

REVIEW

Clara cell secretory protein (CC16): characteristics and perspectives as lung peripheral biomarker

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Summary

Clara cell protein (CC16) is a 15.8-kDa homodimeric protein secreted in large amounts in airways by the non-ciliated bronchiolar Clara cells. This protein increasingly appears to protect the respiratory tract against oxidative stress and inflammation. *In vitro*, CC16 has been shown to modulate the production and/or the activity of various mediators of the inflammatory response including PLA₂, interferon- γ and tumour necrosis factor- α . CC16 has also been found to inhibit fibroblast migration or to bind various endogenous or exogenous substances such as polychlorobiphenyls (PCBs). This protective role is confirmed by studies on transgenic mice, showing that CC16 deficiency is associated with an increased susceptibility of the lung to viral infections and oxidative stress. In humans, a polymorphism of the CC16 gene, localized to a region linked to airway diseases, has recently been discovered in association with an increased risk of developing childhood asthma. Finally, CC16 also presents a major interest as a peripheral marker for assessing the integrity of the lung epithelium. The determination of CC16 in serum is a new non-invasive test to detect Clara cell damage or an increased epithelial permeability in various acute and chronic lung disorders.

Keywords: asthma, biomarker, Clara cell protein, CC10, CC16, CCSP, cytokine, ozone, lung epithelium, lung injury, lung-specific protein, oxidative stress, PCB-binding protein, pneumoproteinaemia, uteroglobin

Introduction

The Clara cell is one of the most heterogeneous and multi-functional cell types of the mammalian lung, showing a great interspecies variability in abundance and spatial distribution. Clara cells are localized mainly in the proximal, or central, portion of the pulmonary acinus, at the junction between the conducting airways and the gas-exchange area. The functions of Clara cells are mainly orientated to the protection of the respiratory tract. Clara cells act as stem cells in the repair of the bronchial epithelium, have a high xenobiotic biotransformation capacity and secrete several substances with important biological activities [1].

One of the major proteins secreted by Clara cells is the 16 kDa Clara cell protein (CC16) [2]. Even though the exact *in vivo* function of the CC16 remains to be clarified, there is growing evidence that this protein plays a protective role against pulmonary inflammatory response. From a diagnostic perspective, CC16 is also at the origin of a novel approach for assessing the integrity of the respiratory epithelium, based on the assay in serum of lung-specific proteins [3–5]. As shown by various clinical and experimental studies, the determination of CC16 in serum is a new sensitive marker to detect early permeability changes of the lung epithelial barrier and/or to evaluate the integrity of Clara cells, a privileged target of many pneumotoxins.

Characteristics of CC16

The Clara protein family encodes a fascinating group of proteins including rodent, monkey and human CC16, human

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mammaglobin and rabbit uteroglobin. Interspecies homologues of the CC16 are referred in the literature by different names which allude to their origin [human protein-1, urine protein-1, uteroglobin (UG), Clara cell secretory protein (CCSP)], their molecular weight [CC16, Clara cell 17 kDa protein, Clara cell 10 kDa protein (CC10)] or their biological property (Blastokinin, Clara cell phospholipid-binding protein, PCB-binding protein). The human CC16, first identified in urine of patients with renal failure [6] and purified later from lung lavage [7], is the counterpart of rabbit UG, a secretory protein of the lung (both sexes) and the uterus (during pregnancy) of lagomorphs (rabbits, hares) [8]. The amino acid sequence of CC16 presents a high similarity with CC16 from other mammalian species (monkey, rodents), indicating a high level of phylogenetic conservation and suggesting a physiological importance [9]. Although unreduced CC16 shows an apparent molecular weight of about 10 kDa on gel electrophoresis its exact molecular mass, determined by electrospray ionization/mass spectrometry, is 15 840 Da [2].

The entire human CC16 gene has been sequenced and localized to chromosome 11, p12-q13, a region occupied by several genes involved in the regulation of allergy and inflammation. The CC16 gene is comprised of three short exons separated by a long first and short second intron and is controlled by the combinatorial action of the cell-specific factor HNF3 α and the ubiquitous factor Sp1 [10]. In both humans and rodents, CC16 mRNAs are found in the respiratory airways (tracheal, bronchial and bronchiolar regions) but not in the alveolar epithelium [11]. We showed by Masterblot mRNA analysis that CC16 is also expressed in the prostate, the endometrium, the fetal lung and the kidney [5]. In the rabbit uterus, UG synthesis is regulated by progesterone whereas in the lung, UG production is completely independent of such compounds. UG gene expression is positively controlled by glucocorticoids in the lung, due to the presence of a glucocorticoid receptor binding site in the DNA region upstream from the 5'-end of UG gene [12].

