

Original article

Inverse relation between nasal fluid Clara Cell Protein 16 levels and symptoms and signs of rhinitis in allergen-challenged patients with intermittent allergic rhinitis

Background: Decreased levels of the anti-inflammatory Clara Cell Protein 16 (CC16) are found in intermittent allergic rhinitis (IAR) and asthma. In asthma this decrease has been associated with hyperreactivity and the A38G single nucleotide polymorphism (SNP). The aim of this study was to examine if IAR is associated with signs and symptoms of rhinitis and the A38G SNP.

Methods: Nasal fluid CC16 was analyzed in 20 patients with IAR before allergen challenge and 1 and 6 h after challenge, and from 28 healthy controls. The A38G SNP was analyzed in 80 patients with IAR and 106 controls. Nasal biopsies were obtained from three subjects in each group for immunohistochemical analysis of CC16.

Results: In the allergen-challenged patients symptoms and rhinoscopic signs of rhinitis increased after 1 h and normalized after 6 h. In contrast, nasal fluid CC16 decreased 1 h after allergen challenge and returned to baseline after 6 h. Nasal fluid CC16 levels did not differ from controls before and 6 h after challenge. Immunohistochemical investigation showed intense CC16 staining in the nasal epithelium of both patients before season and healthy controls, but weak staining in symptomatic patients during season. No significant association between the A38G SNP and IAR was found.

Conclusion: There was an inverse relation between nasal fluid CC16 levels and symptoms and signs of rhinitis in allergen-challenged patients with IAR. However, there was no association between IAR and the A38G SNP.

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Decreased expression of the anti-inflammatory gene Clara Cell Protein 16 (CC16) may contribute to allergic inflammation. This protein, which is also known as CC16 or uteroglobin is secreted by Clara cells in the lungs and by nasal mucosal epithelial cells. Clara Cell Protein 16 has important anti-inflammatory effects, such as the inhibition of leukocyte chemotaxis, proinflammatory cytokines, and protease activity (1). The relevance of CC16 to allergic disease is supported by several observations. Peptides derived from CC16 have been found to be effective in an animal model of allergic conjunctivitis (2). Decreased levels of CC16 have been described in intermittent allergic rhinitis (IAR) and asthma (3–8). In asthma low CC16 has been associated with airway hyperreactivity. However, in IAR the association between CC16 and clinical disease severity is less clear. Lower nasal fluid CC16 levels in symptomatic patients with IAR than in healthy controls support that CC16 could have a role in the disease (7). However, in one study CC16 concentrations were generally low, both before and during season as well as following treatment with

glucocorticoids (8). This makes the relation between CC16 and disease severity less clear. A possible explanation for this could be a primary genetic cause. The CC16 gene is located on chromosome 11q12–13. This region is linked to atopy and contains candidate genes for allergy such as the high-affinity immunoglobulin E receptor. An adenine (A) to guanine (G) substitution in the promoter region of the gene-encoding CC16 (A38G) has been associated with low expression of CC16 and airway hyperreactivity in asthma (6, 9–11). However, the association with this single nucleotide polymorphism (SNP) could not be replicated in other studies of asthma (12, 13). The A38G SNP has not been examined in IAR. An alternative explanation for altered CC16 production in allergy could be that proinflammatory cytokines, such as tumor necrosis factor- α (14–16) regulate CC16 synthesis. As CC16 itself affects cytokine production, it is possible that the balance between the two constitutes a regulatory mechanism in mucosal inflammation. The relation between CC16 and clinical disease can be readily studied in IAR. Signs of rhinitis can be determined

rhinoscopically and symptoms scored by asking the patients. In natural IAR, this is complicated by variable pollen exposure. However, patients can also be challenged with allergen out of season under controlled circumstances using a specific amount of allergen and nasal fluid obtained at defined time points. It is thus possible to examine how nasal fluid CC16 levels relate to signs and symptoms of rhinitis.

The aims of this study were to examine how nasal fluid CC16 levels relate to signs and symptoms of rhinitis as well as if there is an association between IAR and the A38G polymorphism.

