ml for $1x10^5$ cells) respectively. After cultivation for 3, 6, 9, 12, 15 and 18 days for collagen I and Collagen III detection, and 13, 16, 19, 22, 25 and 28 days for α-SMA detection, the cell culture supernatant and cells were collected for the subsequent experiments.

Immunohistochemistry. Immunofluorescence staining was used to determine the levels of collagen I, collagen III and α-SMA proteins in the cFbs. The cFbs were fixed in 4% (v/v) paraformaldehyde (Sigma-Aldrich) for 20 min at 4˚C. To suppress endogenous peroxidase activity, the samples were treated with 3% hydrogen peroxide solution (Wuhan Boster Biological Technology, Ltd., Wuhan, China) for 15 min and rinsed with PBS. To prevent non-specific immune reactions,

Table I. Primers for β-actin, collagen I, collagen III and α-SMA mRNA and their annealing temperatures.

Gene	Primers	Predicted size (bp)	Annealing temperature (˚C)
β-actin	F, 5'-CCCATCTATGAGGGTTACGCT-3' R, 5'-TTTAATGTCACGCACGATTTC-3'	519	54
Collagen I	F, 5'-CCCACCCCAGCCGCAAAGAT-3' R, 5'-TTGGGTCCCTCGACTCCTACA-3'	352	51
Collagen III	F, 5'-TGCCCACAGCCTTCTACACCT-3' R, 5'-CAGCCATTCCTCCCACTCCAG-3'	240	52
α-SMA	F, 5'-CCGAGATCTCACCGACTACC-3' R, 5'-TCCAGAGCGACATAGCACAG-3'	121	51

F, forward; R, reverse.

the samples were treated with 3% normal horse serum (Wuhan Boster Biological Technology, Ltd.) for 20 min. Non-specific staining was prevented by omitting primary antibodies and rat non-immune serum. The slides were gently agitated and then incubated with mouse anti-rat α-SMA monoclonal antibody (dilution 1:50; cat. no. BM0002; Wuhan Boster Biological Technology, Ltd.), mouse anti-rat collagen I polyclonal antibody (dilution, 1:50; cat. no. BA0325; Wuhan Boster Biological Technology, Ltd.) and mouse anti-rat collagen III polyclonal antibody (dilution, 1:50; cat. no. BA0326; Wuhan Boster Biological Technology, Ltd.) at 37˚C for 90 min. Following washing with PBS, horseradish peroxidase-conjugated goat-anti-mouse monoclonal antibody (dilution, 1:200; OriGene Technologies, Inc., Beijing, China) was added and the solution was incubated at 37˚C for 30 min, followed by rinsing with PBS. Subsequently, avidin-biotin complex solution was added to the solution and incubated for 30 min. The slides were rinsed with PBS, stained with 3-3'-diaminobenzidene (Origene Technologies, Inc.) for 5 min and rinsed with the buffer solution, as previously described (18,19).

Extraction and RT-qPCR analysis. RNA extraction and RT-qPCR analysis of the mRNA expression levels of collagen I, collagen III and α-SMA were performed as previously described (20). The primers were designed and synthesized by Takara Biotechnology Co., Ltd. (Dalian, China) and the annealing temperatures used for the PCR are shown in Table I. A PrimerScript RT reagent kit (Tiangen Biotech Co., Ltd., Beijing, China) was used to reverse-transcribe the total RNA according to the manufacturer's instructions. The resulting cDNA was sequentially amplified with 2X PCR Master Mix (Tiangen Biotech Co., Ltd.) and 10 pmol of each primer in a Biometra TPersonal Thermocycler (Biometra GmbH, Goettingen, Germany). The thermocycling conditions were as follows: Initial denaturation at 94˚C for 3 min, and three-step PCR for 30 cycles (denaturation at 94˚C for 30 sec, annealing for 30 sec at the appropriate annealing temperature depending on the primer (51˚C for collagen I, 52˚C for collagen III, 51˚C for α-SMA and 54˚C for β-actin), and extension at 72˚C for 60 sec), with a final extension at 72˚C for 5 min. The PCR products were combined and then separated by 1.0% agarose gels containing ethidium bromide (Sigma-Aldrich). Autoradiographic films of the RT-qPCR assays were subjected to densitometric analyses using a Kodak Gel Logic 100 (Kodak, Rochester, NY, USA). The PCR bands were analyzed using Quantity One 4.3.0 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The amount of gene-specific PCR products was expressed as the ratio of the intensity of each band to that of the corresponding β-actin internal reference.

