Original Paper

Role of MCP1 in the Endothelial-Mesenchymal Transition Induced by Silica

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Key Words

Endothelial-mesenchymal transition • MCP1 • Silicosis • Fibrosis • Autophagy

Abstract

Background: Silicosis is characterized by the accumulation of fibroblasts and the excessive deposition of extracellular matrix. Fibroblast generation via endothelial-mesenchymal transition (EndMT) is one process responsible for this accumulation of fibroblasts. However, the mechanisms underlying EndMT remain unknown. **Methods:** Human umbilical vein endothelial cells (HUVECs) were exposed to SiO₂ (50 µg/cm²). Specific endothelial and mesenchymal markers were evaluated using immunofluorescence and western blot analysis. Functional changes were evaluated by analyzing cell migration and proliferation. LC3-adenovirus transfections were performed, and changes in autophagy were measured using a marker of autophagy. **Results:** SiO₂ induced decreases in the endothelial cell-specific markers in HUVECs while dramatically increasing mesenchymal cell product levels and mesenchymal functions. Although MCP1 expression increased in parallel with the increase in specific mesenchymal cell products, the MCP1 expression level was not consistent with the observed decrease in specific endothelial marker expression. Autophagy mediated the effects of MCP1, as rapamycin and 3-MA enhanced and attenuated the effect of SiO₂ on HUVECs, respectively. MAPKs and the PI3K/Akt pathway were involved in the regulation of MCP1 by SiO₂, and Pyk2 and MLC-2 mediated cell migration. **Conclusion:** Our findings reveal a new potential function of MCP1, suggesting a possible mechanism of fibrosis in pulmonary silicosis.

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Introduction

Silicosis is a disease that is characterized by progressive pulmonary fibrotic reactions [1-4]. The accumulation of activated fibroblasts and the excessive deposition of extracellular

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matrix (ECM) contribute to tissue reconstruction and fibrosis [5]. Traditionally, embryonic mesenchymal cells are considered to be the sources of intrapulmonary fibroblasts via cell proliferation [6]. However, many studies have indicated that during pulmonary fibrosis, endothelial cells can also contribute to the accumulation of fibroblasts via endothelial-mesenchymal transition (EndMT) [7-10].

EndMT has been observed during fibrosis in organs including the kidneys [11], liver, and heart [12] as well as in patients with diabetes [13] and metastatic tumors [12]. EndMT can occur during physiological and pathological processes. As a consequence of EndMT, endothelial cells lose their expression of specific endothelial markers, acquire a mesenchymal phenotype, and begin to express mesenchymal cell products, such as type I collagen, type III collagen and α -smooth muscle actin (α -SMA) [14].

Many factors can induce the EndMT process. The most important of these is transforming growth factor- β (TGF- β), which promotes EndMT through the Smad2/3 and Smad1/5/8 signaling pathways [15]. In contrast to our detailed knowledge of growth factors and their signaling pathways, the roles of chemokines in EndMT are poorly characterized. Previous data produced by our laboratory suggest that monocyte chemotactic protein-induced protein 1 (MCP1) is involved in the regulation of cell proliferation and migration via an miR-9-mediated mechanism. Moreover, we found that MCP1 played an important role in the SiO₂-induced proliferation and migration of pulmonary macrophages and fibroblasts, which are the important effectors of fibrosis [16-20]. On the other hand, MCP1 has been shown to be involved in enhancing angiogenic activity in human umbilical vein endothelial cells (HUVECs) and murine bone marrow-derived mesenchymal stem cells [21-23]. Additionally, whether MCP1 mediates EndMT in silicosis remains unknown.

In this study, we demonstrated that MCP1 causes endothelial cells that are exposed to silica to undergo EndMT via autophagy, and we show that MCP1, a chemokine family member, played a key role in EndMT. Furthermore, our findings may provide researchers with a new understanding of and new methods for treating pulmonary fibrosis associated with silicosis.

Materials and Methods

Reagents

SiO₂ was obtained from Sigma® (S5631), and 80% of the particles were less than 5 μ m in diameter. The particles were selected via sedimentation in a vertical glass tube according to Stokes' law, which yielded particles of 1-5 μ m in diameter [24]. The particles were then acid-hydrolyzed and baked overnight (200°C for 16 h). The silica samples used for the cell experiments were sterilized by autoclaving and then suspended in sterile normal saline (NS) at a concentration of 5 mg/ml. Fetal bovine serum (FBS), normal goat serum (NGS) and Dulbecco's modified Eagle's medium (DMEM; #1200-046) were purchased from Life Technologies™, and PenStrep (15140-122) was obtained from Fisher Scientific. PureCol® type I bovine collagen (3 mg/mL) was obtained from Advanced Biomatrix. Antibodies against MCP1 (SC136750, goat, 1:1000) and β -actin (SC8432, mouse, 1:2000) were obtained from Santa Cruz Biotechnology®, Inc. The collagen I (Col I; BS1530, 1:1000) and collagen III (Col III; BS1531, 1:1000) antibodies were obtained from BioWorld®. Antibodies against Akt (9272S, 1:1000), p-Akt (9271S, 1:1000), ERK (9107S, 1:1000), p-ERK (9101S, 1:1000), JNK (9258S, 1:1000), p-JNK (9251S, 1:1000), p38 (9212S, 1:1000), p-p38 (9211S, 1:1000) and α -SMA (14968S, 1:1000) were obtained from Cell Signaling®, Inc.

