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Research Article

Roles of the ERK, JNK/AP-1/cyclin D1–CDK4 pathway in silica-induced cell cycle changes in human embryo lung fibroblast cells

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Abstract

Silica is a potent occupational fibrogenic agent capable of inducing lung fibrosis and many other lung diseases. Our current study focused on the signalling pathways regulating cell cycle changes in HELF (human embryo lung fibroblast) after silica (α-quartz) exposure. Our results showed silica exposure could lead to cell cycle changes. The cell cycle alternations were accompanied with overexpression of cyclin D1 and CDK4 (cyclin-dependent kinase 4) in a time-dependent manner. Silica exposure also decreased E2F-4 expression in HELF. These changes were blocked by overexpression of dominant-negative mutants of ERK (extracellular signal-regulated protein kinase) or the JNK (stress-activated c-Jun NH_2-terminal kinase), respectively. Moreover, pretreatment of cells with curcumin, an activation of AP-1 (activator protein-1) inhibitor, inhibited silica-induced cell cycle alteration, the decreased expression of E2F-4 and overexpression of cyclin D1 and CDK4. Furthermore, both antisense cyclin D1 and antisense CDK4 can block silica-induced cell cycle changes. These results suggest that silica exposure can induce cell cycle changes, which may be mediated through ERK, JNK/AP-1/cyclin D1–CDK4-dependent pathway.

Keywords: cell cycle; HELF; lung fibrosis; occupational; signalling pathway; silica

1. Introduction

Silica, a major component of the earth's crust, is a ubiquitous occupational fibrogenic agent (Ding et al., 2002). Occupations having a high potential for exposure to crystalline silica dust (respirable quartz) are stone clay, glass production work and agricultural, chemical production and so on (Cherry et al., 1998; De Klerk et al., 1998; Johnston et al., 2000; Rice et al., 2001). Both epidemiological and animal studies support an association between occupational crystalline silica exposure and several diseases such as lung fibrosis and many other lung diseases (Hughes et al., 2001; Knaapen et al., 2002; Porter et al., 2002, 2004; Calvert et al., 2003; Langley et al., 2004; International Agency for Research on Cancer, 1997). It has been accepted that human lung fibroblasts play a role in lung fibrosis. Previous studies showed that lung fibrosis is related to the proliferation of human lung fibroblasts and the increase of the collagen production (Arcangeli et al., 2001; O'Reilly et al., 2005; Luzina et al., 2006). Here, we want to explore the molecular events involved in the proliferation of human lung fibroblasts.

Cell cycle is controlled by the sequential activation and inactivation of CDKs (cyclin-dependent kinases). The G_1-/S-phase checkpoint is the major cell cycle transition point in which cells are susceptible to extracellular mitotic signals. Activation of cyclin D–CDK4/CDK6 and cyclin E–CDK2 is required for cell cycle progression through G_1/S transition. It is well known that cell cycle aberrations often lead to uncontrolled cell proliferation. Hence, it is of great importance to understand the signal transduction mechanism that silica caused cell cycle changes in fibroblasts.

The classic MAPK (mitogen-activated protein kinase) pathway is a key component in the transduction of signals leading to growth and transformation in many cell types. AP-1 (activator protein-1) is a family of transcription factors. AP-1 components are regulated by MAPK signalling cascades, which have been shown to be involved in the regulation of various intracellular responses, including cell proliferation, differentiation and cell death. The biological effects of MAPKs are mediated by downstream phosphorylation substrates, which in the nucleus are often transcription factors (Shen et al., 2001). Previous studies have shown that cyclin D1 transcription is modulated by AP-1 transcription factor (Clarke et al., 1998), and AP-1 is able to promote cell cycle transition by a direct transcriptional up-regulation of cyclin D1 (Chen et al., 2001). Earlier study has also suggested that in a non-transformed alveolar type II epithelial cell line (C10), α-quartz silica causes persistent dose-related increases in phosphorylation of JNKs (the stress-activated c-Jun NH_2-terminal kinases), which is accompanied by elevations in AP-1

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Abbreviations: AP-1, activator protein-1; CDK4, cyclin-dependent kinase 4; DN-ERK, dominant-negative mutant ERK; DN-JNK, dominant-negative mutant JNK; DN-p38, dominant-negative mutant p38; ERK, extracellular signal-regulated protein kinase; FBS, fetal bovine serum; HELF, human embryo lung fibroblast; JNK, the stress-activated c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; ROS, reactive oxygen species.

binding to DNA and increases in the proportion of C10 cells in S-phase (Shukla et al., 2001).

The mechanisms that silica exposure caused lung fibrosis and many other lung diseases have been extensively studied in the last several years. Previous studies have largely focused on silica-induced production of ROS (reactive oxygen species) by lung phagocytes (Ding et al., 2001; Shen et al., 2001). Several studies have reported that, in many cell types, silica could affect DNA including DNA damage caused by silica dusts directly or by ROS released by silica dusts, and p53 and TNF (tumour necrosis factor) gene mutations (Barrett et al., 1999; Seiler et al., 2001; Deshpande et al., 2002; Gambelli et al., 2003; Albrecht et al., 2005; Roy et al., 2005). It has been thought that cell cycle perturbation caused by silica exposure is an important mechanism implicated in its pathogenic effects. However, the signalling pathways that lead to cell cycle alteration after silica exposure have not been well defined. In the present study, we focus on investigating the role of ERK (extracellular signal-regulated protein kinase), JNK/AP-1/cyclin D1–CDK4 pathway in silica-induced cell cycle changes in HELF (human embryo lung fibroblast).