Detection of the protein expression levels of collagen I, collagen III and α-SMA in the cell culture supernatant by ELISA. After cultivation for 3, 6, 9, 12, 15 and 18 days, the levels of collagen I and collagen III in the cell culture supernatant were detected by ELISA, whereas the levels of α-SMA were detected after culture for 13, 16, 19, 22, 25 and 28 days. Collagen I, collagen III and α-SMA in the cFbs culture supernatant were detected using a commercially available ELISA kit (Yixing Qianchen Bioengineering Company, Shanghai, China) according to the manufacturer's instructions.

Statistical analysis. All values are expressed as the mean ± SD. SPSS 12.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Statistical analysis of the differences between treated and untreated groups was performed by one-way analysis of variance, and by Dunnett's test for multigroup comparisons. $P<0.05$ was considered to indicate a statistically significant difference. Graphic representation was performed using GraphPad Prism 5.0 for Windows (GraphPad Software, Inc., La Jolla, CA, USA).

Results

SiO_2 inhibits the growth of rat AMs and cFbs. The primary culture model of rat AMs and cFbs *in vitro* is shown in Fig. 1A. Following treatment of macrophages with various concentrations of SiO_2 (0, 20, 40, 60, 80, 100 and 120 μg/ml) for 24 h, the cell viability was detected using an MTT assay. As shown in Fig. 1B, compared with the control group (0 μg/ml SiO_2), treatment with SiO_2 at concentrations of 100 and 120 μg/ml significantly inhibited the growth of AMs, with a significant decrease in cellular activity ($P<0.05$). To

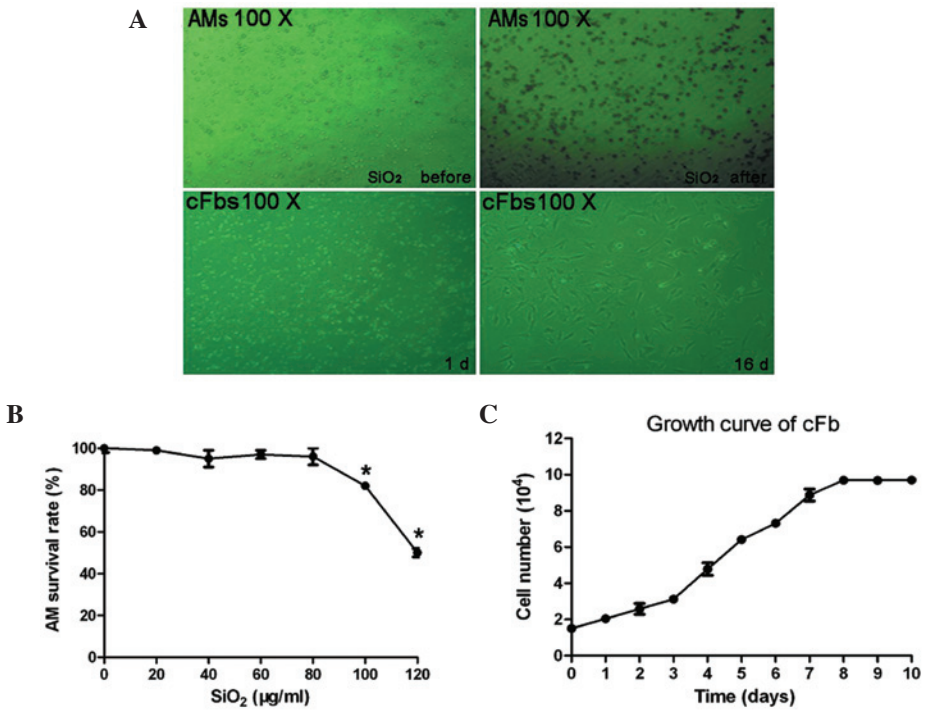

Figure 1. (A) Microscopic images of macrophages treated with SiO_2 for 24 h and of cFbs treated with macrophage supernatant. (B) Growth curve of cFbs. The survival rate of macrophages stimulated with SiO_2 at 0, 20, 40, 60, 80, 100 or 120 μg/ml (n=6 per treatment group) as determined by MTT assay. (C) Assessment the effects of SiO_2 on the proliferative rate of the cFbs. The cFbs were seeded and cultured with 80 μg/ml SiO_2 treated AM supernatant for 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 days. Viable cells were detected by MTT assay and the cells were counted. Values are expressed as the mean ± standard deviation. *P<0.05, vs. the control group (0 μg/ml SiO_2-treated group). cFbs, circulating fibrocytes; AMs, alveolar macrophages; SiO_2, crystalline silica.