Material and methods

Subjects

- 1 Single nucleotide polymorphism study: This part was performed in blood samples from 80 patients (38 women) with birch and/or grass pollen-induced allergic rhinitis and 109 healthy controls (56 women). The median (range) age of patients and controls was 31 (15–61) and 46 (21–65) years, respectively.
- 2 Allergen provocation study: This part was performed in nasal fluids from 20 patients (13 women) with birch and/or grass pollen-induced allergic rhinitis obtained outside the pollen season. The median (range) age of the patients was 37 (18–53) years. In addition, 28 healthy controls (17 women) were seen outside pollen season. The median (range) age of controls was 33 (18–51) years.

The diagnosis of birch and grass pollen-induced allergic rhinitis was based on a positive history of seasonal allergic rhinitis and positive skin prick tests against birch and/or grass pollen. All patients were seen during the late autumn and were asymptomatic at the time of inclusion in the study. Exclusion criteria included a history of perennial symptoms, positive skin prick tests against house dust mite (*Dermatophagoides pteronyssinus* and *D. farinae*) and molds (*Cladosporium* and *Alternaria*), treatment with local or systemic corticosteroids or antihistamines for the last 2 months. The controls were all symptom-free, had no history of allergic rhinitis or any other atopic disease, and had negative skin prick tests against a panel of allergens, including birch, timothy, mugwort, house dust mite (*D. pteronyssinus* and *D. farinae*), horse, dog, cat, molds (*Cladosporium* and *Alternaria*). The study was approved by the Ethics Committee of the Medical Faculty, Lund University.

Allergen challenges

The patients were challenged with birch or timothy pollen extracts; 10 000 SQ/U per nostril of Aquagen (ALK, Denmark) was administered with a mechanical pump spray. Recovery of nasal secretions was done before challenge and 1 and 6 h after challenge.

Nasal lavage fluids were obtained as previously described (17); after clearing excess mucous by forceful exsufflation, sterile normal saline solution of normal room temperature was aerosolized into each nostril, while alternately clearing the other. The nasal fluids were allowed to return passively and collected in a graded test tube that was submerged in iced water, until 7 ml was recovered. The fluids were then centrifuged for 10 min at 1334 g in 4°C. The supernatant was separated from the pellet and stored at –70°C until analysis.

Nasal symptom and rhinoscopy scores

Nasal symptoms, i.e. itching, secretion, and blockage were scored from 0 to 3 (0 = no, 1 = mild, 2 = moderate, 3 = severe symptoms). A total nasal symptom score was calculated by addition of the three scores. Anterior rhinoscopy was performed on all individuals. Edema and secretion in each nostril were scored from 0 to 2 (0 = no, 1 = mild, 2 = severe). Total edema and secretion scores were computed by adding the scores for each sign and each nostril (18).

SNP analysis

The TaqMan chemistry was used to genotype the rs3741240 SNP. This technique is based on a 5'-nuclease assay with a reporter and a quencher molecule. Assays were run on an ABI PRISM® 7900HT sequence detection system from Applied Biosystems (Foster City, CA, USA).

Nasal biopsy procedure

Three patients were randomly chosen from the patients with symptomatic allergic rhinitis and from patients outside pollen season for nasal biopsies. In addition, nasal biopsies were also obtained from three healthy controls for immunohistochemical analysis. Biopsies were taken from the inferior turbinate after topical application of local anesthesia containing lidocainhydrochloride/nafazoline (34/0.17 mg/ml) for 20 min. The nasal biopsies were approximately 2 × 2 × 2 mm.

CC16 immunocytochemistry

The specimens were fixed in a cold fixative solution composed of 2% formaldehyde and 0.2% picric acid, buffered to pH 7.2 with 0.1 M phosphate buffer. They were rinsed twice in a Tyrode solution containing 10% sucrose at 4°C for 48 h, frozen on dry ice, and sectioned at 10 µm in a cryostat at –20°C. The sections were processed for immunocytochemical demonstration of CC16 using an indirect immunofluorescence method. The CC16 antiserum (DakoCytomation Norden A/S, Glostrup, Denmark) was raised in rabbit against natural porcine CC16 and used in a dilution of 1 : 800. The site of the antigen–antibody reaction was revealed by using fluorescein isothiocyanate-labeled goat-antirabbit IgG in a dilution of 1 : 20. Slides were coverslipped in glycerol/phosphate-buffered saline 2 : 1 (v/v) containing DAP 1 (1 mg/µl) and observed under microscope with chromofluorescence filters.

