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Features of mesenchymal transition in the airway epithelium from chronic rhinosinusitis

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Keywords: airway fibrosis, epithelial-to-mesenchymal transition, rhinosinusitis, vimentin

Abstract

Background: Chronic rhinosinusitis (CRS) defines a group of disorders characterized by persistent inflammation of the sinonasal tract. Epithelial changes and structural remodelling are present, but whether epithelial differentiation is altered remains uncertain.

Methods: To evaluate the differentiation state of the sinonasal epithelium in CRS, sinonasal biopsies from patients with CRS with (CRSwNP) or without polyps (CRSsNP), or with allergic rhinitis (AR), as compared to controls, were processed by immunohistochemistry and RT-qPCR for terminal differentiation (E-cadherin, high molecular weight cytokeratins (Hmw CK) and CK5, vimentin) and lineage differentiation (β -tubulin IV+ ciliated cells, MUC5AC+ goblet cells, p63+ basal cells). Findings were correlated to subepithelial fibrosis and clinical CT score.

Results: Expression of E-cadherin was decreased at protein and mRNA levels in CRSwNP and CRSsNP, as compared to controls. Staining for Hmw CKs was also reduced in CRSwNP and CRSsNP, and CK5 mRNA was decreased in CRSwNP. These features were not due to changes in lineage specification, but associated with increases in vimentin-expressing epithelial cells. In addition, vimentin expression correlated with the basement membrane thickening and with CT score, as well as with tissue eosinophils.

Conclusion: Features of epithelial de-differentiation towards a mesenchymal phenotype are observed in CRSwNP and CRSsNP, and correlate with airway fibrosis and inflammation.

Abbreviations:

AR: allergic rhinitis

BCs: basal cells

BM: basement membrane

CCs: ciliated cells

CK: cytokeratin

COPD: chronic obstructive pulmonary disease

CRS: chronic rhinosinusitis

CRSsNP: chronic rhinosinusitis without nasal polyps

CRSwNP: chronic rhinosinusitis with nasal polyps

Hmw CK: high molecular weight cytokeratins

Ig: immunoglobulin

IL: interleukin

RT-qPCR: real time quantitative polymerase chain reaction

INTRODUCTION

Chronic rhinosinusitis (CRS) is defined as a chronic inflammation of the nose and the paranasal sinuses, characterized by persistent symptoms and typical features at nasal

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endoscopy and CT scan¹. CRS has been typically divided into CRS with nasal polyps (CRSwNP) and without nasal polyps (CRSsNP) based on the presence or not of polyps on endoscopic examination, and differ from allergic rhinitis (AR), which represents another very common chronic nasal disease².

Besides inflammation, these chronic upper airways disorders are characterized by remodelling of structural components, especially of the epithelium. The upper airway epithelium provides both a physical and chemical barrier through the formation of a strong mechanical cohesion by apical (tight and adherens) junctions and by secretion of a large array of antimicrobial host defense molecules mediating innate immunity respectively. Tissue remodelling in upper airways is an active process that includes increased extracellular matrix deposition, with subepithelial basement membrane (BM) thickening³.

The normal upper airway epithelium comprises three main cell types, namely ciliated cells (CCs), representing 50% to 90% of the airway epithelial cell population^{4, 5}, mucus-secreting goblet cells (GCs) and basal cells (BCs). BCs represent 6 to 30% of the epithelial population in the lower airways and are considered as stem cells⁶. GCs and CCs act in concert to support mucociliary clearance and remove inhaled potentially harmful substances. Changes in epithelial specification have been described in chronic airway diseases and following injury. Thus the number of CCs is decreased in asthma⁷, after a viral infection⁸ or in response to environmental toxics such as cigarette smoke⁹. In contrast, changes in these epithelial lineages remain unclear in CRS, with controversial findings reported in CRSwNP and CRSsNP regarding MUC5AC expressing GCs^{10, 11}. One study also reported increased BC numbers in CRSwNP¹².