2. Materials and methods

2.1. Reagents

RPMI 1640 medium was obtained from Gibco. Glutamine, gentamicin, curcumin, PI (propidium iodide) and RNase A were obtained from Sigma. Phospho-MAPK antibody and total MAPK antibody were purchased from Cell Signaling Technology Inc. Monoclonal antibodies of cyclin D1, CDK4 and actin were from Santa Cruz Biotechnology, Inc. Nitrocellulose membrane 102 was bought from Bio-Rad. The substrate for the luciferase assay and Transfectam Reagent were purchased from Promega. Antisense cyclin D1 stable transfectants and antisense CDK4 stable transfectants were established in our previous work (Jia et al., 2006). Dominant-negative mutant ERK, JNK, p38 and the AP-1-luciferase reporter plasmid and CMV-neo vector plasmid were kindly donated by Professor Huang of New York University School of Medicine.

2.2. Cell cultures

Human embryonic lung fibroblast cell line was obtained from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. Cells were cultured in RPMI 1640 containing 10% FBS (fetal bovine serum), 2 mM L-glutamine, 50 μg/ml gentamicin. The cells were grown at 37°C in 5% CO_2 atmosphere. Cells were switched to 0.5% FBS-containing medium for 24 h prior to addition of silica at final concentrations of 200 μg/ml.

2.3. Silica exposure

Briefly, standard α-quartz, 95% of which had the diameter of less than 5 μm, was obtained from the National Institute of Occupational Health and Poisons Control, Chinese Center for Diseases Control and Prevention. Crystalline silica was hand ground in an agate mortar with a pestle to produce freshly fractured silica. The mortar ground silica exhibited a wide size distribution pattern. The silica particles were suspended in D-Hanks buffer saline, autoclaved to sterilize and diluted into the needed concentrations.

2.4. Generation of stable transfectants

Cells were cultured in six-well plates until they reached 40–50% confluence. One microgram of CMV-neo vector, 2 μg of AP-1-luciferase reporter plasmid DNA and 12 μg of dominant-negative mutant plasmids [DN-ERK (dominant-negative mutant ERK), DN-JNK (dominant-negative mutant JNK) or DN-p38 (dominant-negative mutant p38), respectively] or vector control, mixed together with 15 μl of Transfectam Reagent and then were used to transfect each well of six-well plate in the absence of serum. After 10–12 h, the medium was replaced with RPMI-1640 containing 10% FBS. Approximately 30–36 h after beginning of the transfection, the cells were dislodged with 0.125% trypsinase, and cell suspensions were plated on to 60-ml culture flasks and cultured for 24–28 days with G418 selection (400 μg/ml). The stable transfectants were identified by measuring both the basal level of luciferase activity and the activities of target proteins (data not shown). Stable transfectants, HELF–AP-1 and HELF–ERK, JNK or p38 were established and cultured in G418-free RPMI-1640 for at least two passages before each experiment.

2.5. Immunofluorescence assays

Cells were cultured with 10% FBS RPMI-1640. After cell density reached 50–60% confluence, the medium was replaced with serum-free RPMI-1640 (supplemented with 0.5% FBS) and cultured for 24 h and then exposed to silica. Cells cultured in a six-well glass slide chamber were fixed with ice-cold methanol for 10 min at 4°C and then permeabilized with 0.2% Triton X-100. After blocking with 5% normal goat serum, they were incubated with a rabbit polyclonal antibody against cyclin D1 overnight at 4°C and then with FITC-conjugated goat anti-rabbit IgG at room temperature for 1 h after extensive washing between each step. The slides were washed three times with PBS. After a final washing with PBS, the slides were mounted using Gel/Mount. An OLYMPUS fluorescence microscope coupled to a digital camera and Adobe Photoshop software was used to view and acquire images.

2.6. Western blot analysis

Cells were cultured with 10% FBS RPMI-1640. After cell density reached 70–80% confluence, the medium was replaced with serum-free RPMI-1640 (supplemented with 0.5% FBS) and cultured for 24 h and then exposed to silica. Cell lysates were extracted with lysis buffer containing 62.5 mM Tris/HCl, pH 6.8, 2% (w/v) SDS, 0.01% Bromophenol Blue, 10% glycerin, 50 mM DTT (1,4-dithiothreitol). The samples were boiled for 10 min, sonicated for 1 min and then centrifuged at 10000 ***g*** for 10 min. Total protein was separated by 5–10% SDS/PAGE and transferred to

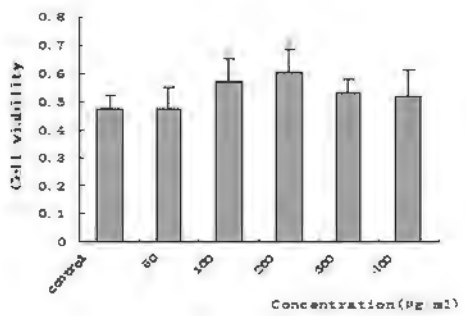

Figure 1 Effect of silica on HELFs cell proliferation measured by MTT assay
Cells (1.0×10^4) were plated in 96-well plates and treated with various concentrations of silica for 24 h. MTT assay was performed as described in Section 2. *$P<0.05$ compared with control.

nitrocellulose membranes. Membranes were blocked in blocking buffer containing 5% (w/v) non-fat dry milk in Tris-buffered saline, pH 7.6, 0.1% (v/v) Tween-20 (TBST) for 1 h at room temperature and incubated with the relevant antibodies (1:1000) in blocking buffer overnight at 4°C. After washing with TBST, blots were incubated with the appropriate peroxidase-conjugated secondary antibody (1:2000) for 1 h at room temperature and developed using ECL (enhanced chemical luminescence) detection system (Amersham) according to the manufacturer's instructions.