Negative controls for nonspecific binding included normal rabbit serum without primary antibody and secondary antibody alone. As cross-reactions with other proteins containing amino acid sequences recognized by the antisera could not be excluded, it is appropriate to refer to the immunoreactive material as 'CC16-like'. For brevity, the immunoreactive material is referred to as CC16 in the text.

Nasal fluid mediator assays

Clara Cell Protein 16 levels in nasal lavage fluids were determined with enzyme-linked immunosorbent assay according to the manufacturer's protocol (Biovendor, Brno, Czech Republic).

Statistics

The Wilcoxon signed rank test was used to make paired comparisons of nasal fluid CC16 among the different patient groups.

The levels of nasal fluid CC16 in nasal fluids from patients and controls were compared using the nonparametric Mann–Whitney *U*-test.

Nomenclature

The revised nomenclature for allergy according to recent EAACI recommendations is used (8).

Results

Nasal symptoms and signs in patients with IAR before and after challenge with allergen

The total nasal symptom score increased from 0.25 ± 0.2 before allergen challenge to 3.7 ± 0.9 at 1 h following challenge ($P < 0.001$). After another 5 h, the total nasal symptom score had decreased to 0.9 ± 0.5 , which did not significantly differ from baseline ($P = 0.09$; Fig. 1). Examination with anterior rhinoscopy revealed a similar pattern. The edema score was 0.05 ± 0.1 before challenge and 2.0 ± 0.7 after 1 h and 0.1 ± 0.1 after 5 h. The corresponding values for secretion were 0.3 ± 0.23 , 2.2 ± 0.7 and 1.0 ± 0.5 (statistical analyses have not been made for rhinoscopic values to avoid multiple inferences).

Nasal fluid CC16 levels

Measurement of CC16 protein in nasal fluids showed no significant difference between patients outside of season and healthy controls, 8.4 ± 1.5 compared with 12.9 ± 2.5 ng/ml. One hour after challenge CC16 levels decreased to 6.2 ± 1.3 ($P < 0.05$). Six hours after challenge the CC16 concentration was 9.7 ± 1.3 ng/ml, which did not differ significantly compared with before challenge (Fig. 1). In comparison, in a previous study (7) using the same methods as in this one, CC16 levels in symptomatic patients during pollen season were 5.4 ± 1.5 ng/ml.

Immunohistochemical investigation of CC16 in the nasal mucosa

Immunohistochemical investigation was performed to examine the expression of CC16 in nasal biopsies from healthy controls and patients with allergic rhinitis before and during the pollen season. In the nasal mucosa from healthy individuals and asymptomatic patients out of season, an intense immunostaining of CC16 was detected in the epithelial cells. In symptomatic patients during the season a similar distribution was seen although the staining of epithelial cells was less intense and a smaller number of cells displayed immunoreactivity (Fig. 2).

Analysis of the A38G SNP in the CC16 promotor region

An SNP (rs3741240) located in the CC16 promotor region causing lower CC16 protein levels in bronchoalveolar lavage fluids of asthmatics has previously been reported (6, 11). To examine if this SNP could account for lower CC16 protein levels in symptomatic patients with IAR blood samples from 80 patients and 109 controls were analyzed for this SNP. The A-allele had an overall frequency of 0.33 and the G-allele had 0.67. No significant difference was found between patients with allergic rhinitis and healthy controls regarding this polymorphism (Table 1).

Discussion

Several reports support that decreased expression of the anti-inflammatory protein CC16 is an important pathogenic mechanism in allergic disease. Decreased levels have been reported in both asthma and allergic rhinitis (4–8). In fact, in a gene profiling study of IAR, CC16 was one of the anti-inflammatory proteins that decreased most in expression (7). Peptides derived from CC16 have been successfully used therapeutically in an animal model of allergic conjunctivitis (19). However, the association between CC16 and symptoms and signs of IAR is not

