**Hypoxia Induced Chemokine Expression in Nasal Epithelial Cells: Development of an *In Vitro* Model for Chronic Rhinosinusitis**

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Summary

Chronic rhinosinusitis (CRS) is defined as an inflammatory condition involving the paranasal sinuses and the lining of the nasal passages that persists longer than 12 weeks. It is one of the most common chronic diseases today, affecting up to 15% of the adult population in the Western world with a dramatic increase in prevalence. One hallmark of chronic inflammation in CRS is the predominance of eosinophils and T lymphocytes in the inflamed tissue. We pursued the hypothesis that the blockage of the paranasal sinuses induces hypoxic conditions, which subsequently lead to the induction of chemotactic activity, attracting inflammatory cells. To this end, we established an in vitro model by showing that hypoxia is able to induce the release of chemokines in nasal epithelial cells. Furthermore, we show that this induction leads to the migration of eosinophils and neutrophils. Finally, we demonstrated the applicability of this in vitro model by showing its sensitivity to the glucocorticoid dexamethasone, which is used in the clinical situation. These results suggest that this nasal epithelial cell culture model may allow the evaluation of novel anti-inflammatory compounds for the treatment of CRS directly on the relevant target cells in vitro. This approach may result in replacing and refining animal experimentation in the screening of new therapeutics for CRS.

Keywords: 3R, rhinosinusitis, hypoxia, epithelial cells, chemokines, inflammation

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1 Introduction

Chronic rhinosinusitis (CRS) is a multifactorial disease defined as inflammation of the nasal cavity and paranasal sinuses with a history of at least 12 weeks duration (Meltzer et al., 2004). It is one of the most common chronic diseases today affecting up to 15% of the adult population in the Western world with a dramatic increase in prevalence (Kaliner et al., 1997). The aetiology, pathophysiology and therapy of chronic rhinosinusitis are largely unknown (Bachert et al., 2003). Microorganisms play a significant role in the persistence and origination of the inflammatory process, although the exact role of these organisms in the pathogenesis of chronic rhinosinusitis is unclear (Kennedy, 2004). Streptococcus pneumoniae, Haemophilus influenzae, and Staphylococcus aureus predominate in acute infection in patients with acute...
frontal sinusitis, and *S. aureus* and anaerobic bacteria are commonly isolated in chronic sinusitis (Brook, 2003). While chronic inflammation, with a predominance of eosinophils and T lymphocytes in the tissues, is being recognised as a hallmark of chronic rhinosinusitis, the causal relationship with bacterial or fungal infection remains elusive (Nguyen et al., 2003).

Inflammation is characterised by a series of cellular and molecular responses that are designed to eliminate foreign agents and promote repair of damaged tissues. It begins with a reaction of blood vessels, leading to the accumulation of fluid and leukocytes in extravascular tissues (Serhan and Savill, 2005). There is increasing evidence that in addition to infection, immunologic inflammatory responses play major roles in the cause and pathophysiology of CRS (Mucha and Baroody, 2003). One hallmark of the inflammation in CRS is the congestion and blockage of the nose and the paranasal sinuses (Pawankar et al., 2004). This blockage leads to the development of hypoxic conditions in the sinuses (Matsune et al., 2003). Therefore it has been hypothesised that this environmental change caused by the blockage of the sinuses, leads to a dysregulation of the nasal epithelium.

We hypothesised, that these hypoxic conditions induce the secretion of chemotactic factors in the nasal epithelium and that these chemotactic factors subsequently lead to the attraction of inflammatory cells. In the present study, we used primary human nasal epithelial cells to test the possible initiation of nasal inflammation by releasing chemokines. These epithelial cells were exposed to hypoxic conditions and the release of chemokines was measured. Furthermore, we investigated whether hypoxia induces the migration of inflammatory cells. The aim of these studies was to establish a novel *in vitro* cellular system to study pathomechanisms underlying the inflammation observed in CRS. Furthermore, this model system allows for the screening of drug candidates without animal experiments.

2 Materials and methods

2.1 Reagents

Dexamethasone was purchased from Sigma (Deisenhofen, Germany). HEPES Buffered Saline Solution, Trypsin/EDTA Solution and Trypsin Neutralizing Solution were from BioWhittaker Europe (Verviers, Belgium). RPMI 1640 medium was obtained from Life Technologies (Heidelberg, Germany). Chemokine measurements in culture supernatants were performed with ELISA kits from BD Pharmingen (Heidelberg, Germany). Unless otherwise indicated, all other chemicals were purchased from Sigma Chemical Co. (Deisenhofen, Germany).

