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Research Article

Epithelial-to-mesenchymal transition and some parameters of extracellular matrix remodeling in chronic rhinosinusitis with nasal polyps

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Abstract. Objective. The purpose of our study was to evaluate vimentin expression in nasal polyp tissue, concentrations of monocyte chemoattractant protein-1 (MCP-1) and matrix metalloproteinase-9 (MMP-9) in blood serum of patients with chronic rhinosinusitis with nasal polyps (CRSwNP).Materials and methods. Levels of MMP-9 and MCP-1 were determined in blood serum of twenty patients with CRSwNP using the commercially produced ELISA kits. Immunohistochemical vimentin staining using specimens of nasal polyp tissue was performed. The results were compared to the control group consisted of twenty relatively healthy subjects.Results. Vimentin was found to be strongly expressed in both the lamina propria and epithelial cells in nasal polyp tissue of patients with CRSwNP. Blood serum concentrations of MMP-9 and MCP-1 were higher in CRSwNP compared to the control individuals. MCP-1/MMP-9 ratio was higher as well. Conclusion. The development of nasal polyps is accompanied by an increase in the amount of vimentin-positive cells in the epithelial layer, which indicates the activation of endothelial-to-mesenchymal transition (EMT). CRSwNP is associated with the imbalance between profibrotic and antifibrotic factors.

Key Words: chronic rhinosinusitis with nasal polyps, vimentin, epithelial-mesenchymal transition, monocyte chemoattractant protein-1, matrix metalloproteinases, extracellular matrix remodeling.

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Introduction

Vimentin is a highly conserved 57 kDa protein made up of 466 amino acid residues whose expression is observed in normally functioning mesenchymal cells (Kidd et al 2014; Satelli & Li 2011). It is a component of the cell cytoskeleton network and vimentin intermediate filaments are involved in providing cellular integrity, maintaining structure and motility during cell migration (Richardson et al 2018; Satelli & Li 2011). Vimentin is considered a biomarker for epithelial-to-mesenchymal transition (EMT), which is associated with the loss of epithelial markers by cells and the gain of mesenchymal markers (Liu et al 2015; Kidd et al 2014). As a result of EMT, epithelial cells lose their polarity, their motility increases. Moreover, EMT cells are able to resist apoptosis and secrete components of the extracellular matrix. When cells undergo EMT, they stop expressing several epithelial markers, including E-cadherin and cytokeratin. However, such cells gain the ability to generate vimentin, fibronectin, matrix metalloproteinases (MMPs) (Liu et al 2015). The major driver of vimentin expression and, therefore, EMT is hypoxia (Kidd et al 2014). It has been reported that vimentin expression is upregulated by hypoxia-inducible factor-1 (HIF-1), produced in hypoxic conditions (Zhang et al 2015; Zhang et al 2013). In addition, EMT is triggered by some transcriptional factors, including Snail1, Slug and ZEB1, and several regulatory microRNAs (Voutsadakis 2016).

EMT is closely related to inflammation and tumorigenesis. There is strong evidence that inflammatory processes of various origins are associated with intense EMT and, therefore, overexpression of vimentin (Suarez-Carmona et al 2017; Kidd et al 2014; López-Novoa & Nieto 2009). One of the diseases associated with such tissue remodeling caused by the chronic inflammatory microenvironment is chronic rhinosinusitis, which is accompanied by the chronic inflammation in paranasal sinuses. The pathogenesis of chronic rhinosinusitis is not fully elucidated, and features of mesenchymal transition in the nasal epithelium, as well as peculiarities of extracellular matrix remodeling, remain unclear.

The aim of the research was to assess vimentin expression in nasal polyp tissues, blood serum levels of monocyte chemoattractant protein-1 (MCP-1) and matrix metalloproteinase-9 (MMP-9) in patients with chronic rhinosinusitis with nasal polyps (CRSwNP) in order to study the EMT of nasal epithelial cells and features of extracellular matrix remodeling.