2.7. Cell cycle analysis

Flow cytometry was performed to analyse cell cycle distribution. Cells were cultured in flasks with 10% FBS RPMI-1640. Cells were serum starved for 24 h prior to silica exposure at a final concentration. Cells were dissociated with trypsin and washed in cold PBS and fixed with 70% cold ethanol on ice for 30 min. The suspensions were centrifuged at 250 ***g*** for 5 min. The pellet was resuspended in a solution containing 50 μg/ml PI, 1 mg/ml sodium citrate, 0.3% nonidet P40 and 5 μg/ml RNase A and stained on ice without light for at least 40 min. Then, the pellets were analysed by a Beckman Coulter Epics XL Flow Cytometer.

2.8. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay for cell proliferation

Cells were seeded in 96-well plates (1×10^4/well) at 37°C overnight and then exposed to silica at various concentrations of 50–300 μg/ml. Following treatment for 24 h, the culture medium was removed and replaced with a medium containing 0.5 mg of MTT dissolved in PBS (pH 7.2). After 4 h, the formed crystals were dissolved with 200 μl of DMSO. The intensity of the colour in each well was measured at a wavelength of 490 nm using a microplate reader (Dynex Technology). The experiment was repeated three times in triplicate.

2.9. Statistical analysis

All data of flow cytometry were shown as means with the S.E.M. Statistical analysis were performed by using one-way ANOVA (analysis of variance) with the probability of $P<0.05$ considered to be significant.

3. Results

3.1. Cell cycle alternation occurred in response to silica treatment

HELFs were spindle or fibrous cells. After being exposed to silica, the basic morphology of the cell almost did not change, except the shape became slightly round. Silica at the concentration of 200 μg/ml can increase cell proliferation compared with the control (Figure 1). To check the effects of silica on cell cycle distribution, HELFs were treated with silica (200 μg/ml) for 24 h, and cell cycle distribution was analysed by flow cytometry. The results showed that there was significant increase in S-phase cells accompanied by significant decrease in G_1-phase cells after silica treatment (Table 1). This data suggests that silica exposure may be able to induce HELFs to progress into S-phase.

3.2. Silica exposure increased levels of cyclin D1 and CDK4, but not E2F-1, and decreased levels of E2F-4 in HELF

To determine whether or not silica could induce changes of the levels of cyclin D1, CDK4, E2F-1/4, in HELFs, a time-dependent experiment was performed. The Western blot analysis showed that significant increases in cyclin D1 and CDK were observed after addition of silica4, peaking at 24 h (Figure 2). In contrast, after exposure to silica, significant decreases in E2F-4 were observed at 12, 24 and 48 h (Figure 2). Silica exposure did not lead to changes of E2F-1 expression at any time point tested (Figure 2).

Table 1 Effects of antisense of cyclin D1 and antisense CDK4 on silica-induced cell cycle changes
Cells were treated with silica (200 μg/ml) for 24 h. Results represent three independent experiments. Data were analysed using one-way ANOVA. *$P<0.05$, compared with 2BS-PXJ.

	Cell cycle distribution (%)		
Group	**G_1**	**S**	**G_2/M**
2BS-PXJ	53.93 ± 3.52	39.13 ± 2.74	6.93 ± 0.86
2BS-PXJ+silica	37.93 ± 4.56*	58.37 ± 3.48*	3.70 ± 1.08
2BS-PXJ-anti-D1	53.70 ± 5.11	41.70 ± 4.60	4.60 ± 0.56
2BS-PXJ-anti-D1+silica	47.83 ± 2.55	46.87 ± 2.02	5.30 ± 0.62
2BS-PXJ-anti-K4	57.00 ± 3.34	39.37 ± 1.99	3.63 ± 1.46
2BS-PXJ-anti-K4+silica	50.97 ± 6.10	45.03 ± 4.16	4.00 ± 2.21

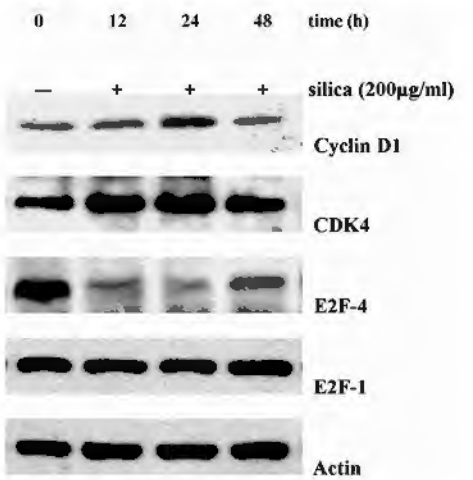

Figure 2 Effects of silica on expression of E2F-1/4, cyclin D1 and CDK4 in HELF

Cells were treated with silica (200 μg/ml) for 12, 24 and 48 h. Results represent three independent experiments.

