Early decrease of serum Clara cell protein in silica-exposed workers

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Early decrease of serum Clara cell protein in silica-exposed workers. A.M. Bernard, J.M. Gonzalez-Lorenzo, E. Siles, G. Trujillano, R. Lauwerys. ©ERS Journals Ltd 1994.

ABSTRACT: Clara cell protein (CC16) is a 16 kDa protein secreted by nonciliated cells of the tracheobronchial tree; it has recently been proposed as a peripheral marker of respiratory epithelial injury.

The concentration of CC16 was measured in the serum and, when available, in the sputum of 86 miners exposed to silica and of 86 control subjects matched for age, body mass index and smoking status (26 lifelong nonsmokers and 60 current smokers in both groups). Workers were exposed to silica-rich dust in a quarry for 15.2 months on average.

No difference between exposed and control workers could be detected with regard to respiratory symptoms, chest radiographs or lung function tests. By contrast, the concentration of CC16 in serum was decreased in silica-exposed workers (geometric mean 12.3 $\mu g \cdot l^{-1}$) compared to controls (16.3 $\mu g \cdot l^{-1}$). The decrease was found both in lifelong nonsmokers (14.7 *vs* 21.9) and current smokers (11.3 *vs* 14.5). In the latter, tobacco smoking caused a decrease of serum CC16 that was additional to that associated with silica exposure. The determination of CC16 in sputum samples, judged to be reliable on the basis of the CC16/alpha-amylase concentration ratio (mostly from smokers), also revealed a reduction of CC16 following silica exposure (46.2 *vs* 106 $mg \cdot l^{-1}$).

We conclude that alterations in the serum concentrations of CC16 probably reflect very early toxic effects of silica particles on the respiratory epithelium. This reinforces the view that serum CC16 is a sensitive marker, which might improve our ability to detect exposure to chemicals potentially harmful to the respiratory tract.
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Clara cell protein is a potentially immunosuppressive protein secreted by nonciliated cells of the tracheobronchial epithelium and by some reproductive system organs, such as the prostate [1–3]. It is a homodimer consisting of 70 amino acid subunits, and has a molecular mass of 15,840 Da (hence the CC16 abbreviation). The protein was previously referred to as the 10 kDa Clara cell protein (CC10) because of an underestimation of its molecular weight by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis [4]. It is identical to protein 1, an alpha-microprotein isolated from the urine of patients with tubular proteinuria [5], and is the counterpart in humans of rabbit uteroglobulin [5–9]. The highest concentrations of CC16 are observed in sputum and bronchoalveolar lavage fluid (BAL), reflecting an intense secretion of the protein in airways [4, 10]. The protein also occurs, but in smaller concentrations, in other fluids such as urine, amniotic fluid or semen. It is present in trace amounts in serum, probably deriving from the respiratory tract by passive transudation [4, 10]. The exact physiological function of CC16 remains unknown, but there are several lines of evidence indicating that it is an immunosuppressive or anti-inflammatory protein protecting the airways from undue activations of the immune system that might cause tissue injury [6, 8, 9].

Recently, we have shown that the concentration of CC16 in serum and BAL was significantly decreased in tobacco smokers [10]. The decrease in serum could be related in a dose-dependent manner with the pack-years smoking history [11, 12]. Interestingly, this effect was already detectable in subjects with normal lung X-ray and function, which led us to postulate that serum CC16 might be a marker of early toxic effects on the bronchial epithelium [10]. The present study further supports this hypothesis, by showing a very early decrease of CC16 in serum and sputum samples of asymptomatic workers exposed to silica-rich dust.

Subjects and methods

Study population

The total population recruited for the study consisted of 100 male subjects working in a quarry for less than 2 yrs, and of 292 manual workers (concrete reinforcement workers, electrification workers and heavy manual workers) with no exposure to silica-containing dust or other pulmonary toxicants (*e.g.* welding fumes, paints, solvents, *etc.*). Of these subjects, we excluded 38 subjects who were suffering from chronic lung (17) or kidney (5) disease, hypertension (11), or diabetes (5), 40 who refused to provide blood samples, 39 for whom not all relevant information could be obtained from the questionnaire, and 20 who smoked only pipes or cigars (3), or were ex-smokers (6), or who regularly took analgesics or anti-inflammatory drugs (11). After this selection, the exposed group included 86 subjects and the control group 157. These two groups were well-matched for age, but not for tobacco smoking and body mass index (BMI) (significantly higher in the exposed group). Since the latter factor is intimately linked to respiratory function parameters, we decided to match each exposed subject with a control subject of the same smoking status and with an age (± 2 yrs) and BMI (± 1 kg·m^{-2}) as close as possible.

