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Research Article

Decreased pulmonary levels of the anti-inflammatory Clara cell 16 kDa protein after induction of airway inflammation in asthmatics

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Abstract. The Clara cell 16 kDa protein (CC16) maps to an atopy-associated region of chromosome 11 and has been ascribed an anti-inflammatory function. Using reverse-phase HPLC and Western blot analysis, we have evaluated the polypeptide pattern in bronchoalveolar lavage (BAL) fluid retrieved from asthmatics, before and after induction of airway inflammation by low-dose allergen inhalation challenge. A prominent decrease of CC16 was seen after induction of inflammation, and a further CC16 decrease was observed in lavage fluid where surfactant had been removed. Reduced levels of pulmonary CC16 may cause loss of anti-inflammatory activity in the airways and contribute to the development of airway inflammation in asthma.

Key words. CC16; reverse-phase HPLC; asthma; BAL fluid proteins.

The Clara cell 16 kDa protein (CC16, also known as CC10 uteroglobin, polychlorinated biphenyl-binding protein (PCB-BP)), or Clara cell secretory protein (CCSP) is a 15.8 kDa homodimer expressed by non-ciliated airway epithelial cells [1]. The protein subunits are linked by two disulphide bridges, and enclose a hydrophobic cavity. Four CC16 isoforms, differing in pI, have recently been detected in bronchoalveolar lavage (BAL) fluid [2]. The in vivo function of CC16 is not yet fully understood, but in vitro experiments have shown that CC16 inhibits fibroblast and leukocyte migration [3, 4] and acts as an inhibitor of phospholipase A_2 (PLA_2) in the synthesis of arachidonic acid metabolites [5]. CC16 knockout mice show aggravated early pro-inflammatory responses to oxidant challenge [6, 7], indicating a protective role of CC16 against acute oxidative stress. In humans, mutation screening of asthma candidate genes has revealed an association between a polymorphism in the CC16 gene and an increased risk of developing asthma. The polymorphism represents a single nucleotide substitution in a non-coding region of exon one [8]. Recently, reduced plasma CC16 levels in individuals with the aberrant genotype were reported, indicating an altered expression of the protein derived from the mutant gene [9]. Asthmatics show lower levels of CC16 in BAL fluid and serum and reduced numbers of CC16 positive cells in the airways compared to healthy individuals [10–12].

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To investigate any alterations in the pulmonary protein pattern in an early stage of allergic airway inflammation in asthmatics, we evaluated the pulmonary polypeptide pattern by reverse-phase high performance liquid chromatography (RP-HPLC) of BAL fluid retrieved before and after low-dose allergen inhalation challenge. The challenge model utilized induces an asymptomatic allergic airway inflammation and mimics a natural exposure to environmental airborne allergens by repeated administration of low doses of inhaled allergen [13].

We found an altered pulmonary RP-HPLC polypeptide pattern following allergen challenge, with the most prominent finding being a reduction of the CC16 levels. In addition, we found by Western blot analysis a further decrease of CC16 in surfactant-depleted lavage fluid. The results also indicated increased pulmonary surfactant levels after induction of airway inflammation. Combined, the observations suggest an altered protein-surfactant interaction in an early stage of allergic airway inflammation.

Materials and methods

BAL fluid supernatants were obtained before and after allergen challenge from eight asthmatic subjects submitted to a low-dose allergen provocation study [13]. The patients had a clinically well-characterized mild, atopic, seasonal asthma and were all sensitive to inhaled birch or grass pollen. Briefly, adequate allergen was administered by inhalation in a dose corresponding to 10% of the dose of allergen provoking a 20% fall in the forced expiratory volume in one second ($FEV_{1.0}$), repeated during seven consecutive weekdays. The patients developed an allergic airway inflammation, which was subclinical in six out of eight subjects. Two patients (two and eight) showed slight airway obstruction during the study. The study was approved by the local ethics committee and written consent was obtained from all subjects.