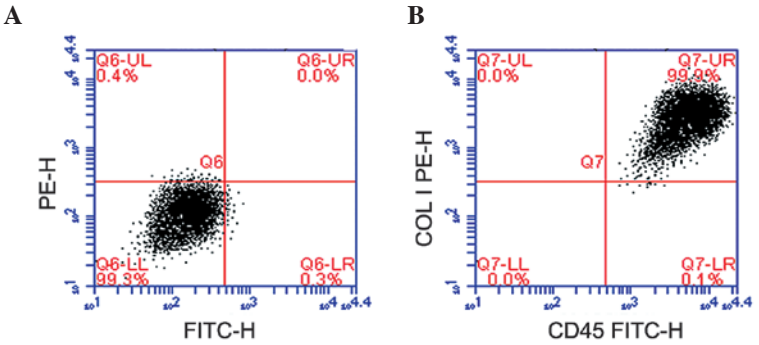

Figure 2. cFbs were identified by flow cytometry after 6 weeks of culture. Representative flow cytometric dot plots of circulating fibrocytes. (A) Isotype control for collagen 1 and CD45 using unstained cells. (B) Positive population for collagen 1 and CD45. COLI, collagen I; PE, phycoerythrin; FITC, fluorescein isothiocyanate; cFbs, circulating fibrocytes.

further confirm the effects of SiO_2 on the proliferative rate of cFbs, the cFbs were cultured with AM supernatant, treated with 80 μg/ml SiO_2 and subjected to an MTT assay at 1 day intervals. The cFbs were seeded and cultured for 1-10 days, and harvested and counted each day. As shown in the growth curve (Fig. 1C), the doubling time of the cells was 96 h, and the proliferative activity of the cFbs reached a peak between the fourth and eighth day.

Characterization of cFbs. cFbs were identified by flow cytometry, and the purity of the cFbs was assayed by staining with collagen I and CD45 antibodies after six weeks of culture. The results demonstrated that 99% of the cultured cells co-expressed collagen I and CD45 (Fig. 2).

SiO_2 stimulates the protein expression of collagen I, collagen III and α-SMA in cFbs. The present study investigated the effects of SiO_2 on collagen I, collagen III and α-SMA protein expression at various concentrations and incubation times by immunohistochemistry. As shown in Fig. 3A, collagen I, collagen III and α-SMA was significantly increased in the cFbs following treatment with the supernatant of AM treated with SiO_2 (0, 20, 40, 60 and 80 μg/ml) for 24 h. A dose-dependent increase in the expression levels of collagen I, collagen III and α-SMA was observed in the cFbs treated with the supernatant of AM treated with 20, 40 and 60 μg/ml SiO_2, as compared with that in the control group (n=6; P<0.05), whereas in the 80 μg/ml SiO_2 group, the expression of the respective proteins was similar to that in the 60 μg/ml SiO_2

