

Club cell secretory protein 16 is a potential biomarker for silica-induced pulmonary fibrosis

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This study was conducted to investigate the changes of Club cell protein 16 (CC16) and surfactant protein D (SP-D) levels in serum and bronchoalveolar lavage fluid (BALF) in silicotic rats and to explore their potential as early biomarkers for silicosis. Pulmonary fibrosis models of rats were constructed by exposing them to silica particles. BALF and serum were collected to determine CC16 and SP-D levels using enzyme-linked immunosorbent assay (ELISA) at different times after the exposure. Hydroxyproline (HYP) level in BALF and CC16 level in the lung tissues were also measured immunohistochemically. The BALF levels of CC16 decreased from 49.65 to 38.02 ng/mg after the rats were exposed to silica for 3 and 28 days, which were all significantly lower as compared with the controls ($P < 0.05$), where the levels remained barely changed during the same period (61.27 to 56.76 ng/mg). The serum CC16 also showed a similar decrease from 9.8 ng/ml to 8.78 ng/ml during the period, while in the controls, the serum CC16 levels remained constantly between 11.04 and 10.96 ng/ml. The levels of SP-D in the serum of silica-exposed rats did not decrease as compared with the controls and BALF SP-D presented a parabolic curve change with silica exposure. Immunohistochemical examinations showed that the lung Club cells were severely damaged and CC16 expression was obviously decreased after silica exposure. BALF HYP level was higher in silica-exposed rats than in control only when the exposure was at 50 mg/ml. Our work demonstrates that expressions of CC16 and SP-D are pulmonary tissue-specific and CC16 expression is down-regulated as a result of silica-exposure. The significant relationship between CC16 and silica dose indicates that CC16 may be exploited as an early biomarker to assess silica-induced pulmonary fibrosis.

Keywords: biomarker; Club cells; silicosis; CC16; surfactant protein D

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Abbreviations: ANOVA, analysis of variance; BALF, bronchoalveolar lavage fluid; CC16, Club cell protein 16; DAB, diaminobenzidine; ELISA, enzyme-linked immunosorbent assay; HYP, hydroxyproline; SD, Sprague Dawley; SP-D, surfactant protein D; SPSS, Statistical Program for Social Sciences; PLA2, phospholipase A2; TBS, tris buffered saline

INTRODUCTION

Silicosis is a chronic and progressive fibrotic lung disease caused by inhaled silica (Ramsgaard *et al.*, 2010). Preventing the inhalation of silica particles through the use of filtered air can help reduce the risk of silicosis

(Castranova & Vallyathan, 2000; Kamp, 2009). For diagnosis of silicosis, biomarkers have been explored using macrophages, fibroblasts, epithelial type II cells and cytokines (Rosenberg & Kalhan, 2012; Wang *et al.*, 2007). However, these biomarkers are not sufficiently sensitive for early diagnosis of silicosis before functional abnormalities in the lung become apparent and visible. To address this issue, Club cells and their secretions are being explored as biomarkers in recent years (McAuley & Matthay, 2009a; Ulvestad *et al.*, 2007; Lakind *et al.*, 2007).

Club cell protein 16 (CC16) is released from the distal respiratory and terminal bronchioles (Broeckaert & Bernard, 2000; Lakind *et al.*, 2007). It interacts with multiple components in the inflammatory and coagulation cascades. For instance, it may act as a phospholipase A2 inhibitor to inhibit inflammation and fibrosis (McAuley & Matthay, 2009a). Studies also show that CC16 inhibits the formation of IFN- γ to constrain antiviral activity and stimulate the key enzyme phospholipase A2 (PLA2) in the inflammatory reaction (Johansson *et al.*, 2007; Ramsay *et al.*, 2003). CC16 can inhibit fibroblast and the activity of synthetic 2cPLA₂ which is related to the signal transduction of the PDGF that induces the chemotaxis of the fibroblast (Broeckaert & Bernard, 2000). Earlier studies showed that the serum levels of CC16 decreased in silicotic patients (Chen *et al.*, 2010). Decreased levels of CC16 have also been reported in bronchoalveolar lavage fluid (BALF) in silica-exposed rats (Liu, 2008). It is likely that reduction in the levels of fibrosis-suppressing factors may contribute to the development of pulmonary fibrosis and these factors are therefore potential biomarker candidates for fibrosis.

The aim of the present study is to test the hypothesis that CC16 may be a potential biomarker for the diagnosis of silicosis. For this purpose, CC16 in serum and BALF was measured following exposing rats to silica via intratracheal instillation to determine the relationship between the levels of lung injury and CC16.