Exposure

Exposed subjects worked in a quarry extracting a quartzite rock made of 90% of crystalline silica (SiO_2). The mean concentrations of SiO_2 in air samples collected at the most representative worksites ranged 6.8–400 mg·m^{-3}. Twenty five to 30% of the SiO_2 particles had a diameter below 5 µm. Waterspray systems were used to reduce dust emissions and the machines (drillers, excavators, loaders, *etc.*) were equipped with air-conditioned cabins. Workers were also requested to wear personal respiratory protection equipment.

Examination

The subjects completed a questionnaire about their medical history, current and past occupations, intake of drugs and tobacco smoking. They also filled in the European Coal and Steel Community Questionnaire on chronic bronchitis, and provided a sample of blood and urine. A sputum sample was also obtained from 141 workers (75 exposed and 66 controls). All subjects underwent a standard spirometric and chest X-ray examination.

Determination of CC16 and other analytes

The concentration of CC16 in serum was determined by the same immunoassay as that recently used for the analysis of the protein in serum and BAL [10]. The assay uses the anti-protein 1 antibody from Dakopatts (Glostrup, Denmark) and, as standard, the protein purified in our laboratory [4, 13]. Sputa were diluted twice (on a weight basis) with physiological saline and after overnight storage at 4°C, they were cleared with Freon 113 and centrifuged at 2,000×g for 10 min. Since saliva does not contain CC16, contamination of sputum by saliva results in a dilution of the sample. Sputum samples excessively diluted by saliva were identified on the basis of the CC16/alpha-amylase concentration ratio. As exclusion criterion, we adopted the median of CC16 concentration in the sputum of smokers not exposed to silica divided by the 5th percentile value of alpha-amylase concentrations in the saliva samples from 20 healthy subjects from the laboratory staff (ratio=0.123). Seventy six sputum samples (39 from exposed and 37 from control workers) having a CC16/alpha-amylase ratio below this value were considered as unreliable and excluded from the analysis.

To account for possible variations in the renal clearance of low molecular weight plasma proteins, we determined the serum concentration of β_2-microglobulin, a microprotein with a size (11.8 kDa) similar to that of CC16. Beta$_2$-microglobulin was also determined in sputum to assess the specificity of changes affecting CC16. The concentrations of β_2-microglobulin and alpha-amylase in serum, sputum or saliva were measured by latex immunoassay [14], using the Dakopatts antibodies and, as standards, purified β_2-microglobulin or pooled saliva samples from 20 healthy subjects from the laboratory staff.

Environmental dust sampling was performed with a Sierra model 305 sampler and the silica content of particles was determined by inductively coupled plasma (ICP) spectroscopy with a Bausch and Lomb model 3520 spectrophotometer. As the use of personal protection devices precluded any individual monitoring of the respirable dust concentrations, an estimation of the individual exposure was attempted on the basis of the urinary silicon excretion. Silicon was measured with a Varian Spectra A-30/40 Zeeman atomic absorption spectrophotometer calibrated by standard additions. Dilutions were made with $NaBO_2$. Urine sampling containers were checked for the absence of silicon contamination before use. Creatinine in urine was measured by the Jaffé's method.

Statistical anlalysis

All statistical tests were performed with the Statview SE software [15]. All parameters except age were log-transformed before statistical analysis, and the normality of their distribution was checked by the Kolmogorov-Smirnov one-sample test. The results are reported as the geometric mean ($\pm$geometric SE in the figures) unless otherwise stated. Comparisons between control and exposed workers were made with a two-tailed Student's t-test, whereas the separate or combined effects of tobacco smoking and silica-exposure were assessed by a

two-way analysis of variance (ANOVA). Determinants of the concentrations of CC16 in serum or sputum and of other effect parameters were traced by stepwise regression analysis, using as predictors age, BMI, smoking history, duration of exposure and the urinary excretion of silicon. The smoking history was introduced in the model categorized as follows: 0, >0–10, >10–20, >20–30 and >30 pack years. Prevalences of abnormally decreased values of CC16 in serum were calculated by using as cut-off the geometric mean minus two geometric SD of the values observed in control lifelong nonsmokers (8.9 $\mu g \cdot l^{-1}$). The level of statistical significance was set at p<0.05.