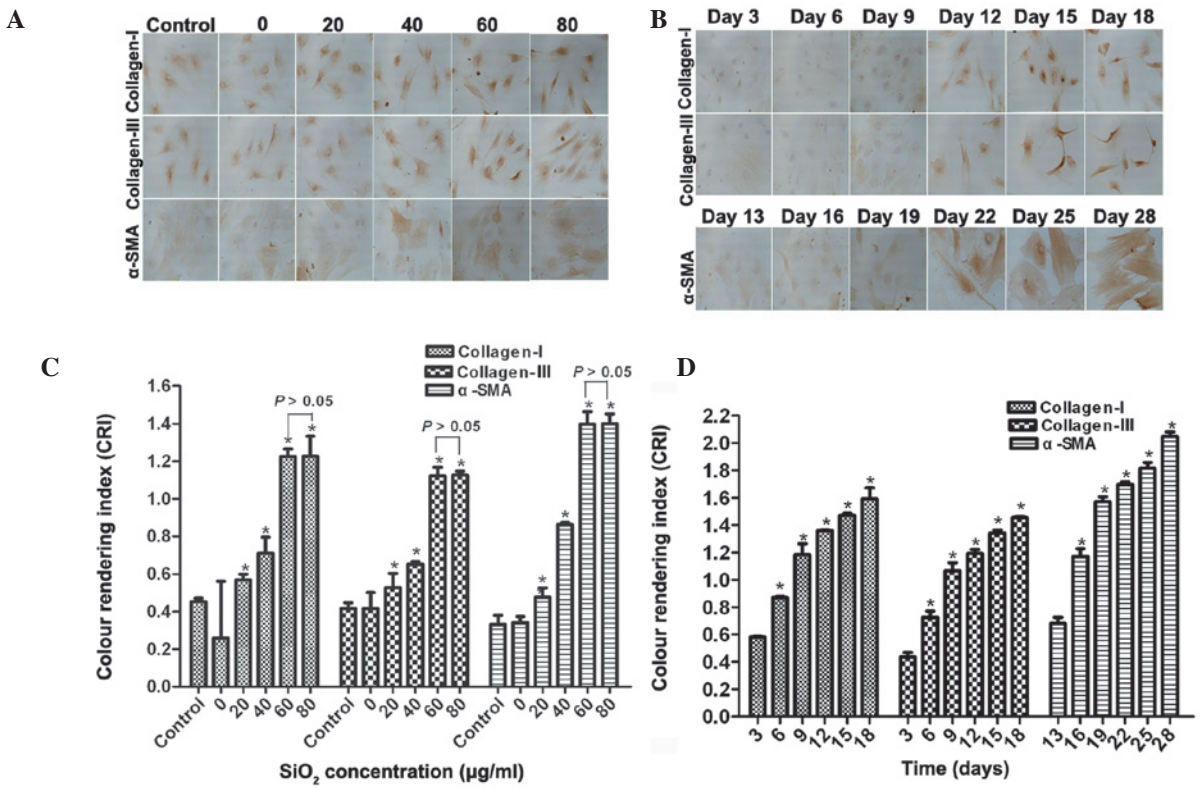

Figure 3. (A and B) Expression levels of collagen I, collagen III and α-SMA in cFbs treated with the supernatant of alveolar macrophages treated with various concentrations of SiO_2 for 24 h, as determined by immunofluorescence (magnification, x100). (C and D) The staining intensity of collagen I, collagen III and α-SMA was quantified by densitometric analysis. Values are expressed as the mean ± standard deviation of three independent experiments (n=6). *P<0.05, vs. the control group. cFbs, circulating fibrocytes; α-SMA, α smooth muscle actin; SiO_2, crystalline silica.

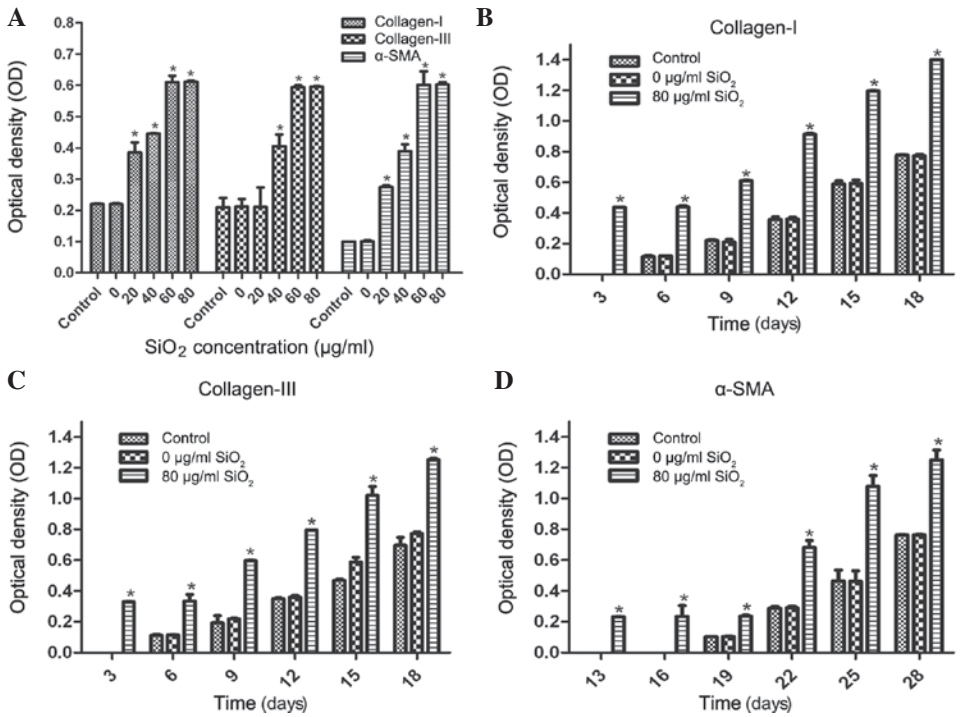

Figure 4. Protein expression levels of collagen I, collagen III and α-SMA in cFbs, as determined by ELISA. (A) The cFbs were treated with the supernatant of AM treated with SiO_2 (0, 20, 40, 60 and 80 μg/ml) for 24 h, and untreated cells served as the control group. The protein expression levels of collagen I, collagen III and α-SMA were quantified and normalized relative to the control group. The bands were quantified by densitometric analysis. (B-D) Expression levels of collagen I, collagen III and α-SMA following treatment with AM supernatant (0 and 80 μg/ml SiO_2) for 3, 6, 9, 12, 15 and 18 days for collagen I and collagen III, and for 13, 16, 19, 22, 25 and 28 days for α-SMA. The cFb supernatant was collected and protein expression was determined by ELISA. The expression levels of collagen I and collagen III were quantified and normalized relative to the control group. The bands were quantified by densitometric analysis. Values are expressed as the mean ± standard deviation from three independent experiments (n=6). *P<0.01, vs. the control. cFbs, circulating fibrocytes; α-SMA, α smooth muscle actin; SiO_2, crystalline silica; AM, alveolar macrophages.

