

Original article

Clara cell 10-kDa protein expression in chronic rhinosinusitis and its cytokine-driven regulation in sinonasal mucosa

Background: Clara cell 10-kDa protein (CC10) is a multifunction protein with anti-inflammatory and immunomodulatory effects; hence we compared the CC10 expression between chronic rhinosinusitis (CRS) patients with and without nasal polyps (NPs), analyzed its association with disease severity and response to surgery, and explored its regulation via cytokines.

Methods: The plasma and tissue CC10 levels were compared between controls and CRS patients with and without NPs by means of quantitative RT-PCR, ELISA, and immunohistochemistry. Computed tomography (CT) scan and endoscopy findings and symptoms were scored. Nasal explant culture was used to explore the effect of TNF- α , IL-1 β , IL-4, INF- γ , and IL-10 on CC10 gene regulation.

Results: Compared with controls, the CC10 expression in sinonasal mucosa was significantly inhibited in both CRS patients with and without NPs. There was a significant further decrease of CC10 expression in patients with NPs and asthma. No difference in CC10 plasma levels was found between controls and patients. CC10 levels inversely correlated with preoperative CT scores, and postoperative endoscopy and symptom scores. TNF- α , IL-1 β and IL-4 inhibited, whereas INF- γ and IL-10 promoted CC10 production in nasal mucosa. A significantly faster decay of CC10 transcripts was seen after IL-1 β treatment. IL-1 β and IL-10 induced thyroid transcription factor-1 expression. INF- γ increased, whereas IL-4 inhibited hepatocyte nuclear factor-3 α expression.

Conclusion: CC10 may take part in the pathogenesis of CRS and correlates with disease severity and response to surgery. Different cytokines can regulate CC10 expression in nasal mucosa differentially through modulating mRNA stability and certain transcriptional factors expression.

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Key words: chronic rhinosinusitis; clara cell 10-kDa protein; cytokine; nasal polyps; regulation.

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Accepted for publication 12 June 2008

Chronic rhinosinusitis (CRS) is a widely prevalent health condition that may severely impact the quality of life of affected individuals. The most severe forms of CRS exhibit nasal polyps (NPs), with a high rate of symptomatic recurrence despite optimal medical and surgical care. Although insights into the pathophysiology of CRS have largely expanded over the last two decades, its exact etiology and mechanism are still a source of extensive controversy (1, 2). To date, one of the most important characteristics of CRS is the prolonged and exaggerated inflammatory reaction in paranasal mucous membrane

(1, 2). Inflammation is essential for clearing out infectious and inflammatory agents, but it can also be harmful to the host if appropriate regulation of the magnitude and duration of the response is disturbed. So, inflammation is subjected to tight control under multiple levels. Dysfunction of the anti-inflammatory network can lead to hyper-inflammatory states associated with inflammatory disease and therefore initiate and exacerbate the chronic disease.

Clara cell 10-kDa protein (CC10), a member of the secretoglobin family, also referred to as uteroglobin, is a steroid-inducible, multifunctional, secreted protein with anti-inflammatory and immunomodulatory effects (3, 4). It is constitutively expressed by the epithelial lining of all organs that communicate with the external environment, including bronchi and nose (3, 5). CC10 can inhibit the activity of phospholipase A2, suppress expression and function of several cytokines, diminish inflammatory cell chemotaxis, downregulate Th2 cell differentiation, and block prostaglandin D2 receptor-mediated nuclear

Abbreviations: CC10, clara cell 10-kDa protein; C/EBP, CCAAT/enhancer-binding protein; COUP-TF, chicken ovalbumin upstream promoter transcription factor; CRS, chronic rhinosinusitis; CT, computed tomography; ELISA, enzyme-linked immunosorbent assay; ESS, endoscopic sinus surgery; HNF, hepatocyte nuclear factor; NP, nasal polyp; RT-PCR, reverse transcriptase polymerase chain reaction; TTF-1, thyroid transcription factor-1; VAS, visual analog score.

factor- κ B activation (3, 6–8). Our previous study showed that the CC10 gene was the one most downregulated in human NPs and CC10-positive cells negatively correlated with total infiltrating inflammatory cells and goblet cells, suggesting that a decreased expression of CC10 may result in the dysfunction of the anti-inflammatory network in the upper airway and contribute to persistent inflammation in NPs (5). CRS with and without NPs are two main types of CRS and often taken together as one disease entity, however, increasing evidence suggests that CRS without NPs and NPs may be two distinct disease entities with different pathophysiological properties (1, 2, 9). Whether CC10 is also involved in the pathogenesis of CRS without NPs has not yet been studied. Moreover, although CC10 gene regulation has been studied in the lower airway (10–13), the factors contributing to CC10 gene modulation in the upper airway is unknown.

The aims of the present study were:

- (1) to analyze the expression of CC10 in CRS patients and compare the difference between CRS patients with and without NPs;
- (2) to explore the association between CC10 expression and disease severity and the response to surgery; and
- (3) to elucidate the impact of CRS relevant inflammatory cytokines on CC10 expression in sinonasal mucosa and the possible mechanisms.

Methods

Subjects

This study was approved by the ethical committee of Tongji Medical College of Huazhong University of Science and Technology. Clinical data of the patients are summarized in Table 1.

CC10 gene expression study. Eighty CRS patients, including 40 CRS patients without NPs and 40 CRS patients with NPs, who suffered from bilateral CRS and underwent endoscopic sinus surgery (ESS) were recruited. CRS with and without NPs was diagnosed according to the clinical criteria of Meltzer et al. (1).

