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ORIGINAL PAPER

Primary role of growth-related oncogene- α and granulocyte chemotactic protein-2 as neutrophil chemoattractants in chronic rhinosinusitis

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Keywords

- ☐ chemokines

Summary

Background The aetiology of chronic rhinosinusitis (CRS) remains unclear. The purpose of this study was to investigate neutrophil-attracting chemokine patterns in CRS without nasal polyposis.

Methods The biological activity of the chemokines was identified using a two-step high-performance liquid chromatography (HPLC) technique combined with a bioassay in extracts from 55 CRS patients, and in the turbinate mucosa (TM) of patients ($N=51$) undergoing septoplasty. The biologic activity of each chemokine was assessed using blocking antibodies to chemokines. Immunolocalization of detected neutrophil chemokines was performed by quantitative evaluation of immunohistochemistry. Besides, PCR analysis was performed to quantify neutrophil chemokine mRNA.

Results In CRS, the chemokines primarily detected by two-step HPLC were growth-related oncogene- α (GRO- α) and the granulocyte chemotactic protein-2 (GCP-2). Blocking of GCP-2 and GRO- α each resulted in chemotaxis inhibition rates of 43.3% and 35.9%, respectively, whereas anti-IL-8 and anti-ENA-78 had no effect. Both GCP-2 and GRO- α were generally synthesized by the surface epithelium and mucosal glands while GRO- α in particular was synthesized by endothelial cells, as shown by immunohistochemistry. The concentrations of the chemokines IL-8 and epithelial cell-derived neutrophil attractant-78 (ENA-78) were low in CRS and TM.

Conclusion It appears that both GRO- α and GCP-2 contribute to neutrophil chemotaxis in CRS, whereas IL-8 and ENA-78 appear to be of secondary importance for the chemotaxis of neutrophils in this condition. The expression of chemokines in mucosal gland cells is the main phenomenon involved in constitutive neutrophil chemotaxis in the TM.

Introduction

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Rhinosinusitis is an inflammation of one or more paranasal sinuses, involving the mucosal lining of the nasal cavity. Owing to its unknown aetiology, the absence of adequate definitions for the entities of sinusitis and insufficient knowledge of the agents triggering sinusitis, rhinosinusitis is diagnosed on the basis of its symptoms. In clinical terms, rhinosinusitis is graded as acute when the signs or symptoms appear within a few minutes or hours, subacute when it spans a few days or weeks and chronic when it occurs over a few weeks or months [1, 2].

The most commonly studied condition in chronic rhinosinusitis (CRS) is nasal polyposis originating from ethmoidal cells and the middle meatus, forming oedematous masses in the nasal and the paranasal cavities and resulting in nasal obstruction and abnormal secretion, loss of olfactory sense and impairment of general well-being [2, 3].

About 30% of patients with symptoms of CRS have no nasal polyposis. Endoscopy of the middle meatus yields no pathological findings [endoscopy-negative (-)], whereas the sinus CT scans of these patients show pathological mucosal swellings [4].

Although aetiological factors contributing to chronic non-polypous rhinosinusitis, e.g. ostial blockage, delayed recovery of mucociliary function, mucus recirculation, and microbial factors in sinusitis have been defined, knowledge concerning the natural history and inflammatory mediators related to CRS is still scarce [5–10]. Studies focusing on alterations in the balance between pro-inflammatory and anti-inflammatory mediators disclosed new aspects in the pathophysiology of this condition, which may well be used as new therapeutic targets.

Neutrophil chemokines belong to the group of pro-inflammatory mediators that attract and activate neutrophils. Owing to their role as first-line defence cells of the innate immune system, neutrophils are equipped with a battery of effector molecules for the destruction of bacteria and other invading microorganisms. In addition, neutrophils can respond very rapidly (within minutes) to signals such as chemotactic gradients generated by ELR-positive CXC chemokines. The expression of chemokines in various tissues is differently regulated during acute and chronic immune response [11].

- ☐ chronic rhinosinusitis
- ☐ epithelium
- ☐ growth-related oncogene- α (GRO- α)
- ☐ neutrophils
- ☐

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Image Previews

Based on the position of conserved cysteines, chemokines are divided into four subfamilies: CXC, CC, C and CX3C chemokines [12, 13]. CXC chemokines containing one amino acid between the two NH₂-terminal cysteine residues can be further divided into two subgroups, depending on the presence or the absence of the sequence Glu–Leu–Arg (ELR) immediately in front of the first cysteine residue. The ELR/CXC chemokines growth-related oncogene- α (GRO- α)/CXCL1, GRO- β /CXCL2, GRO- γ /CXCL3, epithelial cell-derived neutrophil attractant 78 (ENA-78)/CXCL5, granulocyte chemotactic protein-2 (GCP-2)/CXCL6 and IL-8/CXCL8 chemoattract and activate neutrophils, whereas ELR-negative CXC chemokines mainly act on lymphocytes [14–16].

The aim of this study was to investigate the neutrophil chemokine profile of chronic inflamed sinus tissue without nasal polyposis and to compare it with non-chronically inflamed nasal tissue of the inferior turbinate. Chemotactic activity was detected by a biochemical extraction method, as this method facilitates the detection of biologically active neutrophil chemokines that characteristically become activated at the protein level by truncation at their NH₂ terminal end. Furthermore, the immunolocalization and mRNA of neutrophil chemokines were analysed by immunohistochemistry and PCR, respectively.

Methods

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Patients and methods

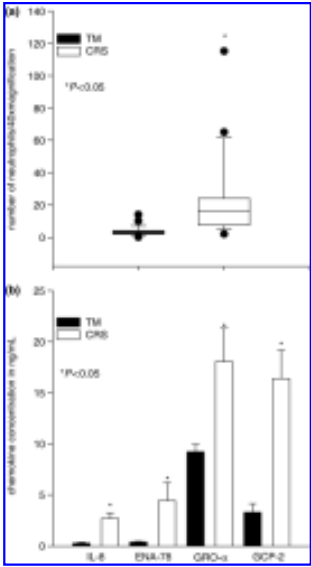
Nasal tissue was obtained from patients with CRS without nasal polyposis. Tissue specimens were taken from the ethmoid or maxillary sinus during routine surgery at the Department of Otorhinolaryngology in the University Hospital in Münster, Germany. CRS was diagnosed on the basis of case histories, clinical examination, nasal endoscopy, sinus CT scans, and the duration of symptoms in 51 subjects (26 males, 25 females). The duration of the illness, based on the patients' medical history was 38.8±4.3 months (mean±SEM); range, 7 months to 6 years. No patient had undergone sinus surgery or had experienced the disease previously, and no patient had received treatment (oral or topical steroids, antibiotics) during 4 weeks before the operation. The atopic status was evaluated by skin prick tests (SPTs) to common inhalant allergens and RAST tests. Eight patients had an allergy (positive SPT and RAST), of whom five had an intermittent allergy with mild symptoms and three had a persistent allergy with mild symptoms. Turbinate mucosa (TM) was obtained from 55 patients, of whom 10 were allergics with a positive SPT and RAST (all of them had intermittent allergy with mild symptoms). These patients were sinus healthy, and underwent septumplasty and conchotomy of the inferior turbinate (in allergics, the procedure was performed outside the allergy season). Informed consent was obtained from all patients and the study was approved by the ethics committee of the University of Münster.

Chemokine extraction method

Tissue samples from patients having CRS (N=51; tissue weight, 30 g) without nasal polyposis, and tissue samples of TM (N=55; 25 g tissue weight) were each pooled in portions of 5 g tissue and suspended in 0.1 m citric acid. After the addition of 96% ethanol, the suspension was homogenized in an ice–water mixture. The homogenized tissue was centrifuged at 1000 *g* for 30 min at 4°C, and the opalescent supernatant was concentrated with ultrafilters (YM3 filters, cut-off of 3 kDa, Amicon, Danvers, MA, USA) to portions of 5–7 mL each [17]. In the crude extract, the chemokine concentration was measured by ELISA as described below.

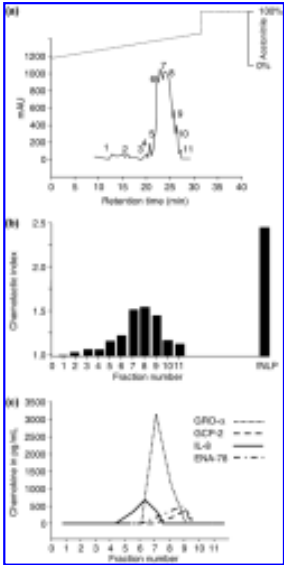
Concentrated supernatants of 20 g tissue CRS or TM were passed through a heparin–sepharose cartridge (Hi-Trap, 10 × 5 mm, Pharmacia, Freiburg, Germany) at 0.5 mL/min. Proteins bound to the column were then eluted using 2 m NaCl in equilibration buffer. The eluate was collected manually as one fraction (15 mL) and tested for neutrophil chemotactic activity.

Proteins stripped from the heparin column were acidified to pH 2–3 with formic acid, concentrated (in the Amicon chamber) and diafiltrated against 0.1% trifluoroacetic acid (TFA) using Amicon YM3 filters (cut-off 3 kDa) and then applied to a preparatory RP-8 high-performance liquid chromatography (HPLC) column (C8 nucleosil with end capping, 250 × 12.6 mm, 7 •m particle size, Marcherey-Nagel, Düren, Germany) previously



[Full Size]

Fig. 1. Number of neutrophils and neutrophil chemokine concentration in TM and CRS. (a) Neutrophil co...



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equilibrated with water/acetonitrile/TFA (90 :10 : 0.1 v/v/v). UV absorbance was monitored at 215 nm, and fractions were taken manually according to peaks or shoulders of UV absorbance at a flow rate of 3 mL/min.