BAL fluid was collected using 250 ml sterile saline as described [14], the fluid was strained through a single layer of Dacron nets, and the cells were pelleted by centrifugation at 400 × g for 10 min at 40 °C. The BAL supernatants were applied to OASIS cartridges (Waters, Milford, MA, USA) equilibrated in 0.1% trifluoroacetic acid (TFA) in order to remove salt and low-molecular weight hydrophilic components, and to obtain a concentrate of the BAL fluid polypeptides. Elution was performed with 80% aqueous acetonitrile in 0.1% TFA and the eluate was lyophilized.

In three subjects, the BAL surfactant fraction was isolated by centrifugation at 5000 × g for 120 min at 4 °C. The supernatants obtained are referred to as surfactant-depleted BAL fluid. To estimate the surfactant phospholipid content, the ethanolamine amount was analyzed by RP-HPLC after hydrolysis and phenylthiocarbamyl (PTC) derivatization as described [15].

Total BAL fluid protein concentrations were measured with the BCA assay (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. Concentrations of albumin in BAL supernatants were measured by nephelometry as described [16]. RP-HPLC (on a 4.6 × 250 mm column of Vydac C_8, 5 μm; Separations group, Hesperia, CA, USA) was performed utilizing a Waters system with detection at 214 nm and 280 nm and a flow of 1 ml/min. The gradient used was 15–60% aqueous acetonitrile in 0.1% TFA for 45 min and 60–80% aqueous acetonitrile in 0.1% TFA for 10 min. Mass determination was performed using a matrix-assisted laser-desorption/ionization instrument (Lasermat, Finnigan MAT, San Jose, CA, USA). Edman degradation was carried out in a Procise HT 494 protein sequencer (PE-Applied Biosystems, Foster City, USA). For Western blot analysis, the material was separated by discontinuous SDS/polyacrylamide gel electrophoresis using 10–20% Tricine Ready Gels (Novex, San Diego, CA, USA) and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes [17]. Immunoreactivity was detected with CC16-specific rabbit antiserum (kindly provided by Professor G. Singh, Dept. of Veteran Affairs, Pittsburgh Health Care Systems, USA) and subsequently anti-rabbit Ig conjugated with horseradish peroxidase (Amersham, Little Chalfont, Buckinghamshire, England). An ECL Western blotting detection system (Amersham) was used to visualize the result.

Statistical correlations were determined using the Pearson's product-moment correlation test.

Results

The concentration of total proteins and albumin in BAL supernatants was measured before and after allergen challenge and showed a slight decrease after induction of airway inflammation (fig. 1). A significant correlation was found between the concentration of total proteins and albumin before ($r = 0.76$, $p < 0.05$) and after ($r = 0.83$, $p < 0.05$) allergen challenge.

A standardized amount (1 mg) of the total lyophilized polypeptides retrieved from BAL fluid, before and after allergen challenge, was analyzed by RP-HPLC. The chromatographic profiles showed similar patterns in all subjects, with pronounced post-challenge decreases of the peaks corresponding to fractions 42 and 48 (fig. 2). The material collected from these fractions was identified by mass spectrometry and amino acid sequence analysis and was shown to represent alpha-1-antitrypsin (α-1-AT) and CC16, respectively. The α-1-AT peak showed a slight post-challenge decrease in four cases.