MATERIALS AND METHODS

Ethics statement

All animal experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Hebei United University. Animals were given free access to food and water and were cared according to guidelines set by the Hebei United University Association for Laboratory Animal Care. All the experiments described in this study were carried out in accordance with the Guide for the Care and Use of Laboratory

Animals as adopted and promulgated by the National Institutes of Health.

Animals and treatments

Male Sprague Dawley (SD) rats (12 weeks), weighing 180–220 g, were purchased from Vital River Laboratory Animal Technology, Beijing. Rats were quarantined at the animal housing facilities at the animal center of Hebei United University for 1 week prior to the beginning of the experiment. All animals were fed with standard mouse chow and tap water ad libitum.

Rat models of fibrosis were constructed. To study the dose-effect, rats were randomly divided into control and silica-exposed groups ($n=8$), and silica was administered by a single intratracheal injection of 1 ml silica suspension at concentrations of 12.5, 25 and 50 mg silica/ml. Control rats were given the same volume of saline. The rats were maintained for 4 weeks after the injection and used for subsequent study.

To study the time effect, rats were randomly divided into control and silica-exposed groups ($n=8$), and silica was administered by a single intratracheal injection of 1 ml silica suspension at a concentration of 50 mg silica/ml. Control rats were given the same volume of saline. The rats were maintained for 3, 7, 14, 21 and 28 days after the injection and used for subsequent study.

Sample collection

Animals were sacrificed at pre-determined time points and the blood samples were collected from the hearts. The serum was separated and stored at -80°C . BALF was also collected. The lung samples were taken from rats and stored at -80°C .

Immunohistochemistry

5 μm tissue sections were deparaffinized, rehydrated and rinsed with tris buffered saline 1X (TBS), reacted to a primary antibody against CC16 for 1 h and subsequently washed with TBS for three times, and incubated with secondary antibody for 30 min. Diaminobenzidine (DAB) and haematoxylin chromogen (Dako, Glostrup, Denmark) method was used to visualize the immunoreactivity. The slides were examined under light microscopy to score CC16-positive cells and drop-off cells. For each sample, 10 randomly selected fields were evaluated.

Enzyme-linked immunosorbent assay (ELISA)

CC16 was measured in the cell-free supernatants of BALF and serum using an ELISA kit (R&D systems Inc, Minneapolis, MN) according to the manufacturer's instructions. Total protein was assessed using the standard Bradford assay.

Measurement of Hydroxyproline (Hyp)

Hyp concentration in BALF and the lung tissue was measured using a standard endpoint measurement at 550 nm using Bradford reagent with a bovine serum albumin standard curve (Thermo Scientific, Rockford, IL) as described previously (Watanabe *et al.*, 2015).

Protein quantification

Protein concentration in BALF and serum was measured using a standard endpoint measurement at 598 nm using Bradford reagent with a bovine serum albumin standard curve (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions.

Table 1. Lung damage was measured by drop-off CC16-positive cells in lung tissue after exposure to silica for 28 days

Silica dose (mg/ml)	No. drop-off cells	%
0	5	1.0
12.5	123	24.6 ^a
25	241	48.2 ^b
50	321	64.2 ^c

^aFigures labelled with different superscripts are different significantly ($P<0.05$).

Table 2. BALF HYP in rats following exposure to different doses of silica.

SiO ₂ (mg/ml)	BALF HYP ($\mu\text{g}/\text{mg}$ protein)	Ratio
0 (Control)	2.02 \pm 0.88	1.00 \pm 0.44
12.5	2.09 \pm 0.57	1.03 \pm 0.27
25	2.63 \pm 0.93	1.30 \pm 0.35
50	2.81 \pm 0.71*	1.39 \pm 0.25

*statistically different ($P<0.05$ in post-hoc analysis) vs control.

Statistical analysis

All data are expressed as means \pm standard deviation (S.D.). The Statistical Program for Social Sciences (SPSS) was used for data analysis. The one-way analysis of variance (ANOVA) was used to examine the differences between groups. Spearman correlations were used to determine the linear relationship between CC16 in serum and BALF. All P -values were 2-tailed, and a $P<0.05$ was considered significant.

RESULTS

Silica exposure increases lung damage

To assess the lung damage caused by silica exposure, we counted the number of CC16-positive cells that were dropped off from the lung tissue after 28 days of exposure. The data indicated that the number of drop-off cells increased with increasing silica doses, while in non-exposed rats, the drop-off was very low (Table 1).