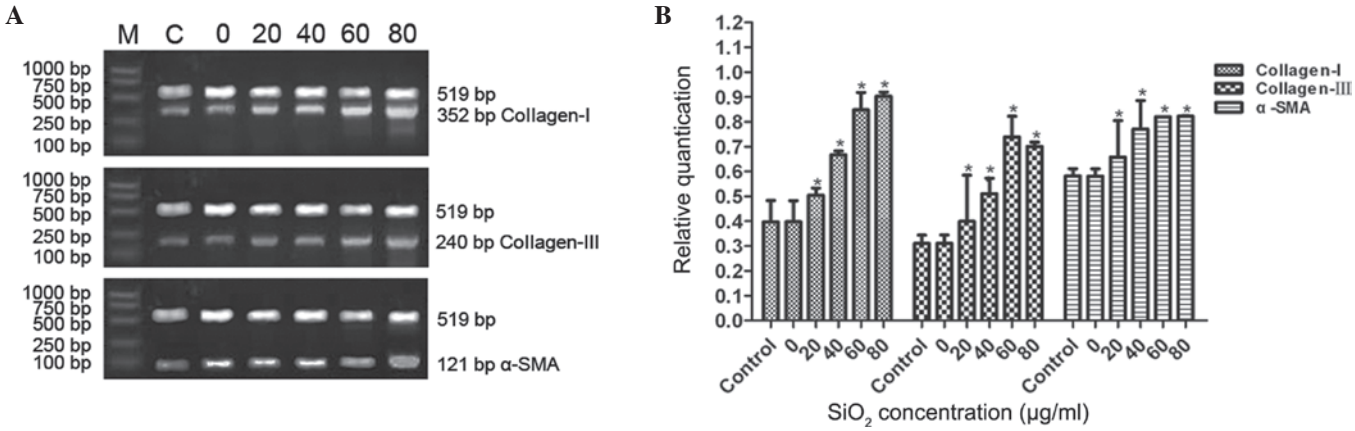

Figure 5. mRNA expression levels of collagen I, collagen III and α-SMA in cFbs treated with the supernatant of AM treated with various concentrations of SiO_2. The cFbs were treated with the supernatant of AM treated with SiO_2 at concentrations of 0, 20, 40, 60 or 80 μg/ml for 24 h, and the control group was not treated. (A) The cFbs were harvested, total RNA was extracted and the mRNA expression levels were determined by reverse transcription-quantitative polymerase chain reaction. (B) The mRNA expression levels of collagen I, collagen III and α-SMA were quantified and normalized relative to internal β-actin mRNA. The bands were quantified by densitometric analysis. Values are expressed as the mean ± standard deviation from three independent experiments (n=6). *P<0.01, vs. the control group. cFbs, circulating fibrocytes; α-SMA, α smooth muscle actin; SiO_2, crystalline silica; AM, alveolar macrophages.

group (P>0.05; Fig. 3C). Fig. 3B shows representative staining for collagen I, collagen III and α-SMA in cFbs following treatment for various durations with the supernatant of AM treated with 80 μg/ml SiO_2. The protein expression levels of collagen I, collagen III began to increase on day 3, and those of α-SMA on day 13. As compared with the control group, the expression levels of collagen I and collagen III were significantly upregulated on days 6, 9, 12, 15 and 18 (P<0.05), whereas the expression levels of α-SMA were significantly upregulated on days 16, 19, 22, 25 and 28 (P<0.05; Fig. 3D). These results suggested that SiO_2 stimulated the expression levels of collagen I, collagen III and α-SMA in a dose- and time-dependent manner.

In a further experiment, an ELISA was used to investigate the effects of SiO_2 on the protein expression levels of collagen I, collagen III and α-SMA in the cFbs cultured with the supernatant of AM treated with various concentrations of SiO_2 for various durations. The cFbs were treated with AM supernatant treated with SiO_2 at concentrations of 0, 20, 40, 60 and 80 μg/ml for 24 h, and untreated cFbs served as a control group. The protein expression levels of collagen I, collagen III and α-SMA were quantified and normalized relative to the control group. As shown in Fig. 4A, a marked increase was observed in collagen I, collagen III and α-SMA expression in the cFbs treated with SiO_2 at concentrations of 20, 40, 60 and 80 μg/ml for 24 h, as compared with that in the control group. No statistically significant difference was observed between the 60 μg/ml and 80 μg/ml treatment groups (P>0.05; n=6). Following cFbs treatment with 80 μg/ml SiO_2 for various durations, the protein expression levels of collagen I and collagen III began to increase on day 3, and those of α-SMA on day 13, whereas the expression levels of collagen I and collagen III in the 0 μg/ml SiO_2 and the control groups began to increase on day 6, and those of α-SMA on day 19. In the treatment group, the expression levels of collagen I and collagen III were significantly upregulated on days 3, 6, 9, 12, 15 and 18, as compared with those in the control group (P<0.05; Fig. 4B and C), whereas the expression of α-SMA was significantly upregulated on days 13, 16, 19, 22, 25 and 28 (P<0.05; Fig. 4D). These results suggested that SiO_2 stimulated collagen I, collagen III and α-SMA expression at the protein level and accelerated the processes of transdifferentiation of cFbs into myofibroblasts in a dose-dependent manner.