Results

The two groups of workers were well-matched for age, BMI and proportion of smokers. The number of pack-years was, however, slightly higher in control than in exposed workers. Urinary silicon excretion (table 1) was higher in the exposed than in the control group, but although the difference was statistically very significant (p=0.001) the ranges of urinary silicon of both groups greatly overlapped. In lifelong nonsmokers, prevalences

Table 1. – Characteristics of populations

	Controls n=86	Exposed n=86	p-value
Age yr	36±9 (19–57)	34±8 (20–57)	NS
Height cm	171.5±6.7 (155–187)	171.2±5.4 (162–184)	NS
Weight kg	70.5±10.5 (56–104)	69.6±8.7 (49–95)	NS
BMI kg·m⁻²	23.9±2.7 (17.8–33.1)	23.7±2.7 (18.2–32.9)	NS
Smokers/nonsmokers	60/26	60/26	
Pack-years	21±15 (2.2–75.0)	17±12* (0.7–68.2)	
Duration of exposure months		15.2±2.4 (11–20)	
Silicon in urine mg·g⁻¹cr	8.6±8.4 (0.9–47)	14.3±9.8** (4.1–77)	

Data are presented as mean±SD, and range in parenthesis. BMI: body mass index; NS: not statistically significant. *: p=0.053, **: p=0.001.

of respiratory symptoms (cough, wheezing, phlegm, dyspnoea) did not differ between control and exposed workers. In current smokers, controls had significantly more complaints of cough (13.3 vs 3.3%) and phlegm (11.7 vs 6.7%) than exposed subjects. One exposed worker and six control workers (all smoker) had symptoms of chronic bronchitis. All chest X-rays were normal and the lung function tests did not reveal any abnormality in relation to silica exposure. The only significant effect which could be disclosed was a slight decrease of forced expiratory volume in one second (FEV_1) and FEV_1/vital capacity (VC) due to smoking (table 2).

Results obtained with CC16 contrasted with the lack of change in the respiratory symptoms and lung function tests. The concentration of CC16 in the serum from exposed workers averaged 12.3 $\mu g \cdot l^{-1}$ (median 13; range 4–40 $\mu g \cdot l^{-1}$) against 16.3 $\mu g \cdot l^{-1}$ (median 16.7; range 2.2–53 $\mu g \cdot l^{-1}$) in controls (p=0.001). The concentrations of serum β_2-microglobulin were similar in both groups (mean 1.39 $mg \cdot l^{-1}$ in exposed and 1.41 $mg \cdot l^{-1}$ in control workers; range 0.85–4.7 and 0.8–3.5 $mg \cdot l^{-1}$, respectively). As shown in figure 1, the reduction of CC16 in workers exposed to silica was significant both in life long nonsmokers and in current smokers. In the latter, the effect of silica exposure was additional to that caused by tobacco smoking. The same pattern was observed in the prevalences of abnormally decreased values of CC16 in serum: 4% in control lifelong nonsmokers; 23.3% in control smokers; 14.9% in exposed lifelong nonsmokers and 31.7% in exposed smokers. The serum concentrations of β_2-microglobulin were unaffected by tobacco smoking and exposure to silica (fig. 1).

The number of lifelong nonsmokers (5 control and 9 exposed) who provided reliable sputum samples on the basis of the CC16/alpha-amylase ratio was too small to be included in the statistical analysis which was, therefore, restricted to current smokers. As shown in figure 2, sputum samples from exposed smokers contained on average half the CC16 (geometric mean 46.2 $mg \cdot l^{-1}$; median 45 $mg \cdot l^{-1}$; range 7.3–590 $mg \cdot l^{-1}$) as those of silica-exposed smokers (geometric mean 106 $mg \cdot l^{-1}$; median 115 $mg \cdot l^{-1}$; range 13–1,900 $mg \cdot l^{-1}$); whereas, they had the same amounts or even slightly more β_2-microglobulin and alpha-amylase.