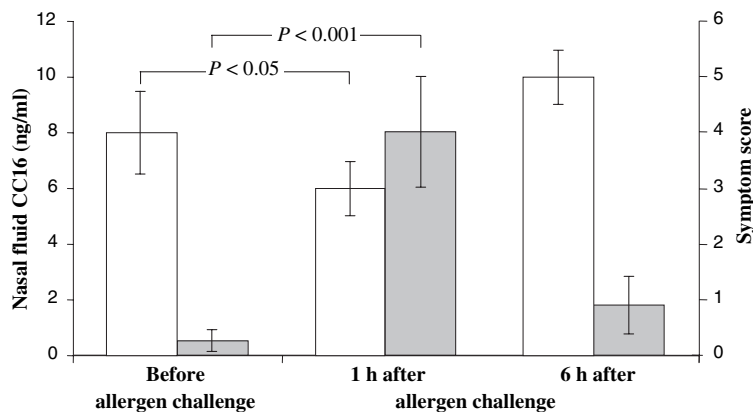


Figure 1. Nasal fluid concentrations of Clara Cell Protein 16 in patients with intermittent allergic rhinitis before allergen challenge, and 1 and 6 h after challenge (empty bars). Total symptom scores for the corresponding time points are indicated with filled bars.

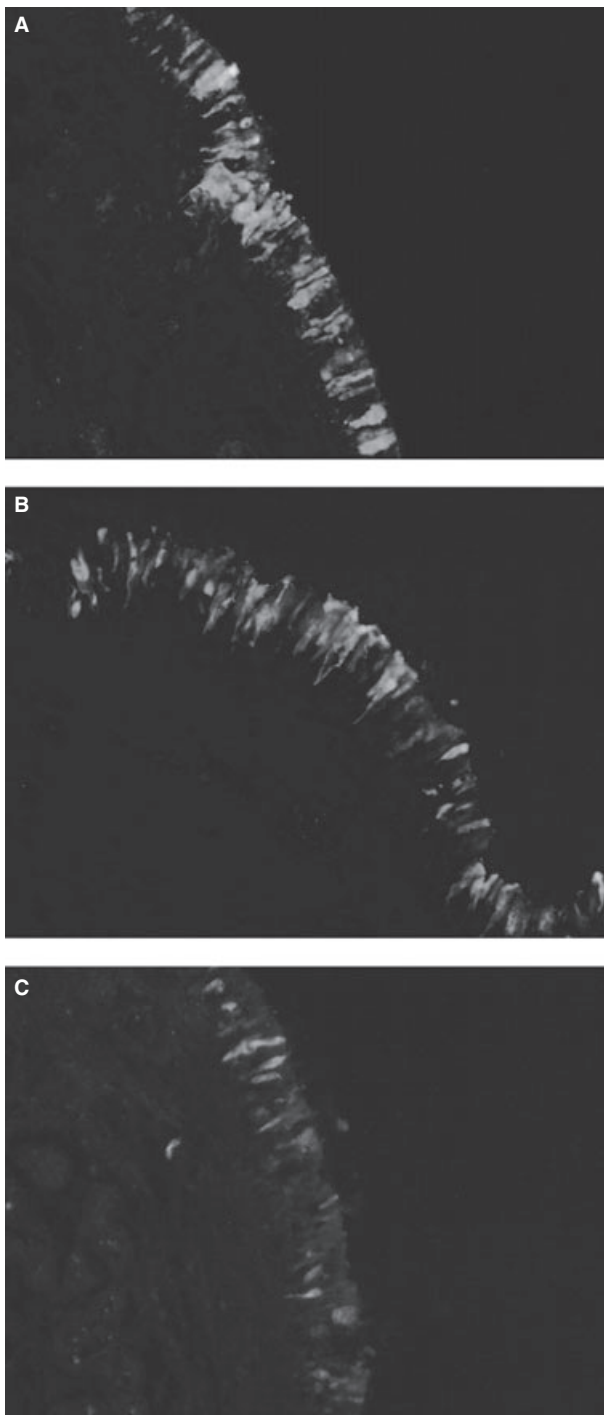


Figure 2. Expression of Clara Cell Protein 16 protein determined by means of immunohistochemistry in the nasal mucosa from one representative healthy control (plate A), one patient with intermittent allergic rhinitis out of season (B) and during season (C) at 200 $\times$ magnification.

clear. In a recent study, low levels of nasal fluid CC16 were reported not only in symptomatic patients during the season, but also before season and following

Table 1. Genotype results for CC16, marker rs3741240 (frequencies of different genotypes in healthy controls and patients with IAR)

	AA or AG	GG	Total*
Controls	0.53	0.47	109
IAR	0.55	0.45	80
Total*	102	87	189
	AA	AG or GG	Total*
Controls	0.10	0.90	109
IAR	0.13	0.88	80
Total*	21	168	189

* Total number of individuals.