SiO_2 stimulates the mRNA expression of collagen I, collagen III and α-SMA in cFbs. To evaluate the effects of SiO_2 on collagen I, collagen III and α-SMA mRNA expression in cFbs, the cFbs were treated with the supernatant of SiO_2-treated AM (20, 40, 60 and 80 μg/ml for 24 h). Treatment with the supernatant of AM treated with various concentrations of SiO_2 increased the mRNA expression levels of collagen I, collagen III and α-SMA in the cFbs. This increase in expression levels became apparent at 20 μg/ml SiO_2 and markedly apparent at 80 μg/ml SiO_2. The expression of collagen I, collagen III and α-SMA was significantly and dose-dependently upregulated following treatment with 20, 40 and 60 μg/ml SiO_2, as compared with that in the control group (P<0.05), while no statistically significant difference was observed between the 60 μg/ml and 80 μg/ml groups (P>0.05; Fig. 5A and B). Following treatment of the cFbs with the supernatant of AM treated with 80 μg/ml SiO_2 for 28 days, the mRNA expression levels of collagen I, collagen III and α-SMA were determined by RT-qPCR. As shown in Fig. 6, increases in collagen I and collagen III expression were detected on day three, and increases in α-SMA expression was observed on day 13 of incubation, whereas in the control and 0 μg/ml groups, elevated collagen I and collagen III expression was detected on day 6, and elevated α-SMA expression on day 19. A significant upregulation of the expression levels of collagen I and collagen III in the treated groups was observed on days 3, 6, 9, 12, 15 and 18 (P<0.05). The expression levels of α-SMA were significantly upregulated on days 13, 16, 19, 22, 25, 28, as compared with those in the control group (P<0.05). These results suggested that SiO_2 was able to stimulate collagen I, collagen III and α-SMA activity at the transcriptional level, and increase the mRNA synthesis of collagen I, collagen III and α-SMA.