Isolation of neutrophils

Venous blood obtained from healthy donors was collected into citric acid/dextran and centrifuged for 20 min. The plasma, as well as the buffy coat (mononuclear cells), were pipetted away. The remaining blood cell sediment was mixed with a gelatin solution pre-heated to 37°C and stored at 37°C for 30 min. The yellowish supernatant was then collected and centrifuged at room temperature. Ammonium chloride (0.15 m) was added in order to lyse red blood cells. The suspension was again centrifuged and the sediment thus obtained was washed with PBS. The final cell preparations contained more than 90% PMN (>85% neutrophils); their viability was greater than 97% as assessed by the trypan blue exclusion method.

Neutrophil chemotaxis assay

HPLC fractions in 60 •L aliquot from the RP-8 column were lyophilized, dissolved in PBS containing 0.1% bovine serum albumin (BSA) [(c) complete phosphate-buffered saline (PBS)] and assayed for neutrophil chemotactic activity. Neutrophil chemotaxis was performed using blind-well Boyden chambers. The Boyden chambers were filled with samples at appropriate dilutions and covered with a polyvinylpyrrolidone-containing filter (pore size 3 •m: Costar, Bodenheim, Germany), as described previously [18]. Neutrophil chemotactic activity was expressed as a chemotactic index, which was calculated as stimulated migration divided by random migration (migration towards buffer). Test series for each fraction were performed in duplicate. When duplicates differed by more than 20%, the duplicate tests were repeated.

Chemotaxis neutralization tests

The inhibitory action of antibodies to IL-8, GRO- α , GCP-2 and ENA-78 on the chemotactic activity of crude extracts (CRS and TM) was tested using the Boyden chamber chemotaxis assay, as described earlier [19]. Serial dilutions of the different antibodies were tested in concentration response curves of all four recombinant chemokines according to the manufacturer's recommendation. Inhibitory concentration was established at a dilution of 1 : 50 for all chemokines (mAb IL-8 from R&D, Wiesbaden, Germany, and anti-rabbit GCP-2, GRO- α , -ENA-78 from PeproTech EC, London, UK), were pre-incubated with 500 •L of lyophilized crude extracts of TM and CRS homogenates. All tests were carried out four times.

Enzyme-linked immunosorbent assay measurements and recombinant chemokines

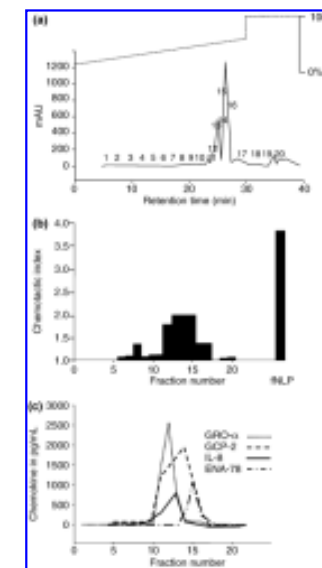
ELISA tests for the chemokines IL-8 (minimal detectable concentration 3.5 pg/mL), GRO- α (minimal detectable concentration 10 pg/mL), ENA-78 (minimal detectable concentration 15 pg/mL) and GCP-2 (minimal detectable concentration 1.6 pg/mL) were carried out according to the manufacturer's recommendations (R&D, Wiesbaden, Germany) in six extracts of CRS and five extracts of TM. The optical density of the samples was measured at a wavelength of 450 nm.

Histochemistry

Tissue preparation. Immediately after surgery, 41 rhinosinusitis specimens and 35 TM specimens were embedded in paraffin. Five-micrometre-thick sections were prepared for haematoxylin–eosin staining in order to perform histologic investigations. Fifteen tissue specimens of CRS and TM sections were then deparaffinized in parasolve, and the slides were hydrated through graded ethanol before use. Tissue sections were then treated with 5% trypsin solution (trypsin, Difco, Hamburg, Germany). The slides were washed in cold Tris-buffered saline (TBS containing 1 vol% CaCl₂; Merck, Darmstadt, Germany), covered in citrate buffer (pH 6.0) and heated in a microwave for 15 min.

The activity of endogenous peroxidase was stopped by sodium azide containing perhydrol in PBS (0.01 m). After blocking for non-specific bindings

Fig. 2. Chemotactic index and concentrations of chemokines in turbinate mucosa. Supernatant collected...



[Full Size]

Fig. 3. Chemotactic index and concentrations of chemokines in mucosa of chronic rhinosinusitis (CRS)....

with 2% BSA (Behring GmbH, Harburg, Germany), the slides were incubated for 45 min at room temperature with primary antibodies as listed in [Table 1](#). These primary antibodies were replaced by a non-immune serum of the first antibodies for the negative control.

Sections were washed twice with PBS for 5 min and incubated for 30 min with a second antibody, as indicated in [Table 1](#). The tissue sections were washed again in PBS. After an additional wash, the slides were stained with a chromogen solution for 30 min. Sections were counterstained in Mayer's haemalum (16) for 90 s, blued in tap water and cover-slipped with Kaiser's glycerol gelatin (Merck).

Four high-power fields were randomly observed on each slide by two blinded observers. Positively stained cells (epithelial cells, neutrophil and mononuclear cells) were counted under a light microscope (Leitz, Oborkocken, Germany, $\times 1000$ magnification). Positively stained endothelial cells and mucosal gland cells were counted in terms of total vessel numbers and mucosal gland complexes at $\times 400$ magnification. The count was divided by the total number of cells (vessels or mucosal gland complexes) in each field. The mean of four fields was the marker expression estimation for each sample. Furthermore, each field was evaluated for the proportion of stained cells (vessels) and the intensity of overall staining. The proportion of stained cells in each field was assessed as follows: 0, no stained cells; 1, <25% stained cells; 2, 25–50% stained cells; and 3, >50% stained cells. Staining intensity was graded as follows: 0, negative staining; 1, mild staining; 2, moderate staining; and 3, intensive staining. A staining-intensity distribution (SID) score was computed for each sample as follows: the score of the proportion of stained cells for each field was multiplied by the score of the staining intensity in that field to yield an SID score for the field. The mean of the four fields was the SID score for the sample.

Semiquantitative reverse transcriptase polymerase chain reaction

RNA extraction. We examined 15 cases of CRS and 15 TM specimens. Total RNA was isolated using the RNeasy minikits from Qiagen (Düsseldorf-Ratingen, Germany) according to the manufacturer's instructions. One microgram RNA was subjected to reverse transcription using the Promega Reverse Transcription System.

Quantitative TaqMan polymerase chain reaction. Quantitative PCR analysis was performed on the ABI Prism® 7900HT detections system (Applied Biosystems, Foster City, CA, USA). Gene expression assays (Assay-on-Demand™, 250 μ L, 20 $\times$) suitable for this system are based on a 5' nuclease chemistry and consist of two unlabelled PCR primers for amplifying the sequence of interest and a 6-FAM dye-labelled TaqMan® MGB probe (Applied Biosystems) for detecting the sequence of interest.

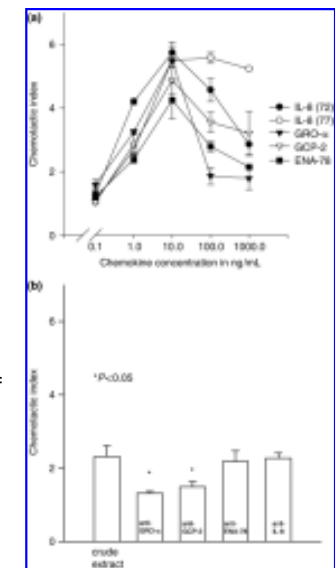
IL-8 and GAPDH-gene expressions were analysed using a commercial gene expression assay from Applied Biosystems (Assay-on-Demand™: IL-8, assay no. Hs 00174103m1 RefSeq NM-000584.2; translated protein NP-000575.1; GAPDH, assay no. Hs 99999905m1; RefSeq NM 002046.2, translated protein NP 002037.2).

Individual gene expression assays were designed for the detection of ENA-78, GRO- α and GCP-2 gene expression. (Assay-by-DesignSM, Applied Biosystems): GRO- α F: CCCCAGTGC GCCCAA, GRO-R: CAAGCTTTCGCCCATCTT, ENA-78F: CCCACAGTGCTCCAAGGT, ENA-78R: TCAAGACAAATTTCTTCCCGTTCT; GCP-2F: CCGCAGTGCTCCAAGGT, GCP-2R: AGACAACTTGCTTCCCGTTCTT).

All primers and probes were functionally tested on a human cDNA pool.

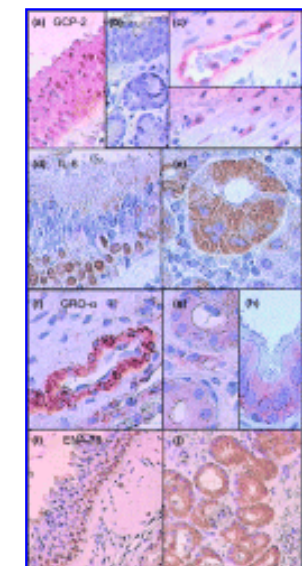
Preparation of polymerase chain reaction

The following components for each PCR were filled in a single well of a 384 PCR plate using a robotic pipette system (TECAN, Männedorf, Switzerland, Genesis Workstation): 4 μ L of 2 $\times$ TaqMan® Universal PCR Master Mix (Applied Biosystems), 0.4 μ L of 20 $\times$ Assay-Mix (Assay-on-Demand™/Assay-by-DesignSM), 2 μ L of diluted cDNA (1 : 100) and 1.6 μ L of RNase-free water. The reaction itself was performed for all gene expression assays according to the following thermocycler protocol: 2 min 50°C, 10 min 95°C, 40 cycles (15 s 95°C, 60 s 60°C). Double



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Fig. 4. Concentration–response curves and neutralization test. (a) Chemokine response curves were esta...