Table 1. Patients' clinical data

	CC10 expression study			Nasal explant culture
	Control	CRS without NPs	CRS with NPs	
Subjects (n)	16	40	40	68
Sex (M/F)	9/7	24/16	21/19	33/35
Age (years)	18–56	20–62	21–62	17–55
Patients with asthma (n)	0	7	9	0
Patients with positive skin prick test results (n)	2	16	21	9
Patients with tobacco smoke exposure* (n)	7	18	18	19

*Active or passive smoking history > 1 year.

CC10, Clara cell 10-kDa protein; CRS, chronic rhinosinusitis; NP, nasal polyp.

Diseased sinus mucosal tissues from most hypertrophied and hyperemic regions or NP tissues from apex part of polyps were collected during surgery. Except for four patients with NPs and six patients without NPs, all CRS patients had complete 1-year postoperative records. Control specimens were retrieved from 16 patients (eight patients with mucus retention cyst of maxillary sinus, four patients with mucocele of maxillary sinus, and four patients with mucocele of sphenoid sinus). These patients did not show obvious anterior ethmoid inflammation on coronal CT scanning and endoscopy and none had a history of persistent mucopurulent drainage. Macroscopically normal anterior ethmoid mucosal tissues were obtained during surgery. Light microscopy of all these specimens confirmed that they consisted of normal sinus mucosa. Surgical samples were used for hematoxylin and eosin staining to assess the degree of inflammatory cells infiltration, and quantitative reverse transcriptase polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) experiments to investigate the mRNA and protein expression of CC10 gene. Immunohistochemical staining of CC10 was only performed on samples of five controls (three men; age range, 18–50 years), five CRS patients without NPs (three men; age range, 27–62 years), and five CRS patients with NPs (two men; age range, 27–57 years). Protein concentrations of TNF- α , IL-1 β , IL-4, INF- γ , and IL-10 in sinonasal mucosa were detected by ELISA in 12 controls (seven men; age range, 19–54 years), 17 CRS patients without NPs (10 men; age range, 21–60 years), and 20 CRS patients with NPs (10 men; age range, 23–60 years). Moreover, a blood sample was collected from each enrolled subject before the surgery to measure plasma CC10 levels.

CC10 gene regulation study. This study population comprised 68 patients undergoing septal surgery and/or turbinectomy because of nasal obstruction. None had a history of persistent mucopurulent drainage, allergic rhinitis, or sinus disease. Inferior turbinate mucosal samples were used for nasal explant culture.

In this study, none of the patients had an acute upper respiratory infection in the four weeks before the operation or a history of aspirin sensitivity. Subjects were excluded if they had received oral steroids or antihistamines three months before the surgery. Topical medications were withheld for a minimum of one month before study. None had received anti-leukotrienes or immunotherapy. Pre- and postoperation medical management strategies were identical in both CRS groups (2).

Assessment of symptoms, CT scan, and endoscopy findings

A symptom questionnaire based on a visual analog score (VAS) of zero to 10 according to severity was used. This focused on five major symptoms: nasal blockage, headache, facial pain, alteration of sense of smell, and nasal discharge. A total VAS score was calculated based on the sum of these five VAS symptom domains. In addition, patients were asked to rate his/her overall burden of CRS symptoms (14).

Findings on sinus coronal CT scans were graded using the Lund-Mackay CT scoring system (15). Endoscopy physical findings were scored according to Lanza and Kennedy (16). Patients were assessed by the questionnaire and endoscopy before and 12 months after the surgery. The CT scan was only performed before the surgery.

Nasal explant culture

Normal inferior turbinate mucosal tissue was collected during surgery and cut into multiple samples of maximum 6 mm³. One was processed for histological evaluation and the others were used for

ex vivo air–liquid interface culture as described previously (17). For dose response experiments, the tissue was incubated with dexamethasone (10^{-6} and 10^{-7} M, Sigma, St Louis, MO, USA), TNF- α (1, 10, and 100 ng/ml), IL-1 β (12.5, 25, and 50 ng/ml), IL-4 (1, 10, and 100 ng/ml), INF- γ (0.1, 1, and 10 ng/ml), or IL-10 (12.5, 25, and 50 ng/ml) for 6 or 12 h. The cytokines were purchased from R&D Systems (Minneapolis, MN, USA). For time course experiments, the tissue was incubated with dexamethasone (10^{-6} M), TNF- α (20 ng/ml), IL-1 β (25 ng/ml), IL-4 (20 ng/ml), INF- γ (10 ng/ml), or IL-10 (25 ng/ml) for various time durations between two and 24 h. In some experiments, tissues were pretreated with either 10 μ g/ml cycloheximide (Sigma) for 2 h or 5 μ g/ml actinomycin D (Sigma) for 1/2 h. The tissue was cultured at 37°C with 5% CO₂ in humidified air.

Quantitative RT-PCR

Freshly obtained tissues were immediately snap frozen in liquid nitrogen after surgery or culture. RNA was extracted and cDNA was reverse transcribed as previously described (17). cDNA equivalent to 25 ng total RNA was used to perform quantitative PCR. CC10 PCR was done with Assay-on-Demand Gene expression product (Applied Biosystems, Foster City, CA, USA) on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) as previously described (5). The PCRs for transcription factors, including hepatocyte nuclear factor-3 (HNF-3) α and β , thyroid transcription factor-1 (TTF-1), CCAAT/enhancer-binding protein (C/EBP)- α , β , γ and δ , and chicken ovalbumin upstream promoter transcription factor (COUP-TF) I and II were performed using the SYBR Premix Ex Taq kit [TaKaRa Biotechnology (Dalian), Dalian, China] with appropriate primers constructed from published sequences (Table S1). The PCRs were performed in the LightCycler system (Roche Diagnosis, Mannheim, Germany) as mentioned elsewhere (17). GAPDH was used as a housekeeping gene for normalization and 'no template' sample was used as a negative control. Relative gene expression was calculated by using the comparative CT method. An anterior ethmoid mucosa sample from a control subject was used as a calibrator in CC10 mRNA expression study, whereas respective control tissues without any stimulation were employed as calibrators for the CC10 gene regulation study.