Silica exposure increases HYP

The BALF concentrations of HYP increased after the rats were exposed to silica and the increase reached statistically significant only at 50 mg silica/ml group as compared to the control group ($P<0.05$, Table 2). In addition, the increase in BALF HYP increased with increasing time following exposure to 50 mg silica /ml for 7, 14, 21 and 28 days ($P<0.05$, Table 3).

Silica exposure decreases CC16

The CC16 concentration decreased in BALF and serum following silica exposure and the reduction increased with silica dose in both BALF and serum (Table 4). In addition, the reduction also increased with increasing time after exposure to 50 mg silica /ml for 7, 14, 21 and 28 days (Table 5). Regression analysis showed that there was a significant and negative correlation between BALF CC16 concentration and silica

Table 3. BALF HYP in rats following exposure to 50 mg/ml silica.

SiO ₂ (mg/ml)	Days after silica injection				
	3	7	14	21	28
0 (Control)	0.63±0.19	0.65±0.35	0.67±0.86	0.60±0.005	0.72±0.88
50	0.92±0.18	1.18±0.21*	1.39±0.18*	2.15±0.15*	2.35±0.19*

*denotes statistically different ($P<0.05$ in post-hoc analysis) vs control.

Table 4. CC16 levels in the serum and BALF of rats following exposure to different doses of silica

Silica dose (mg/ml)	Serum CC16 (ng/ml)	Ratio	BALF CC16 (ng/mg protein)	Ratio
0	5.68±0.10	1.00±0.018	89.51±19.29	1.00±0.21
12.5	4.96±0.31*	0.72±0.0625	69.56±14.18*	0.78±0.20
25	4.97±0.18*	0.72±0.036	43.90±4.01*#	0.49±0.091
50	4.90±0.18*	0.73±0.037	30.49±6.39*#	0.34±0.21

*and # denote statistically different from control at $P<0.05$ and 0.01 in post-hoc analysis.

Table 5. CC16 levels in the serum and BALF of rats following exposure to silica at different times.

Days after silica injection	Serum		BALF	
	Control	Silica	Control	Silica
3	5.60±0.28	5.39±0.39	61.27±3.18	49.65±1.97*
7	5.52±0.41	4.9±0.59*	60.67±3.11	47.10±1.78*
14	5.42±0.33	4.68±0.61*	61.20±2.33	46.15±1.05*
21	5.44±0.35	4.5±0.26*	60.61±2.82	41.63±2.37*
28	5.48±0.59	4.39±0.52*	56.76±2.29	38.02±2.82*

*denotes statistically different ($p<0.05$ in post-hoc analysis) vs control.

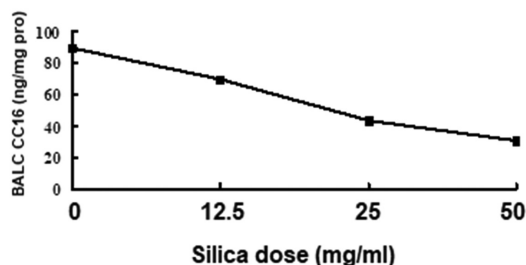


Figure 1. Correlation between BALF CC16 and silica dose.

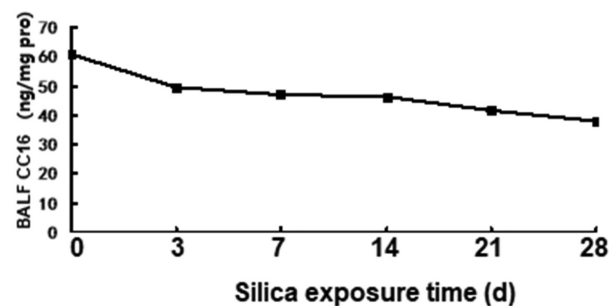


Figure 2. Correlation between BALF CC16 and silica exposure time.

dose (Fig. 1, $r=-0.958$, $P=0.042$) but not between serum CC16 concentration and silica dose. In addition, the exposure time was found to be negatively correlated with BALF (Fig. 2) and positively with serum CC16 levels ($r_s=-0.904$ and 0.940 , respectively. $P=0.013$ and 0.005 , respectively).

BALF and serum CC16 concentrations are positively correlated

In the silica time models, there was a positive correlation between the CC16 levels in BALF and serum ($r=0.882$, $P=0.02$). However, in the silica dose group, there were no such correlations ($P>0.05$).