CC16, Clara Cell Protein 16; IAR, intermittent allergic rhinitis.

treatment with glucocorticoids (8). Thus, low levels of CC16 were found irrespective of the stage and severity of disease. One possible explanation could be a genetically determined inability to produce sufficient amounts of CC16 to control allergic inflammation. In support of this, a SNP in the promotor region of CC16 has been described in asthmatic patients and linked to airway hyperreactivity. In a recent study of acute asthma, plasma CC16 levels were found to increase in patients with 38GG and 38AG genotypes, but not in patients with 38AA (20). These relations were similar to those previously described in a case-control study (6). However, this finding could not be replicated in some studies.

Intermittent allergic rhinitis is a good model of allergic inflammation in that the allergen is known and occurs at a defined time point. Moreover, signs can be determined rhinoscopically and symptoms scored according to standardized protocol. On the other hand, studies of IAR during the pollen season are complicated by variable pollen exposure confounding analysis of the relation between CC16 and symptoms and signs of rhinitis. In this study, we therefore aimed to examine this relation following allergen challenge, which allows administration of a standardized dose of allergen. Moreover, symptoms and signs can be assessed and nasal fluids samples obtained at defined time points. We found that CC16 levels did not differ between asymptomatic patients out of season and healthy controls. Similarly, immunohistochemical investigation showed intense staining of CC16 in the nasal epithelium in both patients before season and healthy controls. This is consistent with previous data implying that CC16 contributes to controlling the inflammatory mechanisms in the nasal mucosa in allergic as well as healthy individuals. In contrast, CC16 decreased significantly in patients 1 h after allergen challenge. This was associated with increased symptoms and signs in all patients. Similar findings were made in symptomatic patients during the pollen season in a previous study performed by us with similar methods as in the present one (7). After 6 h CC16 levels and symptoms and signs normalized. Normalization of symptoms after 6 h is in contrast to some previous studies describing a late phase

reaction after nasal allergen challenge, but not to others (21).

However, taken together our data support that CC16 has an inverse relation to signs and symptoms of IAR. This is in line with a counter-regulatory role of CC16 in relation to the inflammatory response in IAR. In other words, the early inflammatory response leads to decreased release of CC16, while the subsequent restoration of CC16 to baseline levels contributes to normalization of signs and symptoms of IAR. In fact, the possibility that the lack of signs of symptoms observed during the late phase reaction in many studies of IAR depends on CC16 may be an important task for further research. Moreover, the findings that CC16 levels did not differ between controls and patients before challenge and normalized after 6 h support that allergic patients can mount a normal CC16 response. This also agrees with our finding that there was no significant association between the A38G SNP and IAR. However, the material investigated in this study was relatively small and it should be noted that disease-associated SNPs often show small differences between patients and controls. Thus, further studies of larger materials are needed to define the relevance of the A38G SNP in IAR. It should also be noted that there may be a physiologic explanation for decreased nasal fluid CC16 levels following allergen challenge or during natural exposure. Intermittent allergic rhinitis is associated with increased plasma transudation and secretions. This can lead to dilution of nasal fluid CC16 levels. Indeed, decreased levels of CC16 are not specific for allergic inflammation. Studies of other inflammatory diseases have also shown decreased CC16. Examples

include two studies of nasal polyposis, which showed decreased local expression of CC16, both on the mRNA and protein levels (22, 23).

Interestingly, treatment with glucocorticoids led to increased production of CC16 in one of these studies (20). It should also be noted that decreased expression of other anti-inflammatory mediators, such as transforming growth factor- β has been described in IAR (24). Moreover, according to the Th2 hypothesis allergic inflammation is due to an altered balance between Th1 and Th2 cytokines, toward a Th2 predominance. Although this hypothesis is under some debate, it underscores the notion that allergic inflammation can be seen as the net result of counter-regulatory inflammatory mechanisms.

In conclusion, this study shows an inverse relation between nasal fluid CC16 levels and symptoms and signs of IAR, which is more likely due to a counter-regulatory mechanism than a primary genetic deficiency in CC16 secretion.

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