ELISA

Six ml of blood in EDTA were collected and were centrifuged for 10 min at 1000 *g* at room temperature. The supernatant was separated and stored at -70°C until analysis. Freshly obtained tissue specimens were weighed and 1 ml of 0.9% sodium chloride solution supplemented with a protease inhibitor cocktail (Roche Diagnosis) was added per every 0.1 g tissue. The tissues were then homogenized on ice. After homogenization, the suspension was centrifuged at 4°C at 3000 rpm for 10 min and supernatants were stored in aliquots at -70°C for further analysis. CC10 levels in plasma and tissue homogenates were determined with ELISA according to the manufacturer's protocol (BioVendor Laboratory Medicine, Inc., Brno, Czech Republic). Cytokines levels in tissue homogenates were detected using ELISA kits from R&D Systems.

Routine staining, CC10 immunohistochemistry, and quantification

Paraffin sections (4 μ m) were prepared from each block and stained with hematoxylin and eosin. The number of inflammatory cells per high-power field was determined by counting 10 randomly selected fields in a blinded fashion at 400 $\times$ magnification.

As to immunohistochemical staining of CC10, the sections were stained with anti-CC10 antibody (1 : 800, Dako). CC10 was detected using the streptavidin-peroxidase complex method with a histostain-plus kit (Zhongshan Golden Bridge Biotechnology, Beijing, China) as described (17). Color development was achieved with 3',3'-diaminobenzidine, which rendered positive cells brown. Normal rabbit serum and secondary antibody alone were used as negative controls.

Western blotting

After culture, tissue specimens were harvested, weighed, and homogenized in lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Triton) with a protease inhibitor cocktail (Roche Diagnosis). Extracts were subjected to electrophoresis with a 12% SDS-polyacrylamide gel (Bio-Rad, Hercules, CA, USA) and then transferred onto nitrocellulose filters. The membranes were incubated with mouse anti-human γ -tubulin antibody (Sigma) at a 1/30000 dilution and rabbit anti-human HNF-3 α , HNF-3 β , and TTF-1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1/1000 dilution for 24 h at 4°C. Membranes were then washed and probed with peroxidase-conjugated secondary antibodies (1 : 5000, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 2 h at 37°C. Protein bands were developed with enhanced chemiluminescence reagent (Pierce, Rockford, IL, USA).

Statistical analysis

Data are presented as mean $\pm$ SD. Paired sets of RT-PCR and ELISA data were compared with Kruskal–Wallis *H* and Man–Whitey *U*-test. The Spearman test was used to determine correlations. Paired *t*-test was used in tissue culture data analysis. Differences were considered statistically significant at a *P*-value of <0.05.

Results

Inflammatory cells infiltration and cytokines levels in CRS patients with and without NPs

As shown in Table 2, compared with normal tissues, there were significantly more plasma cells, mononuclear cells, and total infiltrating cells in tissues from both CRS patients with and without NPs. However, the number of eosinophils was only significantly increased in CRS patients with NPs. When comparing CRS with and without NPs, the number of eosinophils and plasma cells was found to be significantly higher in CRS with NPs.

The ELISA results were also illustrated in Table 2. The protein levels of TNF- α , IL-1 β , and IL-10 were significantly higher in tissues from both CRS patients with and without NPs than in control tissues, whereas the levels of IL-4 did not differ significantly between CRS patients and controls. The levels of INF- γ were found to be significantly increased only in CRS patients without NPs compared with controls. Among CRS patients, patients with NPs showed significantly higher levels of IL-1 β and lower levels of INF- γ than patients without NPs.

Table 2. The number of inflammatory cells and protein levels of cytokines in sinonasal mucosa

	Controls	vs	CRS without NPs	vs	CRS with NPs	vs controls
Inflammatory cells (number/HP)						
Eosinophil	1.73 ± 1.72	NS	4.07 ± 8.18	<i>P</i> < 0.05	11.29 ± 20.73	<i>P</i> < 0.01
Plasma cell	1.49 ± 1.47	<i>P</i> < 0.01	7.57 ± 9.40	<i>P</i> < 0.05	9.81 ± 10.09	<i>P</i> < 0.01
Mononuclear cell	26.13 ± 15.71	<i>P</i> < 0.01	38.56 ± 21.40	NS	50.27 ± 32.42	<i>P</i> < 0.01
Total infiltrating cell	35.45 ± 16.86	<i>P</i> < 0.01	70.69 ± 46.15	NS	100.55 ± 74.02	<i>P</i> < 0.01
Protein cytokine (pg/ml)						
TNF-α	16.28 ± 11.73	<i>P</i> < 0.05	30.89 ± 22.66	NS	53.26 ± 41.09	<i>P</i> < 0.01
IL-1β	101.11 ± 68.31	<i>P</i> < 0.05	161.64 ± 92.39	<i>P</i> < 0.01	317.9 ± 128.45	<i>P</i> < 0.01
IL-4	34.02 ± 22.92	NS	41.78 ± 26.12	NS	45.77 ± 27.45	NS
INF-γ	49.81 ± 30.59	<i>P</i> < 0.01	259.01 ± 209.99	<i>P</i> < 0.01	70.80 ± 71.81	NS
IL-10	18.60 ± 14.42	<i>P</i> < 0.01	42.96 ± 26.88	NS	50.83 ± 35.17	<i>P</i> < 0.01

P < 0.05 was considered statistically significant. In inflammatory cells counting study, the number of subjects was 16, 40, and 40 for controls, CRS patients without and with NPs, respectively. In cytokine assay study, the number of subjects was 12, 17, and 20 for controls, CRS patients without and with NPs, respectively. CRS, chronic rhinosinusitis; HP, high power field; NP, nasal polyp; NS, no significant.

