REVIEW

Clara cell protein (CC16): characteristics and potential applications as biomarker of lung toxicity

Cedric Hermans and Alfred Bernard

Most biomarkers of lung toxicity presently available require a bronchoalveolar lavage (BAL). Such a procedure cannot be applied for monitoring populations at risk in the industry or environment nor for a regular follow-up of patients with lung disorders. A lung biomarker, measurable in serum, BAL fluid and sputum has recently been identified. This biomarker is a microprotein initially isolated from urine (Urine Protein 1) and subsequently identified as the major secretory product of lung Clara cells which are non-ciliated cells localized predominantly in terminal bronchioles. This protein called Clara cell protein (CC16) is a homodimer of 15.8 kDA. Several lines of evidence indicate that CC16 is a natural immunoregulator protecting the respiratory tract from unwanted inflammatory reactions. CC16 secreted in the respiratory tract diffuses passively by transudation into plasma from where it is rapidly eliminated by glomerular filtration before being taken up and catabolized in proximal tubule cells. Studies reviewed here suggest that CC16 in BAL fluid or serum is a sensitive indicator of acute or chronic bronchial epithelium injury. A significant reduction of CC16 has been found in serum and BAL fluid of asymptomatic smokers. On average serum CC16 decreases by 15% for each 10 pack-year smoking history. Serum CC16 was also found to be decreased in several occupational groups chronically exposed to different air pollutants (silica, dust, welding fumes). A dose–effect relationship with the intensity of exposure to dust has been found in one study on foundry workers. The concentration of CC16 in serum can also be used to detect an acute or chronic disruption of the bronchoalveolar/blood barrier integrity. While confirming the potential interest of CC16 as a lung biomarker, clinical investigations indicate that CC16 might be an important mediator in the development of lung injury. These findings open new perspectives in the assessment of lung toxicity by suggesting that readily diffusible lung-specific proteins may serve as peripheral markers of pneumotoxicity.

Keywords: *biomarkers, Clara cell protein, Protein 1, low-molecular-weight proteins, lung toxicity.*

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Introduction

Diagnosis of lung toxic injury mainly relies on exposure history, clinical examination, functional tests, imaging techniques and blood gases analysis. Invasive techniques such as BAL and pulmonary biopsy are sometimes required. There is at present no toxicity biomarker to monitor populations exposed to pneumotoxicants at the workplace or in the environment. Recent studies have suggested that low-molecular-weight proteins (LMWP) specific for the lung might serve as peripheral biomarkers of lung toxicity. Owing to their small size (molecular weight between 5 and 40 kDa) LMWP are indeed readily exchangeable and might therefore mirror in serum, toxic events taking place in relatively inaccessible organs such as the lung. Rapidly cleared from the plasma by glomerular filtration, they are eliminated with half-lives of a few hours and therefore might be sensitive to both acute and chronic effects. This new approach for detecting lung toxicity stems from observations on Clara cell protein, a LMWP initially isolated from urine (Urine Protein 1) and subsequently identified as the major protein secreted by the lung Clara cells.

Urine Protein 1 or Clara cell protein: identification

Urine Protein 1 is an alpha-microprotein which was first isolated from urine of patients with renal tubular dysfunction by Kjaervig Broe M. in 1974 (Dakopatts A/S. Product list (1990) p. 62 Glostrup, Denmark). The first immunoassay which allowed study of the occurrence of this protein in biological fluids was developed in 1989 (Bernard *et al.* 1989). Protein 1 was found to be a sex-dependent marker of proximal tubule dysfunction, being excreted in greater amounts by males than females after puberty and by patients with tubular proteinuria (Bernard *et al.* 1989, 1991).

Independently of these investigations, Singh and coworkers described in rodents and later in humans a lung secretory protein produced by Clara cells (Singh and Katyal 1984, Singh *et al.* 1985a, b, 1986, 1988a, Bedetti *et al.* 1987). The protein was referred to as the 10 kDa Clara cell protein (CC10) but this was based upon an underestimation of its size by SDS–polyacrylamide gel electrophoresis. Only minute amounts of CC10 were detected in BAL fluid and it was concluded that CC10 was a minor constituent of BAL fluid accounting for only 0.14% of the total protein content (Singh *et al.* 1988a). In 1992, we reported that Urine Protein 1 and the lung secretory protein CC10 actually correspond to the same protein (Bernard *et al.* 1992a). Evidence for identity between both proteins was based on the amino acid sequence and confirmed by the finding of elevated concentrations of Protein 1 in sputum and BAL fluid. Protein 1 was found to be one of the most abundant lung-specific proteins secreted in BAL fluid (2% of the total protein content) (Bernard *et al.* 1992b).