3.3. Cyclin D1 and CDK4 were involved in cell cycle changes

Passage through the cell cycle is a highly regulated event, and a variety of safeguards have been incorporated into this process. In order to examine whether silica-induced cell cycle changes in a cyclin D1-dependent manner or a CDK4-dependent manner in HELFs, we used RNA transfection techniques (we established stable antisense cyclin D1 and antisense CDK4 transfectants in our previous work). For detection of changes of cell cycle, we performed flow cytometry. Antisense cyclin D1 or antisense CDK4 inhibited cell cycle changes, as reflected by the higher percentage of G_1 phase cells (Table 1).

3.4. The role of AP-1 in cell cycle changes, cyclin D1 and CDK4 induction and the decrease of E2F-4 expression

To investigate the role of AP-1 in the cell cycle changes induced by silica, curcumin, the chemical inhibitor of AP-1, was used. The

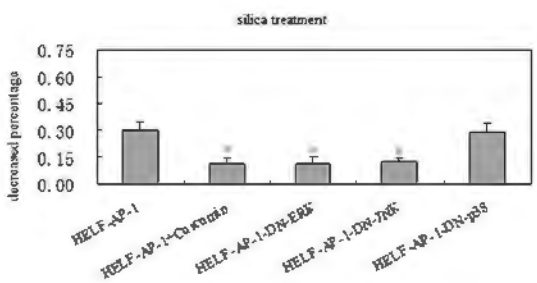

Figure 3 Effects of curcumin and expression of DN-ERK, DN-JNK and DN-p38 on silica-induced cell cycle changes

The decreased percentages of cells in G_1-phase after treatment with silica (200 μg/ml) for 24 h. Results represent three independent experiments. Data were analysed using one-way ANOVA. *$P<0.05$, compared with HELF–AP-1.

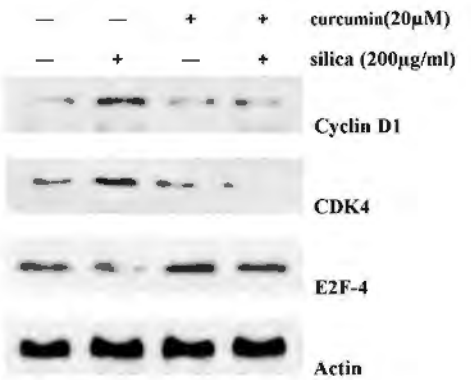

Figure 4 Effects of curcumin on silica-induced cyclin D1 and CDK4 induction and expression of E2F-4

Cells were pretreated with curcumin (20 μM) for 1 h and exposed to silica (200 μg/ml) for 24 h. Results represent three independent experiments.

silica-induced cell cycle progression from G_1-phase to S-phase was slower in HELFs exposed to curcumin than in control cells (Figure 3).

Furthermore, we investigated whether AP-1 was involved in silica-induced up-regulation of cyclin D1, CDK4 and down-regulation of E2F-4. Pretreatment with curcumin before exposure to silica, consistent with down-regulation of AP-1 activation (Jia et al., 2008), expressions of cyclin D1 and CDK4 were decreased and E2F-4 expression was showed an increase (Figure 4).

3.5. DN-ERK and DN-JNK, but not DN-p38, are required for silica-induced cell cycle changes, AP-1 activation and the induction of cyclin D1, CDK4 and the decrease of E2F-4

The increasing evidence has indicated the importance of MAPK pathway in cell proliferation. To confirm the role of ERK, JNK and p38 in silica-induced cell cycle changes, DN-ERK, DN-JNK and DN-p38 were used in performing flow cytometry analysis in the presence of silica. The stable transfectants were identified using Western blot. It was proved that ERK, JNK and p38 were efficiently silenced in their transfected cells, respectively. The silica-induced decrease in the population of cells in G_1-phase was

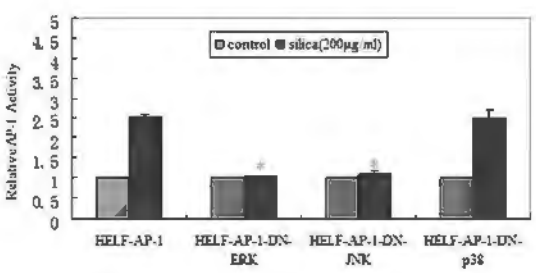

Figure 5 Effects of expression of DN-ERK, DN-JNK and DN-p38 on silica-induced AP-1 activation

Cells were treated with silica (200 μg/ml) for12 h. The luciferase activity was measured, and the results are presented as relative AP-1 activity. Each bar indicates the mean and S.D. of three repeat assay wells. Data were analysed using one-way ANOVA. *$P<0.05$, compared with HELF–AP-1+silica.

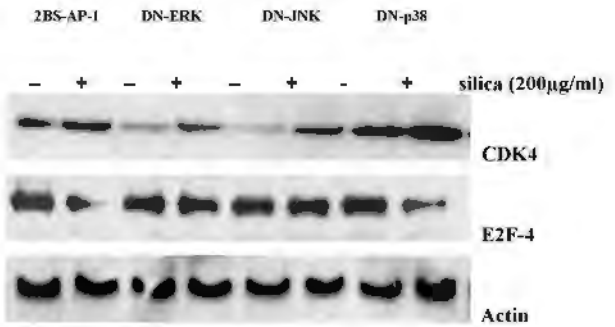

Figure 6 Effects of expression of DN-ERK, DN-JNK and DN-p38 on silica-induced CDK4 induction and expression of E2F-4
Cells were treated with silica (200 μg/ml) for 24 h. Results represent three independent experiments.

slower in DN-ERK and DN-JNK stable transfectants than in control cells similarly treated (Figure 3).