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appeared in the same concentration range: about two to threefold lower than that of GCP-2 and GRO- α . Although a minor aliquot had been taken for chemokine quantification, the concentration of chemokines detected here was higher than that in the TM. Neutrophil chemotactic activity in the Boyden chamber test was the highest in fractions 13–15, using 60 μ L volume lyophilized from each fraction for the Boyden chamber test (Fig. 3). The chemokine profile in TM was mainly characterized by GRO- α , followed by IL-8, GCP-2 and ENA-78, whereas GCP-2, in addition to GRO- α , was detected in a higher concentration in CRS.

Concentration–response curves

Concentration–response curves were established for each chemokine protein using two isoforms of recombinant IL-8 ([Ser⁷²], [Ala⁷⁷]), ENA-78, GRO- α and GCP-2, in order to determine the individual chemokine action.

The curves ($n=8$ for each chemokine) showed different bell-shaped, non-sigmoid forms with maximal chemotactic activities in different concentration ranges (Fig. 4a). Recombinant 72 amino-acid IL-8 was a more potent stimulator of neutrophil chemotaxis than the respective 77-residue [2, 20]. GRO- α , GCP-2 and ENA-78 are less potent than IL-8 isoforms (effective concentration for maximal chemotactic activity: GRO- α >GCP-2>ENA-78).

Neutralization tests

Neutralization test were performed on the crude extracts of TM and CRS (Fig. 4b) in order to determine the individual chemoattractant activity of each chemokine. As the overall chemotactic indexes of TM extracts (data not shown) were very low (CI below 1.5), the inhibition tests could not be performed. In CRS crude extracts, the overall chemotactic index was about 2.2. Anti-GRO- α and anti-GCP-2 blocked 43.3% and 35.9% of the chemotactic activity, respectively. No inhibition was achieved by anti-IL-8 or anti-ENA-78.

Immunostaining of chemokines

Turbinate mucosa. Different structural components of the mucosa were evaluated for quantitative immunostaining of the chemokines, with a distinction being made between the different cell types such as mucosal gland cells, surface epithelium, vessel endothelium, neutrophils and mononuclear cells (Tables 2A and B).

In general, all four chemokines were mainly detected in mucosal gland cells and the surface epithelium of TM. GCP-2 showed stronger expression in mucosal gland cells than IL-8 or ENA-78. Nearly 60% of the mucosal glands were GCP-2 positive, whereas neither IL-8 nor ENA-78 was regularly found in mucosal glands within one specimen, and no IL-8 and ENA-78 were detected in four of 15 specimens. IL-8 and GRO- α were detected in about 20–35% of neutrophils and mononuclear cells.

The basal cell layer of the epithelium in TM expressed IL-8 and ENA-78 protein, but both chemokines showed equally low expression. GRO- α staining was more pronounced than GCP-2 staining in the epithelium (Fig. 5).

In addition, GRO- α was found to exhibit strong positive immunostaining of endothelial cells in TM. Approximately 80% of the vessel endothelium was GRO- α positive, whereas GCP-2 was very weakly detected.

Mucosa of chronic rhinosinusitis. In CRS, GCP-2-positive cells were strongly represented in the basal and upper layer of the epithelium, in contrast to the SID score ($P<0.005$) and the number of stained cells in the TM ($P<0.0005$) (Fig. 5, Table 2).



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Table 2. Immunohistochemical staining expression and staining intensity distribution (SID) scores for...

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Primary role of growth-related oncogene- α and granulocyte chemotactic protein-2 as neutrophil chemoattractants in chronic rhinosinusitis.

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As in TM, IL-8 and ENA-78 were co-expressed in the basal epithelial layer of CRS, but not regularly found in the basal epithelial layer within one slide. The number of IL-8-positive epithelial cells and the SID score differed significantly from those measured in TM ($P<0.005$).

GRO- α expression in the epithelium of CRS was significantly higher, based on the number of positive cells and the SID score. More neutrophils expressed IL-8 and GRO- α compared with TM ($P<0.0005$), but the SID score was not significantly different.

Interestingly, mononuclear cells were positive for GCP-2 in 60% (cell count, $P<0.0005$). The SID score was also significantly higher for GCP-2 in mononuclear cells.

Semiquantitative Taqman® polymerase chain reaction of chemokines

Comparing mRNA levels in CRS and TM, we registered the lowest levels of all chemokines in TM (Fig. 6). In CRS, we measured significantly up-regulated mRNA levels for GRO- α and GCP-2, whereas IL-8 and ENA-78 mRNA seemed to be higher, but without significance. mRNA levels corresponded to protein levels.

Discussion

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Inflammatory mediators occurring in the mucosal linings of the nose and the paranasal sinuses might play a significant role in the pathophysiology of CRS without nasal polyposis. It remains unclear whether an imbalance of pro-inflammatory and anti-inflammatory mediators is responsible for the chronification of rhinosinusitis. The aim of the study was to determine the neutrophil-attracting pattern of chemokines according to their biological potency on the protein level, mRNA level and their expression in tissue, compared with non-chronically inflamed mucosa of nasal turbinates.

In tissue specimens of CRS, we registered a significantly higher number of neutrophils than in TM; the majority of the neutrophils in CRS were positive for neutrophil elastase. The latter is a marker for the activation of neutrophils. As neutrophil elastase has been functionally shown to increase vascular permeability in inflamed tissue, we recently measured the albumin content in CRS specimens [20, 21]. It was found that albumin was significantly elevated compared with the TM. Comparison of IL-8 levels in tissue specimens of CRS and TM in the past had also revealed no differences [22]. These data led us to consider a more differentiated investigation of neutrophil-attracting chemokine patterns.

To establish the biological function of neutrophil-attracting chemokines in CRS, we extracted leucocyte chemotactic factors from tissue specimens. Given the known biological properties of chemokines with a low molecular mass, it was essential to extract the proteins by adding ethanolic citric acid, as the extraction at a neutral pH would be disturbed by high-molecular-mass proteins. The addition of ethanolic citric acid concentrates low-molecular-mass proteins and allows better extraction of extracellular-matrix-bound chemokines [23].

Large quantities of GRO- α and GCP-2 were found in crude extracts of CRS, whereas both were less in TM. The outcome of HPLC revealed that in CRS, GRO- α dominates the chemokine profile in terms of concentration, followed by GCP-2, ENA-78 and IL-8. In TM, GRO- α also outweighs all other chemokines with regard to the level of protein expression, followed by lower levels of IL-8, GCP-2 and ENA-78. Biological activity was low due to generally low concentrations of the chemokines, as shown in crude extracts of TM. The results of HPLC chemokine extraction were in concordance with the measured mRNA levels, thus confirming the validity of our results.

In order to determine the individual chemokine activity, standardized concentration response curves were established. IL-8 showed the strongest chemotactic activity (bell-shaped curves) with respect to their NH₂-terminal truncation, as shown by the example of IL-8 in the 72-amino acid sequence vs. the 77-amino acid sequence [23]. The chemotactic activity of IL-8 was followed by GRO- α , GCP-2 and ENA-78. These results showed

that the extracted chemokine concentrations GCP-2 and GRO- α were below the maximum of the respective chemotactic potential. Thus, the impact of the extracted quantities of GCP-2 and GRO- α on chemotaxis was confirmed by the neutralization tests on crude extracts.

These results led to the hypothesis that, among all neutrophil chemokines, GRO- α and GCP-2 are likely to play a primary neutrophil-attracting role in CRS, whereas IL-8 seems to be of minor importance. The concentrations and chemotactic potencies of GCP-2 and GRO- α differed between the two types of tissue; both were more pronounced in CRS. A striking phenomenon was the large quantities as well as the biological activity of GCP-2 in CRS compared with TM, which had never been previously detected in the paranasal mucosa or described in the published literature.

Similar data were obtained when comparing the chemokine profiles of acute and chronic inflammatory conditions in tonsils. Here, GRO- α outweighed IL-8, whereas IL-8 was detected as the most potent neutrophil chemotactic and activating protein in peritonsillar abscesses [18]. These data, and the quantification of IL-8 levels in tissue specimens of acute rhinosinusitis [22] clearly indicate the function of IL-8 in the regulation of acute inflammatory processes. However, no data are as yet available concerning proteins that regulate the termination processes in acute inflammatory disorders or the perpetuation of chronic inflammatory disorders.

The second body of evidence for GRO- α and GCP-2 playing a primary role in CRS was obtained by immunohistochemistry. GCP-2 was strongly expressed in mononuclear cells, endothelial cells, and on the surface epithelium in CRS, whereas in TM GCP-2 was regularly found in mucosal gland cells. In both tissues, i.e. TM and CRS mucosa, neutrophils are positive for IL-8 and GRO- α ; however, the number of positive neutrophils is significantly higher in CRS. Similar observations were made for nasal polyposis: Klein et al. [24] registered GRO- α -positive neutrophils and eosinophils.

From induction experiments, it was deduced that GCP-2 is produced by fibroblasts and macrophages, and not by blood monocytes or neutrophils. Based on these experiments, it was concluded that GCP-2 protein is up-regulated over a period of 144 h of stimulation with IL-1 β , whereas IL-8 levels peak at 24 h [25]. This is in accordance with our findings in respect of nasal fibroblasts, where no detectable quantities of GCP-2 were observed after 24 h of TNF- α stimulation [17]. Interestingly, GCP-2 is regulated in a different way compared with IL-8, both *in vitro* and in CRS. One explanation for this phenomenon may be that the constitutive or inducible GCP-2 production is disturbed by inhibitory mediators [26].