CC16 is a homodimer formed by two identical antiparallel chains of 70 amino acid residues, each joined by two disulphide bonds. The appearance of the homodimer is globular, with a large hydrophobic pocket in the central position between the two chains [13]. CC16 is one of the major proteins secreted by the respiratory epithelium. In human bronchoalveolar lavage fluid (BALF) the concentration of CC16 is, on average, between 1 and 2 mg/l, which represents 2–3% of the total soluble protein content of BALF [2]. Since the lavage technique results in an approximately 100-fold dilution, CC16 concentrations in the epithelium lining fluid (ELF) can be estimated at about 100 mg/L. In serum from healthy non-smokers, CC16 concentrations are, on average, between 10 and 15 μ g/l, i.e. a value

approximately 10 000 times lower than in ELF. This huge concentration gradient probably provides the driving force for the movement of CC16 from the lung into the blood [5]. In rodents, CC16 follows the same metabolic pathway as in humans, also leaking from the respiratory tract into serum from where it is rapidly cleared by the kidneys [14].

Biological properties of CC16

In vitro studies

One of the first studied properties of CC16 (UG) is its ability to carry progesterone, allowing the transfer of this molecule between the uterus and the blastocysts [13]. The affinity of CC16 for progesterone shows a great interspecies variation which seems to be linked to differences in the amino-acid sequences. Human CC16 can bind progesterone whereas for the monkey CC16 the substitution of two polar residues abolishes the affinity of the protein for progesterone [9]. Although human CC16 does not bind steroids (or corticosteroids), the hydrophobic pocket may be occupied by lipids (phosphatidylcholine and phosphatidylinositol) or lypophilic xenobiotics, among which are the widespread pollutants polychlorinated biphenyls (PCBs) [15]. This led to the hypothesis that CC16 could facilitate in the accumulation of PCBs in the respiratory tract, explaining chronic pulmonary disease observed in PCBs-exposed workers [16].

CC16 is a potent natural immunosuppressor and anti-inflammatory agent. *In vitro*, CC16 inhibits both monocyte and polymorphonuclear neutrophils chemotaxis and phagocytosis [13]. CC16 also inhibits fibroblast chemotaxis in a dose-dependent manner, an effect which is related to a blockage of cytosolic PLA₂ activity [17]. Human and rat CC16 as well as rabbit UG have been shown to inhibit the activity of secretory and intracellular PLA₂ [18]. Interestingly, PLA₂ enzymes are a family of esterases which are involved in various physiological and pathological processes, controlling the production of arachidonic acid and the availability of prostaglandins and leukotrienes [19]. In this way, PLA₂ inhibition could attenuate the progression of the inflammation and also reduce the degradation of lung surfactant phospholipids, thereby preventing the development of chronic lung disease [20]. In addition, by inhibiting secretory PLA₂ activity and decreasing the level of lysophosphatidic acid in the alveolar spaces, CC16 may indirectly reduce the activation of integrins (e.g. $\alpha 5 \beta 1$) that enhance abnormal tissue deposition of fibronectin (Fn) and fibroblast migration. CC16 presents a natural affinity for fibronectin but the physiopathological significance of this property is controversial at present [21,22]. A recent study has also raised the possibility that CC16, through its binding properties, plays critical roles in the regulation of cellular motility and extracellular matrix invasion by cancer cells [23].

Several studies show a link between CC16 and the cytokine network. CC16 inhibits *in vitro* both the production and the biological activity of interferon-gamma (IFN- γ), a potent multi-functional cytokine produced in the course of viral infections and inflammation [24]. Conversely, IFN- γ has been found to increase the expression of CC16 mRNA in the mouse lung and the CC16 protein levels in cultured human airway epithelial cells [25,26]. Another cytokine, tumour necrosis factor- α (TNF- α), stimulates CC16 production by bronchial epithelial cells *in vitro* [27]. Interestingly, IFN- γ has been shown to induce the activation of cytosolic PLA₂ in a human bronchial epithelial cell line [28] whereas TNF- α priming causes the secretion of PLA₂ by inflammatory cells [29].