Furthermore, we studied whether ERK, JNK and p38 were involved in silica-induced AP-1 activation; the effects of MAPK on AP-1 activation were evaluated in HELF transfected with dominant-negative mutant ERK, JNK and p38, respectively. Overexpression of DN-ERK and DN-JNK, but not DN-p38, impaired AP-1 activation (Figure 5).

Additionally, our result shows that overexpression of DN-ERK and DN-JNK blocked silica-induced down-regulation of E2F-4 and up-regulation of cyclin D1 and CDK4 (Figures 6 and 7). DN-p38, however, did not show any inhibitory effect on these gene expression induced by silica (Figures 6 and 7).

4. Discussion

Silica exposure received worldwide concerns for its impact on human health. Silicosis is thought to be the main occupational disease, which is a major threat for workers in both developed and developing countries. Previous studies show that macrophages, lung fibroblasts and their soluble mediators are responsible for the onset and development of pulmonary fibrosis (O'Reilly et al., 2005; Liu et al., 2007). However, the molecular and cellular mechanisms involved in the silica-induced pathogenesis are not fully understood. Therefore, we focus on exploring the signalling pathways leading to cell cycle changes in HELF after silica exposure directly.

Our recent study has shown that silica can lead to cell cycle changes in HELFs, which is reflected by the decrease in G_1-phase cells. In the present study, our results revealed that silica exposure could increase the expression of cyclin D1 and CDK4 and decrease the expression of E2F-4. There were no changes in expression of E2F-1 at all time points tested in this study. Cyclin D1 and CDK4 are the important genes implicated in G_1 to S progression (Kim et al., 2005). Our previous study had demonstrated that cyclin D1 and CDK4 were increased in S-HELF (Shen et al., 2006).

Cyclin D1 and CDK4 are found to be involved in cell cycle changes caused by various stimuli (Kim et al., 2005; Youn et al., 2007). Progression through the first gap phase (G_1) requires CDK4 and CDK6 and cyclin E/CDK2 activities. The ability of pRb (retinoblastoma protein) to bind E2F is regulated by the CDK4/6–cyclin D-activated kinase complex, which can hyperphosphorylate pRb and release it from E2F (Dimova et al., 2003; Lin et al., 2004; Kim et al., 2005). To determine whether silica induced cell cycle changes in a cyclin D1- or CDK4-dependent manner in HELFs, we used the antisense techniques. Introduction of antisense cyclin D1 or antisense CDK4 could slow the silica-induced cell cycle progression from the G_1-phase to S-phase, as reflected by the higher percentage of G_1-phase cells in HELFs. These results suggest that cyclin D1 and CDK4 were involved in cell cycle changes induced by silica.

It is well known that cell cycle is mediated through many regulatory proteins. MAPK is considered to play an important role in regulating protein synthesis and cell proliferation in various cell types (Ding et al., 1999; Buder-Hoffmann et al., 2001; Joslin et al.,

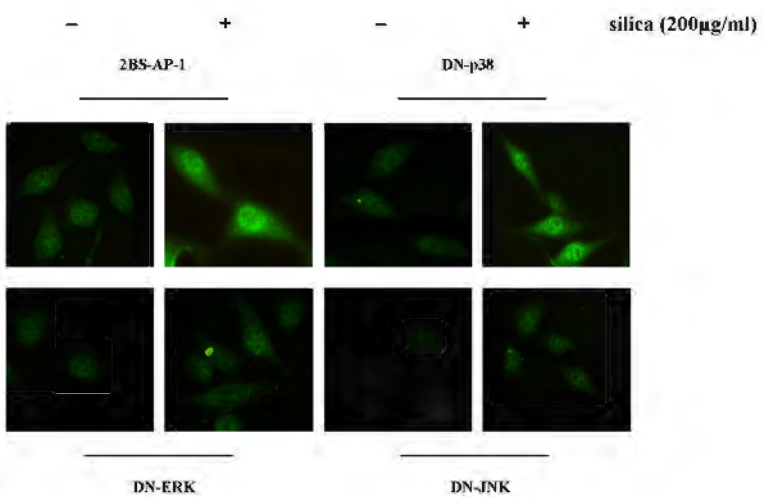

Figure 7 Effects of expression of DN-ERK, DN-JNK and DN-p38 on silica-induced cyclin D1 induction
Cells were treated with silica (200 μg/ml) for 24 h. Secondary antibodies were conjugated to FITC (green).

2007). Previous studies from our laboratory showed that silica exposure can induce phosphorylation of MAPK family, including p38, ERKs and JNKs in HELF (Shen et al., 2006). We also indicated that silica induced a marked activation of AP-1 in a time-dependent manner, and the maximum induction of AP-1 activity occurred at 12 h after exposure (Jia et al., 2008). A series of evidences in *in vitro* studies have shown that AP-1 is thought to play an important role in the regulation of cell cycle progression (Leaner et al., 2005; Stepniak et al., 2006). All of which suggest that there might be some association among the MAPK, AP-1 activation and cell cycle alternation in cells treated with silica.