Cell culture

HUVECs were purchased from ScienCell® and maintained in T75 flasks in DMEM supplemented with 10% FBS. HUVECs from passages 3-7 (P3-7) were stored in liquid nitrogen. A vial of P3-7 HUVECs was thawed, plated, and passaged upon confluence to perform each experiment, and each experiment was performed using HUVECs between P10 and P15 [23].

Lentiviral transduction of HUVECs with GFP

HUVECs were transduced using LV-GFP lentiviruses (Hanbio Inc., Shanghai, China) as previously described [25]. Briefly, P3-4 HUVECs were cultured in 24-well plates at 1×10^4 cells/well in DMEM supplemented with 10% FBS for 48 h. The medium was then replaced with 1 ml of fresh medium and 8 μ g/ml polybrene. Then, 100 μ L of lentivirus solution (107 IU/ml) was added to each well, and the cells were incubated at 37°C in 5% CO₂ for 24 h. After the incubation period, the treatment medium was replaced with fresh DMEM supplemented with 10% FBS, and the cells were cultured at 37°C in 5% CO₂ until they reached >50% confluence. Transduced cells were selected using puromycin as follows: the medium was replaced with DMEM containing 10 μ g/ml puromycin and 10% FBS, and the cells were cultured at 37°C in 5% CO₂ for 24 h. The cells were then washed twice with fresh DMEM supplemented with 10% FBS. Purified transduced HUVEC cultures were expanded and/or stored in liquid nitrogen as previously described [26].

Transduction of HUVECs with dually fluorescent mRFP-GFP-MAP1LC3-adenovirus

The relative level of cellular autophagy was quantified using the fluorescent mRFP-GFP-MAP1LC3-adenovirus transduction technique as described by Zhu [23]. Briefly, HUVECs were transfected with dually fluorescent mRFP-GFP-MAP1LC3-adenovirus (Hanbio, Inc., Shanghai, China), which expressed a specific marker of autophagosome formation, to detect autophagy according to the manufacturer's instructions [27]. Five fields from three different cell preparations were chosen for analysis. GFP- and mRFP-expressing spots, which were visualized as fluorescent puncta, and DAPI-stained nuclei were counted manually. The number of GFP- and mRFP-expressing spots per cell was determined by dividing the total number of spots by the number of nuclei in each field.

MTT assay

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, the cells were collected and seeded in 96-well plates. Different seeding densities were used at the beginning of the experiments. The cells were exposed to SiO₂ (50 μ g/cm²). The cells were incubated for different periods of time (from 24-72 h), and 20 μ L of MTT dissolved in Hank's balanced salt solution at a final concentration of 5 μ g/ml was added to each well. The plates were then incubated in a 5% CO₂ incubator for 1-4 h. Afterwards, the medium was aspirated from each well, and 200 μ L of dimethyl sulfoxide was added to dissolve the formazan crystals. Finally, the absorbance of each well at reference wavelengths of 570 and 630 nm was measured using a plate reader. Each experiment was repeated at least three times.

Immunocytochemistry

HUVECs were fixed in 4% paraformaldehyde in PBS at 4°C overnight. The fixed samples were permeabilized for 30 min at room temperature (RT) with 0.3% Triton X-100 in PBS. The permeabilized samples were blocked in PBS containing 10% NGS (Life Technologies) and 0.3% Triton X-100 at RT for 2 h. The blocked samples were incubated in primary antibodies diluted in PBS containing 10% NGS and 0.3% Triton X-100 at 4°C overnight. Then, the samples were washed three times with PBS and incubated in donkey anti-rabbit (conjugated to Alexa-Fluor® 488) and donkey anti-mouse (conjugated to Alexa-Fluor® 576) secondary antibodies for 2 h at RT. After the samples were washed three times in PBS, they were mounted using mounting solution (Prolong® Gold antifade reagent with DAPI; P36931, Life Technologies). The slides were imaged using a fluorescence microscope (Olympus IX70, Olympus America, Inc., Center Valley, PA, USA).

Western blot analysis

Immunoblotting was performed as previously described [26], with minor modifications. HUVECs were collected from the culture dishes, washed with PBS and then lysed using a mammalian cell lysis kit (MCL1-1KT, Sigma-Aldrich®) according to the manufacturer's instructions. The western blot membranes were probed using primary antibodies. Alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit IgG secondary antibodies were then used (1:5,000). Signals were detected via chemiluminescence (SuperSignal West Dura Chemiluminescent Substrate, Thermo Scientific). Each western blot experiment was repeated using cells from three different donors. A single representative immunoblot is shown in each Figure.