2.2 Cell culture

Human nasal epithelial cells (HNECs) were obtained from Oligene (Berlin, Germany). The cells were cultured in Nasal Epithelial Cell Growth Medium (Oligene, Berlin, Germany) in a humidified atmosphere containing 5% CO2 or in hypoxic conditions (95% N2; 5% CO2) at 37°C. The measured pO2 in the medium was 18-20 mm Hg under hypoxic conditions. When confluent, the cells were washed and resuspended at 10^6 cells per ml and incubated in 500 µl aliquots in 24-well tissue plates (Falcon Becton Dickinson Labware, Heidelberg, Germany). Cells were harvested at different time points. Supernatants were kept frozen at -80°C until assayed.

2.3 Enzyme-linked immunosorbent assay

Chemokine measurements in culture supernatants were done by sandwich ELISA using matched antibody pairs (BD Pharmingen, Heidelberg, Germany). ELISA plates (Maxisorb, Nunc) were coated overnight with anti-cytokine mAb in 0.1 M carbonate buffer, pH 9.5. After washing, plates were blocked with Assay Diluent (Pharmingen, Heidelberg, Germany). Chemokine measurements in culture supernatants were performed with ELISA kits from BD Pharmingen (Heidelberg, Germany). Unless otherwise indicated, all other

![Fig. 1: Secretion of chemokines in the supernatant of HNECs.](image-url)

HNECs were incubated under either normal or hypoxic conditions and harvested at different time points. IL-8 (A) and MCP-1 (B) chemokine protein levels were determined by ELISA. Each data point represents mean ± SEM of five independent experiments; *p<0.05 (normal versus hypoxic conditions).
and the plates were incubated for 2 h at room temperature. Plates were washed, incubated for 1 h with working detector (biotinylated anti-cytokine Ab and avidin-horseradish peroxidase conjugate). After washing, substrate (TMB and hydrogen peroxide) was added. The reaction was stopped by addition of 1 M H₃PO₄. Plates were read at 450 nm (reference 570 nm) in a microplate reader (Dynatech).

2.4 Chemotaxis assay
Human eosinophils and neutrophils were prepared from human blood as described (Murray et al., 2003). Cells were diluted 1:1 with 2.5 μM BCECF-AM (2,7-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester) (Molecular Probes, Eugene, OR) in RPMI 1640 for 1 h at 37°C. Next, the cells were washed twice and resuspended at 5 x 10⁵ cells per millilitre in chemotaxis medium (RPMI 1640, 1% bovine serum albumin). Chemotaxis assays were performed on plastic chemotaxis chambers (Chemicon, Hoheim, Germany) containing HNEC supernatant in chemotaxis media in the lower chamber and 20 μL of the neutrophil or eosinophil suspension (10,000 cells total) in the upper chamber. The chamber was then placed in a tissue-culture incubator (37°C, 5% CO₂) for 1 h. Cells that had transmigrated to the bottom chamber were counted using a Cytofluor fluorescence plate reader (480 nm excitation, 530 nm emission) and cell numbers were quantified using a standard curve. Results were confirmed by manual count of at least one of the triplicate wells in each group.

3 Results
3.1 Hypoxia increases chemokine production of human nasal epithelial cells
HNECs were either incubated under normal ambient conditions or under hypoxic conditions. The secretion of the chemokines IL-8 and MCP-1 was measured over several days of incubation. The amount of IL-8 and MCP-1 increased in the supernatant during the incubation period at ambient conditions over several days (fig.1). In comparison, incubation of HNECs under hypoxic conditions increased protein levels of IL-8 and MCP-1 even more (fig.1).

3.2 Hypoxia induces chemotaxis of granulocytes
We analysed whether the induction of chemokines in HNECs has functional relevance. To this end, chemotaxis assays were performed using supernatants of cells exposed to hypoxic conditions. The migration of primary human eosinophils and neutrophils, which were separated by a semi-permeable membrane from the supernatant of HNECs, was analysed. Supernatants from HNECs incubated at normal conditions induced chemotaxis of neutrophils and eosinophils (fig. 2A and B). The number of cells that had migrated increased with the duration of incubation. The incubation of HNECs under hypoxic conditions led to an even higher number of migrated cells.