In summary, in CRS the chemokine profile was dominated by GRO- α and GCP, followed by IL-8 and ENA-78. In TM, the chemokine profile was dominated by GRO- α , followed by IL-8, GCP-2 and ENA-78. Concentrations and chemotactic potencies of GRO- α and GCP-2 differed between the two types of tissue; both were more pronounced in CRS. GCP-2 had never been previously identified in nasal or paranasal mucosa.

In conclusion, we showed that in CRS, neutrophil chemokine patterns differ in terms of biological activity and immunoreactivity. Whereas the chemokines GRO- α , IL-8, GCP-2 and ENA-78 appear to be involved in constitutive expression in TM, GRO- α and GCP-2 protein levels are up-regulated. The localization of chemokines in nasal mucosal gland cells and surface epithelial cells led us to hypothesize that chemokines may be transported within the secretions of nasal mucosal glands into the sinuses, where they might be responsible for the passage of neutrophils into sinus secretions. In addition, the expression of GRO- α on endothelial cells in the nasal mucosa supports the theory that GRO- α acts as part of the adhesion cascade.

Acknowledgements

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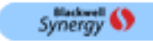
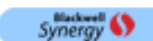
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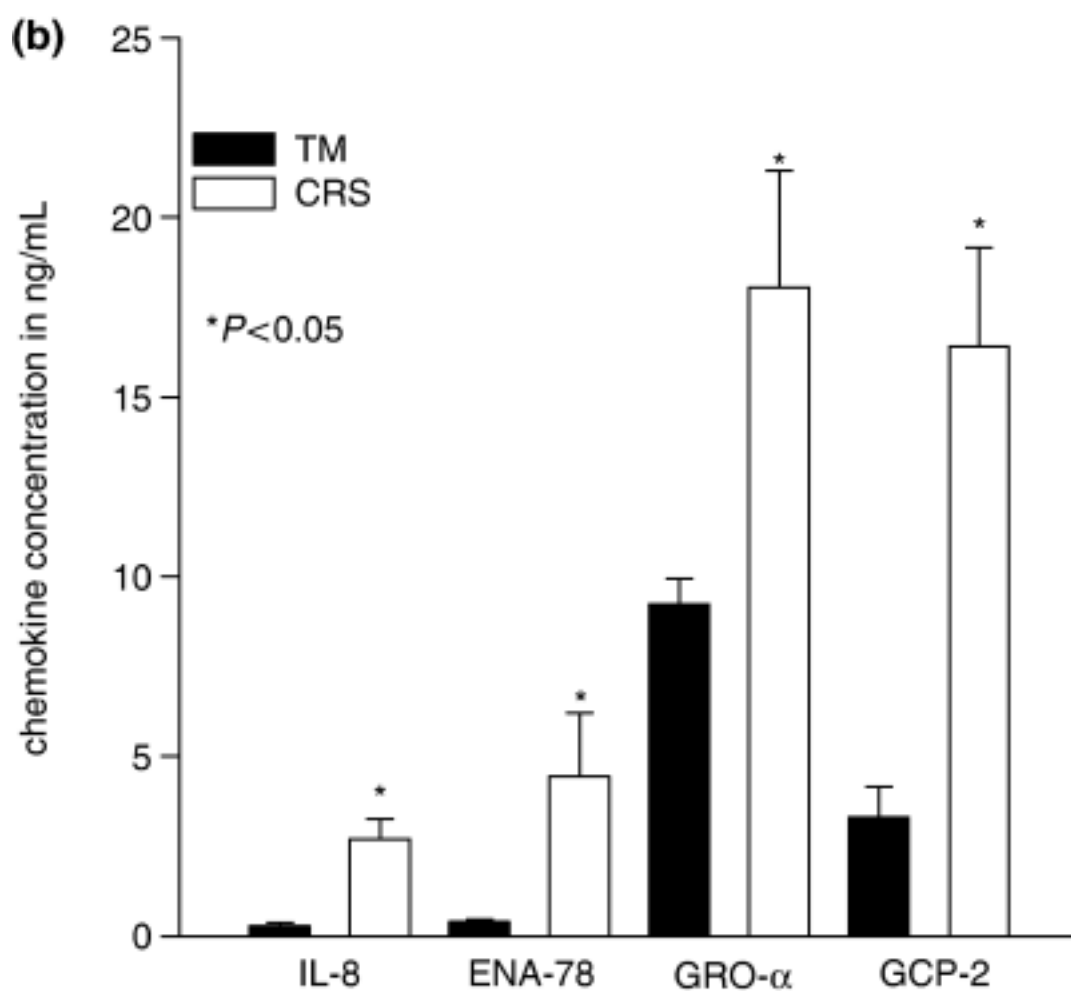
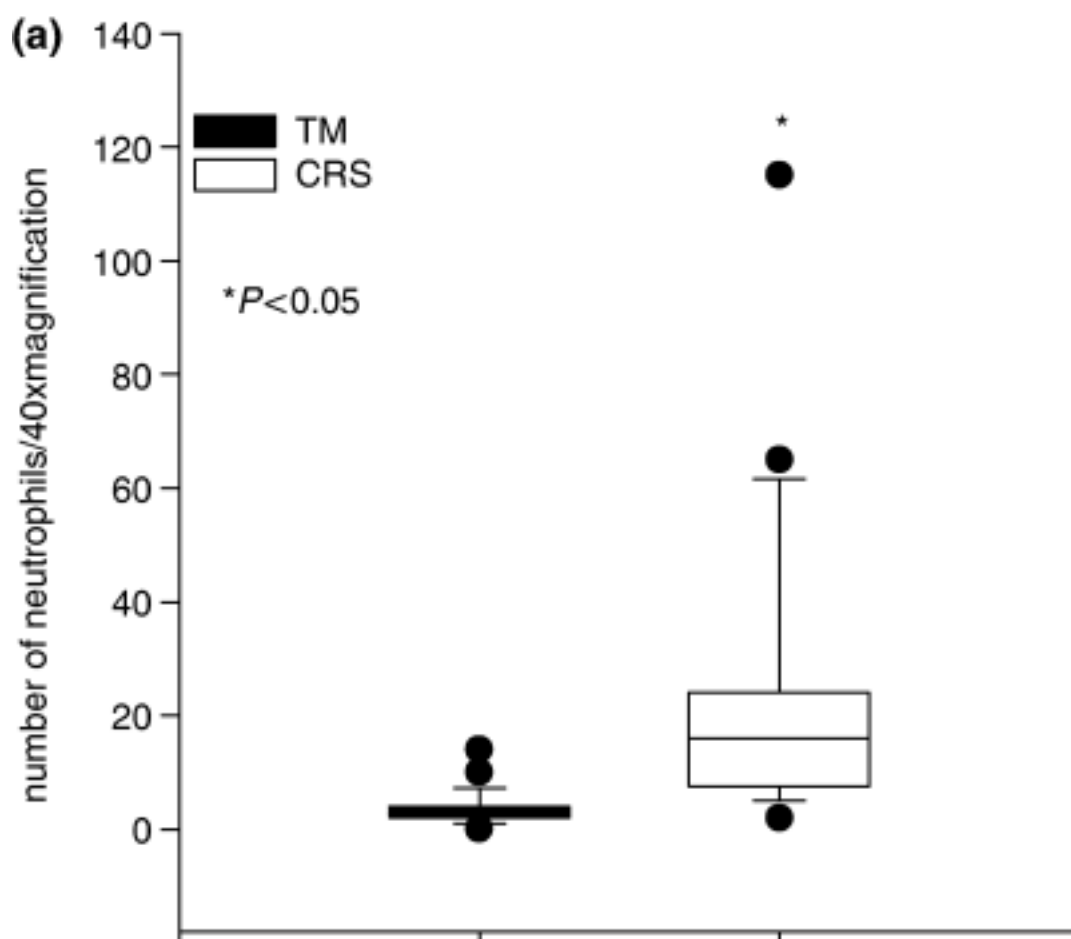
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Fig. 1. Number of neutrophils and neutrophil chemokine concentration in TM and CRS. (a) Neutrophil counts in TM ($n=41$) and CRS tissue (chronic rhinosinusitis; $n=35$) were determined at $\times 400$ magnification and are presented as boxplots. The boundary of the box closest to zero indicates the 25th percentile. A line within the box marks the median and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers above and below the box indicate the 90th and 10th percentiles. Outlying points are graphed as solid dots. $*P<0.05$ indicates the level of significance for the Mann–Whitney U -test. (b) Concentration of neutrophil chemokines in crude extracts of CRS obtained from $n=6$, (tissue weight of each, 5 g) and TM from $n=5$ (tissue weight of each, 5 g) presented as median and standard deviation. $*P<0.05$ indicates the level of significance for the Mann–Whitney U -test. TM, turbinate mucosa; CRS, chronic rhinosinusitis.