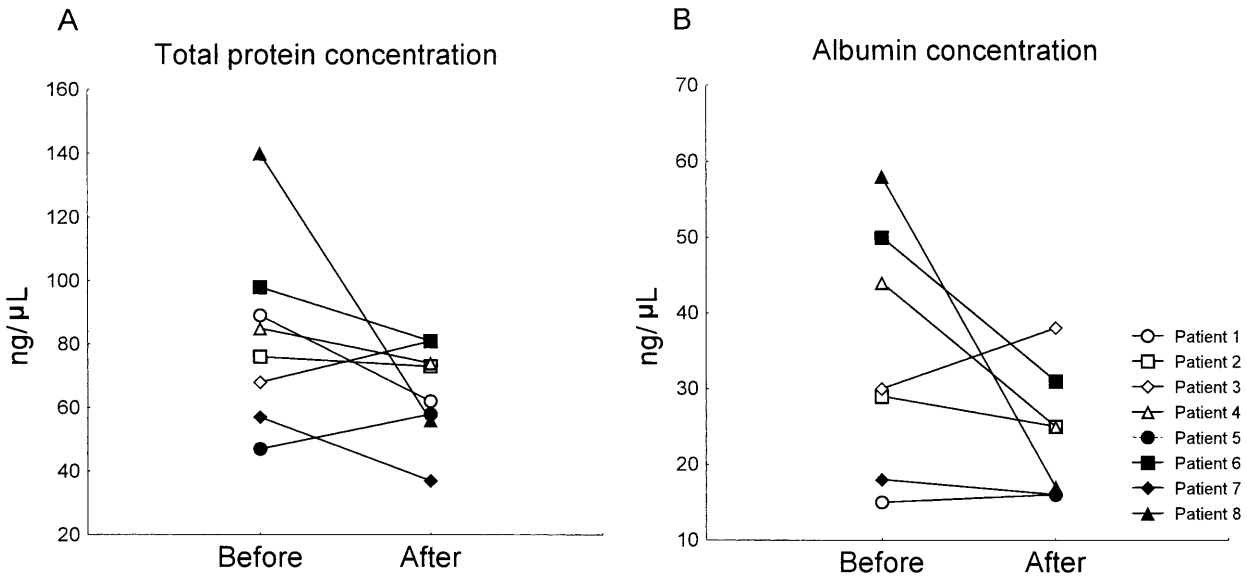

Figure 1. Total protein concentration (*A*) and albumin concentration (n = 7) (*B*) in BAL fluid before and after allergen challenge.

Edman degradation for 20 cycles of the material collected in fraction 48 showed only one sequence, identical to that of the N-terminal part of CC16. The material had a mass value of 15823 Da, in agreement with that of the CC16 protein. Thus, the material collected in fraction 48 was shown to constitute a highly pure eluate of CC16. This material was furthermore shown to decrease after allergen challenge in all patients (fig. 2, table 1). The peak corresponding to fractions 32–35 represents a large proportion of the BAL fluid proteins and predominantly represents albumin (fig. 2). To determine the decrease of CC16 relative to that of other BAL proteins, the peak areas of fraction 48, representing CC16 were expressed in relation to those of the albumin-containing fraction. In 7 out of 8 patients, CC16 showed a decrease relative to the albumin containing protein fraction after induction of airway inflammation (table 1).

To investigate whether the post-challenge reduction of CC16 was related to a re-distribution between CC16 isoforms [2], CC16 immunoreactivity was determined in HPLC-fractionated BAL fluid using dot blot and Western blot analyses. CC16 was found to elute in three separate HPLC fractions, indicating the presence of CC16 isoforms. CC16 was predominantly detected in the peak indicated in fig. 2. No quantitative redistribution could be detected between CC16 isoforms after allergen challenge.

We hypothesized that the reduction of CC16, seen after induction of airway inflammation, could be related to an altered interaction with pulmonary surfactant [18,