The determinants of CC16 concentration in serum or sputum were examined separately in control and exposed

Table 2. – Lung function tests

	Control		Exposed		Two way Anova		
	Longterm Nonsmokers	Smokers	Longterm Nonsmokers	Smokers	Tobacco	Silica	Interaction
n	26	60	26	60			
Age yrs	36±10	36±8	36±9	33±7	NS	NS	NS
VC l	4.09±0.63	4.15±1.13	4.31±0.64	4.32±0.83	NS	NS	NS
FEV_1 l·s⁻¹	3.61±0.58	3.43±0.88	3.79±0.73	3.46±0.54	0.03	NS	NS
FEV_1/VC %	89±10.5	83±13.5	86.5±21.9	81.5±10.7	0.02	NS	NS
FVC l	4.06±0.85	4.02±1.0	3.71±0.59	3.96±0.92	NS	NS	NS

Data are presented as mean±SD. VC: vital capacity; FEV_1: forced expiratory volume in one second; FVC: forced vital capacity; ANOVA: analysis of variance; NS: nonsignificant.
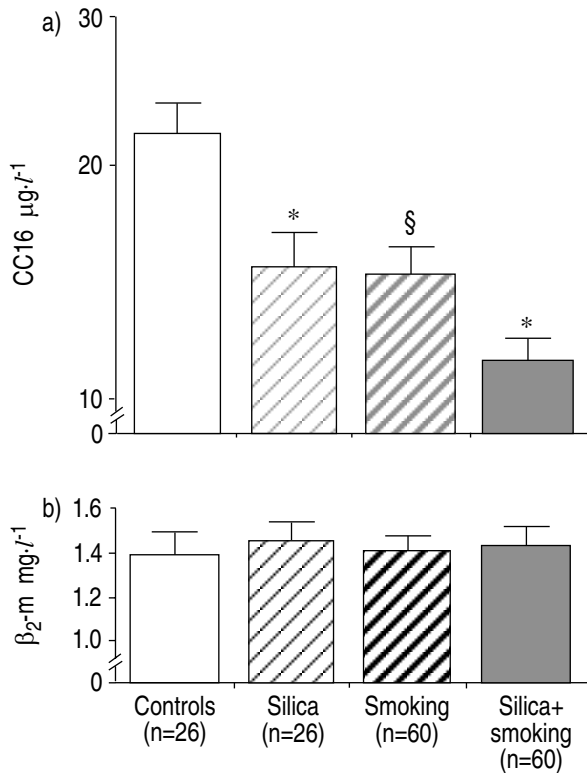
Fig. 1. – Effects of silica exposure and/or smoking on the concentrations of: a) Clara cell protein (CC16); and b) β_2-microglobulin (β_2-m) in serum of quarry workers. *: significantly different from the respective controls (lifelong nonsmokers, p=0.04; and smokers, p=0.012). §: significantly different from lifelong nonsmoker controls (p=0.008). Values are given as the geometric mean±geometric SE.

respectively; p=0.004) and the BMI in exposed workers (partial r^2 and regression coefficient: 0.098 and 0.071, respectively; p=0.003). In silica-exposed workers, no association was found between serum CC16 and the duration of exposure or the urinary excretion of silicon. None of the determinants tested had an influence on CC16 in sputum.

In simple regression analysis, several associations emerged that, although weak, are worth mentioning. In control workers, the serum level of CC16 was positively correlated with the FEV_1 (r=0.29; p=0.008) or FEV_1/VC (r=0.25; p=0.02). Similar correlations were also found with the CC16/β_2-microglobulin concentration ratio in serum. These associations were, however, not found in exposed workers. The concentration of CC16 in sputum of control or silica-exposed workers and that in serum did not correlate, although a tendency was apparent in the total population (r=0.21; p=0.1). Of note also, a weak correlation was found between CC16 in sputum and the serum CC16/β_2-microglobulin concentration ratio in the total population of workers (n=64; r=0.30; p=0.01) as well as in control workers (n=29; r=0.41; p=0.03).