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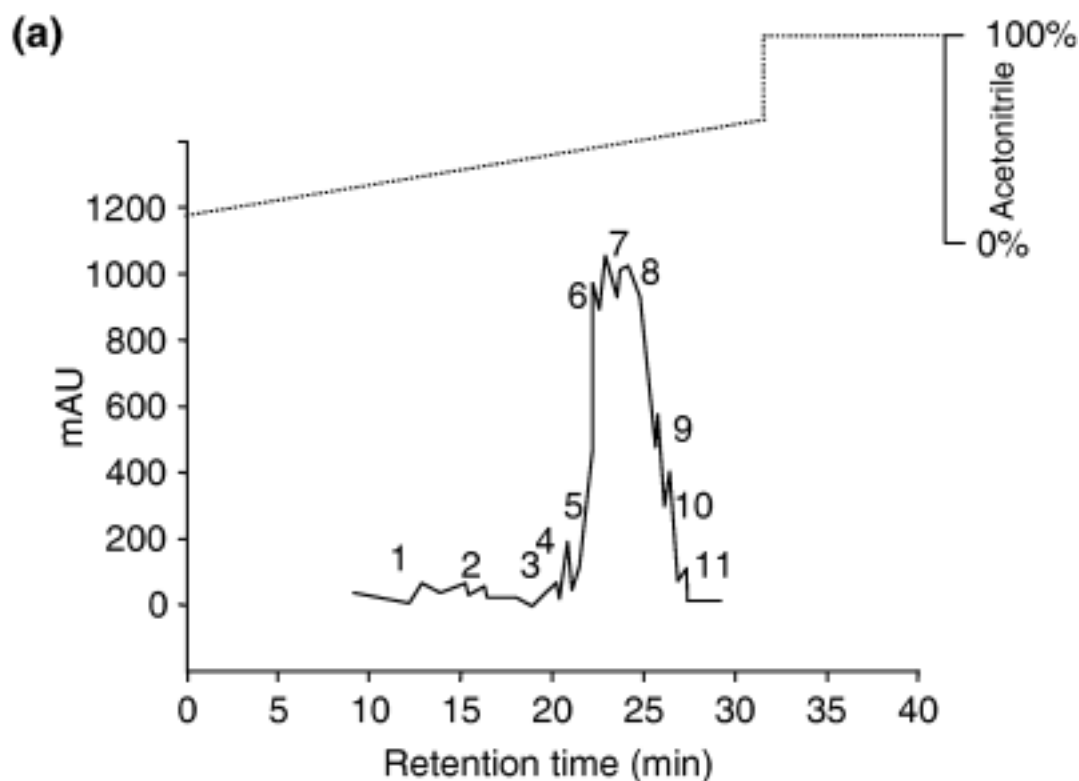
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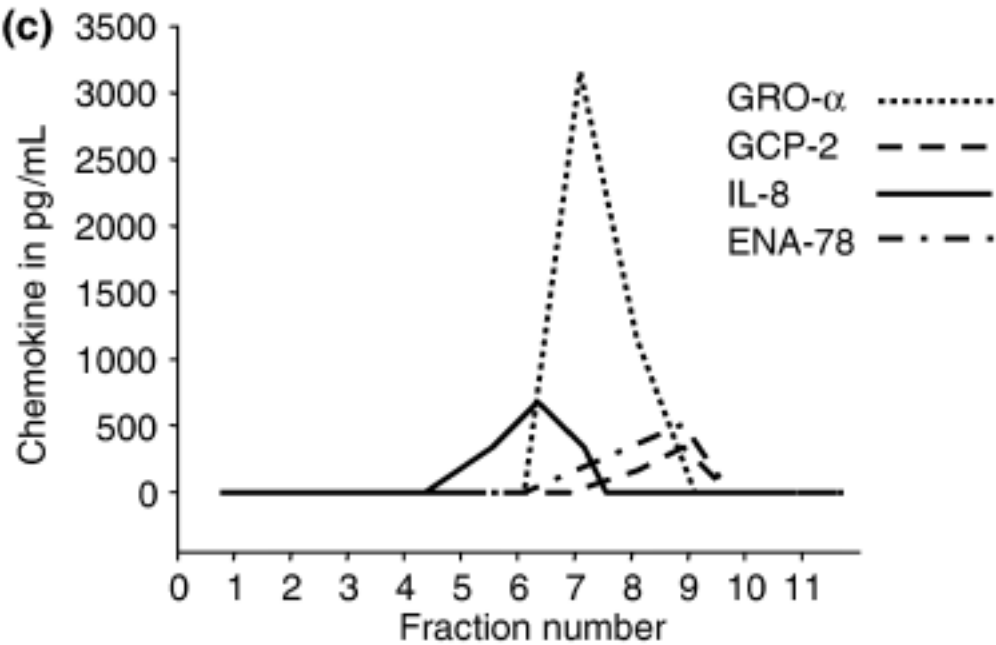
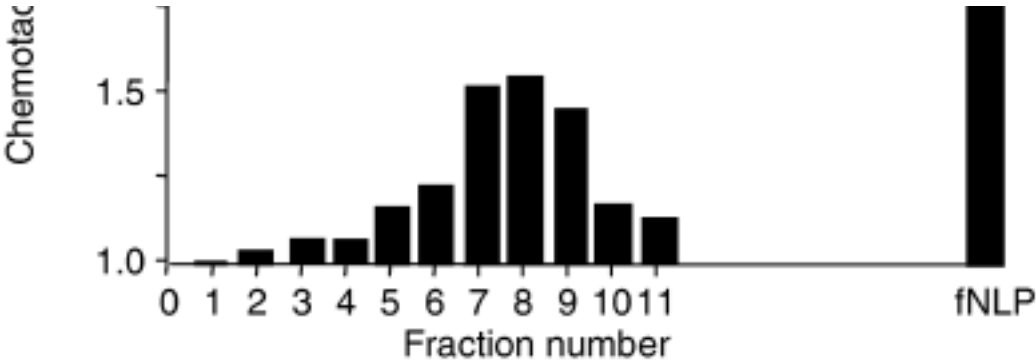
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Fig. 2. Chemotactic index and concentrations of chemokines in turbinate mucosa. Supernatant collected from tissue homogenization of turbinate mucosa was concentrated and applied to a heparin–sepharose cartridge. The eluate was collected as one fraction and subjected to size exclusion chromatography (RP-8-column). (a) shows the chromatogram and the collected protein fractions labelled with numbers. (b) These protein fractions were subjected to the chemotaxis assay in order to detect chemotactic activity. In each test series, both positive and negative controls were included at appropriate concentrations. FNLP (formyl–Nle–Leu–Phe) served as positive control for chemotactic responsiveness of PMN, while phosphate-buffered saline was used as negative control. (c) The ELISA data refer to the 60 μ L fraction aliquots transferred to the Boyden chamber. All tested fraction volumes were freeze-dried before testing. In addition, an ELISA was performed to identify the different chemokines in 60 μ L aliquots. GRO- α , growth-related -oncogene; ENA-78, epithelial cell-derived neutrophil attractant-78; GCP-2, granulocyte chemotactic protein-2.





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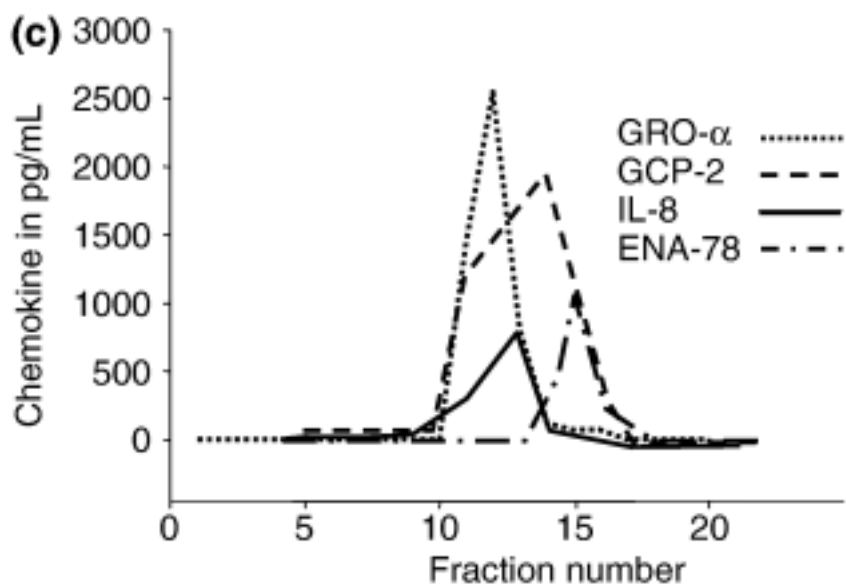
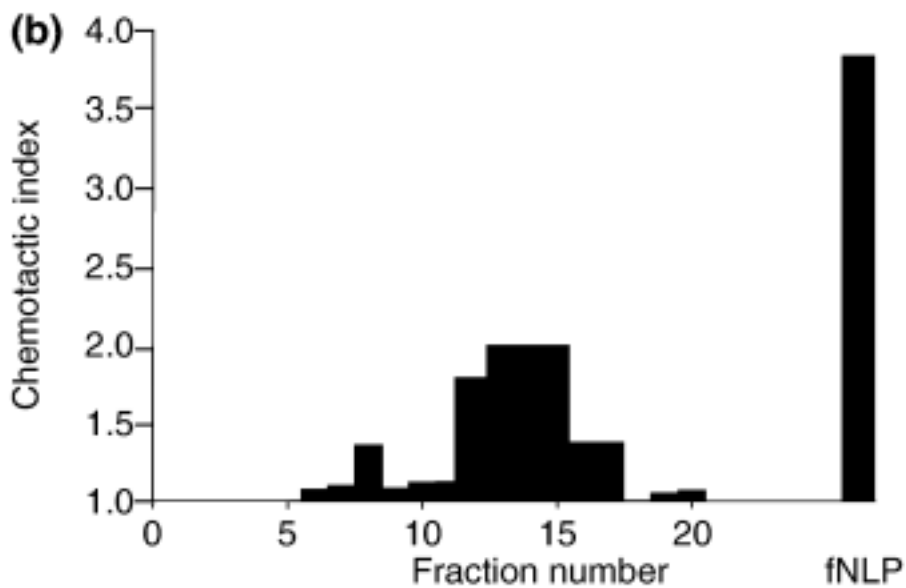
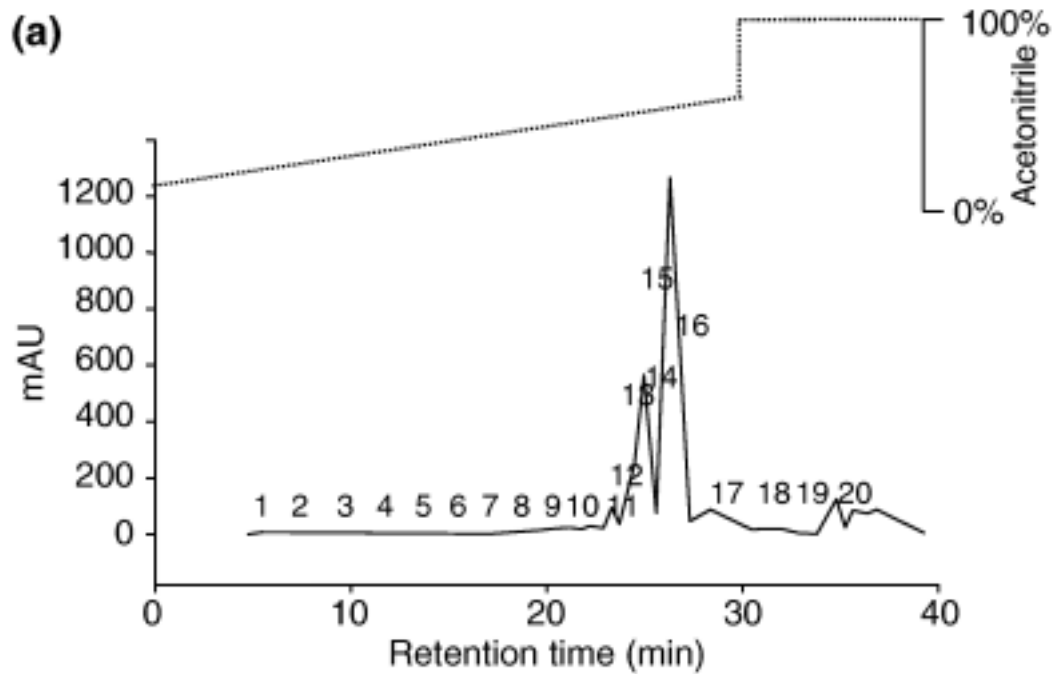
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Fig. 3. Chemotactic index and concentrations of chemokines in mucosa of chronic rhinosinusitis (CRS). Supernatant collected from tissue homogenization of CRS mucosa was concentrated and applied to a heparin–sepharose cartridge. The eluate was collected as one fraction and subjected to size exclusion chromatography (RP-8-column). (a) shows the chromatogram and the collected protein fractions labelled with numbers. (b) The manually collected protein fractions were subjected to the chemotaxis assay in order to detect chemotactic activity. In each test series, both positive and negative controls were included at appropriate concentrations. FNLP (formyl–Nle–Leu–Phe) served as positive control for the chemotactic responsiveness of PMN, while phosphate-buffered saline was used as negative control. (c) The data shown refer to the 60 μ L fraction aliquots transferred to the Boyden chamber. All tested fraction volumes were freeze-dried before testing. In addition, an ELISA was performed to identify the different chemokines in 30 μ L aliquots. GRO- α , growth-related oncogene; ENA-78, epithelial cell-derived neutrophil attractant-78; GCP-2, granulocyte chemotactic protein-2.