3.3 Inhibition of hypoxia induced chemokine secretion and chemotaxis by an anti-inflammary drug
The action of the anti-inflammatory glucocorticoid dexamethasone was analysed in this model to compare this in vitro method with in vivo conditions. First, the effect of hypoxia induced chemokine secretion was investigated. After three days under hypoxic conditions, the HNEC supernatant was harvested and the effect of various concentrations of dexamethasone was analysed (fig. 3A). This drug dose dependently inhibited the hypoxia induced secretion of IL-8 as well as of MCP-1. The IC₅₀ of this effect was 4.2 nM for IL-8 and 8.4 nM for MCP-1. Furthermore, we analysed whether this inhibition was reflected by a reduction of cell migration. Therefore, we subjected the same supernatants to chemotaxis assays. Similar to the reduction of secreted chemokines, dexamethasone inhibited the migration of eosinophils and neutrophils (fig. 3B).

![Fig. 2](image-url)

*Fig. 2: Hypoxia conditioned medium from HNECs increases chemotaxis. HNECs were incubated under either normal or hypoxic conditions and harvested at different time points. The chemotaxis of primary human neutrophils (A) or eosinophils (B) toward supernatants of so treated HNECs was assayed. The migration of cells towards supernatant from day 0 was set to 1. Each bar represents mean ± SEM of five independent experiments; *p<0.05 (normal versus hypoxic conditions).
4 Discussion

Nasal epithelial cells form a tight barrier that protects underlying tissue from the external environment. As such, nasal epithelial cells have been described classically as barrier cells that are involved in homeostasis. These cells respond to a variety of environmental stimuli, resulting in alteration of their cellular functions. Recent evidence suggests that airway epithelial cells might also act as immune effector cells in response to noxious endogenous or exogenous stimuli. Several studies have shown that airway epithelial cells express and secrete various immune molecules, such as lipid mediators, oxygen radicals, adhesion molecules, and a wide variety of cytokines, including chemokines (reviewed in Takizawa, 2005). Based on expression and production of these immune molecules, the epithelium is now thought to be important in the initiation and exacerbation of inflammatory responses within the airways.

We could indeed show that the pathophysiology of sinusitis is intriguing, as it involves many disturbed physiological functions. Our in vitro model focuses on one specific aspect of CRS. It has been shown that congestion and blockage of the nose and the paranasal sinuses leads to the development of hypoxic conditions in the sinuses (Matsune et al., 2003). Therefore we investigated whether this environmental change causes changes in the inflammatory response of nasal epithelial cells. We found that hypoxic conditions increase the release of chemokines from these epithelial cells. Chemokines are a group of small molecules that act on different types of leukocytes via a chemotactic gradient. They have a wide range of effects, mainly cellular recruitment, activation and differentiation. They are fundamental regulators of leukocyte homeostasis and inflammation (Subroe et al., 2002). Accordingly, increased chemotaxis of inflammatory cells such as eosinophils and neutrophils was detected when supernatants from hypoxic nasal epithelial cells were applied. By linking these two methods, we were able to provide an in vitro system modeling a key symptom of CRS for the first time: blockage of the sinuses induces inflammation.

Non-surgical and surgical approaches constitute the general strategies in the therapeutic management of rhinosinusitis. For pharmacotherapy, glucocorticosteroids are the mainstay of anti-inflammatory treatment (van Cauwenberge et al., 2005). It has been observed that glucocorticoids decrease airway infiltration by eosinophils (Barnes, 1998). It has been proposed, that glucocorticoids decrease the secretion of chemotactic cytokines in nasal mucosa. Our in vitro system provides further evidence for this assumption. The release of chemokines as well as the migration of inflammatory cells was inhibited by dexamethasone. The inhibition constant for both effects is in the low nanomolar range. This sensitivity reflects the clinical situation.

The cell culture model presented here may provide an in vitro system to investigate the mechanism by which nasal blockage and subsequent hypoxia induces inflammation in CRS. Our results suggest that this model can be further developed to predict the effectiveness of novel anti-inflammatory compounds for CRS treatment. However, additional data with other compounds are needed to validate this model. By using primary nasal epithelial cells the experiments can target the relevant cells directly. Animals are not required as direct objects of such experiments or as donors of tissue or cells.

Altogether, our present data suggest that primary nasal epithelial cells exposed to hypoxic conditions represent an interesting in vitro model for investigating the pathomechanisms of CRS and for seeking novel compounds suitable for the treatment of inflammation in CRS.

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**Fig. 3: Inhibition of chemokine secretion and chemotaxis by dexamethasone.** HNECs were preincubated with vehicle or various concentrations of dexamethasone. After 30 minutes cells were exposed to hypoxic conditions for 3 days. IL-8 and MCP-1 chemokine protein levels were determined by ELISA and were referenced to vehicle treated cells (A). The chemotaxis of primary human neutrophils and eosinophils toward supernatants of so treated HNECs was assayed (B). The migration of cells towards supernatant from day 0 was set to 1. Each data point or bar represents mean ± SEM of three independent experiments.
References


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