Silica exposure causes reduced CC16 expression

Immunohistochemical analysis showed that CC16 was highly expressed in the Club cells of bronchioles. The cells were intact and regularly arranged in the unexposed lung. After exposure to 12.5 mg/ml silica, the wall of bronchioles was thickened, and some Club cells were damaged and dropped off and CC16 expression was decreased. After exposure to 25 mg/ml silica, many Club cells have degenerated and CC16 expression was remarkably reduced. Only a few Club cells remained intact while most of them were broken and fibrous nodules become visible appeared after the rats were exposed to 50 mg/ml silica (Fig. 3). When examined after silica exposure, the wall of bronchioles began to thicken and Club cells were seen damaged 7 days after the exposure. The thickening and damage were more remarkable 14 days after the exposure, when most Club cells were dropped off

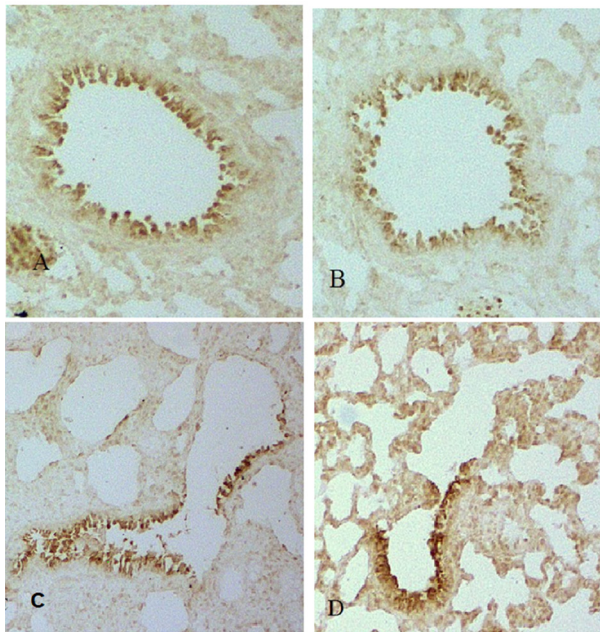


Figure 3. Immunohistochemical analysis of the Club cells and CC16 in the lung after exposure to different concentrations of silica.

A–D, 0, 12.5, 25 and 50 mg/ml silica.

into the alveolar cavity. By day 21, only a few broken Club cells were still attached to the wall of bronchioles and most Club cells were replaced by a large number of collagen fibers and fibroblasts and the lung tissue was highly fibrotic. The expression of CC16 decreased constantly after the exposure and was barely detectable by day 21 (Fig. 4).

DISCUSSION

It has been demonstrated that CC16 is a potential biomarker of lung injury in many diseases, including idiopathic pulmonary fibrosis (Ye *et al.*, 2004a), sarcoidosis (Janssen *et al.*, 2003; McAuley & Matthay, 2009a; Provost *et al.*, 2014; Ye *et al.*, 2004b), COPD (Ye *et al.*, 2004c; McAuley & Matthay, 2009b), occupational or environmental lung injury (Petrek *et al.*, 2002; Ulvestad *et al.*, 2007; Wang *et al.*, 2007), bronchiolitis obliterans (Mattsson *et al.*, 2005; Nord *et al.*, 2002), chronic tobacco use (McAuley & Matthay, 2009a) and ALI/ARDS (Geerts *et al.*, 2001; Lesur *et al.*, 2006; McAuley & Matthay, 2009a; Michel *et al.*, 2005). Studies also showed that there are significant differences in serum CC16 in workers exposed to different levels of silica dust (Michel *et al.*, 2005). However, the relationship between CC16 expression and silica-induced lung injury is unclear, although a previous study on works of stone mines and stone quarries showed that lung damage is inversely associated with the duration of silica exposure to likely increased amount of silica dust inhaled over a year and serum CC16 level (Sarkar *et al.*, 2021).

This study was conducted to examine the relationship and investigated whether CC16 is a potential biomarker of silicosis. The results of the present study confirmed that lung damage is positively associated with silica exposure doses and BALF CC6 expression is negatively related the silica-induced lung damage and could therefore be exploited as a biomarker to measure lung injury.