Materials and methods

1. Patients, groups and tissue sample collection

The study comprised 20 patients (13 males; 7 females) whose age varied from 25 to 77 years with a clinical diagnosis of CRSwNP who were enrolled from the Kharkiv Regional Clinical Hospital. Diagnosis of CRSwNP was verified in accordance

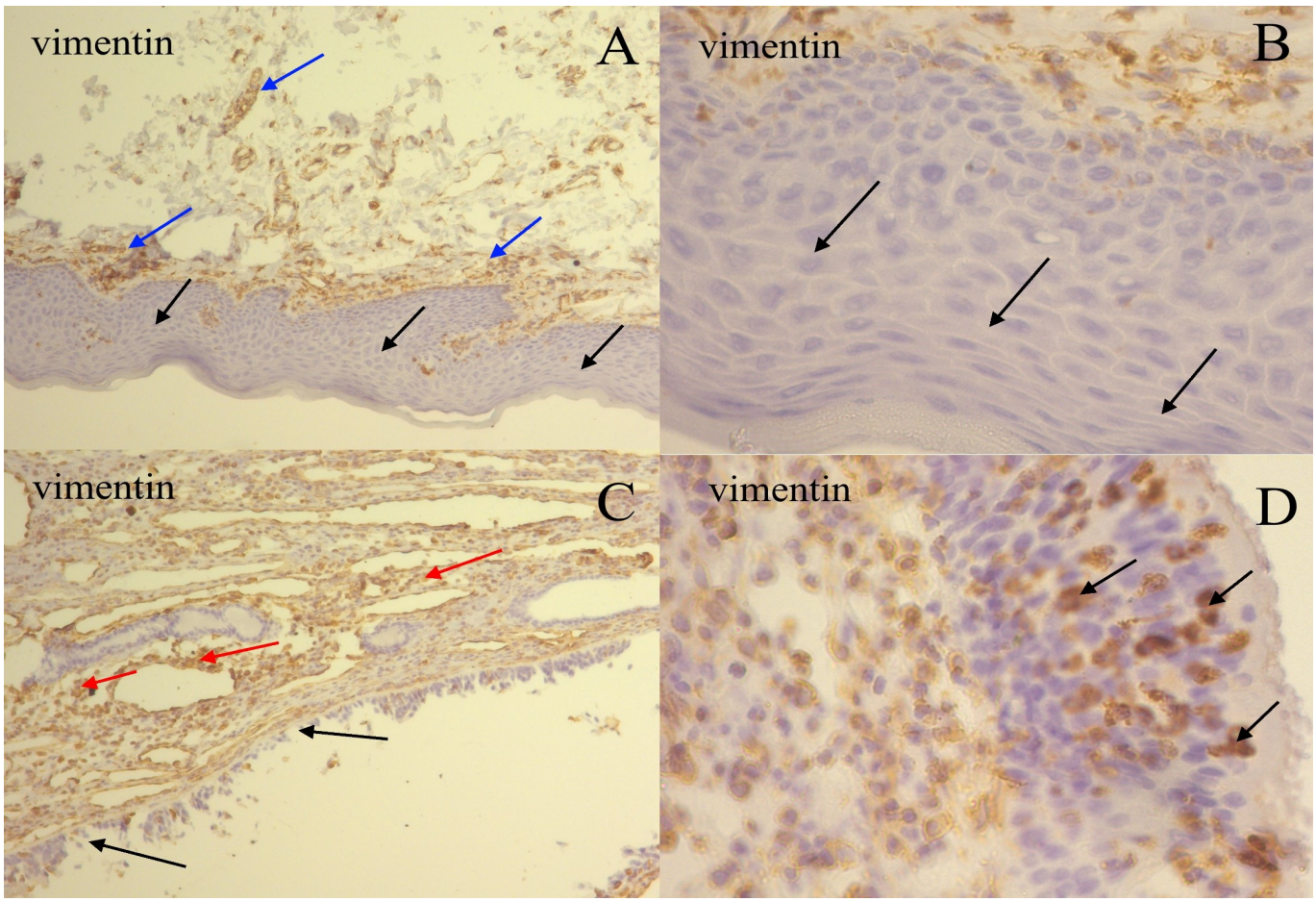

Fig. 1. Nasal epithelial immunostaining. A) Nasal tissue of an individual from the control group. Vimentin expression is virtually not observed in the epithelium (marked with black arrows). Some vimentin-positive cells are clearly visible in the lamina propria (marked with blue arrows). Immunohistochemical reaction with antibodies to vimentin. x100. B) Nasal tissue of an individual from the control group. There are no signs of vimentin expression in nasal epithelial cells. Vimentin-negative nasal epithelial cells are marked with black arrows. Immunohistochemical reaction with antibodies to vimentin. x400. C) Nasal tissue of a patient with CRSwNP. Atrophy of nasal epithelium is observed (marked with black arrows). Overexpression of vimentin in the lamina propria is revealed (marked with red arrows). Immunohistochemical reaction with antibodies to vimentin. x100. D) Nasal tissue of a patient with CRSwNP. Vimentin is expressed in cells of the epithelial layer, indicating the epithelial-mesenchymal transition (marked with black arrows). Immunohistochemical reaction with antibodies to vimentin. x400.

with the criteria of "EPOS 2012: European Position Paper on Rhinosinusitis and NPs 2012" guidelines (Fokkens et al 2012). Their mean age was 42.55±3.42 years.

Twenty individuals (16 males; 4 females) with mean age of 30.1±2.21 years ranging from 18 to 55 years who underwent surgery due to deviated nasal septum under combined general and regional anesthesia without any clinical sign of sinonasal inflammation were used as controls. Those patients who had been diagnosed with other acute or chronic inflammatory diseases, immunodeficiency, endocrine diseases, chronic cardiovascular pathology, any kind of tumors, pregnancy, and cystic fibrosis were excluded from the study. The patients did not exert any signs of atopic diseases and asthma at the moment of research beginning.