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CC16: biochemical aspects and localization

CC16 is a homodimer consisting of 70 aminoacid subunits, in antiparallel orientation and connected by two disulphide bonds (Singh *et al.* 1988b). The molecular weight by SDS–polyacrylamide gel electrophoresis is about 10 kDa which explains why CC16 has long been referred to as CC10. The exact molecular mass of the protein determined by electrospray ionization/mass spectrometry is 15 840 (Bernard *et al.* 1993a). CC16 is an anionic microprotein with a pI value of 4.7 (Bernard *et al.* 1992a). The entire human CC16 gene has recently been isolated, sequenced and localized to chromosome 11 (Hay *et al.* 1995). CC16 has been shown to be the human counterpart of uteroglobulin, a secretory protein of the lung and the endometrium in rabbits (Miele *et al.* 1987, Jackson *et al.* 1988, Mantile *et al.* 1993). The two proteins indeed show a 61% sequence homology and also similarities in their tertiary structures (Singh *et al.* 1990). Interestingly, uteroglobulin is a globular protein with a central hydrophobic pocket which, as discussed later on, is probably involved in important interactions with endogenous or exogenous lipophilic compounds (Nordlund-Möller *et al.* 1990, Andersson *et al.* 1991).

CC16, however, is not an entirely specific and exclusive product of lung Clara cells. Molecular biology techniques have shown that CC16 is also expressed by non-ciliated cells all along the tracheobronchial epithelium (Broers *et al.* 1992). Although all evidence available so far points to the respiratory epithelium as the major site of CC16 secretion, the pattern of distribution is more complex and rather similar to that of its animal counterpart, uteroglobin (Peri *et al.* 1993). CC16 is also secreted by the male urogenital tract, presumably the prostate, which explains its sex-dependent urinary excretion as well as its presence in semen and prostatic secretions (Bernard *et al.* 1991, 1992a).

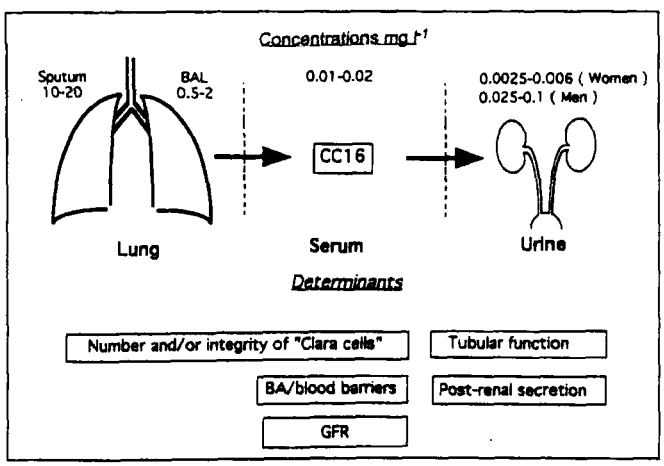

Figure 1. Concentrations and determinants of CC16 in the respiratory tract secretions (BAL and sputum), serum and urine. The values correspond to 'never-smokers' since the concentrations of CC16 both in serum and respiratory tract secretions are reduced by tobacco smoking. BAL, bronchoalveolar lavage; BA, bronchoalveolar; GFR, glomerular filtration.