Besides commitment of epithelial cells to ciliated or goblet phenotypes, terminal differentiation is critical to epithelial homeostasis. Upon injury, epithelial cells may dedifferentiate through squamous metaplasia or so-called epithelial-to-mesenchymal transition (EMT), which describes a rapid and normally reversible modulation of the epithelial phenotype towards mesenchymal cells¹³. Epithelial cells undergoing EMT are losing cell-cell polarity and adhesion to become migratory, and usually downregulate junctional proteins (such as E-cadherin) whilst modulating their cytoskeleton organization and acquiring mesenchymal features such as vimentin filaments. EMT occurs during development and normal wound repair, while deregulated EMT may occur during cancer metastasis. In addition, it is increasingly recognized that chronic inflammation resulting from repeated injury may also induce persistent EMT, thereby contributing to tissue fibrosis¹⁴. In the upper airways, downregulation of tight junction-related proteins has been recently described in CRSwNP¹⁵⁻¹⁷, whereas it remains unclear whether this relates to dedifferentiation of the sinonasal epithelium through EMT.

The aim of this study was to evaluate whether the sinonasal epithelium undergoes dedifferentiation in CRS and, if so, its relation to lineage specification and to EMT. Findings were also correlated to airway remodeling and clinical CT score.

MATERIAL AND METHODS

Additional supporting information may be found in the online version of this article.

Subjects and surgical sampling

Patients with CRSsNP (n = 11), CRSwNP (n = 11), AR (n = 10) and controls (n = 13) were recruited at the outpatient clinic of our Department of Otorhinolaryngology. The diagnosis of CRSwNP and CRSsNP was, accordingly to EPOS criteria, based on history, clinical examination, nasal endoscopy and CT scanning¹. AR was diagnosed based on symptoms and positive skin prick tests and/or serum specific IgE (> 0.35 kU/L) to common aeroallergens, following the ARIA guidelines². Clinical characteristics of the patients are described in Table 1. Controls and AR patients underwent surgery for anatomical obstruction, whilst CRS patients underwent surgery as clinically indicated. All patients were weaned of corticosteroids or antibiotics for at least 3 weeks before surgery, and underwent a preoperative CTscan as part of the clinical work-up, which was graded according to the Lund-Mackay score¹⁸.

Biopsies from inferior turbinates (AR and controls) and from ethmoidal mucosa (CRS) were processed for immunohistochemistry or RT-qPCR.

An informed consent was obtained from each subject, and the study was approved by the local ethical committee (Cliniques Universitaires Saint-Luc, Brussels, Belgium).

Immunohistochemistry for epithelial differentiation and phenotyping

Five- μ m sections of turbinal and ethmoidal biopsies were stained for E-cadherin and Hmw CK as epithelial markers and for vimentin as mesenchymal marker. Lineage markers

comprised p63, MUC5AC and β -tubulin IV for BCs, GCs and CCs, respectively. To quantify the epithelial markers, immunostained sections were scanned using a SCN400 scanner (Leica) and analyzed using TissueIA software (SlidePath). Counting of vimentin-expressing columnar cells was carried out manually by a blinded observer (CH). Serial stainings for vimentin and CD45 allowed confirming the presence of vimentin-expressing epithelial cells, in addition to intraepithelial leukocytes (Figure E1). Five random epithelial fields were examined per slide, at high magnification (x400). Data are reported for all patients, except when tissue and/or epithelial areas were not sufficiently preserved. BM thickness was measured by multiple point-to-point repeated measurements, as previously described¹⁹.

Quantitative RT-PCR for E-cadherin, CK5, vimentin, β -tubulin IV, MUC5AC and p63 gene expression

In order to confirm the staining for epithelial markers, mRNA expression levels were quantified in sinonasal tissue and normalized to GAPDH housekeeping gene by using the UPL-probe system (Roche Diagnostics, Basel, Switzerland).

Statistics

Statistical analyses were performed using IBM SPSS Statistics 21.0 (SPSS, Inc, Chicago, USA) and figures were designed using GraphPad Prism 5.00 (GraphPad Software, San Diego, USA). Results are shown as scatter dot plots with mean and standard deviation. Normality was assessed for each data set. For non-parametric data, Kruskal-Wallis test followed by Dunn post hoc test were carried out for multiple comparisons. Mann-Whitney U test was used for the analysis of differences between 2 groups of unpaired data. Normal data

were assessed by one-way Anova followed by Tukey post-hoc test for multiple comparisons. Correlation coefficients were calculated using Spearman's rank method. P-values <0.05 were considered as statistically significant.