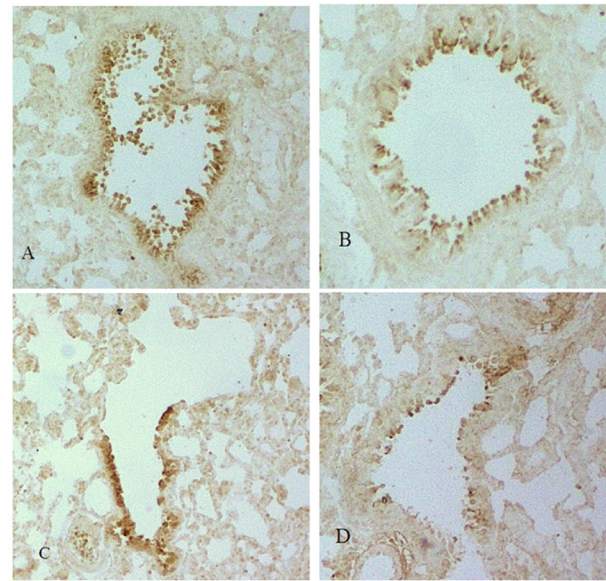


Figure 4. Immunohistochemical analysis of the Club cells and CC16 in the lung at different times after exposure to 25 mg/ml silica.

A–D, 0, 7, 14 and 21 days after exposure.

In this study, both doses and the time effect of silica exposure were measured. BALF and serum CC16 levels decreased as the dose and time increased, while BALF HYP tended to increase. This is consistent with results obtained in acute respiratory distress syndrome, asthma and pulmonary fibrosis diseases (Sarafidis *et al.*, 2008; Zhang *et al.*, 2009). However, the decline in serum CC16 appeared to be not silica dose-dependent. The reason for this is unclear, but might be attributed to the dilution effect of blood, which has a relatively large volume that would “buffer” the change. The significant negative correlation between CC16 and silica dose and exposure time indicates that there is a dose and time effect of silica exposure on CC16 expression, and CC15 can specifically respond to silica exposure and could therefore be further exploited as a biomarker for silica-induced damage.

On other hand, the levels of SP-D in the serum of silica-exposed rats did not decrease as compared with the controls and BALF SP-D presented a parabolic curve change with silica exposure, not in a linear way, suggesting that while SP-D is responsive to silica exposure, it might not be a sensitive biomarker for silica-induced injury. Previously, SP-D was found to respond to fungal infection in the lung and bind glucan and mannose residues from the fungal cell wall to play an antifungal role (Carreto-Binaghi *et al.*, 2016) through innate immunity mediated by pattern recognition (Pandit *et al.*, 2012). However, since fungi and silica particle might have a different model of action against lung tissue, it is likely that SP-D responds differently, although it was found accumulated silica-induced pulmonary lipoproteinosis (LeVine *et al.*, 2004).

HYP increased only after exposure to the highest silica dose (50 mg/ml) in this study, indicating that it is not very sensitive in response to silica. HYP is a unique amino acid in collagen, accounting for 13.4% of collagen. Except for elastin which contains very little HYP (about 1%), almost all HYP is present in collagen, the main component of extracellular substrate for fibrosis. Therefore, HYP is an indicator of hepatic fibrosis (Toyoki *et*

al., 1998) and lung fibrosis (Ren *et al.*, 2017). Since fibrosis is a chronic progress and can be measured by HYP change in a relatively long-term, it is not surprising that HYP did at increased after low level silica exposure.

CC16 is immunosuppressive, anti-inflammatory, anti-fibrosis, anti-tumor, and could clear the hazardous substances deposited in the respiratory tract and the surface of the lung (Sarafidis *et al.*, 2011). Once silica dust enters the lung, they would first activate macrophages in the lung to swallow silica particles and release toxins to damage the Club cells, leading to reduced expression and secretion of CC16, and subsequently decreased serum and BALF CC16 levels. In addition, the toxicity of silica particle and free radicals on the surface of silica particles can damage the lung epithelial cells, resulting in a reduced number of Club cells and CC16 expression (Christensen *et al.*, 2010). In addition, when the silica dust enters the human body, it would cause an inflammation reaction, particularly in the lung tissue. Long-term exposure to high concentrations of silica dust would lead to fibrosis in the lung tissue. On the other hand, the Club cells have anti-inflammatory and anti-fibrosis, fibrosis would inhibit these activities and reduce CC16 release from the Club cells.

In conclusion, our works demonstrate that the serum level of CC16 is negatively related to lung injury and silicosis. This negative correlation indicates that CC16 might be further exploited as an early biomarker for silicosis.

Declarations

Ethics approval and consent to participate. All animal experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Hebei United University. Animals were given free access to food and water and were cared according to guidelines set by the Hebei United University Association for Laboratory Animal Care. All the experiments described in this study were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Availability of data and material. The datasets used during the current study are available from the corresponding author upon reasonable request.

Competing interests. The authors declare that they have no competing interests

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Authors' contributions. LY, HW and ML designed the study. HW, ML, CW and YZ collected the data and performed analysis. LY, HW, ML, CW, YZ and ZZ drafted the manuscript. All authors read and approved the final version of the manuscript.

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