Densitometric analysis was performed using ImageJ software, and the results from all of the repeated experiments were combined into one plot.

RNA interference using MCP1 siRNA

RNA interference targeting MCP1 was performed using HUVECs as previously described [28], with some modifications. The RNA interference protocol that was applied to individual wells in a 24-well plate was as follows. Briefly, 49 μ l of serum-free DMEM was combined with 1 μ l of transfection reagent, and 1 μ l of siRNA stock solution was added to another aliquot of 49 μ l of serum-free DMEM. These two solutions were incubated separately at RT for 15 min. The transfection reagent and siRNA solutions were then mixed together, and the resulting solution was incubated at RT for an additional 15 min. HUVECs were seeded at a concentration of 5.0×10^5 cells/100 μ l/well in serum-free DMEM. Afterwards, the siRNA-vehicle solution was added to the plated cells. The transfected HUVECs were cultured in serum-free DMEM for 24 h. Then, the medium was replaced with DMEM containing 10% FBS for 48 h before further experiments were conducted. The knockdown efficiency of the siRNA was determined via western blot analysis at 2 days after transfection.

MCP1 plasmid transfection

The Flag-tagged MCP1 expression plasmid was generated by inserting the human MCP1 coding fragment into the pCMV-MAT-Tag®-FLAG®-1 vector (Sigma-Aldrich®, catalog number C5989) at its HindIII and BamHI sites. The expressed fusion protein consisted of MCP1 with the 15- amino acid MAT-Tag-Flag sequence attached to the N-terminus of MCP1. Plasmid transfection was performed as previously described [29].

In vitro scratch assay

Cell migratory ability in a 2D culture system was tested via an *in vitro* scratch assay. Briefly, 1×10^5 HUVECs were seeded in 24-well tissue culture plates and cultured in growth medium for 24 h, at which time the HUVECs were approximately 70-80% confluent. Using a sterile 200- μ l pipette tip, a straight line was carefully scratched in a single direction in the monolayer across the center of the well while maintaining the tip in a position perpendicular to the plate bottom. A second straight line was scratched perpendicular to the first line to create a cross-shaped gap between the cells within each well. Each well was washed twice with 1 ml of fresh growth medium to remove any detached cells. Digital images of the cell gap were captured at different time points, and the gap width was quantitatively evaluated using ImageJ software.

Nested matrix model and cell migration assay

A nested collagen matrix model was used as previously described [30], with some modifications. For the nested attached matrix, a standard fibroblast-populated collagen matrix (FPCM) was incubated in an attached state for 72 h in DMEM containing 10% FBS. Then, the FPCM was removed from the culture well and placed in a 60- μ l aliquot of fresh acellular collagen matrix solution (a NeoMatrix solution) that was centered within a 12-mm diameter score on the bottom of a new culture well. Next, a 140- μ l aliquot of NeoMatrix solution was used to cover the newly transferred FPCM. The NeoMatrix was allowed to polymerize for 1 h at 37°C in 5% CO₂, and 2 ml of DMEM containing 10% FBS was then added to the well.

Cell migration from the nested FPCM to the acellular NeoMatrix was quantified via fluorescence microscopy at 24 h after nesting. Digital images (with constant dimensions of 1000×800 μ m) of 3-5 randomly selected microscopic fields at the interface of the nested FPCM with the acellular NeoMatrix were captured using an EVOS® FL Cell Imaging microscope (Life Technologies, Grand Island, NY, USA). Cell migration from the nested FPCM was quantified by counting the number of cells that had clearly migrated from the nested matrix to the cell-free matrix. The number of cells per field that had migrated from the nested matrix was averaged across these digital micrographs.

Statistics

The data are presented as the means $\pm$ SEM. Unpaired numerical data were compared using unpaired t-tests (two groups) or ANOVA (more than two groups), and statistical significance was set at $p < 0.05$.

Results

SiO₂ induced EndMT in HUVECs

EndMT is characterized by the loss of expression of endothelial markers, such as VEGF receptors (VEGFRs), and the acquisition of the ability to produce mesenchymal products, such as Col I, Col III and α -SMA [14]. The HUVECs were seeded in 24-well plates (Costar® 3524, 1×10^5 cell/ml, 1 ml/well). SiO₂ in NS was added to each well at a final concentration of 50 $\mu\text{g}/\text{cm}^2$. As shown in Fig. 1A-E, SiO₂ induced the expression of Col I, Col III and α -SMA over 12 hours in a time-dependent manner. In contrast, the expression levels of endothelial markers, except for VEGFR1, were significantly decreased after 12 h of exposure to SiO₂ (Fig. 1F-J). These results were confirmed using immunocytochemistry staining (Fig. 1A, K). This

Fig. 1. SiO₂ induced EndMT in HUVECs.