CC16 metabolism

As depicted in Figure 1, CC16 is secreted in large amounts in airways from where it passively diffuses into serum across the broncho- and alveolo-capillary membranes. The hypothesis of a diffusional equilibrium between respiratory tract and blood is supported by the existence in healthy subjects of a correlation between CC16 in serum and in BAL fluid (Bernard *et al.* 1992b). The huge difference between the concentrations of CC16 in serum and at the surface of respiratory epithelium (see below) probably provides the driving force to this diffusion process. The integrity of the bronchoalveolar/capillary membranes is an important determinant of this diffusional equilibrium as indicated by situations in which this barrier is compromised (e.g. sarcoidosis). Like other LMWP, plasma CC16 is rapidly eliminated by glomerular filtration and reabsorbed by renal tubules. As a corollary, serum CC16 rises as the GFR declines (Bernard *et al.* 1993b) and therefore the status of renal function must be taken into account when interpreting variations of serum CC16. The renal disposal of CC16 takes place mainly in the proximal tubule cells and a defect in the tubule reabsorptive capacity results in a greatly increased excretion of the protein (Bernard *et al.* 1993b). The half-life of CC16 in plasma has not yet been determined but one may logically assume that it is of the same order of magnitude as other plasma LMWP (i.e. a few hours).

CC16 functions

The exact functions of CC16 are still unknown but several observations support the hypothesis that CC16 plays a role in lung inflammatory processes. It has been suggested that like uteroglobin, CC16 is a natural immunosuppressor and anti-inflammatory agent (Levin *et al.* 1986, Miele *et al.* 1987, Singh *et al.* 1990, Mantile *et al.* 1993). CC16 has been shown to inhibit the activity of cytosolic phospholipase A2, a key enzyme in inflammatory phenomena (Mantile *et al.* 1993). Phospholipase A2 is the rate-limiting enzyme in the production of arachidonic acid, the substrate for the synthesis of prostaglandin and leukotriene mediators of inflammation. Interestingly, the glucocorticoid inducible lipocortin 1 is also a potent inhibitor of cytosolic phospholipase A2 and synthetic peptides corresponding to the regions of highest similarity between human lipocortin 1 and uteroglobin, the animal counterpart of human CC16, and referred to as antiflammins, have been demonstrated to inhibit phospholipase A2 (Miele *et al.* 1988, Facchiano *et al.* 1991). Glucocorticoids have also been shown to induce the synthesis of uteroglobulin in rabbit foetal lung (Lopez de Haro and Nieto 1985). CC16 is able to inhibit interferon-gamma production by peripheral blood mononuclear cells (Dierynck *et al.* 1995). The biological action of interferon-gamma, namely its antiviral activity as well as its phagocytosis-stimulating activity are also diminished by the addition of CC16 (Dierynck *et al.* 1995). CC16 produces a dose-dependent inhibition of PDGF-induced chemotaxis of foetal lung fibroblasts (Lesur *et al.* 1995a) and it has been speculated that the decreased availability of CC16, as observed in fibrosing lung disorders, might facilitate the recruitment of fibroblasts in such diseases. By inhibiting phospholipase A2 CC16 could also

prevent the degradation of lung surfactant phospholipids (Guy *et al.* 1992). Lastly, CC16 contains a central hydrophobic cavity which has been shown to bind xenobiotics including the widespread pollutants polychlorinated biphenyls (Nordlund-Möller *et al.* 1990). CC16 could thus also play an important role in the sequestration or clearance of some harmful substances deposited in the respiratory tract.

Normal values of CC16 concentrations in biological fluids

The ranges of mean values of CC16 in biological fluids reported so far are given in Figure 1. The highest concentrations have been observed in sputum with average values between 10 and 20 mg l^{-1}. CC16 concentrations in sputum may greatly vary depending on the degree of contamination by saliva which contains no CC16. To identify excessively diluted samples, we use a criterion based on the CC16/alpha-amylase concentration ratio (Bernard *et al.* 1994a). In BAL fluid mean CC16 concentrations are usually between 1 and 2 mg l^{-1} depending on the technique of lung lavage. In healthy subjects, 60–80% of proteins present in BAL derive from serum by passive transudation (Reynolds and Newball 1974), the remainder comprising locally secreted proteins such as CC16. CC16 concentrations in BAL fluids of healthy subjects represent on average 6.3% of that of albumin and 2.3% of that of total protein (Bernard *et al.* 1992b).