RESULTS

Decreased expression of epithelial cadherin and keratins in CRS

To assess the differentiation state of the sinonasal epithelium, tissue sections of control subjects, CRSsNP, CRSwNP and AR patients were first immunostained for E-cadherin and Hmw CK (Figure 1A). E-cadherin was significantly decreased in patients with CRSsNP ($p = 0.03$) and CRSwNP ($p = 0.02$), when compared to controls (Figure 1B). Similarly, Hmw CK expression was decreased in CRSsNP ($p = 0.04$) and CRSwNP ($p = 0.01$), as compared to control subjects (Figure 1C). These results were confirmed by RT-PCR, showing decreased transcription of E-cadherin in CRSwNP and CRSsNP, while CK5 mRNA (a major Hmw CK, preferentially expressed by basal cells) was also significantly decreased in CRSwNP (Figure 1D).

Numbers of ciliated and goblet cells are not significantly changed in CRS

To address whether these epithelial changes related to changes in epithelial specification, numbers of CCs, GCs and BCs were evaluated in the CRS sinonasal epithelium by immunohistochemistry and RT-qPCR (Figure 2A). No significant changes were observed for β -tubulin IV + CCs and MUC5AC+ GCs, and this was confirmed at the mRNA level for these markers (Figure 2B-C). The number of p63+ BCs was decreased in CRSsNP, as

compared to controls ($p = 0.008$) (Figure 2B), while this change was not observed at the mRNA level (Figure 2C).

These data suggested that the epithelial de-differentiation may not be related to changes in the lineage specification of the upper airway epithelium.

Increased numbers of vimentin-expressing epithelial cells in CRS

We then evaluated whether epithelial de-differentiation could relate to the acquisition of a mesenchymal phenotype, by assessing the number of vimentin positive epithelial cells in the sinonasal epithelium of our patients. The number of vimentin expressing epithelial cells was significantly increased in CRSsNP ($p=0.001$) and CRSwNP ($p<0.0001$), as compared with controls (Figure 3A-C). In addition, increased vimentin-expressing epithelial cell numbers correlated with the radiological (Lund-McKay) score ($r = 0.59$, $p<0.0001$) (Figure E2-C). It was also correlated with the number of eosinophils ($r = 0.5$, $p = 0.004$) assessed in our previous study of the same series of patients²⁰ (figure E2-A).

Increased subepithelial fibrosis in CRS

We then evaluated whether this EMT feature was associated with subepithelial fibrosis, by measuring the BM thickness in the same epithelial areas (Figure 3B). We observed increased BM thickness in CRSsNP ($p = 0.04$) and CRSwNP ($p = 0.01$) (Figure 3D). In addition, BM thickening correlated with the CT score ($r = 0.48$, $p = 0.003$) (Figure E2-D) and with

vimentin-expressing epithelial cells ($r = 0.54$, $p = 0.0001$) (Figure 3F). Moreover, the BM thickness correlated with tissue eosinophilia ($r=0.36$, $p=0.04$) (Figure E2-B).

Discussion

This study shows in a large series of well-characterized patients that the sinonasal epithelium undergoes de-differentiation in CRS, which does not relate to changes in lineage differentiation (CCs/GCs), but to mesenchymal transition. In addition, percentage of vimentin-expressing epithelial cells correlates with subepithelial fibrosis evidenced by BM thickness and with the disease severity (CT scoring), suggesting a role in disease pathogenesis.

E-cadherin is a transmembrane glycoprotein that establishes and stabilizes cellular contacts in adherens junctions between adjacent epithelial cells. E-cadherin is involved in the control of several aspects of epithelial homeostasis, including morphological differentiation, cell proliferation and cell motility, notably during wound healing and inflammation²¹. A defective epithelial barrier has been recently described in patients with CRSwNP, along with a decreased expression of tight junction proteins, such as claudin-4, occludin, zonula occludens-1^{15, 16}. We confirm a defect in junctional complexes, by showing decreased expression of adherens junction-associated E-cadherin in CRSwNP. In addition, this epithelial defect was also observed in CRSsNP, suggesting that this feature may occur in different phenotypes of chronic sinusal disorders.