In vivo studies

Recently, it has been proposed that tolerance to long-term ozone (O₃) exposure might be due to a site-specific elevation of CC16 in the Clara cells, as observed in rats exposed for 90 days to 0.12 p.p.m. O₃ [30]. Studies using CC16-deficient mice reinforce the hypothesis that CC16 is involved in the protection against oxidative stress, and also in the modulation of lung inflammation during viral infection. In mice, CC16 deficiency is associated with increased susceptibility to lung epithelial damage induced by O₃ [31] and hyperoxia [32], as evidenced by a higher incidence of mortality, early onset of lung oedema and induction of pro-inflammatory cytokine mRNAs. Mice deficient for CC16 develop also a much more severe lung inflammation following acute viral infection. Interestingly, histological evidence of pulmonary inflammation in deficient mice was associated with increased production of interleukin-1 β and -6 and TNF- α , as well as chemokine (macrophage inflammatory proteins and monocyte chemo-attractant protein-1) mRNAs expression within the lung [33].

CC16 in asthma and other lung disorders

The CC16 gene is localized to a region of the chromosome 11 known to be occupied by the β -subunit of the high-affinity immunoglobulin E receptor [34] and various genes involved in the regulation of inflammation [11]. A polymorphism in the 5'-untranslated region of exon 1 of the CC16 gene has recently been associated with an increased risk of asthma in childhood, homozygous and heterozygous subjects demonstrating a 6.9-fold and 4.2-fold increased risk of developing asthma, respectively [35]. Of interest, the mutation is associated with a significant decrease of the serum concentration of CC16 [36]. This observation is in agreement with a previous work, showing that the mean

CC16 concentration in BALF of asthmatics was significantly decreased compared with control healthy subjects [37]. In serum, asthmatic patients with a long duration of the disease (≥ 10 years) had significantly lower CC16 levels than those with a short duration of the disease (< 10 years) [38].

Changes of CC16 concentrations in BALF and/or serum have also been studied in other human lung diseases (for a review, see [5]). Pulmonary fibrosis has been associated with decreased BALF CC16 levels [39]. In the acute respiratory distress syndrome, CC16 was found in higher concentrations in BALF from patients who survived compared to those who did not [40].

CC16 as lung peripheral biomarker

Most biomarkers of lung inflammation or injury described so far are constituents of the epithelial lining fluid sampled by bronchoalveolar lavage techniques. These biomarkers consist of plasma-derived proteins or molecules produced locally by the epithelial and inflammatory cells such as cytokines, enzymes or growth factors. Although of great potential for evaluating the extent of inflammation or tissue damage, markers in BAL are not applicable for monitoring populations exposed to ambient air pollutants in the environment or in the workplace. Also, they cannot be used for assessing health risks in children and patients with compromised cardiopulmonary function, who are the most vulnerable to air pollution.

Studies carried out with CC16 have led to the development of a new non-invasive approach for detecting acute or chronic effects on the respiratory epithelium. This approach is based on the concept that lung secretory proteins move passively across the epithelial barrier into serum where they may serve as peripheral indicators of epithelium damage [3–5,40]. As reviewed hereafter, the concentration of CC16 in serum is a sensitive marker of increased permeability of the lung epithelial barrier or, when the latter is intact, of a loss of integrity or a reduction of Clara cells.

Acute lung injury

Acute damage to Clara cells can be produced experimentally by various pneumotoxicants such as MMT or 4-ipomeanol [41]. These chemicals induce a decrease of Clara cell numbers with an ensuing reduction of both CC16 mRNA and CC16 protein levels in the lung. In contrast, these effects are associated with an increase of serum concentration of CC16, which correlates with the concentration of albumin in BALF. Similar observations have been made with O₃. Acute exposure of mice and rats to O₃ produces a transient dose-dependent elevation of CC16