Since the BAL techniques result in approximately a 100-fold dilution, CC16 concentrations in the lining fluid at the surface of airways can be estimated at about 100 mg l^{-1}. It is noteworthy that CC16 is slightly less concentrated in alveolar than in bronchial lavage fluid, which agrees with the predominant localization of Clara cells in terminal bronchioles. CC16 concentrations in serum are about 50-times lower than in BAL fluid. The main determinants of serum CC16 identified so far are: the glomerular filtration rate (dependent on the age), the secretion and/or production by Clara cells and the integrity of the bronchoalveolar capillary barrier. It should be noted that in contrast to major plasma LMWP (e.g. β_2-microglobulin, cystatin C and retinol-binding protein) which fluctuate within narrow ranges, CC16 shows considerable variations in serum of healthy subjects with values differing by a factor of 10 or more. We have at present no explanation for this great variability. Perhaps, this could represent inter-individual differences in the transudation of CC16 in plasma or in the synthesis/secretion of CC16 in the respiratory tract. The latter hypothesis is supported by the fact that the concentration of CC16 in BAL of healthy subjects also shows a great dispersion which cannot be explained solely by the variable dilution of BAL sample (Bernard *et al.* 1992b).

The concentrations of CC16 in urine of women are normally very low, around 5 μg l^{-1}. The post-renal secretion of CC16 in the male urogenital tract results in concentrations that can be 10- to 100-times higher than in females according to the age and the mode of urine collection (Bernard *et al.* 1991). CC16 also occurs in amniotic fluid where it is detected from the 15th week of pregnancy and steadily increases until delivery to a mean value of about 1 mg l^{-1}. (Bernard *et al.* 1994b).

Applications of CC16 as a marker of lung injury

General aspects

At present, the toxicological interest of CC16 mainly stems from the fact that it is the major secretory product of the Clara cells which appear to play an important role in the development of lung injury. First, following bronchiolar epithelium damage, Clara cells can act as progenitor cells for themselves as well as for ciliated cells (Evans *et al.* 1978, Plopper *et al.* 1992). A depletion of Clara cells is thus likely to compromise the bronchial epithelium repair mechanisms. Second, Clara cells are particularly sensitive to toxic lung injury (Richards *et al.* 1990). They contain indeed most of the lung cytochrome P-450 activity which confers them a high xenobiotic metabolizing capacity. The cytochrome P-450 system plays a major role in the activation of numerous cytotoxic and carcinogenic chemicals (Boyd 1977, 1984). This sensitivity of Clara cells has been illustrated by the many pulmonary toxicants that have been shown to damage electively Clara cells in animals exposed by inhalation or systemic routes (e.g. 4-ipomeanol, 3-methylfuran, naphthalene, trichloroethylene, 1,2-dichloroethylene). Acute exposure to these toxicants causes a rapid destruction of Clara cells which in turn as suggested by our studies leads to a decreased production of CC16 (unpublished data). Third, if CC16 does play an important protective role in the lung as increasingly emerges, it is logical to assume that a decrease of its production may contribute to the further development of lung injury.

CC16 in smokers

In a study involving 134 current smokers (65 female and 69 male) and a sex- and age-matched control group of 135 'never-smokers', we observed a reduction of CC16 in the serum of smokers of both sexes which negatively correlated both with the current and life-time cigarette consumption and with 24-h urinary excretion of thiocyanate (Bernard *et al.* 1992c, 1994c). After adjustment for age, a linear dose–response relationship was apparent between smoking history and serum CC16, the latter decreasing on average by about 15% for each 10 pack-year smoking history. All these observations suggest that CC16 may serve as a peripheral biomarker of bronchial epithelium alterations caused by tobacco smoke. The smoking-induced decrease of CC16 is probably a reflection of the reduction in Clara cell number in smokers. Histologically, indeed, a significant decrease in the Clara cell population in distal airways has been described among smokers (Lumsden *et al.* 1984). Clara cells might be progressively destroyed either by toxic metabolites of tobacco smoke generated via the cytochrome P450 system or by irritating substances present in smoke. Whatever the underlying mechanism, the alterations of Clara cells caused by tobacco smoke with an ensuing decrease of the production of CC16 might be involved in the progressive destruction of lung parenchyma in smokers. Interestingly, an increased dose-dependent synthesis of TxB2 and PGF2 alpha has been reported in BAL fluid of healthy smokers (Zijlstra *et al.* 1992). Reduction of CC16 observed in

smokers could account for the mechanism by which tobacco could stimulate the recruitment and/or the production of mediators of inflammation in the respiratory tract of smokers.