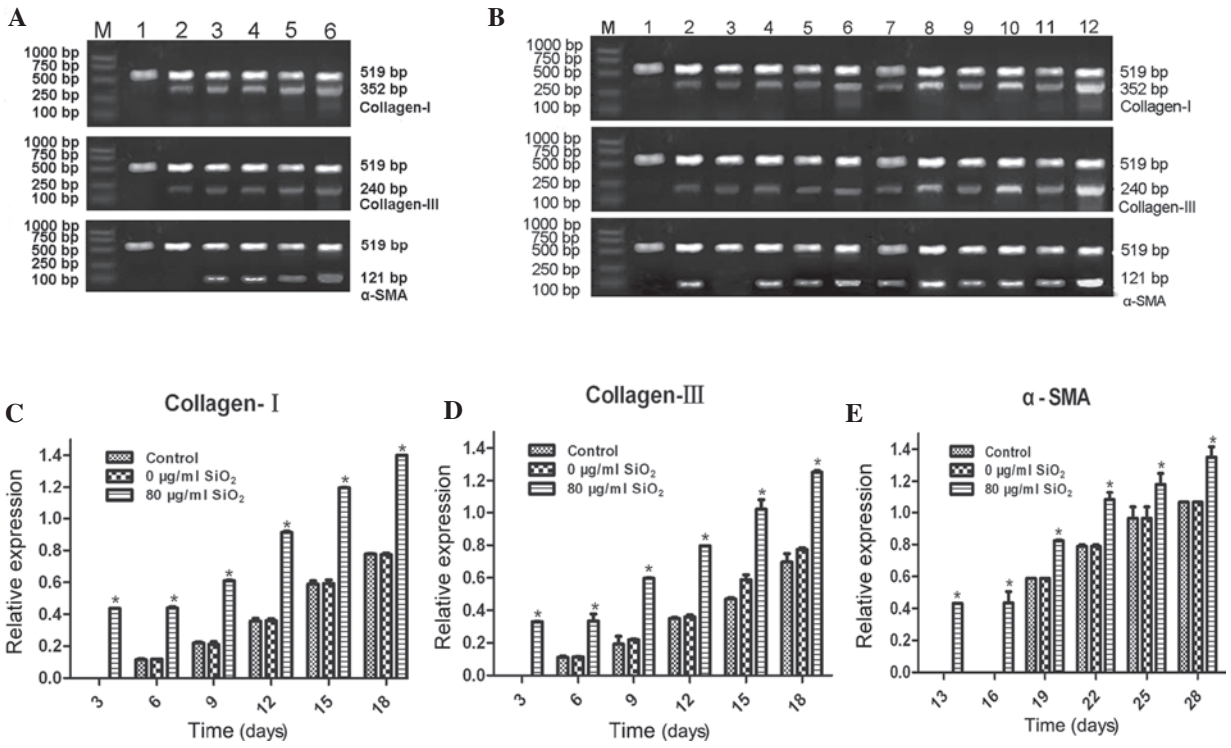

Figure 6. mRNA expression levels of collagen I, collagen III and α-SMA in cFbs. The cFbs were treated with alveolar macrophage supernatant treated with SiO_2 at concentrations of 0 and 80 μg/ml for 24 h, and untreated cells served as the control group. (A) Collagen I and collagen III detection was performed following cultivation with AM supernatant (0 μg/ml SiO_2) for (1) 3, (2) 6, (3) 9, (4) 12, (5) 15 and (6) 18 days, and α-SMA detection was performed following cultivation for (1) 13, (2) 16, (3) 19, (4) 22, (5) 25 and (6) 28 days. (B) Collagen I and collagen III detection was performed following treatment with AM supernatant (80 μg/ml SiO_2) for (1) 3, (2) 6, (3) 9, (4) 12, (5) 15 and (6) 18 days, and α-SMA detection was performed following cultivation for (1) 13, (2) 16, (3) 19, (4) 22, (5) 25 and (6) 28 days. Total RNA was extracted and quantified by reverse transcription-quantitative polymerase chain reaction. The mRNA expression levels of collagen I, collagen III and α-SMA were quantified and normalized relative to the mRNA expression levels of β-actin. (C-E) The bands were quantified by densitometric analysis and are shown in histograms. Values are expressed as the mean ± standard deviation from three independent experiments (n=6). *P<0.01, vs. the control group. cFbs, circulating fibrocytes; α-SMA, α smooth muscle actin; SiO_2, crystalline silica.

Discussion

The present study demonstrated that SiO_2 treatment accelerated cFb differentiation in a concentration- and time-dependent manner. In patients with ongoing lung fibrosis, blood monocytes may penetrate into the alveolar space, where they differentiate into fibrocytes (21). cFbs have previously been reported to be increased in patients with idiopathic pulmonary fibrosis (7). Silicosis is characterized by persistent inflammation, leading to fibroblast formation and excessive collagen deposition, which causes interstitial fibrosis and silicotic nodule formation (22). The presence of cFbs may serve as a prognostic biomarker for silicosis.

In healthy individuals, fibrocytes comprise 0.1-1.0% of the peripheral blood nucleated cells, and are present in a variety of healthy as well as diseased tissue types (5). Fibrocytes from human peripheral blood are circulating progenitor cells with the ability to differentiate into osteoblasts and chondrocyte-like cells (23). These cells remain active during tissue remodeling through the elaboration of extracellular matrix (ECM) proteins (collagen I and collagen III) and secrete various inflammatory mediators (cytokines, chemokines and growth factors) (5). Of note, fibrocytes deposit ECM components during various fibroproliferative disorders of the lung (14). The present study aimed to investigate differentiated fibrocytic cell lines expressing the myofibroblast marker α-SMA. The present study observed changes in collagen I, collagen III and α-SMA expression associated with the development of cFbs treated with SiO_2. The proliferative activity levels of AMs treated with SiO_2 were assessed using an MTT assay and the effects of their supernatants on cFbs were investigated by observing the mRNA and protein expression levels of collagen I, collagen III and α-SMA over the course of 28 days. A previous study reported that there are three stages in the process of cFbs cultivation *in vitro*: An early developmental stage over the course of the first three days, a differentiation stage from the fourth to the sixth day, and a ripening stage from the seventh day to the 18th day (17). The results of the present study demonstrated that the expression levels of collagen I, collagen III and α-SMA in cFbs increased at different time-points: Collagen I and collagen III expression increased on day 3, while α-SMA expression increased on day 13, and these expression levels continued to increase on days 3, 5, 7, 9 and 12 for collagen I and collagen III, and on days 13, 15, 17, 19 and 23 for α-SMA.

The mRNA expression levels of collagen I, collagen III and α-SMA in the experimental groups were significantly higher as compared with those in the control group, and the expression levels were increased by SiO_2 treatment in a dose-dependent manner. No statistically significant difference was observed between the 0 μg/ml SiO_2 treatment group and the control group (P>0.05). Therefore, the mRNA expression levels of collagen I, collagen III and α-SMA were increased

by SiO_2 in a dose-dependent manner. As compared with the control group, marked increases were observed in the mRNA expression levels of collagen I, collagen III and α-SMA in the treated groups. These results indicated that SiO_2 promoted the transdifferentiation of cFbs into myofibroblasts, as deduced from the marked increment of α-SMA expression. Increases in the mRNA expression levels of collagen I and collagen III were detected on day 6, those of α-SMA were detected on day 19, and mRNA levels continued to increase in a time-dependent manner.