CC10 expression in CRS patients with and without NPs

As illustrated in Fig. 1A,B, CC10 expression at mRNA and protein levels was significantly decreased in CRS patients compared with controls. Within the CRS group, there was a significant further decrease of CC10 expression in patients with NPs. Comparing asthmatic and non-asthmatic patients, a significant decrease of CC10 expression was found in asthmatic patients in both CRS with and without NPs group (Fig. 1C,D). Immunohistochemical staining showed that CC10 was expressed by epithelial cells and weaker staining was found in CRS patients (Fig. 1E–G).

Contrast to the findings in sinonasal mucosa, no significant difference in plasma CC10 levels was found between controls, CRS patients with and without NPs [control (*n* = 16) vs CRS without NPs (*n* = 40) vs CRS with NPs (*n* = 40): 14.87 ± 4.25 ng/ml vs 13.86 ± 3.52 ng/ml vs 13.53 ± 3.2 ng/ml]. Furthermore, no correlation was found between CC10 levels in plasma and CC10 levels in sinonasal mucosa in all studied groups. However, when comparing the asthmatic and non-asthmatic patients, a significantly lower CC10 plasma concentration was found in asthmatic patients in both CRS with and without NPs group [asthmatic (*n* = 7) vs non-asthmatic (*n* = 33) in CRS without NPs group: 10.61 ± 4.3 ng/ml vs 14.54 ± 2.97 ng/ml; asthmatic (*n* = 9) vs non-asthmatic (*n* = 31) in CRS with NPs group: 11.72 ± 3.81 ng/ml vs 14.06 ± 2.86 ng/ml; *P* < 0.05 for both]. No difference in CC10 level in mucosa and plasma was found between atopic and nonatopic patients, and between smoking and nonsmoking patients (data not shown).

Correlation between CC10 local expression and CRS clinical features

A significant negative correlation was found between CC10 protein levels and preoperative CT scores in both CRS with

and without NPs patients [*r* = −0.49 (*n* = 40) and *r* = −0.40 (*n* = 40) in CRS without and with NPs group, respectively; *P* < 0.01 for both]. In addition, CC10 protein levels inversely correlated with endoscopy scores [*r* = −0.45 (*n* = 34) and *r* = −0.48 (*n* = 36) in CRS without and with NPs group, respectively; *P* < 0.01 for both], overall VAS symptom scores [*r* = −0.43 (*n* = 34) and *r* = −0.34 (*n* = 36) in CRS without and with NPs group, respectively; *P* < 0.05 for both], and total VAS symptom scores [*r* = −0.38 (*n* = 34) and *r* = −0.49 (*n* = 36) in CRS without and with NPs group, respectively; *P* < 0.05 for CRS without NPs and *P* < 0.01 for CRS with NPs] 12 months after the surgery, but not before the surgery in both CRS with and without NPs group.

Effect of cytokines on CC10 expression in sinonasal mucosa

The effect of TNF-α, IL-1β, IL-4, INF-γ, and IL-10 on CC10 gene expression was studied by means of nasal explant culture. Since previous studies showed that glucocorticoids could induce CC10 expression (3), we used dexamethasone as a positive control for our *ex vivo* culture experiment. As shown in Fig. 2, dexamethasone induced the CC10 mRNA expression in a time and dose dependent manner. Dose response and time course studies showed that TNF-α, IL-1β, and IL-4 inhibited, whereas INF-γ and IL-10 promoted CC10 mRNA expression (Fig. 2A,B). The maximum change of CC10 mRNA expression was observed between 12 and 24 h after stimulation. To further confirm the effect of these cytokines, we performed ELISA to detect protein levels of CC10 in cultured tissues after 24 h incubation. These results confirmed the data from studies of the mRNA (Fig. 2C).

Effect of cytokines on CC10 mRNA stability

Nasal inferior turbinate tissues were pretreated with actinomycin, and then incubated in the presence or