(A) Representative images from immunocytochemistry experiments showing that SiO₂ increased the expression of Col III in HUVECs. Scale bar=20 μm . (B). Representative western blot showing the effects of SiO₂ on the expression of the mesenchymal markers Col I, Col III and α -SMA. Densitometric analyses of five separate experiments suggested that SiO₂ induced the expression of Col I (C), Col III (D) and α -SMA (E) in a time-dependent manner. * $p < 0.05$ vs the 0-hour group. (F) Representative western blot showing the effects of SiO₂ on the expression of the endothelial markers VEGFR-1, VEGFR-2, VEGFR-3 and VE-cad in HUVECs. Densitometric analyses of five separate experiments indicating the effect of SiO₂ exposure on the levels of VEGFR-1 (G), VEGFR-2 (H), VEGFR-3 (I) and VE-cad (J). * $p < 0.05$ vs the 0-hour group. (K) Representative images from immunocytochemistry experiments showing that SiO₂ decreased the expression of VEGFR-2 in HUVECs. Scale bar=20 μm .

evidence suggests that the HUVECs that were exposed to SiO_2 were induced to change from an endothelial to a mesenchymal phenotype.

MCP1 is involved in the SiO_2 -induced acquisition of mesenchymal characteristics by HUVECs

Previous data from our lab indicated that MCP1 played an important role in SiO_2 -induced fibrosis [16-19]. Here, we sought to determine whether MCP1 is also involved in SiO_2 -induced acquisition of mesenchymal characteristics. First, we measured the expression

Fig. 2. MCP1 is involved in the SiO_2 -induced acquisition of a mesenchymal phenotype by HUVECs. (A) Representative Western blot showing the effects of SiO_2 on the expression of MCP1 in HUVECs. (B) Densitometric analyses of data from five separate experiments suggested that SiO_2 induced MCP1 expression in a time-dependent manner. * $p < 0.05$ vs the 0-hour group. (C) Representative immunocytochemical images showing that SiO_2 increased the expression of MCP1 and Col III in HUVECs. Scale bar = 20 μm . (D) Representative Western blot showing the effects of RNAi targeting MCP1 on SiO_2 -induced Col I, Col III and α -SMA expression in HUVECs. The results of densitometric analyses from five separate experiments suggested that RNAi targeting MCP1 (E) attenuated the SiO_2 -induced increase in Col I (F), Col III (G) and α -SMA expression (H). * $p < 0.05$ vs the control group at 0 hours; # $p < 0.05$ vs the SiO_2 group at 0 hours.

of MCP1 in HUVECs after exposure to SiO_2 . As shown in Fig. 2A, B, MCP1 expression increased in a time-dependent manner in HUVECs that were treated with SiO_2 . Consistent with the results of western blot analysis, immunocytochemical staining of HUVECs revealed co-localization between MCP1 and Col III after 24 hours of exposure to SiO_2 (Fig. 2C), indicating an interaction between MCP1 and Col III. Furthermore, specifically silencing

Fig. 3. The role of MCP1 in the SiO_2 -induced loss of an endothelial phenotype by HUVECs. (A) Representative images from immunocytochemistry experiments showing that SiO_2 increased the expression of MCP1 and decreased the expression of VEGFR-2 in HUVECs. Scale bar=20 μm . (B) Representative western blot showing the effect of RNAi targeting MCP1 on SiO_2 -induced VEGFR-1, VEGFR-2, VEGFR-3 and VE-cad expression in HUVECs. Densitometric analyses from five separate experiments showing the effect of RNAi targeting MCP1 on SiO_2 -induced VEGFR-1 (C), VEGFR-2 (D), VEGFR-3 (E) and VE-cad expression in HUVECs (F). * $p < 0.05$ vs the control group at 0 hours; # $p < 0.05$ vs the SiO_2 group at 0 hours. (G) Representative western blot showing the effects of transfecting HUVECs with MCP1-Flag on the SiO_2 -induced changes in the expression of VEGFR-1, VEGFR-2, VEGFR-3 and VE-cad. Densitometric analyses from five separate experiments showing the effect of transfection of HUVECs with MCP1-Flag (H) on the SiO_2 -induced changes in the expression of VEGFR-1 (I), VEGFR-2 (J), VEGFR-3 (K) and VE-cad (L). * $p < 0.05$ vs the control group at 0 hours; # $p < 0.05$ vs the SiO_2 group at 0 hours.

MCPIP1 in HUVECs using siRNA alleviated the increase in Col I, Col III and α -SMA expression that was induced by SiO_2 in the control cells (Fig. 2D-H). All of these data suggest that MCPIP1 plays a direct role in the acquisition of mesenchymal characteristics.