The majority of liver injuries feature collagen accumulation and the destruction of normal liver architecture by fibrosis, a process that involves activated myofibroblasts that arose from local fibroblast precursors (24). α-SMA is expressed on the surface of fibrocytes and is a surface marker of myofibroblasts (7,8). As compared with the control group, following treatment with AM supernatant treated with SiO_2 at concentrations of 0, 20, 40, 60 and 80 μg/ml for 24 h, a marked increase in the expression levels of collagen I, collagen III and α-SMA was observed in the cFbs, as determined by immunohistochemistry and ELISA. No statistically significant difference was observed between the 60 μg/ml and 80 μg/ml treatment groups. These results suggested that 80 μg/ml SiO_2 may exhibit toxicity to cFbs, inhibiting the growth of cFbs. As determined by immunohistochemistry, following cFb treatment with 80 μg/ml SiO_2 for various durations, the expression levels in the 80 μg/ml treatment group were markedly elevated as compared with those in the control group, and these expression levels increased in a dose-dependent manner with increasing SiO_2 concentration ($P<0.05$). In the 0 μg/ml treatment and the control group, the protein expression levels of collagen I and collagen III were markedly increased on day 3, whereas those of α-SMA were markedly increased on day 16, as determined by ELISA. The protein expression levels of collagen I and collagen III in the 0 mg/kg treatment and the control groups markedly increased on day 6, whereas those of α-SMA increased on day 19. The variation in the results obtained by immunohistochemical analysis and ELISA indicated that collagen I, collagen III and α-SMA were expressed earlier in the cells, as compared with their presence in the culture supernatant. These results suggested that SiO_2 was able to stimulate translational expression of collagen I, collagen III and α-SMA and stimulate cFb transformation in a dose-dependent manner and the protein expressions of α-SMA, collagen I and collagen III in cells were similar to that of 3 days earlier in the culture supernatant at the same concentration of SiO_2.

The expression levels of collagen I and collagen III observed in the cFbs treated with 80 μg/ml SiO_2 were significantly higher, as compared with those in the 0 μg/ml treatment or control groups. Similar cellular distribution and appearance was observed in cells expressing α-SMA, as demonstrated by immunohistochemical analysis of cFbs. These results indicated upon treatment with the supernatant of AM treated with SiO_2, cFbs produced more collagen I, collagen III and α-SMA. It has been proposed that fibrocytes may be a transitional stage between a monocyte and a fibroblast (25). Previous studies have demonstrated the role of myofibroblasts in pulmonary fibrotic processes (26), and cFbs are capable of differentiating into myofibroblasts *in vitro* and *in vivo* (27). Concordant with these previous observations, in the present study, the presence of significantly higher levels of α-SMA expression by fibrocytes treated with the supernatant of AM treated with SiO_2, as compared with those in the control group (untreated), suggested and important role for α-SMA in the transformation of cFbs. The observations of the present study suggested that the time required for leukocytes to differentiate into cFbs was accelerated by SiO_2 treatment in a concentration-dependent manner.

The present study provided numerous important implications which serve as a basis for future studies. Considering that the silicosis-induced fibrotic process is similar to that of other fibrotic pulmonary diseases, the fibroproliferative effects of SiO_2 on cFbs are of paramount importance. In addition, the effects of cFbs in the development and progression of silicosis prompt further investigation. Future studies investigating the mechanisms underlying the role of cFbs in silicosis in the human body would be of great value.

Despite the significant aforementioned results, the present study has notable limitations. It remains elusive whether cFbs differentiate into myofibroblasts during the development and progression of silicosis *in vivo*. As the present study only provided results based on *in vitro* findings, future studies are required to further characterize the changes in cFbs that occur during the development and progression of silicosis. However, the present study represents an important contribution to scientific research and indicated that cFbs may represent a novel therapeutic target in the treatment of silicosis.

In conclusion, the present study provided a novel therapeutic approach, and determined that the overexpression of collagen I, collagen III and α-SMA protein as well as mRNA in cFbs in the peripheral blood may have an important role in the development of silicosis. In addition, the results of the present study may provide novel methods of silicosis prevention and control. The present study investigated the effects of SiO_2 on collagen I, collagen III and α-SMA protein and mRNA in cFbs *in vitro*. To the best of our knowledge, the present study was the first to demonstrate that the transdifferentiation of cFbs into myofibroblasts was accelerated by SiO_2. cFbs may represent a novel therapeutic target for developing more effective treatments against silicosis

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