CC16 in occupationally exposed populations

Several cross-sectional studies have been carried out on workers exposed to various lung toxicants to determine whether serum CC16 might be of value to detect pulmonary effects of air pollutants. The first study involved a group of 86 miners exposed to high concentrations of silica-rich particles for 15.2 months on average (Bernard *et al.* 1994a). These workers were compared with a group of 86 control subjects matched for age, smoking status and body mass index. No difference between exposed and control workers was apparent in respiratory symptoms, radiography and lung function tests. By contrast, mean serum concentrations of CC16 were significantly reduced in workers exposed to silica in both smokers and 'never-smokers'. A significant effect of tobacco smoking was also found that was additive to that caused by silica. It is noteworthy that serum concentrations of β_2-microglobulin, a LMWP similar in size to CC16, were unaffected either by silica exposure or by tobacco smoking which testifies to the specificity of changes affecting serum CC16. Since workers were exposed for less than 2 years and had no sign of silica-induced lung impairment, these data demonstrate that the assay of CC16 can detect very early toxic effects on the airways of asymptomatic subjects with normal radiographic and spirometric tests. In the absence of information on CC16 concentrations in BAL fluid, it is difficult to speculate on the mechanism by which silica could reduce the concentration of CC16 in the serum of these miners. One might formulate the hypothesis that, as for tobacco smoking, this decrease reflects a reduced secretion/production of the protein by damaged or altered Clara cells. This explanation is supported by the significant reduction of CC16 concentration which was found in the sputum of smokers exposed to silica compared with that of non-exposed smokers (the 'never-smokers' provided very few reliable sputum samples in that study). The alternative explanation of a reduced diffusion of CC16 through the bronchoalveolar/capillary barrier is not supported by experimental data (Merchant *et al.* 1990).

We have also examined a group of 52 welders and 47 controls matched for age and smoking habits (Bernard and Lauwerys 1995). No change in lung function attributable to exposure to welding fumes could be detected among these workers. Nor was any difference found between the serum levels of CC16 of smoking welders and their controls. In 'never-smokers', by contrast, the concentration of CC16 in serum of welders was on average half that of controls. This difference which was not statistically significant because of the very low numbers of subjects (11 and 12 respectively) is, however, in the line of our previous observations.

More recently we determined CC16 concentrations in the serum of 47 foundry workers and 41 control workers matched for smoking habits and body mass index. Foundry workers were mainly exposed to mineral and metallic dusts for 15.2 years on average. No difference in respiratory symptoms, radiological findings and lung function tests was apparent between exposed and control workers. Serum concentration of CC16 by contrast, was on average significantly decreased among exposed-workers compared with control subjects. Interestingly, as illustrated in Figure 2, the decrease of serum CC16 was significantly correlated with the level of dust exposure.

CC16 in lung diseases

Asbestosis is associated with an increase of mean CC16 concentration in BAL fluid. Of note is that this increase was less important among asbestosis patients who were smokers compared with non-smokers (Lesur *et al.* 1995b). The mechanism of this increase of BAL fluid CC16 in asbestosis, which did not correlate with the stage of the disease, remains to be determined. In the BAL fluid of asthmatic patients, by contrast, CC16 has been found to be decreased (Van Vyve *et al.* 1995). Interestingly, the CC16 gene has been located to a 36 centimorgan region of chromosome 11 (Hay *et al.* 1995). This region has been linked to atopy (Cookson *et al.* 1989) and to the high-affinity immunoglobulin E (IgE) receptor (Sandford *et al.* 1993), which are both suspected to be associated with asthma. Lung cancer (Bernard *et al.* 1992b) and pulmonary fibrosis (Lesur *et al.* 1995a) were found to be associated with decreased BAL levels of CC16. Acute respiratory distress syndrome, however, was associated with an increased concentration of CC16 in BAL from patients who survived compared with those who died (Jorens *et al.* 1995). This observation reinforces the hypothesis of a protective role of CC16 in lung injury. Increased serum levels of CC16 have been observed in sarcoidosis (Bernard *et al.* 1992b) and pulmonary