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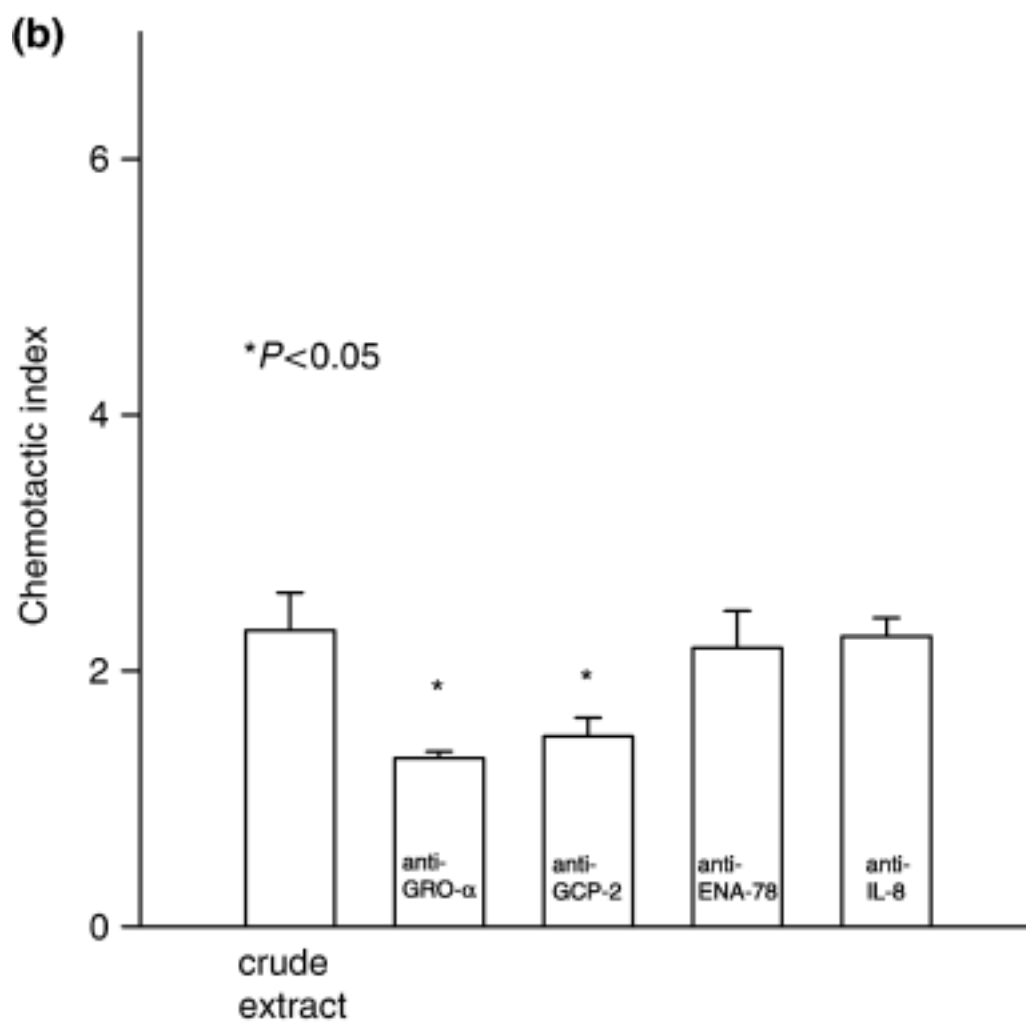
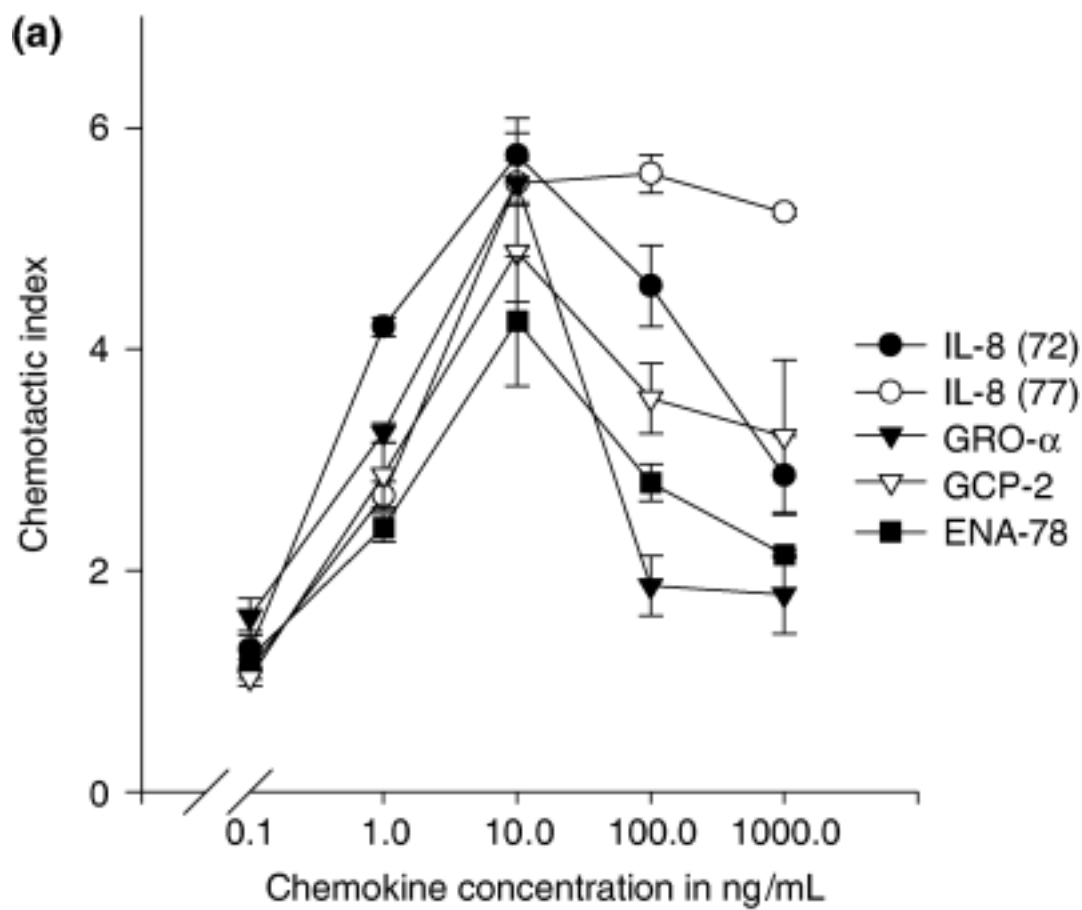
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Fig. 4. Concentration–response curves and neutralization test. (a) Chemokine response curves were established using five different recombinant chemokines and are presented as means (symbols) and standard deviation ($n=8$ for each chemokine). Two isoforms of IL-8 were tested: one containing 72 amino acids [IL-8(72)] and the other containing 77 amino acids [IL-8(77)]. GRO- α , growth-related oncogene; ENA-78, epithelial cell-derived neutrophil attractant-78; GCP-2, granulocyte chemotactic protein-2. (b) Neutralization chemotaxis assays were performed using crude extracts of chronic rhinosinusitis specimens ($n=4$). Antibodies directed against the four different chemokines were used at concentrations of 1 : 50 dilution to inhibit chemotactic activity. * $P<0.05$ indicates the level of significance for the Wilcoxon-rank test. anti-GRO- α , blocking antibody directed against GRO- α ; anti-IL-8, blocking antibody directed against IL-8; anti-ENA-78, blocking antibody directed against ENA-78; anti-GCP-2, blocking antibody directed against GCP-2.