Role of MCPIP1 in SiO_2 -induced loss of endothelial characteristics by HUVECs

After exploring the role of MCPIP1 in the acquisition of a mesenchymal phenotype, we next sought to elucidate the function of MCPIP1 in another step of EndMT: loss of an endothelial phenotype. Therefore, we first measured the extent of co-localization between MCPIP1 and VEGFR-2 in HUVECs using immunocytochemical staining. As shown in Fig. 3A, exposure to SiO_2 induced an increase in the expression of MCPIP1 and a decrease in the expression of VEGFR-2 in HUVECs. To further define the correlation between the changes in MCPIP1 and VEGFR-2 levels, MCPIP1 was specifically silenced in HUVECs using siRNA. Instead of inducing recovery of the expression of endothelial markers, down-regulation of MCPIP1 resulted in a stronger decrease in the expression of VEGFR2-3 and VE-cad than

Fig. 4. The role of autophagy in the SiO_2 -induced loss of an endothelial phenotype by HUVECs. (A) Representative western blot showing the effects of SiO_2 on the expression of the autophagy markers BECN, ATG5 and LC3B in HUVECs. The results of densitometric analyses from five separate experiments suggested that SiO_2 induced the expression of BECN (B) ATG5 (C) and LC3B (D) in a time-dependent manner. * $p < 0.05$ vs the 0-hour group. (E) Representative images showing the effect of SiO_2 on the appearance of RFP-GFP-MAP1LC3 puncta. Scale bar = 10 μm . (F) Quantification of RFP- and GFP-fluorescent puncta of RFP-GFP-MAP1LC3; the results demonstrated that SiO_2 induced autophagy. * $p < 0.05$ vs the control group. (G) Quantification of RFP-fluorescent puncta and both RFP- and GFP-fluorescent puncta of RFP-GFP-MAP1LC3; the results demonstrated that SiO_2 induced autophagic flux. * $p < 0.05$ vs the control group.

that induced by SiO_2 ; furthermore VEGFR-1 expression was decreased only in the MCP1 siRNA-treated group (Fig. 3B-F). To validate this finding, we used a MCP1-Flag plasmid [29] to upregulate the expression of MCP1 in HUVECs. As shown in Fig. 3G-L, transfecting HUVECs with MCP1-Flag increased the expression of MCP1 within 24 hours, whereas the SiO_2 -induced decreases in VEGFR-2, VEGFR-3 and VE-cad were ameliorated. VEGFR-1 was not affected by transfection with MCP1-Flag, and these findings were consistent with the results shown in Fig. 1. These data indicated that the mechanism responsible for the loss of endothelial characteristics was different from the mechanisms responsible for the acquisition of a mesenchymal phenotype.

SiO_2 induced autophagy in HUVECs

Previous data suggested that MCP1-induced autophagy plays a role in the functions of adipogenesis [23]. It appeared relevant to determine whether autophagy occurred in HUVECs following exposure to SiO_2 since cell proliferation is an important aspect of silicosis. As shown in Fig. 4A-D, exposing HUVECs to SiO_2 significantly up-regulated the expression of the autophagy markers BECN, ATG5 and LC3B (according to the guidelines on the evaluation of autophagy [31]) in a time-dependent manner. Autophagic flux was also monitored in HUVECs that were transduced with adenovirus carrying dually fluorescent mRFP-GFP-MAP1LC3, which expresses MAP1LC3, a specific marker of autophagosome formation [23]. Differences in the nature of how GFP and RFP fluorescence is induced under acidic conditions were used to determine the results of these experiments [27]. GFP fluorescence is sensitive to the acidic conditions within the lysosomal lumen, whereas RFP fluorescence is relatively

Fig. 5. Role of MCP1 in the SiO_2 -induced loss of an endothelial phenotype by HUVECs. (A) Representative Western blot showing the effects of RNAi targeting MCP1 on SiO_2 -induced BECN, ATG5 and LC3B expression in HUVECs. Densitometric analyses from five separate experiments suggesting that RNAi targeting MCP1 attenuated the SiO_2 -induced expression of BECN (B), ATG5 (C) and LC3B (D). * $p < 0.05$ vs the control group at 0 hours; # $p < 0.05$ vs the control group at the corresponding time point. (E) Representative Western blots demonstrating the effects of 3-MA and Rapa application on the SiO_2 -induced changes in the expression of VEGFR-1, VEGFR-2 and VEGFR-3. Densitometric analyses from five separate experiments showing the effects of 3-MA and Rapa on the SiO_2 -induced changes in the expression of VEGFR-1 (F), VEGFR-2 (G) and VEGFR-3 (H) in HUVECs. * $p < 0.05$ vs the control group; # $p < 0.05$ vs the SiO_2 group.

stable under acidic conditions. Thus, the colocalization of GFP and RFP signals (yellow dots) indicates the presence of phagophores or autophagosomes that have not fused with lysosomes, whereas RFP-only signals (red puncta) indicate autolysosomes [23]. As shown in Fig. 4E-G, SiO_2 induced a significant increase in the number of yellow dots (Fig. 4G) and concomitantly enhanced the increase in RFP-only MAP1LC3-positive dots in the HUVECs of the SiO_2 -treated group (Fig. 4F, G), indicating that SiO_2 induced autophagic flux.