Our data suggest that EMT occurs in the upper airways of patients with CRS. In the lung, EMT has been shown in lung fibrosis¹⁴ and obliterans bronchiolitis²² and is debated in

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COPD²³ and asthma²⁴. During EMT, epithelial cells undergo phenotypic transition to mesenchymal cells, acquiring the intermediate filament vimentin in their cytoskeleton, subsequently become spindle shaped, and exhibiting front-to-back, leading-edge polarity¹³. EMT has not been specifically studied in chronic sinonasal diseases. One previous study showed the presence of vimentin+ epithelial cells in both normal nasal mucosa and CRS, and suggested that these cells may arise from EMT²⁵. A recent paper showed a trend to a higher, but not significantly, number of vimentin+ cells in the turbinate part of nasal polyps, as compared to normal mucosa¹⁶. Our study shows that the vimentin expression is increased in the epithelium from patients with CRSwNP and CRSsNP, as well as to a lesser extent (only at the transcriptional level) with AR. BM thickening has been described in asthma²⁶, COPD²⁷, and CRS²⁸. Accordingly, we found a thickening of the BM in CRSsNP and CRSwNP, while clefts identified within the BM from COPD patients²⁷ were not observed (not shown). The low number of vimentin-expressing epithelial cells may indicate that EMT selectively concerns certain cells and, although quantitatively limited, may reflect a profound reprogramming of the upper airway epithelium in CRS also characterized by the loss of junctional proteins (Figure 4). In addition, epithelial vimentin expression correlated with BM thickening, and both vimentin and BM thickness correlated with the radiological score, suggesting that EMT correlates to disease severity in CRS and could contribute to its pathogenesis.

In contrast to these differentiation features, no significant change in lineage differentiation was observed in CRS when assessing the two main differentiated cell types, namely GCs and CCs⁵. BCs are relatively undifferentiated cells, considered as a multipotent progenitor population and a common feature of stratified and pseudostratified epithelia throughout the body⁶. During normal epithelial turnover and repair, they progressively differentiate into

other cell types, such as GCs or CCs, reconstituting a functional respiratory epithelium⁶. An increase of BCs has been reported in CRSwNP¹². In contrast, we observed a decrease in p63+ BCs in patients with CRSsNP, while p63 mRNA expression was not affected, and a decrease in mRNA expression of CK5 (a CK preferentially expressed by BCs, along CK14) in CRSwNP. It remains thus unclear whether BC numbers are contributing to epithelial changes. In addition, whereas mucus-secreting GCs show varying density in the nasal mucosa (with a peak of 10.000 cells/mm² in the maxillary sinus²⁹) and changes in patterns of mucin gene expression^{11, 29}, studies showed contradictory results with either a decrease¹¹ or an increase¹⁰ of MUC5AC expression in CRSwNP. In our study, no significant change in the percentage of MUC5AC+ epithelial cells was observed. Similarly, no change was observed for β -tubulin IV+ CCs, considered as terminally differentiated columnar cells, both at protein and mRNA levels. These data suggest that, although the number of BCs is probably highly variable according to the turnover of the upper airway epithelium and may be affected in CRS, the differentiation programming into the two main cell types (CCs and GCs) is preserved in CRS.

In contrast, de-differentiation and EMT features were observed both in CRSwNP and CRSsNP. As these two disorders generally differ in terms of immune profile, this observation may indicate that these changes could occur independently of the nature of the inflammatory reaction. Similarly, EMT has been suggested in the bronchial epithelium both in asthma and COPD^{23, 24}. However, we observed that vimentin expression correlated with tissue eosinophils. It is known that damage to the epithelium closely correlates with the degree of eosinophil infiltration, and that eosinophil products can cause epithelial damage and submucosal oedema³⁰. Activated eosinophils contribute to CRSwNP through the release of major basic protein in the production of cytokines³¹. It could therefore be speculated, although several pathways could be involved, that eosinophils may contribute to epithelial

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de-differentiation and EMT. Alternatively, epithelial dysfunction could favour eosinophilia through several mechanisms such as impaired exclusion of proeosinophilic superantigens from *Staphylococcus Aureus*³². We recently reported decreased levels of IgA antibodies to *Staphylococcus Aureus* enterotoxin B in the nasal fluid from patients with CRSwNP²⁰, as the putative result of impaired pIgR expression in the sinonasal epithelium, which closely relates to epithelial differentiation (*Gohy AJRCCM 2014* provisionally accepted on 30 July 2014).

It is also very likely that EMT *per se* affects the epithelial barrier against pathogens, by defects in junctional complexes and in epithelial secretion of antimicrobial factors.