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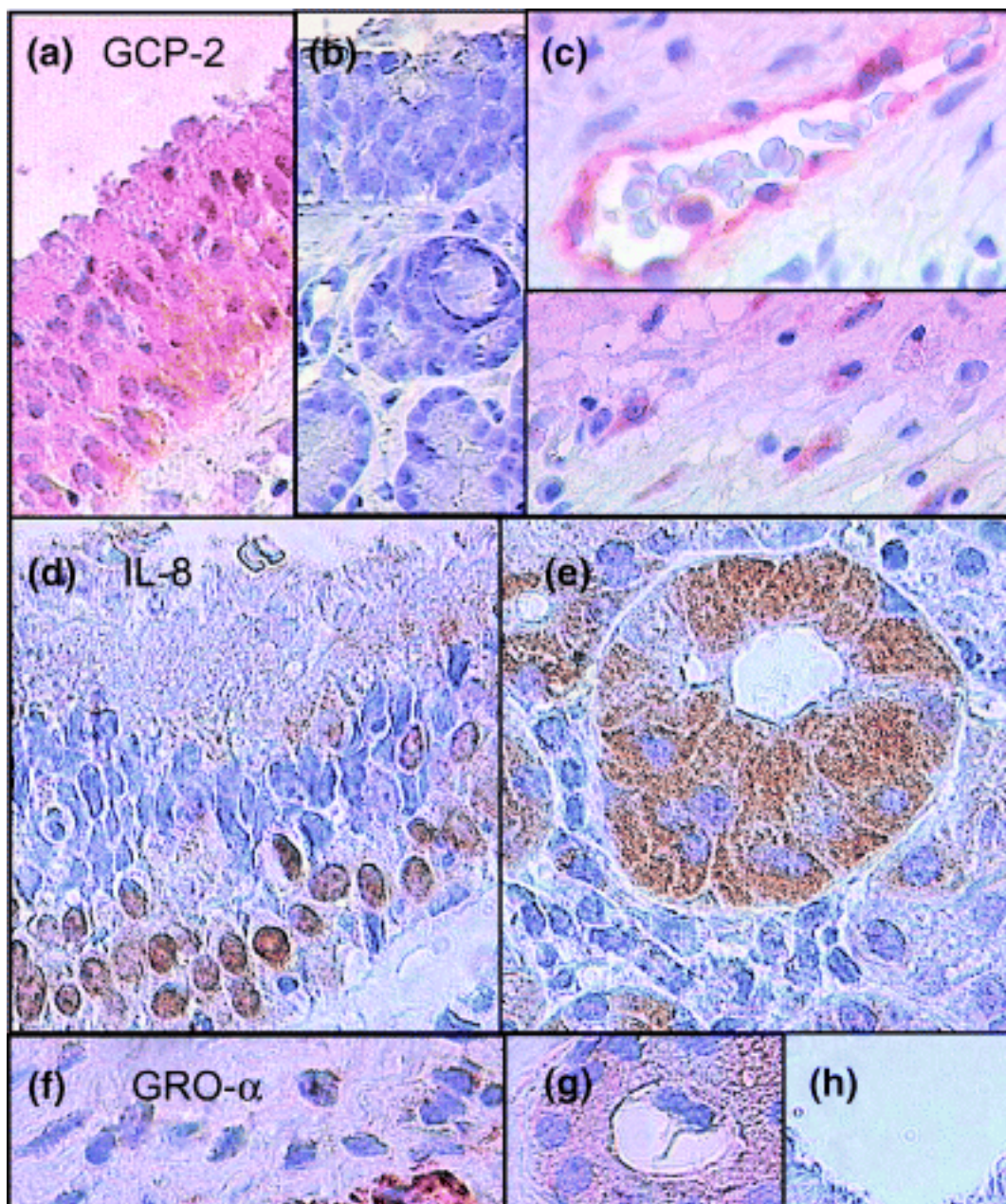
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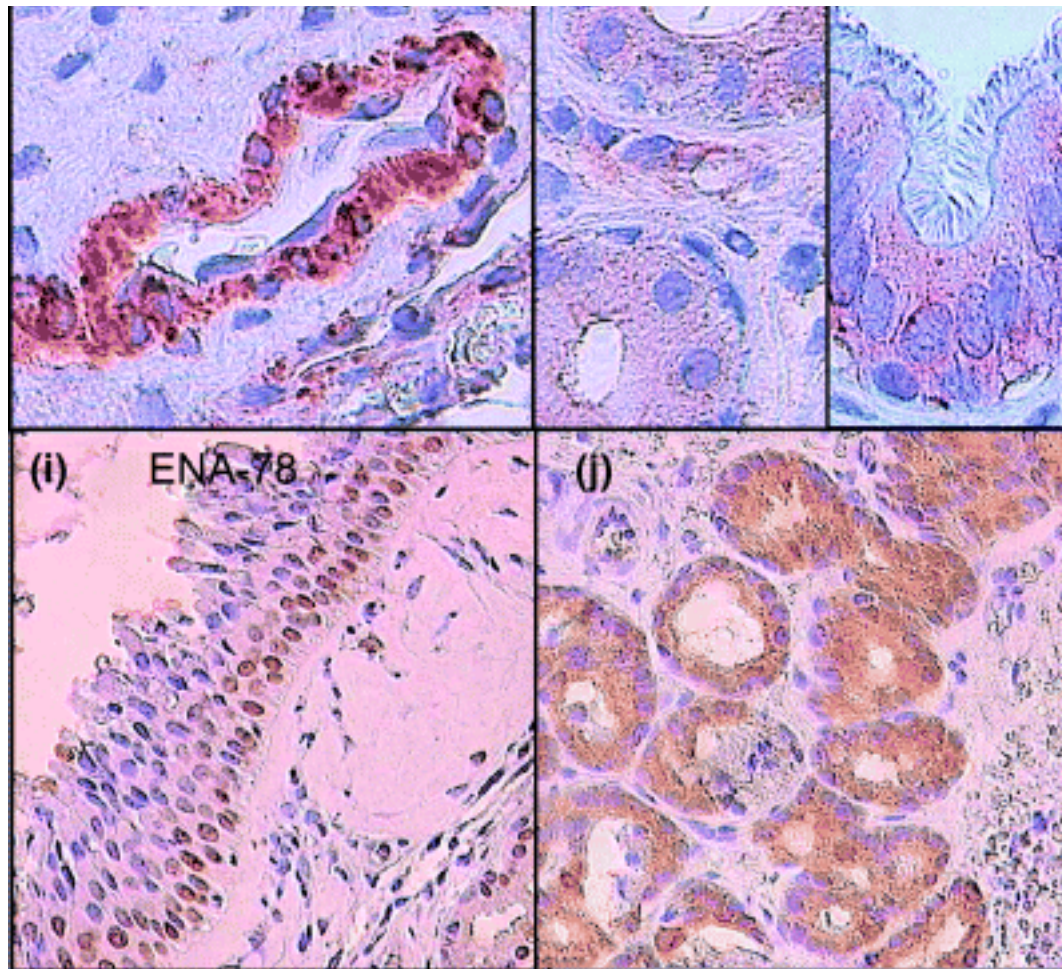
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Fig. 5. Immunohistochemistry of chemokines in chronic rhinosinusitis (CRS). The expression of the different chemokines in CRS: granulocyte chemotactic protein-2 (GCP-2) in surface epithelium (a) negative control (b) upper part GCP-2-positive endothelial cells and lower part GCP-2-positive fibroblasts (c) at $\times 1000$ magnification in red; IL-8 in surface epithelium (d) and mucosal gland cells (e) at $\times 1000$ magnification in brown; GRO- α on endothelial cells (f), in mucosal glands (g) and on the surface epithelium (h) at $\times 400$ magnification in red; ENA-78 overview at $\times 200$ magnification (i) and zoomed at $\times 400$ magnification in mucosal gland cells (j) in brown. GRO- α , growth-related oncogene; ENA-78, epithelial cell-derived neutrophil attractant-78; GCP-2, granulocyte chemotactic protein-2.