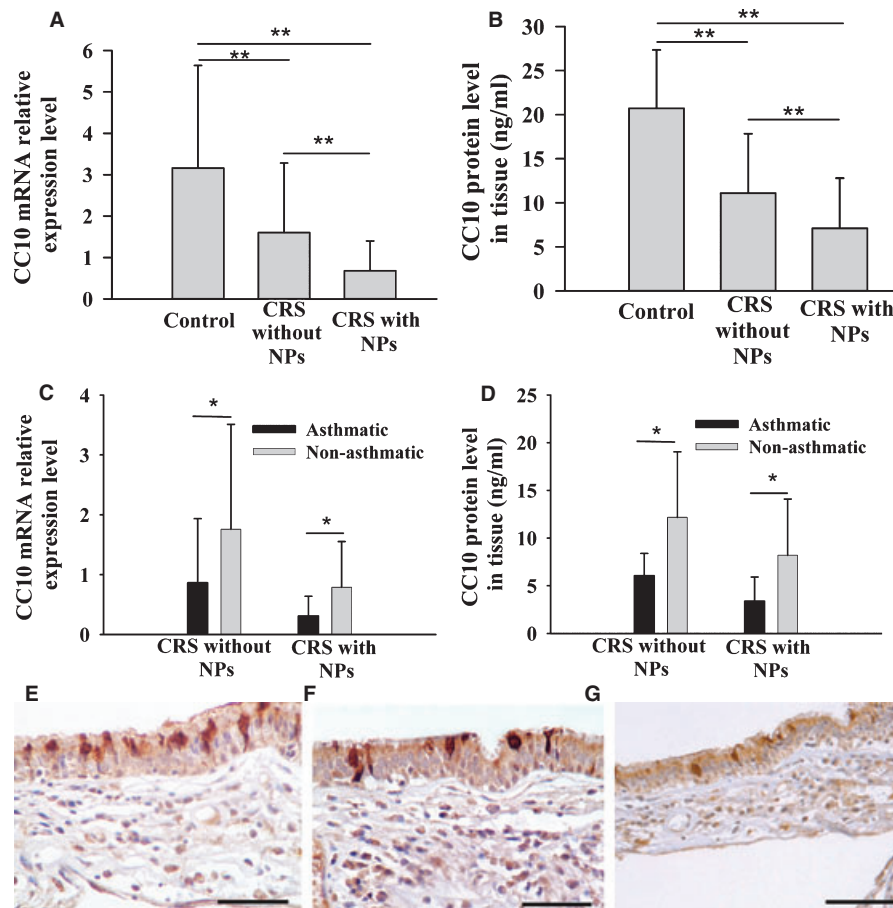


Figure 1. Expression of Clara cell 10-kDa protein (CC10) in sinonasal mucosa. (A–D) Quantification of CC10 mRNA levels in tissues with real-time reverse transcriptase polymerase chain reaction (RT-PCR) assay (A, C) and CC10 protein levels in tissues with enzyme-linked immunosorbent assay (ELISA) (B, D). In control group, $n = 16$; in chronic rhinosinusitis (CRS) without nasal polyps (NPs) group, $n = 40$ (7 asthmatic/33 nonasthmatic); and in CRS with NPs group, $n = 40$ (9 asthmatic/31 nonasthmatic). * $P < 0.05$; ** $P < 0.01$. (E–G) Representative photomicrographs of CC10 immunohistochemical staining of sinonasal tissue sections from controls (E), CRS patients without NPs (F) and with NPs (G). Scale bars, 50 μm .

absence of various cytokines. Total RNAs were isolated immediately (time = 0) or at 2, 4, 6, and 12 h after the addition of cytokine, and quantitative RT-PCR of CC10 was performed. A decay curve was generated by plotting the ratios of normalized intensities relative to the respective samples at time = 0. No significant alteration was seen in decay kinetics of CC10 transcripts in tissues treated with or without TNF- α , INF- γ , IL-4, or IL-10 (data not shown). In contrast, a significantly faster decay of CC10 transcripts was seen in IL-1 β treated tissues compared with untreated tissues (Fig. 3).

Effect of cytokines on the expression of CC10 gene relevant transcriptional factors

Since the maximum effect of cytokines on regulation of CC10 mRNA expression was achieved after 12 h treatment, we examined whether *de novo* protein synthesis is

required for the regulatory effect of cytokines. The tissues were pretreated with or without cycloheximide, and then incubated with various cytokines. We found that the effect of studied cytokines, except for IL-1 β , was abrogated by the protein synthesis inhibitor (data not shown). Next, we explored the expression of CC10 gene relevant transcriptional factors. We observed mRNA for HNF-3 α , β , C/EBP- β , γ , δ , and TTF-1 with the exception of C/EBP- α , COUP-TF I and II in normal inferior turbinate. IL-1 β and IL-10 induced TFF1 mRNA in a dose- and time-dependent manner. INF- γ increased, whereas IL-4 decreased mRNA for HNF-3 α (Fig. 4A,B). No change of other transcriptional factors and no effect of TNF- α on these transcriptional factors could be found (data not shown). The change reached maximum around 2–6 h and disappeared at 24 h after stimulation. We next performed Western blot analysis, and the protein data further confirmed the results from studies of the mRNA (Fig. 4C).