In summary, our data indicate for the first time that epithelial de-differentiation towards a more mesenchymal phenotype occurs in the upper airways of patients with CRSsNP and CRSwNP, independently of lineage differentiation into ciliated and goblet cells. This epithelial reprogramming correlates with subepithelial fibrosis and with disease severity. These findings also highlight common mechanisms of epithelial dysfunction between upper and lower airways, occurring during chronic inflammatory diseases. Future studies should address the underlying mechanisms, which could represent future therapeutic targets in CRS.

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Table 1. Patient characteristics.

	Controls	CRSsNP	CRSwNP	AR
N	13	11	11	10
Sex (M/F)	11/2	7/4	9/2	7/3
Age, years (range)	36.4 (18-62)	37.2 (20-51)	48.2 (28-73)	35.2 (20-48)
History of asthma	1	0	3	1
Atopy	0	0	4	10
Smoking	4 (30%)	4 (36%)	4 (36%)	3 (30%)
CT score	1 (0-2)	12 (7-21)**	17 (14-23)***	1 (0-2)

Patients were assigned to disease groups based on criteria described in Methods, and associated features (asthma, atopic background, cigarette smoking) are stated. Asthma diagnosis was based on the report of typical symptoms and lung function tests, according to GINA³³, and atopy was defined by the presence of skin-prick test or serum IgE responses to at least one common aeroallergen. ** p<0.01, *** p<0.0001 (Kruskal-Wallis test followed by Dunn post hoc test) for CT scores (Lund-MacKay), as compared to control subjects.

FIGURE LEGENDS

Figure 1: Immunohistochemistry for epithelial markers in sinonasal tissue.

- A. Immunostaining for E-cadherin (left) and Hmw CK (CK50) (right) in sinonasal epithelium. Representative images from each group are shown (scale bar, 100 μ m).
- B. Quantification of E-cadherin staining using TissueIA software (SlidePath) (n= 45).
- C. Quantification of the Hmw CK staining using TissueIA software (SlidePath) (n=41).
- D. E-cadherine (left) and CK5 (right) mRNA expression in sinonasal mucosa from patients with CRSwNP, CRSsNP and AR, as compared to controls, and normalized to GAPDH housekeeping gene.

Figure 2: Immunohistochemistry for different cell types in sinonasal tissue.

- A. Immunostaining for p63 (basal cells, left), MUC5AC (goblet cells, middle) and β -tubulin IV (ciliated cells, right). Representative images from each group are shown (scale bar, 100 μ m).
- B. Quantification of p63 (n=42), MUC5AC (n = 36) and β -tubulin IV (n=40) staining in sinonasal epithelium, expressed as percentage of positive cells (p63) and percentage of stained area (MUC5AC and β -tubulin IV).
- C. p63, MUC5AC and β -tubulin IV mRNA expression in sinonasal mucosa from patients with CRSwNP, CRSsNP and AR, as compared to controls, and normalized to GAPDH housekeeping gene.

Figure 3: Vimentin and fibrosis in sinonasal tissue.

- A. Immunostaining for vimentin in sinonasal epithelium. Representative images from each group are shown (scale bar, 100 μ m) (n=45).

- B. Evaluation of BM thickness; representative hematoxylin-and-eosin stained images from each group are shown.
- C. Quantification of vimentin staining in sinonasal epithelium (n=45), expressed as percentage of positive cells.
- D. Measurement of the BM height using TissueIA software (SlidePath) (n=45).
- E. Vimentin mRNA expression in sinonasal mucosa from patients with CRSwNP, CRSsNP and AR, as compared to controls, and normalized to GAPDH housekeeping gene.
- F. Correlation of vimentin expression to BM thickness.

Figure 4: Epithelial-to-mesenchymal transition in chronic rhinosinusitis

De-differentiation and EMT features are observed in CRSwNP and CRSsNP, characterized by decreased expression of junctional proteins associated with tight and adherens junctions, increase of intracellular vimentin filaments, and thickening of the basement membrane.

Figure E1: Serial immunostaining (5 μ m between sections) for vimentin (left) and CD45 (right). Red arrows show intraepithelial leukocytes that were not taken in account for vimentin quantification. Scale bar, 100 μ m.

Figure E2: Correlations between epithelial vimentin expression, eosinophils, subepithelial fibrosis and clinical CT score of sinusitis.

- A. Correlation between vimentin-expressing epithelial cells and eosinophil numbers.
- B. Correlation between BM thickness and eosinophil numbers.
- C. Correlation between vimentin-expressing epithelial cells and Lund-McKay CT score.
- D. Correlation of BM thickness and Lund-McKay CT score.

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