Discussion

Biomarkers of toxicity are increasingly used for screening early toxic effects of occupational or environmental pollutants. However, most biomarkers presently available for the lung require a bronchoalveolar lavage, a procedure which cannot be applied for monitoring populations at risk. A few peripheral markers measurable in serum have been proposed, but they are either not specific to the lung or lack sensitivity [16–19]. The situation by contrast appears more promising with CC16, which presents several features that may be suitable to a noninvasive assessment of lung toxicity: 1) the high concentration of CC16 in the respiratory tract secretions and its small size permit the diffusion into serum
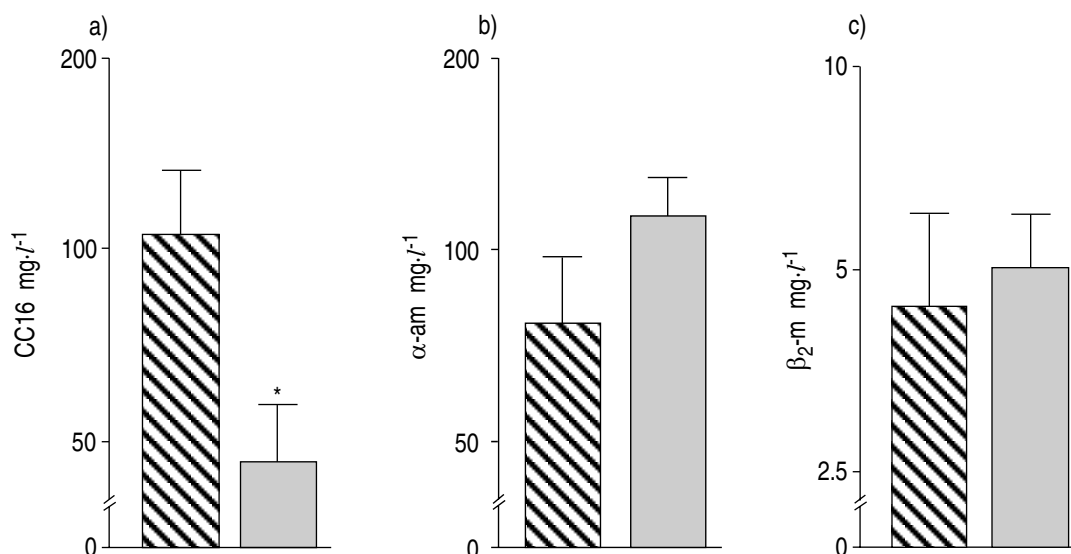
workers. The predictors tested in the model were age, pack-year smoking history and BMI, to which were added in exposed subjects the urinary silicon excretion and the duration of exposure. The only significant determinants of serum CC16 which emerged from this analysis were the pack-year smoking history in controls (partial r^2 and regression coefficient: 0.094 and -0.056,

Fig. 2. – Concentrations of: a) Clara cell protein (CC16); b) alpha-amylase (α-am); and c) β_2-microglobulin (β_2-m) in the sputum of smokers exposed to silica and their smoker controls. Note expanded axis for β_2-m. *: p=0.04, geometric mean±geometric SE. ▧: control smokers (n=24); ▥: silica-exposed smokers (n=27).

of sufficient amounts of protein to be detected by conventional immunoassays [10]; 2) although CC16 messenger ribonucleic acid (mRNA) is also expressed in several nonrespiratory organs (*e.g.* prostate), all data available so far point to the lung as the principal source of CC16 in serum [9]; 3) CC16 is the major secretory product of an epithelial cell type which, owing to its xenobiotic-metabolizing potential, is likely to be the primary target of a number of pulmonary toxicants [20, 21]. This makes CC16 a potentially sensitive marker of lesions involving the respiratory epithelium; and 4) in view of its immunosuppressive properties, CC16 might also signal or predict inflammatory reactions that may be important in the development of lung injury. The present study supports these theories by demonstrating a significant reduction of serum CC16 in workers inhaling silica-rich dust for less than 2 yrs and with no radiographic or functional signs of lung impairment.