Autophagy mediated by MCP1 promoted the SiO_2 -induced loss of an endothelial phenotype by HUVECs

To further elucidate whether autophagy mediates the MCP1-induced loss of an endothelial phenotype by HUVECs, we used MCP1 siRNA to explore the relationship

Fig. 6. SiO_2 induced functional changes in HUVECs. (A) MTT assay showing that the SiO_2 -induced increase in cell viability was time-dependent in HUVECs. * $p < 0.05$ vs the 0-hour group. (B) Representative images showing the effects of SiO_2 on GFP-labeled HUVEC migration in scratch assays. Scale bar = 80 μm . (C) Quantification of the scratch gap distances from six separate experiments. * $p < 0.05$ vs the control group at the corresponding time point. (D) Representative images showing the effects of SiO_2 on GFP-labeled HUVEC migration in a nested matrix. Scale bar = 80 μm . (E) Quantification of cell migration from six separate experiments. * $p < 0.05$ vs the control group at the corresponding time point.

between MCP1 and autophagy. As shown in Fig. 5A-D, silencing MCP1 in HUVECs significantly inhibited the SiO₂-induced increase in the expression of BECN, ATG5 and LC3B.

After we determined that MCP1 mediated SiO₂-induced autophagy in HUVECs, a specific inhibitor of autophagy, 3-methyladenine (3-MA, 1 mmol/L), and a specific activator of autophagy, rapamycin (Rapa, 1 μmol/L), were applied to the cells. [32] As shown in Fig. 5E-H, pretreatment with 3-MA for 1 hour attenuated the SiO₂-induced decreases in VEGFR-2 and VEGFR-3 levels observed in the control HUVECs. However, pretreatment with Rapa for 1 hour did not further decrease the expression of VEGFR-2 and VEGFR-3 beyond that induced by SiO₂. This result may be because SiO₂ treatment exerted a maximal effect. These data suggest that autophagy induced the loss of an endothelial phenotype by HUVECs upon exposure to SiO₂.

Fig. 7. Pyk2 and MLC-2 mediated the effect of MCP1 on SiO₂-induced HUVEC migration. (A) Representative images showing the effects of RNAi targeting MCP1 on the SiO₂-induced migration of GFP-labeled HUVECs in scratch assays. Scale bar=80 μm. (B) Quantification of the scratch gap distances in six separate experiments. * p<0.05 vs the control group at the corresponding time point. (C) MTT assay showing that RNAi targeting MCP1 attenuated the SiO₂-induced increase in HUVEC viability. * p<0.05 vs the control group at the corresponding time point. (D) Representative western blot showing the effects of SiO₂ on the phosphorylation of Pyk2 and MLC-2 in HUVECs. Densitometric analyses from five separate experiments suggested that SiO₂ induced the phosphorylation of Pyk2 (E) and MLC-2 (F) in HUVECs. * p<0.05 vs the 0-hour group. (G) Representative western blot showing the effects of RNAi targeting MCP1 on the SiO₂-induced phosphorylation of Pyk2 and MLC-2 in HUVECs. Densitometric analyses from five separate experiments suggested that RNAi targeting MCP1 attenuated the SiO₂-induced phosphorylation of Pyk2 (H) and MLC-2 (I) in HUVECs. * p<0.05 vs the control group at 0 hours; # p<0.05 vs the control group at the corresponding time point.

blot showing the effects of RNAi targeting MCP1 on the SiO₂-induced phosphorylation of Pyk2 and MLC-2 in HUVECs. Densitometric analyses from five separate experiments suggested that RNAi targeting MCP1 attenuated the SiO₂-induced phosphorylation of Pyk2 (H) and MLC-2 (I) in HUVECs. * p<0.05 vs the control group at 0 hours; # p<0.05 vs the control group at the corresponding time point.

Fig. 8. SiO₂ induced the phosphorylation of MAPK and PI3K/Akt in HUVECs. (A) Representative western blot showing that SiO₂ induced the phosphorylation of Akt, p38, JNK and ERK in HUVECs. Densitometric analyses of the expression of p-Akt (B), p-p38 (C), p-JNK (D) and p-ERK (E) from five separate experiments. * p<0.05 vs the 0-min group. (F) Representative western blot showing that MCP1, Col I, Col III and α-SMA expression was attenuated by pre-treating HUVECs with a MAPK or PI3K/Akt inhibitor. Densitometric analyses of MCP1 (G), Col I (H), Col III (I) and α-SMA (J) from five separate experiments. * p<0.05 vs the control group; # p<0.05 vs the SiO₂ group.