The signal transduction pathways leading to cell cycle changes have been extensively studied in the last several years. The classic MAPK pathway is a key component in the transduction of signals leading to growth and transformation in many cell types. It is believed that MAPK pathways were activated by various kinds of environmental stresses that induced cell cycle changes (Buder-Hoffmann et al., 2001; Ramos-Nino et al., 2002; Collins et al., 2005; Brown et al., 2005). Hence, we explored the role of MAPK in silica-induced cell cycle alternation, AP-1 activation and the regulation of the expression of cell cycle regulatory proteins by using dominant-negative mutants. Expression of the dominant-negative mutant ERK and JNK can substantially inhibit silica-induced cell cycle alteration and the activation of AP-1. It has been reported that silica exposure induced phosphorylation of the p38 and ERK, but not JNK, and both p38 and ERK were involved in AP-1 activation in a rat epidermal cell line (Ding et al., 1999). However, studies by Shukla et al. (2001) have shown that silica induced dose-related increases in phosphorylated JNK and ERK protein and that JNK was involved in AP-1 activation in a non-transformed alveolar type II epithelial cell line. Our result is consistent with previous findings that MAPK phosphorylation is the upstream kinase event responsible for mediation of cell cycle changes and AP-1 activation induced by silica (Shukla et al., 2001; Øvrevik et al., 2004; Shen et al., 2006). These studies suggested that the precise roles of each of the MAPK depend on the type of cell at the specific stimuli.

AP-1 is an important transcription factor that governs the expression of genes involved in intercellular communication, amplification and primary pathogenic signals spreading as well as initiation and acceleration of tumorigenesis (Mitsuno et al., 2001; Sawai et al., 2005; Zhou et al., 2005). Using curcumin, inhibitor of AP-1, can impair cell cycle alteration remarkably, which suggested AP-1 was required for silica-induced cell cycle changes in HELF. Our study proved that application of curcumin attenuated the overexpression of cyclin D1 and CDK4 and the decreased expression of E2F-4 induced by silica. Thus, we anticipate here that MAPK/AP-1 pathway might mediate the expression of cell cycle regulatory proteins. As we expected, silica significantly elevated the expression of cyclin D1 and CDK4 and decreased the expression of E2F-4, using dominant-negative mutant of ERK and JNK, respectively, obviously inhibiting silica-induced overexpression of cyclin D1 and CDK4 and the decreased expression of E2F-4. These results indicate that ERK and JNK are upstream kinase of cyclin D1, CDK4 and E2F-4. Silica-induced cell cycle alternation is regulated by ERK, JNK/AP-1/cyclin D1–CDK4 pathway.

In the experiments performed by other members of our group, we found an interesting phenomenon that expressions of cyclin D1 and CDK4 were decreased after addition of 400 μg/ml silica for 2 h (Shen et al., 2008). We were very interested with regard to the different results after using different concentrations of silica and detecting the changes in different times. Further study has been currently conducted to explore if these results should be related to different exposure times and concentrations, or there may be additional molecules regulating these processes in our laboratory.

5. Conclusion

In summary, the present study demonstrated that silica exposure could induce cell cycle changes in HELF. These changes were mediated through JNK, ERK/AP-1/cyclin D1–CDK4-dependent pathway and p38-independent pathway. This pathway may be involved in the mechanism of silica-induced fibrosis and other occupational lung diseases. These findings will help us to understand the signal transduction mechanisms involved in the pathogenic effects of silica at the cell cycle level.

Author contribution

Bingci Liu and Xiaowei Jia were responsible for study design, planning, data analysis, doing the experiments, interpretation and writing. Xianglin Shi contributed to planning and interpretation of data. Meng Ye, Fengmei Zhang and Haifeng Liu contributed by doing the experiments and data analysis. All authors read and approved the final manuscript.

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References

Albrecht C, Knaapen AM, Becker A, Höhr D, Haberzettl P, van Schooten FJ et al. The crucial role of particle surface reactivity in respirable quartz-induced reactive oxygen/nitrogen species formation and APE/Ref-1 induction in rat lung. Respir Res 2005;6:129.

Arcangeli G, Cupelli V, Giuliano G. Effects of silica on human lung fibroblast in culture. Sci Total Environ 2001;270:135–9.

Barrett EG, Johnston C, Oberdörster G, Finkelstein JN. Silica-induced chemokine expression in alveolar type II cells is mediated by TNF-alpha-induced oxidant stress. Am J Physiol 1999;276:979–88.

Brown L, Benchimol S. The involvement of MAPK signaling pathways in determining the cellular response to p53 activation – cell cycle arrest or apoptosis. J Biol Chem 2005;281:3832–40.

Buder-Hoffmann S, Palmer C, Vacek P, Taatjes D, Mossman B. Different accumulation of activated extracellular signal-regulated kinases (ERK 1/2) and role in cell-cycle alterations by epidermal growth factor, hydrogen peroxide, or asbestos in pulmonary epithelial cells. Am J Respir Cell Mol Biol 2001;24:405–13.

Calvert GM, Rice FL, Boiano JM, Sheehy JW, Sanderson WT. Occupational silica exposure and risk of various diseases: an analysis using death certificates from 27 states of the United States. Occup Environ Med 2003;60:122–9.