A significant effect of tobacco smoking was also found that was additional to that associated with silica exposure. The lack of change in the serum levels of β_2-microglobulin, which has a size close to that of CC16, makes it possible to exclude the possibility of an altered clearance of low molecular weight plasma proteins. That the decreases of serum CC16 are related to changes in the respiratory tract is also corroborated by the weak, but yet statistically significant, correlations with FEV_1 or FEV_1/VC emerging in the group of control subjects. The fact that these correlations are not found in exposed workers suggests that silica exposure decreases serum CC16 before causing significant effects on lung ventilatory parameters.

On the basis of the current understanding of CC16 metabolism and behaviour in man, the only plausible explanation which can be proposed for the decreased levels of serum CC16 in silica-exposed workers is either a reduced release from secreting cells or a decreased transudation from the respiratory tract. The latter explanation seems, however, very unlikely because experimental evidence suggests that if silica can alter the bronchoalveolar/capillary barrier permeability it is more towards an increase rather than a decrease [22]. The only hypothesis which can, thus, be retained at present is a reduced secretion of CC16 into the respiratory tract, which is mirrored by serum levels. In smokers, this hypothesis is supported by the fact that the CC16 concentration also decreases in sputum, presumably as a reflection of changes in serum. It is also consistent with the current views on the mechanisms of the respiratory toxicity of silica. There is indeed ample evidence that the toxic action of silica on the lung structures is closely linked to the ability of silica particles to damage cell membranes.

The exact molecular processes by which silica particles distort or damage cell membranes are not yet fully understood, but they probably occur through electrostatic bonding or *via* free-radical-mediated reactions, or both [23, 24]. The number or integrity of CC16-secreting cells might then be compromised in different ways. Firstly, silica particles might directly damage or impair Clara cells, with an ensuing decreased secretion of CC16. It has been shown, for instance, that the surface structure of rat Clara cells is electively and very rapidly damaged following ozone exposure, indicating that Clara cells are very sensitive to membrane-reactive toxins [25]. Secondly, reduction of CC16-secreting cells might also be consecutive to lesions affecting other cell types of the respiratory epithelium known to be sensitive to lung irritants. Clara cells indeed act as stem cells in the renewal of bronchiolar epithelium [26], which may lead to a transient decline of their number, as has been described in animals exposed to sulphur dioxide [27]. Thirdly, Clara cells may be damaged by cytotoxic mediators released from activated macrophages that have engulfed silica particles. In support of these silica-cell interactions, it should be recalled that drilling, milling and other operations carried out on silica-rich rocks (90% of crystalline silica in the present study) generate fresh silica particles that are highly reactive to lung tissue. Lastly, the hypothesis of a toxicity of silica on epithelial cells of airways is supported by the experimental evidence of small airways lesions induced by silica in rats [28], and consistent with several epidemiological studies in miners showing associations between airways alterations and exposure to silica-rich dust [29–33].

A weakness of the present study is that changes affecting CC16 in serum or sputum could not be related to any indicator of exposure. The range of exposure durations was too narrow for a meaningful assessment of the influence of this parameter. Also, no association was found with the urinary excretion of silicon, but this is probably due to the inadequacy of this exposure index, which reflects the very recent exposure to silica and can be confounded by nonoccupational factors (*e.g.* dietary habits). The interest of this measurement was mainly to confirm objectively a higher absorption of silica, presumably by inhalation, in the exposed workers despite the use of personal protection devices. In the absence of dose-response relations, the association between silica exposure and CC16 changes, thus, relies essentially on comparison with a control group. However, the indisputable acute and chronic pulmonary toxicity of silica-rich dust and the careful matching of exposed and control workers for all other factors likely to affect the lungs leaves little doubt as to the causal nature of this association. Actually, the pack-year smoking history was slightly higher in control than in exposed smokers, which means that the effect of silica has perhaps been underestimated in the latter and the existence of an interaction overlooked.

In conclusion, our data show an early decrease of serum CC16 in asymptomatic silica-exposed workers with no sign of silicosis or lung function impairment. Since crystalline silica represents an important occupational hazard, the finding of a potential peripheral biomarker of early toxicity might improve our capability to detect groups at risk. Further studies are, however, needed to assess the health significance and the validity (specificity and sensitivity) of this new marker. Information on the relationships between the inhalation or pulmonary retention of silica particles and changes of CC16 in serum or sputum would be particularly relevant in that respect.

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