Samples of sinonasal tissues with polyps for the immunohistochemical study were collected from eight patients with CRSwNP (4 males; 4 females) with mean age of 39.13±3.76 years ranging from 29 to 56 years. Specimens of nasal mucous membranes were taken from seven control subjects with deviated nasal septum during surgery (6 males; 1 female). The mean age of control group was 35.43±4.86 years varying from 23 to 59 years.

Blood was collected from twenty patients and twenty controls in order to prepare serum for biochemical analysis of MCP-1 and MMP-9 concentrations.

2. Immunohistochemistry and morphology

Samples of nasal polyps were fixed in 10% neutral formalin solution. Tissue sections whose thickness was 4 μm were prepared from paraffin-embedded polyp tissues. The tissue sections were stained with picrofuchsin by Van Gieson. Imunohistochemical analysis was carried out using mouse monoclonal antibodies to vimentin manufactured by Thermo Fischer Scientific (UK). After incubation with the primary antibodies to vimentin, microslides were treated with anti-(mouse IgG)–horseradish peroxidase conjugate. Visualization was performed using 3,3′-diaminobenzidine (DAB).

3. ELISA

To determine the accurate concentrations of MCP-1 and MMP-9 in blood serum of control subjects and patients with CRSwNP, we used the ELISA kits from eBioscience (Vienna, Austria). Both kits were based on the quantitative sandwich enzyme

Table 1. Levels of MCP-1 and MMP-9 in blood serum of patients with CRSwNP

Group	Monocyte chemoattractant protein-1 (MCP-1), pg/ml	Matrix metalloproteinase - 9 (MMP-9), ng/ml	MCP-1-to-MMP-9 ratio
Control (n=20)	51.5 [49.26; 61.28]	2.73 [1.52; 4.75]	18.9
CRSwNP (n=20)	497.2 75.13; 624.2] p<0.001	4.77 [4.07;5.63] p<0.01	104.2