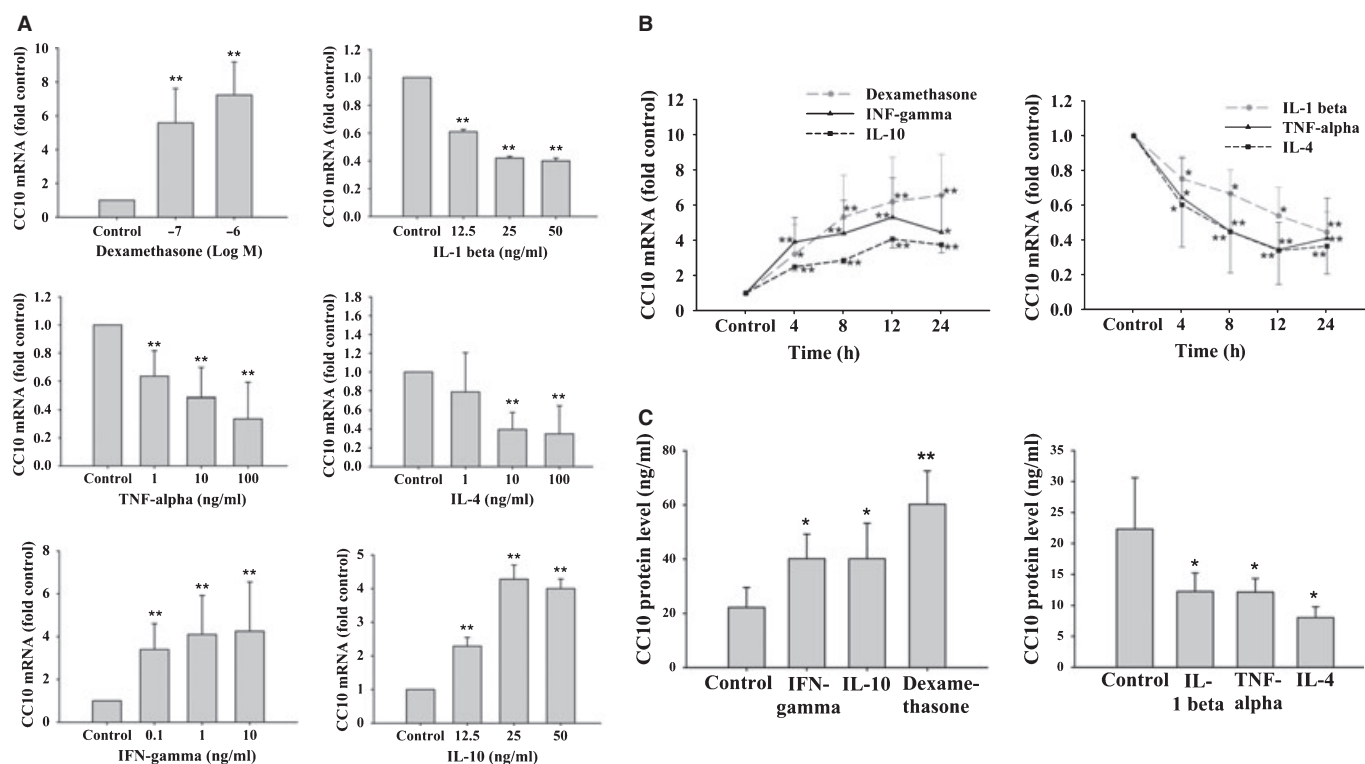


Figure 2. Clara cell 10-kDa protein gene regulation is differentially regulated by cytokines in normal nasal tissue. (A) Tissues were incubated with dexamethasone or cytokine at various concentrations for 12 h (at least three independent experiments). (B) Tissues were incubated with dexamethasone (10^{-6} M), TNF- α (20 ng/mL), IL-1 β (25 ng/mL), IL-4 (20 ng/mL), INF- γ (10 ng/mL), or IL-10 (25 ng/mL) for various time durations (at least four independent experiments). (C) Tissue was incubation with dexamethasone (10^{-6} M), TNF- α (20 ng/mL), IL-1 β (25 ng/mL), IL-4 (20 ng/mL), INF- γ (10 ng/mL), or IL-10 (25 ng/mL) for 24 h (at least four independent experiments). mRNA levels and protein levels in sinonasal mucosa were analyzed by means of real-time reverse transcriptase polymerase chain reaction assay and enzyme-linked immunosorbent assay, respectively. * $P < 0.05$; ** $P < 0.01$.

Discussion

Although the etiology of CRS with and without NPs is not completely understood, one important recognized

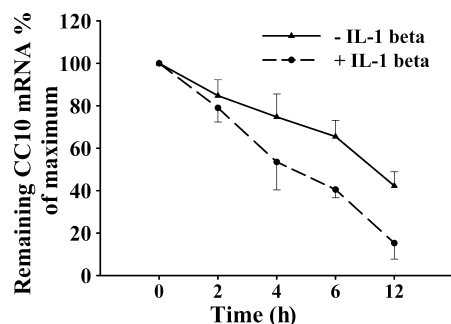


Figure 3. Decay kinetics of Clara cell 10-kDa protein transcripts. Nasal inferior turbinate tissues were pretreated with 5 μ g/mL actinomycin D for $\frac{1}{2}$ h, then incubated in the presence or absence of IL-1 β (25 ng/mL) for various time durations. Afterwards, quantitative reverse transcriptase polymerase chain reaction assay of CC10 was performed ($n = 4$).

feature of CRS is the persistent inflammation in sinonasal mucosa. In present study, significantly increased inflammatory cells infiltration and inflammatory cytokines expression were found in both CRS with and without NPs, which is consistent with previous reports (1, 2). Recent study has shown that CRS with and without NPs can be differentiated on the basis of the expression of inflammatory mediators. CRS without NPs manifests a Th1 polarization with high INF- γ concentration, whereas NPs presents a Th2 polarization with eosinophilic inflammation (9). In our present study, similar results were found. Comparing CRS with and without NPs, we found that the number of eosinophils and plasma cells was significantly higher in CRS with NPs, whereas INF- γ levels were significantly higher in CRS without NPs.

The factors inducing persistent inflammation in CRS remain uncertain, CC10 is a multifunctional protein with anti-inflammatory and immunomodulatory effects (3, 4). Recently, studies from us and others have shown that CC10 is also expressed in the upper airway and CC10 might also be involved in the pathogenesis of upper airway diseases (5, 18). Our previous microarray study showed that the CC10