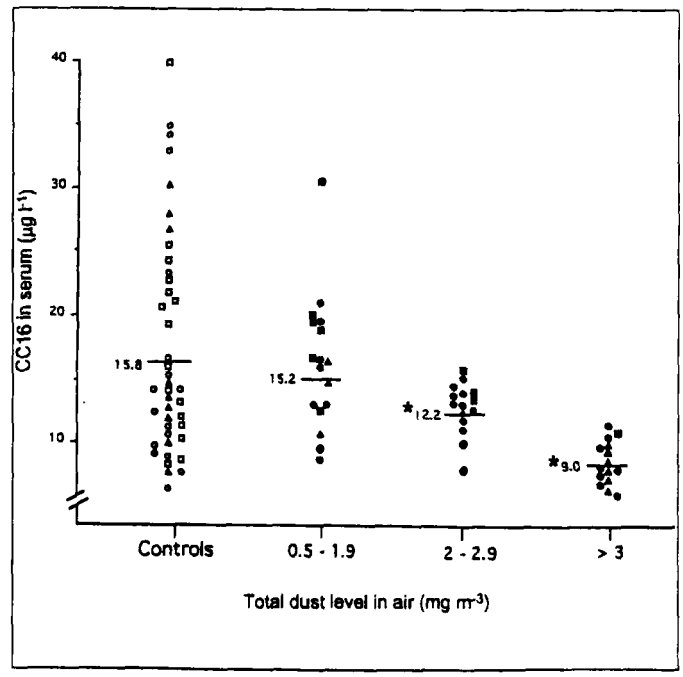

Figure 2. Correlation between serum CC16 and the total dust concentration in air in a group of foundry workers. Bars represent the geometric means. *: Significantly different from controls ($p < 0.05$), using one-way ANOVA followed by Dunnetts multiple comparison test. □, ■, 'Never-smokers'; △, ▲, ex-smokers; ○, ● smokers.

	Serum		BAL fluid	
	$\mu g\ l^{-1}$	% of controls	$mg\ l^{-1}$	% of controls
Smoking (Bernard *et al.* 1992b)	21.2	77.6	0.56	46
COPD (Bernard *et al.* 1992b)	9.6	25.1	0.33	27.5
Silica exposure (Bernard *et al.* 1994a)	12.3	75.4	–	–
Asbestosis (Lesur *et al.* 1995b)	13.6	125	3.1	163.1
Sarcoidosis (Bernard *et al.* 1992b)	44.6	163	1.16	96
Smoke exposure (unpublished data)	51.6	348	–	–
Idiopathic pulmonary fibrosis (Lesur *et al.* 1995a)	34	188	1.3	61.9
Bleomycin lung injury (Lesur *et al.* 1995a)	19	105	1.15	54.7
Asthma (Van Vyve *et al.* 1995)	–	–	2.13	66.9

Table 1. Concentrations of CC16 in serum or BAL fluid of patients with lung diseases or of subjects exposed to various pneumotoxicants. The results are given as the geometric mean and as a percentage of the geometric mean in the respective control group.

Key: COPD, chronic obstructive pulmonary airway disease.

fibrosis (Lesur *et al.* 1995a). The changes in serum and BAL fluid concentrations of CC16 in different lung disorders are summarized in Table 1. While confirming the potential interest of CC16 as lung biomarker, clinical investigations indicate that CC16 might be an important mediator in the development of lung injury.

Other applications

In amniotic fluid, CC16 has been shown to be a potential marker of foetal lung growth (Bernard *et al.* 1994b). The potential usefulness of CC16 to detect transplacental lung toxicity is under investigation in a group of smoking pregnant women. As CC16 is handled by the kidneys in the same way as other LMWP, it can also be used to detect proximal tubular dysfunction (Bernard *et al.* 1989, 1990, 1991). In that respect, urinary CC16 presents a unique sensitivity allowing detection, especially in women, of very subtle defects of proximal tubular dysfunction that pass unseen when screening is based on classical urinary microproteins such as β_2-microglobluin and retinol-binding protein (Bernard *et al.* 1994d). The low affinity of CC16 for tubular binding sites combined with its very low concentration in the tubular fluid might account for its exceptional sensitivity to proximal tubular dysfunction. CC16 is also exceptionally stable in acid urine, with a resistance to proteolysis by acid proteases even superior to that of retinol-binding protein (Bernard *et al.* 1990).

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