Chen EY, Mazure NM, Cooper JA, Giaccia AJ. Hypoxia activates a platelet-derived growth factor receptor/phosphatidylinositol 3-kinase/Akt pathway that results in glycogen synthase kinase-3 inactivation. Cancer Res 2001;61:2429–33.

Cherry NM, Burgess GL, Turner S, McDonald JC. Crystalline silica and risk of lung cancer in the potteries. Occup Environ Med 1998;55:779–85.

Clarke N, Arenzana N, Hai T, Minden A, Prywes R. Epidermal growth factor induction of the c-jun promoter by a Rac pathway. Mol Cell Biol 1998;18:1065–73.

Collins NL, Reginato MJ, Paulus JK, Sgroi DC, Labaer J, Brugge JS. G1/S cell cycle arrest provides anoikis resistance through Erk-mediated Bim suppression. Mol Cell Biol 2005;25:5282–91.

De Klerk NH, Musk AW. Silica, compensated silicosis, and lung cancer in Western Australian goldminers. Occup Environ Med 1998;55:243–8.

Deshpande A, Narayanan PK, Lehnert BE. Silica-induced generation of extracellular factor(s) increases reactive oxygen species in human bronchial epithelial cells. Toxicol Sci 2002;67:275–83.

Ding M, Shi X, Dong Z, Chen F, Lu Y, Castranova V, Vallyathan V. Freshly fractured crystalline silica induces activator protein-1 activation through ERKs and p38 MAPK. J Biol Chem 1999;274:30611–16.

Ding M, Shi X, Lu Y, Huang C, Leonard S, Roberts J, Antonini J, Castranova V, Vallyathan V. Induction of activator protein-1 through reactive oxygen species by crystalline silica in JB6 cells. J Biol Chem 2001;276:9108–14.

Ding M, Chen F, Shi X, Yucesoy B, Mossman B, Vallyathan V. Diseases caused by silica: mechanisms of injury and disease development. Int Immunopharmacol 2002;2:173–82.

Dimova DK, Stevaux O, Frolov MV, Dyson NJ. Cell cycle-dependent and cell cycle-independent control of transcription by the *Drosophila* E2F/RB pathway. Genes Dev 2003;17:2308–20.

Gambelli F, Di P, Niu X, Friedman M, Hammond T, Riches DW, Ortiz LA. Phosphorylation of tumor necrosis factor receptor 1 (p55) protects macrophages from silica-induced apoptosis. J Biol Chem 2003;279:2020–9.

Hughes JM, Weill H, Rando RJ, Shi R, McDonald AD, McDonald JC. Cohort mortality study of North American industrial sand workers. II. Case-referent analysis of lung cancer and silicosis deaths. Ann Occup Hyg 2001;45:201–7.

International Agency for Research on Cancer. IARC Monographs on the evaluation of the carcinogenic risk of chemicals to humans. Silica, some silicates, coal dust and para-aramid fibrils, vol. 68 Lyon: IARC; 1997. p. 1–475

Jia X, Liu B, Shi X, Gao A, You B, Ye M, Shen F, Du H. Inhibition of benzo(a)pyrene-induced cell cycle progression by all-trans retinoic acid partly through cyclin D1/E2F-1 pathway in human embryo lung fibroblasts. Cell Biol Int 2006;30:183–9.

Jia X, Liu B, Shi X, Gao A, You B, Ye M, Zhang F, Liu H. ERK, JNK/AP-1 pathway was involved in silica-induced cell cycle changes. Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi 2008;26:3–6.

Johnston CJ, Driscoll KE, Finkelstein JN, Baggs R, O'Reilly MA, Carter J, Gelein R, Oberdörster G. Pulmonary chemokine and mutagenic responses in rats after subchronic inhalation of amorphous and crystalline silica. Toxicol Sci 2000;56:405–13.

Joslin EJ, Opresko LK, Wells A, Wiley HS, Lauffenburger DA. EGF-receptor-mediated mammary epithelial cell migration is driven by sustained ERK signaling from autocrine stimulation. J Cell Sci 2007;120:3688–99.

Kim YT, Zhao M. Aberrant cell cycle regulation in cervical carcinoma. Yonsei Med J 2005;46:597–613.

Knaapen AM, Albrecht C, Becker A, Höhr D, Winzer A, Haenen GR, Borm PJ, Schins RP. DNA damage in lung epithelial cells isolated from rats exposed to quartz: role of surface reactivity and neutrophilic inflammation. Carcinogenesis 2002;23:1111–20.

Langley RJ, Kalra R, Mishra NC, Hahn FF, Razani-Boroujerdi S, Singh SP, Benson JM, Peña-Philippides JC, Barr EB, Sopori ML. A biphasic response to silica: I. Immunostimulation is restricted to the early stage of silicosis in Lewis rats. Am J Respir Cell Mol Biol 2004;30:823–9.

Leaner VD, Donninger H, Ellis CA, Clark GJ, Birrer MJ. p75-Ras-GRF1 is a c-Jun/AP-1 target protein: its up regulation results in increased Ras activity and is necessary for c-Jun-induced nonadherent growth of Rat1a cells. Mol Cell Biol 2005;25:3324–37.

Lin YC, Wu MY, Li DR, Wu XY, Zheng RM. Prognostic and clinicopathological features of E-cadherin, alpha-catenin, beta-catenin, gamma-catenin and cyclin D1 expression in human esophageal squamous cell carcinoma. World J Gastroenterol 2004;10:3235–9.