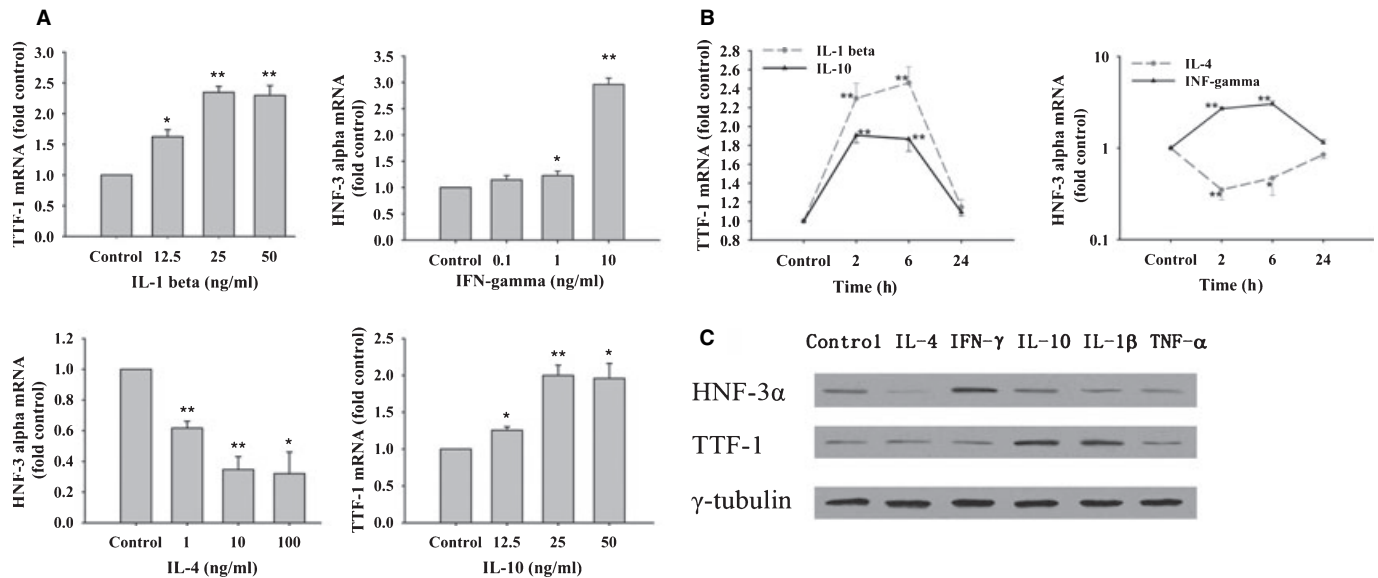


Figure 4. Expression of relevant transcriptional factors in the cytokine-driven Clara cell 10-kDa protein gene regulation. (A) Tissues were incubated with cytokines at various concentrations for 6 h ($n = 3$). (B) Tissues were incubated with IL-1 β (25 ng/mL), IL-4 (20 ng/mL), INF- γ (10 ng/mL), or IL-10 (25 ng/mL) for various time durations ($n = 3$). mRNA levels were analyzed by means of real-time reverse transcriptase polymerase chain reaction assay. (C) Tissues were incubated with TNF- α (20 ng/mL), IL-1 β (25 ng/mL), INF- γ (10 ng/mL), IL-4 (20 ng/mL), or IL-10 (25 ng/mL) for 12 h and then protein expression of transcriptional factors was assessed by using western blotting. One representative experiment of three independent experiments is shown. * $P < 0.05$; ** $P < 0.01$.

gene was the one most downregulated in human NP tissues (5). In the present study, we extended our previous study and found CC10 production was reduced in CRS without NPs too. However, when comparing CRS with and without NPs, a significant further decrease of CC10 expression was found in NPs. Moreover, asthmatic patients had a lower level of CC10 expression compared with nonasthmatic patients. Since CRS patients with concomitant NPs and asthma had higher CT scores and less improvement after ESS (16), our results suggest that CC10 may correlate with disease severity and may be a predictor of surgical response. Further study on these possible relationships supported this hypothesis. We found that CC10 levels in sinonasal mucosa negatively correlated with preoperative CT scores and postoperative symptom and endoscopy scores. However, CC10 did not correlate with preoperative symptoms and endoscopy findings. The reason for this discrepancy is unclear. One possible explanation might be that most symptoms and endoscopic appearance are improved after ESS and the variation between different individuals is reduced, then the relationship becomes apparent. Although ESS has clearly emerged as the treatment of choice for medically refractory CRS with excellent success rates, objectively identifying patients for whom ESS is likely to be successful remains a difficult task (16). Our results indicate the possibility of measuring CC10 levels in diseased sinonasal mucosa to predict the patients' response to ESS. However, further randomized controlled studies are needed to determine the prognostic value of CC10.

Previous studies on asthma showed that the plasma CC10 concentrations were significantly reduced in asthmatic patients compared with normal controls (4). As a corollary, we found that CC10 plasma levels were significantly lower in asthmatic patients than in nonasthmatic patients in both CRS patients with and without NPs. However, no significant difference was found between controls and CRS patients with or without NPs. Furthermore, no correlation between CC10 levels in plasma and CC10 levels in sinonasal mucosa could be found. These data suggest that CC10 production in the nose has minimal effect on plasma levels.

Previous studies have demonstrated that CC10 expression in lung tissue and pulmonary cell lines could be modulated by some cytokines, such as IL-4, INF- γ , and TNF- α (3, 11–13). However, compared with lung tissue, CC10 is produced by different types of cells in upper airways, and whether cytokines are also involved in CC10 gene regulation in nasal mucosa is unknown. In present study, we detect the effect of proinflammatory (TNF- α and IL-1 β), Th1 (INF- γ), Th2 (IL-4), and anti-inflammatory (IL-10) cytokines, which have been implicated in the pathogenesis of inflammatory and allergic upper airway diseases, on CC10 gene regulation in normal sinonasal mucosa. Our previous study showed that CC10 was expressed by goblet cells and nonmucus cells, but not by ciliated epithelial cells in sinonasal mucosa (5). Therefore, we generated nasal mucosa explants for *ex vivo* air-liquid interface culture, which simulated the *in vivo* nasal environment. We found INF- γ and IL-10