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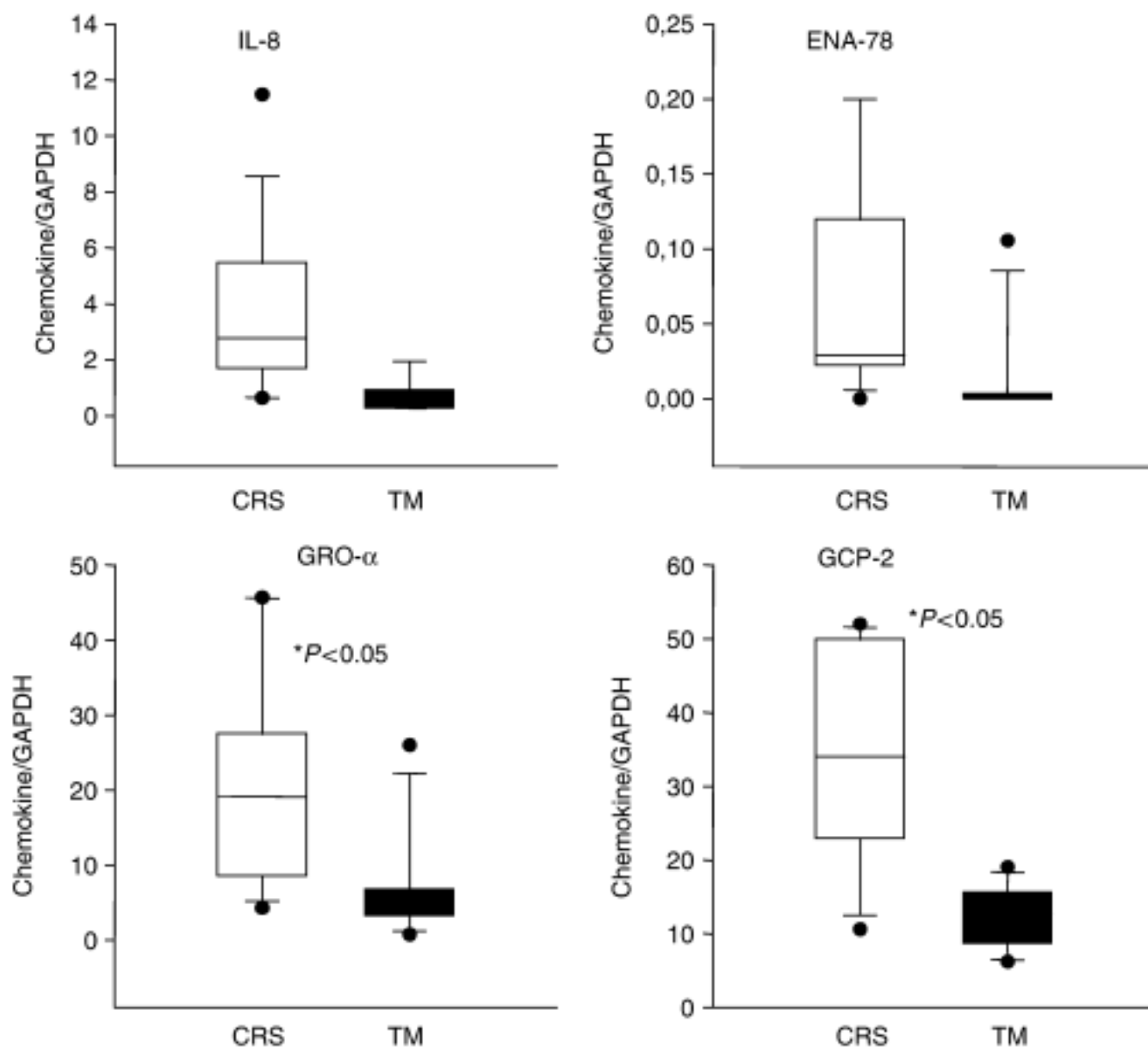
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Fig. 6. mRNA expression of chemokines in turbinate mucosa and chronic rhinosinusitis. The levels of gene expression of chemokine mRNA are expressed as ratios of chemokine/GAPDH in box plots. The boundary of the box closest to zero indicates the 25th percentile. A line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers above and below the box indicate the 90th and 10th percentiles. Outlying points are graphed as solid dots. * $P < 0.05$ indicates the level of significance for the Mann–Whitney U -test.



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Table 1. Antibodies and chromogens used for immunohistochemistry of chemokines

Chemokines	Primary antibody	Secondary antibody	Chromogen
IL-8	Polyclonal goat anti-human, diluted 1 : 50 (Biosource, Belgium)	Avidin–biotin technique LSAB kit/peroxidase	3,3' diaminobenzidine DAB (DAKO Germany)
GRO- α	Polyclonal goat anti-human, diluted 1 : 50, diluted (Santa Cruz, USA)	Peroxidase-conjugated affinipure rabbit anti-goat IgG (H+L) (Jackson Immunoresearch., USA), diluted 1 : 100	3-amino-9-ethylcarbazole AEC, substrate–chromogen system (DAKO)
ENA-78	Polyclonal goat anti-human, diluted 1 : 200 (R&D, Germany)	Avidin–biotin technique LSAB kit/peroxidase (DAKO)	3,3' diaminobenzidine DAB (DAKO)
GCP-2	Polyclonal goat anti-human, diluted 1 : 50 (Santa Cruz)	Avidin–biotin technique LSAB kit/peroxidase (DAKO)	3-amino-9-ethylcarbazole AEC, substrate–chromogen system (DAKO)
GRO- α , growth-related oncogene- α ; ENA-78, epithelial cell-derived neutrophil attractant-78; GCP-2, granulocyte chemotactic protein-2.			

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Table 2. Immunohistochemical staining expression and staining intensity distribution (SID) scores for chemokines

	Cells	Stained cells/ total cell count: mean $\pm$ SD	P (stained cells/ total cell count)	SID score: mean $\pm$ SD	P SID score
(A) Results for ENA-78 and IL-8					
IL-8	n=15				
TM					
	Epithelium	0.277 $\pm$ 0.168		1.929 $\pm$ 1.179	
	Endothelium	0.000 $\pm$ 0.000		0.000 $\pm$ 0.000	
	Mucosal glands	0.393 $\pm$ 0.133		5.250 $\pm$ 2.500	
	Neutrophils	0.270 $\pm$ 0.061		3.455 $\pm$ 1.214	
	Mononuclear cells	0.233 $\pm$ 0.120		2.545 $\pm$ 1.695	
CRS			CRS vs. TM		CRS vs. TM
	Epithelium	0.582 $\pm$ 0.124	**	3.833 $\pm$ 1.323	*
	Endothelium	0.000 $\pm$ 0.000	ND	0.000 $\pm$ 0.000	ND
	Mucosal glands	0.440 $\pm$ 0.036	NS	4.000 $\pm$ 0.000	ND
	Neutrophils	0.548 $\pm$ 0.100	***	5.000 $\pm$ 2.944	NS
	Mononuclear cells	0.717 $\pm$ 0.194	***	8.333 $\pm$ 1.759	***
ENA-78	n=15				
TM					
	Epithelium	0.138 $\pm$ 0.033		2.250 $\pm$ 0.354	
	Endothelium	0.000 $\pm$ 0.000		0.000 $\pm$ 0.000	
	Mucosal glands	0.000 $\pm$ 0.000		0.000 $\pm$ 0.000	
	Neutrophils	0.000 $\pm$ 0.000		0.000 $\pm$ 0.000	

	Mononuclear cells	0.000±0.000		0.000±0.000	
CRS			CRS vs. TM		CRS vs. TM
	Epithelium	0.158±0.046	NS	1.750±0.540	NS
	Endothelium	0.000±0.000	ND	0.000±0.000	ND
	Mucosal glands	0.000±0.000	ND	0.000±0.000	ND
	Neutrophils	0.000±0.000	ND	0.000±0.000	ND
	Mononuclear cells	0.143±0.044	***	1.154±0.376	***
<i>(B) Results for GCP-2 and GRO-α.</i>					
GRO-α	n=15				
TM					
	Epithelium	0.280±0.061		2.250±1.188	
	Endothelium	0.771±0.115		4.636±1.567	
	Mucosal glands	0.838±0.145		1.545±0.522	
	Neutrophils	0.321±0.087		3.455±1.214	
	Mononuclear cells	0.363±0.128		4.636±2.335	
CRS			CRS vs. TM		CRS vs. TM
	Epithelium	0.679±0.119	***	7.083±1.158	***
	Endothelium	0.947±0.075	***	5.750±2.379	ns
	Mucosal glands	0.927±0.110	Ns	6.750±1.357	***
	Neutrophils	0.568±0.108	***	6.583±1.564	***
	Mononuclear cells	0.639±0.186	**	6.750±1.357	*
GCP-2	n=15				
TM					
	Epithelium	0.206±0.033		1.813±1.068	
	Endothelium	0.190±0.046		2.500±1.732	
	Mucosal glands	0.487±0.077		3.000±1.549	
	Neutrophils	0.000±0.000		0.000±0.000	
	Mononuclear cells	0.212±0.069		2.636±1.567	
CRS			CRS vs. TM		CRS vs. TM
	Epithelium	0.609±0.085	***	6.250±1.425	**
	Endothelium	0.208±0.077	NS	1.385±0.506	*
	Mucosal glands	0.763±0.245	**	3.556±1.424	NS

Neutrophils	0.000±0.000	ND	0.000±0.000	ND
Mononuclear cells	0.597±0.190	***	6.769±1.641	***

The expression of positive-stained cells and the staining intensity distribution score are given as means and SD. The non-parametric Mann–Whitney *U*-test at the significance levels.

P*<0.05, *P*<0.005 and ****P*<0.0005 was used to compare the results of immunohistochemistry from TM specimens of the epithelium, mucosal glands, neutrophils and mononuclear cells obtained from sinus healthy subjects, with the immunohistochemistry data obtained from the sinus tissue of patients having CRS.

TM, turbinate mucosa; CRS, chronic rhinosinusitis; ENA-78, epithelial cell-derived neutrophil attractant-78; GRO- α , growth-related oncogene- α ; GCP-2, granulocyte chemotactic protein-2; SD, standard deviation; ND, not done; NS, not significant.