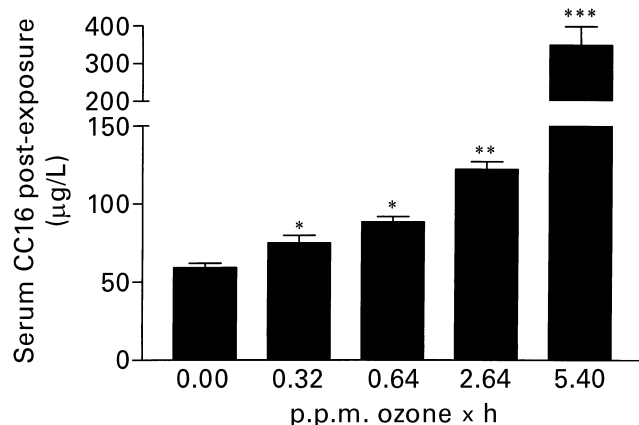


Fig. 1. Concentration of Clara cell protein (CC16, µg/L) in serum of C₅₇Bl/6 mice (Iffa Credo, L'Asbresle, France) exposed to different ozone concentration–time values (0.08 p.p.m. O₃ during 4 or 8 h, 0.11 p.p.m. O₃ during 24 h and 1.8 p.p.m. O₃ during 3 h). O₃ was produced from dried and filtered air by a high voltage generator (Anseros Ozomat, Tübingen, Germany) and monitored every minute by a ultraviolet photometric analyser (Signal Instrument Company, Faringdon, Oxon, UK). Bars represent mean ± SE of six to seven animals compared statistically by one-way ANOVA followed by the Dunnett's *post-hoc* test (**P* < 0.05).

in serum (Fig. 1), which also parallels the elevation of albumin in BAL. At high exposure levels, this effect is found despite a decreased production of CC16 from damaged Clara cells. The O₃-induced intravascular leakage of CC16 correlates with mouse susceptibility to O₃. Still more interesting, in the most sensitive strain, this effect was detectable before the elevation of albumin in BAL from exposure levels to O₃ as low as 0.08 p.p.m. during 2 h. These observations indicate that the assay of serum CC16 allows the detection of very subtle defects in the epithelial barrier permeability which are not reflected accurately by the levels of albumin in BALF. The explanation for the greater sensitivity of serum CC16 to increased permeability of the epithelial barrier most probably lies in the small size and the huge transepithelial concentration gradient of CC16 compared to that of albumin.

In humans, acute exposure to lung irritants also produces an increase of CC16 in serum. A transient elevation of serum CC16 has been found in firefighters after 20 min of smoke inhalation [42]. Of interest, this increase was found in the absence of any functional signs of lung impairment. The high sensitivity of serum CC16 to lung epithelium injury was confirmed in a recent study on cyclists exposed to ambient O₃ (Fig. 2; [43]). The determination of CC16 before and after a 2-h ride during episodes of photochemical smog (O₃ levels between 0.033 and 0.103 p.p.m.) revealed that ambient O₃ exposure produces a dose-dependent increase of airway permeability at exposure levels below

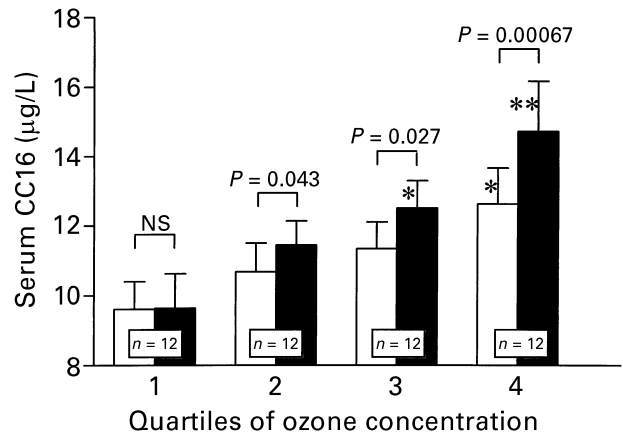


Fig. 2. Serum Clara cell protein (CC16, µg/L) in cyclists before (pre-run) and after (post-run) a 2-h run in Parma (Italy). Values of CC16 are given as mean ± SE. Quartiles correspond to the following ranges of O₃ concentrations: 0.0325–0.0595, 0.0605–0.084, 0.084–0.0925, 0.0925–0.103 p.p.m. Mean O₃ concentrations of the quartiles were 0.048, 0.072, 0.089 and 0.096 p.p.m., respectively. The *P*-values refer to the comparison of pre- and post-run concentrations by the paired-samples Student's *t*-test whereas asterisks indicate means which are significantly different from the first quartile (NS, not significant; **P* < 0.05; ***P* < 0.01; one-way ANOVA followed by Dunnett's *post-hoc* test). □, Pre-run; ■, post-run.

air quality guidelines. However, the most remarkable finding was that in humans serum CC16 shows a sensitivity to O₃ almost identical to that observed in mice. Indeed, in both species, the protein rose in serum after only a few hours of exposure to average O₃ levels around 0.08 p.p.m.