could induce, whereas TNF- α , IL-1 β , and IL-4 could inhibit CC10 expression in nasal mucosa. Our present study showed that TNF- α and IL-1 β concentrations were significantly elevated in both CRS with and without NPs; therefore, the CC10 gene down-regulation in CRS may result from, at least in part, enhanced expression of TNF- α and IL-1 β . Moreover, compared with CRS without NPs, significance higher levels of IL-1 β and lower levels of INF- γ were found in CRS with NPs, which may contribute to the more prominent downregulated CC10 expression in NPs. IL-10, whose expression was also up-regulated in CRS, was found to promote the expression of CC10, indicating a possible negative feedback role of IL-10 in CRS. The opposing role of proinflammatory, Th1, Th2, and anti-inflammatory cytokines in CC10 gene regulation may have significance in the pathogenesis of various inflammatory responses in upper airways, wherein decreased CC10 expression is noted. Moreover, the mutual regulatory axis between cytokines and CC10 may exaggerate and perpetuate the inflammatory reaction in the upper airway. CC10 expression is highly regulated and the mechanisms underlying the regulation are very intriguing. Since it is well established that mRNA stability can play a central role in the regulation of gene expression, we first examined the effect of cytokines on CC10 mRNA stability. We found that the suppressive effect of IL-1 β on CC10 expression is mediated, at least in part, by a decrease in CC10 mRNA stability, whereas TNF- α , INF- γ , IL-4, and IL-10 modulate CC10 expression at the level of transcription. Analysis of the lung-specific expression of CC10 in rodents has resulted in the identification of several trans-acting factors, including HNF-3, TTF-1, and C/EBP, which positively regulate the activity of this gene (10), and COUP-TF, which negatively regulate the activity of this gene (19). Since we found *de novo* protein synthesis is required for the regulatory effect of cytokines except for IL-1 β , we explored the involvement of these transcriptional factors in CC10 gene regulation in human upper airway. In normal nasal mucosa, we could not detect the mRNA expression for C/EBP- α , COUP-TF I and II before or after cytokine stimulation, indicating these three transcription factors might not be involved in CC10 gene regulation in the upper airway. Although the mRNA expression of HNF-3 β , C/EBP- β , γ , and δ did not change after stimulation with cytokines, we can not preclude the possible involvement of these transcription factors in CC10 gene regulation, since the DNA-binding of these transcription factors may be altered without change in protein levels. As to TNF- α , no change in all these studied transcription factors was noted, indicating other transcription factors or mechanisms may be involved. IL-10 could induce TTF-1 expression. Surprisingly, IL-1 β was found to increase TTF-1 expression too, which seems to contradict to its effect on CC10 expression. It indicates

other mechanisms may be more important in the IL-1 β -driven CC10 gene regulation, such as alteration of mRNA stability as demonstrated in our present study. Interestingly, we found IL-4 inhibited, whereas INF- γ enhanced the HNF-3 α expression, which agrees with their effect on CC10 expression. The coincidence between the change in transcript factors and CC10 gene expression suggests that these factors may be involved in the CC10 gene regulation in upper airways. However, obviously, additional functional studies are needed to define their exact role, such as analyzing DNA binding activity (electrophoretic mobility shift analysis), observing the influence of altering transcription factors levels artificially on CC10 gene expression, and exploring the effect of mutation of transcription factor binding site on CC10 gene regulation, etc. Unfortunately, currently, these analyses have been hampered by the lack of a cell line in which the endogenous expression of human CC10 and a full complement of specific trans-acting factors are present to mirror the *in vivo* CC10 expressing cell.

Taken together, our results suggest that reduced production of CC10, perhaps in response to a Th2 environment, accompanies both CRS with and without NPs and correlates with disease severity and response to ESS.

Funding

This study was supported by National Nature Science Foundation of China (NSFC) grant 30500557, scientific research foundation for the returned overseas Chinese scholars of State Education Ministry (SRF for ROCS, SEM) [2006]331, and program for New Century Excellent Talents in University from State Education Ministry (NCET-07-0326) to Dr Zheng Liu.

Conflict of interest

None

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primers used for quantitative polymerase chain reaction analysis of transcriptional factor gene expression.

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SUPPLEMENTARY MATERIAL

Manuscript ALL-1847

Allergy 2008 DOI:10.1111/j.1398-9995.2008.01847.x

Clara cell 10-kd protein expression in chronic rhinosinusitis and its cytokine-driven regulation in sinonasal mucosa

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SUPPLEMENTARY MATERIAL

TABLE S1. Primers used for quantitative polymerase chain reaction analysis of transcriptional factor gene expression

Primer	Sequence	Annealing temperature (°C)
HNF-3 α ¹	[s] 5'- CATTGCCATCGTGTGCTTGT -3' [a] 5'- CCCGTCTGGCTATACTAACACCAT -3'	60
HNF-3 β ²	[s] 5'- AGTTAATATGCTGGGAGCGGTG -3' [a] 5'- TGTACGTGTTTCATGCCATTCA -3'	60
C/EBP- α ³	[s] 5'- GGGCCAGGTCACATTGTAAA -3' [a] 5'- AGTAAGTCACCCCCTTAGGGTAAGA -3'	60
C/EBP- β ⁴	[s] 5'- TGATAAACTCTCTGCTCTCCC -3' [a] 5'- AAACATCAACAGCAACAAGCC -3'	55
C/EBP- γ ⁴	[s] 5'- AACGACCCATACCTCAGAC -3' [a] 5'- ACAAATGTACCTTAGCTGCATC A-3'	55
C/EBP- δ ⁴	[s] 5'- AACGACCCATACCTCAGAC -3' [a] 5'- ACAAATGTACCTTAGCTGCATC-3'	55
TTF-1 ⁵	[s] 5'- AAGTTCTCACAGATCAGCAGTCAAAG -3' [a] 5'- TTCTTCGACAGCCTTGATTAGTTTC -3'	60
COUP-TFI ⁶	[s] 5'- GCTGCCTCAAAGCCATCGTG -3' [a] 5'- AAGGTCTAGGAGCACTGGATGGA -3'	60
COUP-TFII ⁶	[s] 5'- GACAAGCAGCAGCAGCAGCAA -3' [a] 5'- CGAACTGCCCCGTCGTGGGTCGG -3'	66
GAPDH ⁷	[s] 5'- GAAGGTGAAGGTCGGAGTC -3' [a] 5'- GGAAGATGGTGATGGGATT -3'	66

HNF, hepatocyte nuclear factor; C/EBP, CCAAT/enhancer-binding protein; TTF-1, thyroid transcription factor-1; COUP-TF, chicken ovalbumin upstream promoter

transcription factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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