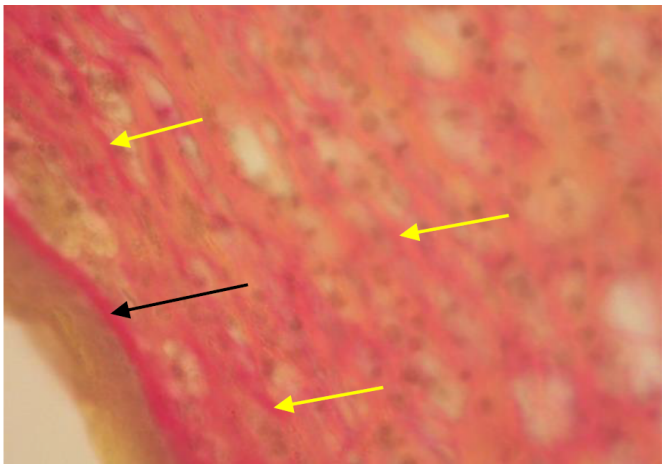

Fig. 2. Nasal tissue of a patient with CRSwNP. Basement membrane is thickened and collagen type IV is replaced by interstitial collagen fibers (marked with a black arrow). A lot of collagen is found in the lamina propria (marked with yellow arrows). Van Gieson's picrofuchsin staining. x100

immunoassay technique. All procedures were carried out in accordance with the instructions provided by the manufacturer. As soon as the color development was stopped, the optical density of the solutions was determined with the help of the Awareness Technology Stat Fax 303 Plus Microstrip Reader (USA). The concentration of MCP-1 in blood serum was expressed in pg/ml, while the level of MMP-9 was expressed in ng/ml.

4. Bioethics

All procedures and manipulations were carried out in accordance with the ethical standards of the Committee of Ethics and Bioethics of Kharkiv National Medical University and the revised Declaration of Helsinki (2000). All subjects from both groups signed a written informed consent.

5. Statistical analysis

Statistical analysis of numerical data obtained in our study was performed using GraphPad Prism 5 software. Mann-Whitney U test was used to process results of ELISA analysis and compare two groups. P value less than 0.05 was considered to be statistically significant.

Results

Mucous membrane of the nasal cavity in the control group is represented by a multirow epithelium and its lamina propria (Fig. 1a, Fig. 1b), separated by the thin basement membrane. Contour of epithelium connection with the lamina propria is slightly convoluted, i.e. the acanthotic epithelial projections are visualized as wide and shallow. The mucosal lamina propria contains a large number of both blood and lymphatic microcirculatory vessels. Lymphocytes and macrophages prevail in a non-abundant leukocyte infiltrate.

The epithelial layer of nasal polyps in patients with CRSwNP has pronounced signs of atrophy. Epithelial cells cover somewhere the lamina propria in one or two layers, and the basement membrane is either not observed or, on the contrary, is replaced by a thick layer of interstitial collagen (Fig. 2). In other areas, the epithelial layer of the mucosa is thicker. It consists of numerous epitheliocytes, and epithelial papillae can be seen on the surface of the epithelium, indicating an increased growth of epithelial cells highly likely due to the influence of growth factors. The lamina propria is abundantly infiltrated with leukocytes. There are many neutrophils among them (Fig. 1c).

In this study, we revealed that the layer of epithelium covering polyps was characterized by various morphofunctional states, both hyperplastic and atrophic. The latter was accompanied by a thick collagen interlayer between the epithelium and the mucosal lamina propria. Lymphocytes and macrophages prevail among the multiple leukocytes in the lamina propria. It is clearly visible on microslides stained with picrofuchsin by Van Gieson that the process of epithelial atrophy occurs simultaneously with the accumulation of collagen along the stroma of the polyp, i.e. collagen fibers are not abundant when the epithelium is hyperplastic, whereas when it is atrophic, the density of collagen fibers in the stroma of the polyp rises sharply (Fig. 2).

Vimentin immunostaining was almost not observed in nasal epithelial cells of control subjects (Fig. 1a, Fig. 1b). However, vimentin was markedly expressed in the nasal epithelium of patients with CRSwNP (Fig. 1d).

Vimentin-positive cells were found in the lamina propria of nasal tissues in both groups. In control subjects, vimentin was weakly expressed in the lamina propria (Fig. 1a). In contrast to the control group, vimentin was strongly expressed in the stromal tissue in patients with CRSwNP (Fig. 1d).