Chronic lung injury and occupational disorders

The determination of serum CC16 can also be used to evaluate the chronic effects of air pollutants on the respiratory epithelium. The concentration of CC16 in serum is decreased in a dose-dependent manner by tobacco smoking, the concentration of the protein decreasing on average by 15% for each 10 pack-years [44]. This effect which has been found consistently in several studies is the reflection of a concomitant reduction of CC16 in lung lavage due to the progressive decline of Clara cell numbers (Fig. 3 [45]). A significant reduction of serum CC16 has also been found in workers exposed to crystalline silica [46] and foundry dust (to be published). In most studies these changes were found in asymptomatic subjects with a normal or only a slightly impaired spirometric tests.

Conclusions

The studies on CC16 open exciting perspectives both as a mediator of inflammation and as a new peripheral lung

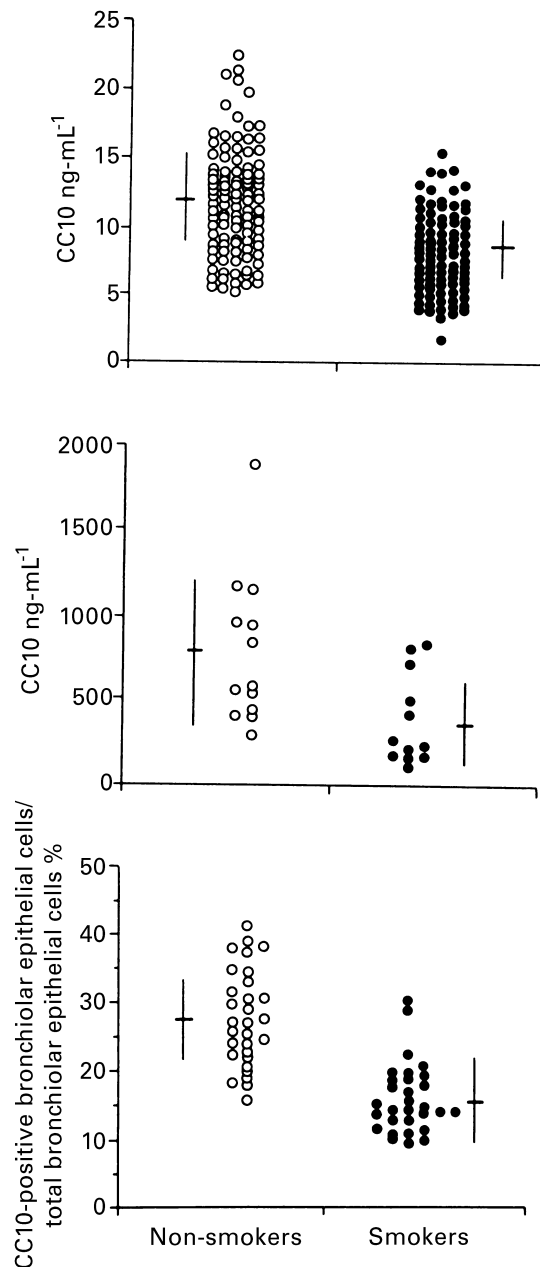


Fig. 3. Concentrations of Clara cell protein (CC10, CC16) in serum (upper graph) and BALF (middle graph) and Clara cell density in bronchiolar epithelium (lower graph) from healthy non-smokers and smokers. Horizontal and vertical bars show mean and SD, respectively. Significant differences were found between non-smokers and smokers in serum CC16 ($P < 0.0001$), BALF CC16 ($P < 0.01$) and CC16-positive cells ($P < 0.001$). Reproduced from Shijubo *et al.* with permission [45].

marker. *In vitro* and *in vivo* experimental studies show that it presents interesting biological properties, suggesting an important protective role of this protein in inflammation and oxidative stress. Studies in humans and animals

also indicate that the assay of CC16 is a sensitive non-invasive test allowing the detection of early damage to the respiratory epithelium caused by acute or chronic exposure. Advantages of this new test include sensitivity, specificity, repeatability and applicability to the whole population in, respectively, their age, condition of exposure and type and severity of lung disorders.

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