SiO₂ induced functional changes in HUVECs

Accumulating evidence indicates that the onset of pulmonary fibrosis is accompanied by changes in cell proliferation and migration [33-36]. To further understand the functional effects of endothelial marker loss and mesenchymal marker acquisition by these cells, HUVEC migration and viability were evaluated upon exposure to SiO₂. As shown in Fig. 6A, 48 hours of exposure to SiO₂ induced a significant increase in HUVEC viability. Moreover, the migratory ability of HUVECs began to increase after 12 hours of SiO₂ exposure, and the gap distance in scratch assays reached 0 after 24 hours of exposure to SiO₂ (Fig. 6B, C). However, this enhancement of cell migration did not result from an increase in cell abundance because cell viability was not changed at any time point up to 24 hours of SiO₂ exposure (Fig. 6A). Increasing evidence suggests that there are significant discrepancies in cell behavior between 2D and 3D culture systems [16, 29, 37] and that cell migration in a 3D matrix is a complicated process that involves cell-matrix adhesion, cell-matrix interactions, and global/local matrix remodeling [38]. We therefore measured cell migration using a nested matrix model that better represented the microenvironment of living tissue [29]. As shown in Fig. 6D, E, SiO₂ induced a similar effect on cell migration in the 3D model to the effect that we observed in the scratch assay. Taken together, SiO₂ induced a rapid and sustained increase in HUVEC migration.

MCP1 mediated the effect of SiO₂ on HUVEC migration and population abundance

After demonstrating that endothelial cells undergoing EndMT acquire mesenchymal properties and increased migratory ability, which is a critical characteristic of pulmonary fibrosis, we explored whether MCP1 mediated cell migration and proliferation. HUVECs

treated with SiO₂ showed a significant increase in migration on scratch assays, while silencing MCP1 in HUVECs attenuated the SiO₂-induced increase in cell migration (Fig. 7A, B). Furthermore, specifically silencing MCP1 in HUVECs abolished the SiO₂-induced increase in cell viability (Fig. 7C). To further explore the mechanism underlying the observed increase in the migration of HUVECs undergoing EndMT, markers that are relevant to cell migration, such as protein-tyrosine kinase 2 (Pyk2) and myosin light-chain 2 (MLC-2), were examined via western blot analysis. As shown in Fig. 7D-F, SiO₂ induced rapid and sustained phosphorylation of Pyk2 and MLC-2, and the phosphorylation of both factors were attenuated when MCP1 siRNA was applied to HUVECs (Fig. 7G-I).

Involvement of MAPKs and the PI3K/Akt pathway in SiO₂-induced EndMT in HUVECs

To improve our understanding of the molecular mechanism underlying SiO₂-induced EndMT, we investigated the potential association between kinase activation and EndMT by measuring the phosphorylation of MAPKs and PI3K/Akt over 3 h of exposure to SiO₂. As shown in Fig. 8A-E, within 5 min of SiO₂ exposure, Akt, p38, JNK and Erk underwent phosphorylation. While the phosphorylation of Akt, p38 and Erk persisted up to 3 hours, SiO₂ induced only rapid and transient phosphorylation of JNK.

After confirming that MAPK and PI3K/Akt activities were enhanced after exposure to SiO₂, pharmacological inhibitors of MAPK and PI3K/Akt was applied to determine whether these pathways are involved in EndMT in HUVECs that were exposed to SiO₂. As shown in Fig. 8F-J, pretreating the HUVECs with the commercially available small molecules LY294002 (an Akt inhibitor, 25 μmol/L), SB203580 (a p38 inhibitor, 10 μmol/L), SP600125 (a JNK inhibitor, 20 μmol/L) or U0126 (a MEK inhibitor, 25 μmol/L) attenuated the SiO₂-induced up-regulation of MCP1, Col I, Col III and α-SMA.

Discussion

Silicosis, an occupational disease caused by inhaling silica, is characterized by progressive pulmonary fibrotic reactions [1, 2, 39, 40]. Although EndMT has been shown to play a role in fibrosis in various settings [7, 8], the role of EndMT in pulmonary fibrosis induced by SiO₂ were unclear. Our findings suggest that MCP1 is involved in the process of EndMT.

Inhaled SiO₂ is not effectively eliminated from the body [2]. SiO₂ destroys the barrier between the air and blood, which exposes endothelial cells to silica. [41] These breaks in the air-blood barrier result in the formation of deposits of SiO₂ in organs such as the liver, kidneys, spleen, heart, and abdominal lymph nodes [42-48]. Moreover, stimuli such as bleomycin not only induced the activation of endothelial cells but also caused endothelial cells to undergo EndMT, exacerbating endothelial cell dysfunction [8]. We therefore hypothesized that endothelial cells stimulated with silica may undergo EndMT.