We determined concentrations of MCP-1 and MMP-9 in blood serum of patients with CRSwNP and control subjects. The former is tightly related to fibrotic processes stimulating them via the induction of collagen secretion, while the latter is involved in the destruction of collagen fibers due to its collagenase activity. It was found that MCP-1 was upregulated in CRSwNP. Its level in blood serum of patients was approximately 8-fold elevated in comparison with the control group. The elevation of MMP-9 blood serum concentrations was less pronounced (Table 1). We calculated the MCP-1-to-MMP-9 ratio to evaluate the state of fibrosis/antifibrosis system. This ratio was found to be 5.5-fold higher in CRSwNP than in the control group (104.2 versus 18.9).

Discussion

Our findings suggest that CRSwNP is accompanied by EMT in the nasal epithelium, evidenced by vimentin upregulation in nasal epithelial cells. Our results are consistent with data of other researchers confirming the overexpression of vimentin in the airway epithelium of patients with CRSwNP (Hupin et al 2014; Shi et al 2012). However, the role of pro-fibrotic and

anti-fibrotic factors in the EMT mechanisms in airway remodeling was not described.

The increase in the amount of collagen fibers and overexpression of vimentin in the lamina propria of paranasal mucous membrane found by us can be explained by the extracellular matrix remodeling induced by inflammation with the predominance of fibrosis-stimulating factors over the fibrosis-inhibiting ones. In particular, MCP-1 is a chemokine with powerful stimulatory effects on collagen synthesis whose upregulation has been reported to occur in numerous fibrosis-associated diseases (Shuiai et al 2017; Li et al 2005). Its elevation in blood serum of patients with CRSwNP may contribute to the excess deposition of collagen fibers in the extracellular matrix. However, matrix metalloproteinases involved in degradation of the extracellular matrix due to their collagenase activity have anti-fibrotic properties and seem to counterbalance the overactivation of pro-fibrotic factors, including MCP-1 (Giannandrea & Parks 2014). Taking into account a significantly higher MCP-1-to-MMP-9 ratio in patients with CRSwNP compared to the control group, we can presume that the activation of MMP-9 is not sufficient to counteract the action of pro-fibrotic MCP-1, which results in the accumulation of collagen fibers. In addition to its ability to directly increase collagen synthesis, MCP-1 promotes EMT in different tissues (Li et al 2017; Lee et al 2015). However, there are no data on the effects of MCP-1 on EMT in the nasal epithelium in chronic rhinosinusitis. We hypothesized that MCP-1 might be involved in the promotion of EMT in nasal polyps in patients with CRSwNP. However, this hypothesis is to be verified.

Converging lines of evidence indicate that EMT is involved in fibrosis in different organs and tissues (Stone et al 2016). In particular, Li M et al have demonstrated that mesenchymal cells derived from the epithelial ones may promote tissue fibrosis via the secretion of extracellular matrix components (Li et al 2016). Thus, EMT may contribute to the development of fibrosis in patients with nasal polyposis via an increase in the number of fibroblasts/myofibroblasts that derive from epithelial cells and are able to generate extracellular matrix structural components. This becomes possible due to the ability of the former epitheliocytes differentiated into mesencymal cells to move to the lamina propria thanks to their migratory capacity. Their migration may be facilitated by the destruction of basement membrane observed by us in some regions of polyp tissues.

We believe that the prevention of EMT may reverse or stop the progression of fibrosis in polyp tissue of patients with CRSwNP. Thus, EMT can be considered a potential target for treatment of CRSwNP.

Conclusions

During the nasal polyp development, the phase of proliferation followed by its atrophy, evidenced by epithelial hyperplasia and less evident stromal sclerosis, is observed at the beginning of its formation. Epithelial atrophy against the background of progressive sclerosis is revealed later. Vimentin immunohistochemical staining, which is used to detect mesenchymal cells, allowed us to reveal the presence of vimentin-positive cells in the hyperplastic epithelial layer. The number of vimentin-positive cells is also higher in the stroma. The imbalance between pro-fibrotic MCP-1 and anti-fibrotic MMP-9 confirms the intensification of stromal sclerosis evidenced by morphological studies.

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