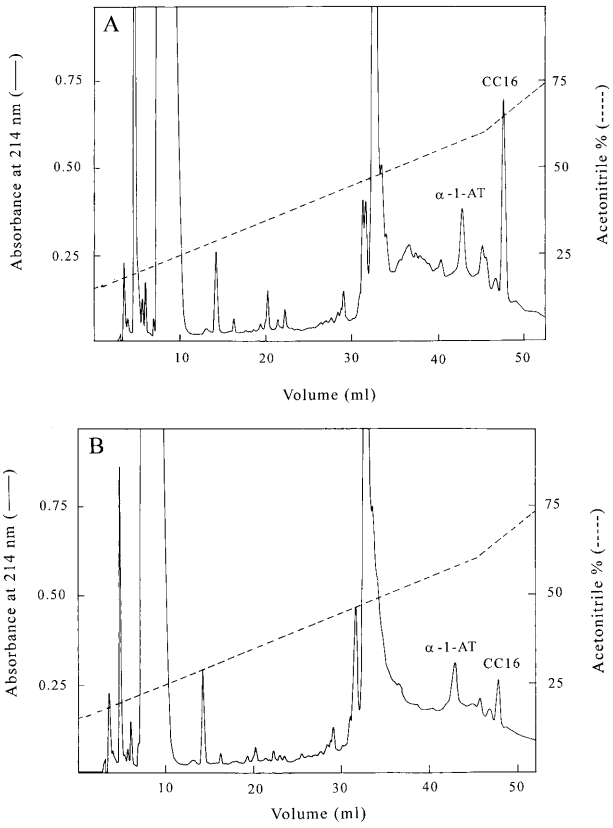

Figure 2*A–B*. Reverse-phase HPLC fractionation of BAL supernatants before (*A*) and after (*B*) allergen challenge. The peaks indicated represent alpha-1-antitrypsin and CC16.

Table 1. HPLC peak areas of albumin, α_1-antitrypsin (α_1-AT), CC16, CC16 relative to albumin and the amount of ethanolamine in the surfactant fraction before and after allergen challenge (B/A).

Patient	Albumin (cm^2) (B/A)	α_1-AT (cm^2) (B/A)	CC16 (cm^2) (B/A)	CC16/Albumin (%) (B/A)	Ethanolamine (pmol) (B/A)
1	12.32/11.47	1.68/2.16	0.51/0.41	4.1/3.6	
2	14.85/16.78	1.58/1.60	1.63/1.16	11.0/6.9	
3	18.17/24.67	2.64/1.68	1.52/0.72	8.4/2.9	
4	22.56/11.88	0.88/0.60	1.26/0.72	5.6/6.1	
5	16.38/13.64	0.65/0.13	1.82/0.44	11.1/3.2	
6	15.48/8.78	3.04/1.53	2.08/0.56	13.4/6.4	33/603
7	14.49/14.63	0.86/0.70	1.85/1.52	12.8/10.4	706/507
8	9.1/18.38	1.83/0.90	4.97/0.86	54.6/4.8	21/289

19]. To elucidate this possibility, surfactant was separated from BAL supernatants from three patients and the fractions were further analyzed by Western blot and dot blot analyses. Surfactant-depleted BAL supernatants showed an altered Western blot CC16 immunoreactivity after allergen challenge compared to supernatants containing surfactant (fig. 3). In surfactant-depleted supernatants retrieved after allergen challenge, a further CC16 decrease was observed, indicating an increased association of CC16 with surfactant after induction of airway inflammation (fig. 3). To investigate whether such an altered protein/surfactant interaction could be attributed to alterations in surfactant content following allergen-induced airway inflammation, the BAL surfactant fractions were analyzed for ethanolamine amount by hydrolysis, PTC derivatization, and RP-HPLC [15]. In BAL surfactants retrieved from patients six and eight after allergen challenge, a clear increase in the ethanolamine level was found, while surfactant retrieved from patient seven showed a moderate decrease of ethanolamine (table 1). Patients six and eight showed an increase of ethanolamine, as well as an explicit decrease in CC16, following allergen challenge, while only a slight decrease of CC16 with a reduced ethanolamine content was observed in patient seven.

Discussion

The increased risk of developing asthma in individuals with a polymorphic allele of the CC16 gene and the anti-inflammatory properties of the protein suggest an important role for CC16 in the pathogenesis of asthma [3, 8, 20]. Using RP-HPLC, we analyzed the BAL fluid polypeptide pattern after low-dose allergen challenge of asthmatic individuals. Post-challenge alterations were pronounced for CC16, where a clear decrease was found in all patients after induction of airway inflammation (table 1, fig. 2). Our results show that in addition to the previously reported reduced expression of pulmonary CC16 in asthmatics, exposure of atopic individuals to low doses of relevant allergen causes a further decrease in CC16 levels detectable by RP-HPLC.