EndMT is first observed during embryonic development in the heart [49, 50]. During the course of physiological and pathological processes, EndMT results in the loss of an endothelial phenotype and the acquisition of a mesenchymal phenotype. Each step of EndMT is characterized by changes in the levels of different sets of specific markers in addition to changes in the functions of the cell, including proliferation and migration [14]. In the current study, HUVECs exposed to SiO₂ underwent EndMT, as indicated by a decrease in expression of endothelial cell-specific markers and an increase in expression of mesenchymal cell products (Fig. 1). Interestingly, SiO₂ did not change the expression of VEGFR1 in HUVECs. The results of studies published to date indicate that VEGFR1 might act as a silent receptor for VEGF because it transmits a weak growth signal. Moreover, VEGFR1 might inhibit most of the effects of VEGF/VEGFR2 signaling in endothelial cells [51, 52]. VEGFR1 knockout mice die as a result of deficiencies in the structural organization of vessel walls, demonstrating that VEGFR1 plays an important role in embryonic vascularization [53]. Recent studies have suggested that the critical role of VEGFR1 in endothelial cells is not the regulation of angiogenesis but rather the induction of the paracrine release of tissue-specific

Fig. 9. Schematic showing the role of MCP1-mediated autophagy in SiO_2 -initiated fibrosis.

growth factors [54]. In contrast to VEGFR1, VEGFR2 plays a pivotal role in endothelial cell proliferation, NO and prostacyclin production, angiogenesis, and vascular permeability [55-57] showed that activating VEGFR2 in HUVECs inhibited apoptosis. Hence, the silica-induced change in VEGFR2 expression in HUVECs is more meaningful than the silica-induced change in VEGFR1 expression.

Many factors can induce the EndMT process. During EndMT, the most important factor is transforming growth factor- β (TGF- β), which promotes EndMT through the Smad2/3 and Smad1/5/8 signaling pathways [15]. In addition to the classical TGF- β signaling pathways, other factors such as Notch, Wnt and miR-21 are also involved in EndMT processes [58, 59]. Not only traditional cytokines, but also chemokines have attracted more attention for their roles in EndMT. Chemokine (C-C motif) ligand 2 (CCL2) can orchestrate the migration of monocytes across the endothelium of blood vessels to induce a late fibrogenic reaction [60, 61]. CCR2, which is the receptor of CCL2, is expressed on endothelial cells [62]. MCP1 is a novel CCCH zinc-finger-containing protein that is significantly induced by CCL2 in human peripheral blood monocytes [63]. Previous data from our lab suggest that MCP1 plays a role in HUVEC migration and proliferation in the context of ischemia and reperfusion [23]. Other studies have suggested that MCP1 is also involved in enhancing angiogenic activity, and specifically silencing MCP1 resulted in the suppression of VEGFR in HUVECs, suggesting a positive correlation between MCP1 and VEGFR expression [21]. We propose that MCP1 may be involved in SiO_2 -induced EndMT. In accordance with this hypothesis, MCP1 was found to be involved in the SiO_2 -induced acquisition of a mesenchymal phenotype.

To our surprise, although we observed a negative correlation between MCP1 and VEGFR expression upon SiO_2 exposure, specifically silencing MCP1 further decreased VEGFR expression instead of rescuing its expression. In fact, our and other laboratories have shown that MCP1 promotes angiogenesis via its RNase activity, suppressing the production of the antiangiogenic microRNA (miR)-20b and miR-34a [21, 64], which are thought to sustain endothelial cell properties. Our findings suggest that SiO_2 induces HUVECs to lose of their endothelial phenotype rather than undergo angiogenesis, indicating that different mechanisms may be involved in the loss of an endothelial phenotype and the acquisition of a mesenchymal phenotype. Endothelial cells first lose their endothelial characteristics as a result of adaptation to an environment that is changed by catabolic and anabolic processes that are collectively referred to as autophagy. MCP1 has been found to induce autophagy [65] and differentiation [66] through its transcription factor activity, RNase activity, and deubiquitinase activity, consistent with our findings. However, the detailed mechanism by which MCP1 participates in the loss of an endothelial phenotype must be further explored.

EndMT by endothelial cells leads to microvascular deficiency and endothelial dysfunction, which contribute to cardiac fibrosis [12]. In the current study, MCP1 mediated the proliferation and migration of HUVECs, indicating dysfunction of these endothelial cells. Morphometric studies of idiopathic pulmonary fibrosis (IPF) showed that capillary density was decreased in the fibrotic region [67].

In conclusion, MCP1 appeared to participate in the induction of EndMT in endothelial cells that were exposed to SiO_2 via a pathway that appeared to involve autophagy, MAPKs and Akt. These processes subsequently led to increased cellular viability and migration. Our results suggest that MCP1-induced EndMT in endothelial cells plays an important role in the development of silicosis. Targeting the MCP1/autophagy signaling axis may represent a therapeutic strategy for preventing silicosis (Fig. 9).

Acknowledgements

This study is the result of work that was partially supported by the resources and facilities at the core laboratory of the Medical School of Southeast University. The National Natural Science Foundation of China (No. 81473263, 81400300, 81600045 and 81301870) and the Natural Science Foundation of Jiangsu Province, China (No. BK20141347 and BK20130642).

Disclosure Statement

The authors have no conflicts of interest to declare.

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