Liu H, Zhang H, Forman HJ. Silica induces macrophage cytokines through phosphatidylcholine-specific phospholipase C with hydrogen peroxide. Am J Respir Cell Mol Biol 2007;36:594–9.

Luzina IG., Highsmith K, Pochetuhen K, Nacu N, Rao JN, Atamas SP. PKCalpha mediates CCL18-stimulated collagen production in pulmonary fibroblasts. Am J Respir Cell Mol Biol 2006;35:298–305.

Mitsuno Y, Yoshida H, Maeda S, Ogura K, Hirata Y, Kawabe T, Shiratori Y, Omata M. *Helicobacter pylori* induced transactivation of SRE and AP-1 through the ERK signalling pathway in gastric cancer cells. Gut 2001;49:18–22.

O'Reilly KM, Phipps RP, Thatcher TH, Graf BA, Van Kirk J, Sime PJ. Crystalline and amorphous silica differentially regulate the cyclooxygenase–prostaglandin pathway in pulmonary fibroblasts: implications for pulmonary fibrosis. Am J Physiol Lung Cell Mol Physiol 2005;288:L1010–6.

Øvrevik J, Låg M, Schwarze P, Refsnes M. p38 and Src-ERK1/2 pathways regulate crystalline silica-induced chemokine release in pulmonary epithelial cells. Toxicol Sci 2004;81:480–90.

Porter DW, Millecchia L, Robinson VA, Hubbs A, Willard P, Pack D, Ramsey D, McLaurin J, Khan A, Landsittel D, Teass A, Castranova V. Enhanced nitric oxide and reactive oxygen species production and damage after inhalation of silica. Am J Physiol Lung Cell Mol Physiol 2002;283:485–93.

Porter DW, Hubbs AF, Mercer R, Robinson VA, Ramsey D, McLaurin J, Khan A, Battelli L, Brumbaugh K, Teass A, Castranova V. Progression of lung inflammation and damage in rats after cessation of silica inhalation. Toxicol Sci 2004;79:370–80.

Ramos-Nino ME, Haegens A, Shukla A, Mossman BT. Role of mitogen-activated protein kinases (MAPK) in cell injury and proliferation by environmental particulates. Mol Cell Biochem 2002;234–5:111–8.

Rice FL, Park R, Park R, Stayner L, Smith R, Gilbert S, Checkoway H. Crystalline silica exposure and lung cancer mortality in diatomaceous earth industry workers: a quantitative risk assessment. Occup Environ Med 2001;58:38–45.

Roy I, Ohulchanskyy TY, Bharali DJ, Pudavar HE, Mistretta RA, Kaur N, Prasad PN. Optical tracking of organically modified silica nanoparticles as DNA carriers: a nonviral, nanomedicine approach for gene delivery. Proc Natl Acad Sci USA 2005;102:279–84.

Sawai H, Funahashi H, Okada Y, Matsuo Y, Sakamoto M, Yamamoto M, Takeyama H, Manabe T. Interleukin-1alpha enhances IL-8 secretion through p38 mitogen-activated protein kinase and reactive oxygen species signaling in human pancreatic cancer cells. Med Sci Monit 2005;11:343–50.

Seiler F, Rehn B, Rehn S, Hermann M, Bruch J. Quartz exposure of the rat lung leads to a linear dose response in inflammation but not in oxidative DNA damage and mutagenicity. Am J Respir Cell Mol Biol 2001;24:492–8.

Shen F, Fan X, Liu B, Jia X, Du H, You B, Ye M, Huang C, Shi X. Overexpression of cyclin D1–CDK4 in silica-induced transformed cells is due to activation of ERKs, JNKs/AP-1 pathway. Toxicol Lett 2006;160:185–95.

Shen F, Fan X, Liu B, Jia X, Gao A, Du H, Ye M, You B, Huang C, Shi X. Downregulation of cyclin D1–CDK4 protein in human embryonic lung fibroblasts (HELF) induced by silica is mediated through the ERK and JNK pathway. Cell Biol Int 2008;32:1284–92.

Shen HM, Zhang Z, Zhang QF, Ong CN. Reactive oxygen species and caspase activation mediate silica-induced apoptosis in alveolar macrophages. Am J Respir Cell Mol Biol 2001;280:10–7.

Shukla A, Timblin CR, Hubbard AK, Bravman J, Mossman BT. Silica-induced activation of c-Jun-NH2-terminal amino kinases, protracted expression of the activator protein-1 proto-oncogene, fra-1, and S-phase alterations are mediated via oxidative stress. Cancer Res 2001;61:1791–5.

Stepniak E, Ricci R, Eferl R, Sumara G, Sumara I, Rath M, Hui L, Wagner EF. C-Jun/AP-1 controls liver regeneration by repressing p53/p21 and p38 MAPK activity. Genes Dev 2006;20:2306–14.

Youn MJ, Kim JK, Park SY, Kim Y, Kim SJ, Lee JS, Chai KY, Kim HJ, Cui MX, So HS, Kim KY, Park R. Chaga mushroom (*Inonotus obliquus*) induces G0/G1 arrest and apoptosis in human hepatoma HepG2 cells. World J Gastroenterol 2007;14:511–7.

Zhou H, Zarubin T, Ji Z, Min Z, Zhu W, Downey JS, Lin S, Han J. Frequency and distribution of AP-1 sites in the human genome. DNA Res 2005;12:139–50.

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