The concentration of total BAL fluid proteins and albumin showed a slight decrease after induction of inflammation by low-dose allergen inhalation challenge (fig. 1). This finding contrasts previous studies on exudative responses in the airways of asthmatics showing dose-dependent increases of airway protein levels following allergen challenge [21–23]. However, these studies are based on challenge models where high doses of allergen are administered locally at the airway mucosa or by aerosol, not comparable to the low-dose allergen challenge model we utilized. Our finding may represent a dilution of the epithelial lining fluid following a selective transudation of serum to the airways. Such a mechanism is however not likely to explain our finding of decreased CC16 levels since a standardized weight of lyophilized polypeptides was used for HPLC analysis to compensate for any dilution. Furthermore, we analyzed the pulmonary CC16 levels in relation to albumin by evaluating peak areas in HPLC separated BAL fluid

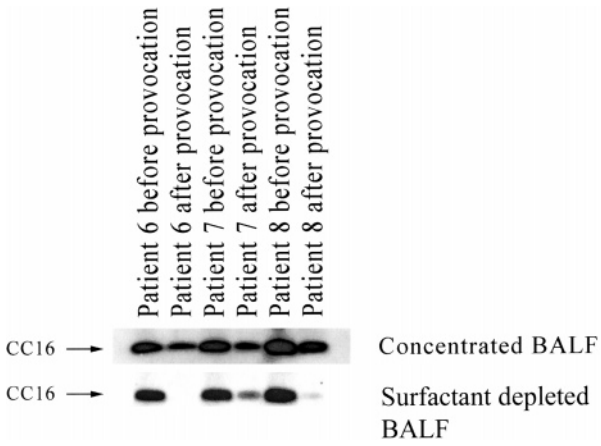

Figure 3. Western blot analysis of CC16 before and after allergen challenge in non-separated BAL supernatants and in surfactant-depleted supernatants.

(table 1). The decrease in size of the HPLC peaks corresponding to CC16 was not accompanied by any general reduction of proteins.

Recently, the presence of four monomeric 6.5 kD isoforms of CC16 in BAL fluid with a decreased level of the major isoform was reported in samples from a patient with occupational asthma [2]. To investigate whether the reduction of CC16 found after induction of airway inflammation was related to a redistribution of CC16 isoforms, dot blot and Western blot analyses for CC16 were performed on HPLC fractionated BAL. No redistribution between CC16 isoforms could be detected and redistribution can therefore not explain the reduction of the major CC16 form.

CC16 has been shown to bind pulmonary surfactant phospholipids in its hydrophobic cavity. Crystal structure and nuclear magnetic resonance show that this binding cavity is formed at the interface between the two protein monomers [18]. Access to the binding site is controlled by reduction of the inter-chain disulphide bonds between Cys 3–Cys 69′ and Cys 69–Cys 3′ [18]. Breakage of these bonds induces a local unfolding, creating a channel into the binding site [24]. It has been suggested that an increased ligand binding is associated with a reduction of the CC16 disulphide bridges, in specific environments [18, 24]. In addition, CC16 has been demonstrated to interact with surfactant phospholipids in a calcium-dependent manner through a calcium binding-site on the surface of the protein [19]. To investigate whether the reduction in CC16 levels was related to surfactant interaction, surfactant-depleted BAL supernatants were investigated for CC16 immunoreactivity. Surfactant-depleted BAL supernatants retrieved after allergen challenge showed a clearly decreased CC16 immunoreactivity, indicating an increased association of CC16 with surfactant components after allergen challenge. To investigate whether an altered protein-surfactant interaction could be attributed to alterations in pulmonary surfactant following allergen-induced airway inflammation, the BAL surfactant fractions were analyzed for ethanolamine content. As ethanolamine is a substituent in phosphatidyl ethanolamine, a major surfactant phospholipid, ethanolamine levels reflect the phospholipid content of surfactant. Interestingly, we found increased ethanolamine levels, indicating an altered surfactant composition in subjects showing an explicit decrease of CC16. Our findings thus indicate that allergen-induced airway inflammation in asthmatic individuals may be associated with increased levels of pulmonary surfactant, rendering an altered interaction with the anti-inflammatory protein CC16.

We conclude that early stage allergic airway inflammation is associated with a reduction of pulmonary levels of CC16 in asthmatics, which may cause a diminished pulmonary anti-inflammatory activity and contribute to the further development of airway inflammation in asthma.

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