Different activation of toll-like receptors and antimicrobial peptides in chronic rhinosinusitis with or without nasal polyposis

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Key words: antimicrobial peptide, cathelicidin, chronic rhinosinusitis, defensin, lactoferrin, lysozyme, nasal polyp, toll-like receptor.

Abstract

Background: Both up- and down-regulation of the Toll-like receptors (TLRs) and antimicrobial peptides (AMPs) of the sinonasal mucosa have already been associated with the pathogenesis of chronic rhinosinusitis with (CRSwNP) or without (CRSsNP) nasal polyps.

Objective: Determination of the expression of all known TLR and several AMP genes and some selected proteins in association with allergy, asthma and aspirin intolerance (ASA) in CRS subgroups.

Methods: Patients with CRSsNP (n=19), CRSwNP (ASA[-]:17; ASA[+]:7) and control subjects (n=12) were selected. Sinonasal samples were taken during endonasal surgery and RT-PCR was used to measure mRNA expressions of 10 TLRs, 4 defesins, lysozyme, cathelicidin and lactoferrin (LTF). In several cases immunohistochemistry was performed to evaluate protein expressions. Statistical analysis was done with the Kruskall-Wallis ANOVA, Mann-Whitney U and Student-t-test.

Results: TLR2, TLR5, TLR6, TLR7, TLR8, TLR9, β-defensins 1 and 4, cathelicidin and LTF mRNA expressions were significantly ($p<0.05$) increased in CRSwNP, whereas only TLR2 and LTF were up-regulated in CRSsNP compared to controls. There was no statistical difference in respect of allergy, aspirin intolerance and smoking between CRSsNP, ASA(-) and ASA(+) CRSwNP patients. TLR2, TLR3, TLR4, LTF, β defensin 2 and lysozyme protein expression was found to be increased in CRSwNP stainings ($p<0.05$) dominantly in macrophages.

Conclusions: Gene expression analysis showed markedly different expressions in CRSwNP (6 out of 10 TLR and 4 out of 7 AMP genes were up-regulated) compared to CRSsNP (1/10, 1/7). The distinct activation of the innate immunity may support the concept, that CRSsNP and CRSwNP are different endotypes of CRS. These findings were found to be independent from allergy, asthma, smoking, aspirin intolerance and systemic steroid application.

Introduction

Chronic rhinosinusitis (CRS) is characterized by the presence of at least two symptoms from nasal blockage, anterior/posterior nasal discharge, facial pain/pressure and reduction of smelling. Either nasal blockage or nasal discharge should be part of the symptoms, which persist for at least 12 weeks. The diagnosis is completed by positive endoscopic and/or imaging findings. According to the most recent guidelines [1] CRS is classified into two subgroups: CRS without nasal polyps (CRSsNP) and CRS with nasal polyps (CRSwNP). Besides the key phenotypes of CRS – on the basis of special biological markers - further endotypic differentiation of CRS subgroups has been suggested [2,3,4]. Eosinophil infiltration of the epithelial and subepithelial tissues is considered as the hallmark of bilateral diffuse nasal polyposis as compared with CRSsNP. Th1-biased adaptive immune polarization has been reported in CRS, while Th2-biased route with high tissue level of IL-5 has been demonstrated in nasal polyposis as compared with healthy controls [3]. Systemic and local factors such as allergy, mucociliary dysfunctions, asthma, aspirin-intolerance, immune deficiencies, gastro-oesophageal reflux disease, anatomical variations, biofilm formation and osteitis are often associated with CRS with or without nasal polyps [1]. Persistent mucosal inflammation and microbial dysbiosis, as well as reduced bacterial diversity have also been implicated with CRS, though the relationship between sinus microbiome and CRS is still unclear [5]. Several recent theories have postulated the participation of fungal and bacterial superantigens, as well as a defective interaction between the host organism and the environment characterized by an altered link between the innate and adaptive immune systems especially on the border-line mucosal surfaces [6,7]. Besides the physical barrier function, the epithelial cells secrete human antimicrobial peptides (AMPs), which exert a variety of antimicrobial, chemotactic and suppressive effects, and also affect established biofilms. Moreover, the epithelial, dendritic and B-cells and also macrophages exhibit cell-surface and endosomal Toll-like receptors (TLRs), which recognize pathogen associated

molecular patterns (PAMPs). TLRs are triggered by a variety of microbial ligands such as lipid-based elements (lipopolysaccharide, lipoteichoic acid), nucleic acids (genomic double- and single-stranded RNS) and proteins (lipopeptide) from Gram-positive and Gram-negative bacteria, fungi and viruses (8). Several TLRs can function in heterodimer formation (TLR2 with TLR1 or TLR6) to identify special structures (triacylated and diacylated lipopeptides). AMPs and the TLRs are key features of the innate immune system, thus their dysfunctions may be associated with improper signalling to the adaptive immune system as well as with disrupted microbial load in the sinus mucosal surfaces. In spite of the previous studies it has remained controversial, whether increased or decreased expressions of TLRs and AMPs are associated with the pathogenesis of CRS. In Table 1 we demonstrated a variety of the controversial results of different authors [9-20] regarding TLR gene and protein expressions in CRSsNP and CRSwNP. Diversity in the selection criteria, application of systemic steroids and different sites of tissue biopsies used for investigations may be responsible for the conflicting results. Allergy and aspirin-intolerance may also be considered as modifying factors. Moreover, our previous study on the -308 G>A SNP of the TNFA gene [21] suggest, that the aspirin-sensitive CRSwNP subjects differ not only in the exhibited clinical symptoms, but also in the genetic predisposing factors and possibly also in the molecular pathogenesis leading to the development of various forms of CRS.

In respect to the conflicting results in the literature we investigated in our current study the expression profiles of all the 10 TLRs and several AMPs (cathelicidin /CAMP/, lysozyme /LYZ/, lactoferrin (LTF) and human β-defensin 1-4 /HBDs/) in a context, standardized study and the results have been correlated between the different CRS subgroups of patients (CRSsNP, CRSwNP, aspirin tolerant and intolerant CRSwNP) and healthy controls. Clinical data of allergy, asthma, systemic steroid application and smoking were also used in the analysis of the results.

Materials and Methods

Nasal tissue was taken from 43 patients with CRS and from 12 controls. Polyp tissue was used in CRSwNP subjects, which was harvested from the uncinate process of the ethmoid during routine endoscopic sinus surgery. Sinus tissue was gained in the same way from the ethmoid cells of CRSsNP patients. Patients undergoing nasal surgery because of non-inflammatory problems (septal surgery or turbinate reduction) were selected as controls and samples were taken from the inferior turbinates. The allergic status was evaluated by means of skin prick test to common inhalant allergens and with the data obtained from the patient's history. Asthma was diagnosed by a pulmonologist, whereas aspirin intolerance was identified from the medical history and with the specialists' assessment. The diagnosis of CRS was established in conformity with the European guidelines [1]: besides history and symptomatology nasal endoscopy and paranasal sinus CT scan were performed in all patients to assess CRSsNP and CRSwNP subgroups. CRSwNP patients were selected into aspirin-tolerant and aspirin-intolerant subjects. The following conditions were regarded as exclusion criteria: congenital mucociliary problems, cystic fibrosis, immunodeficiency, granulomatous and invasive fungal disease, cocaine abuse, neoplasms, acute infections, pregnancy and oral corticosteroid consumption within the last 3 months. Allergic and non-allergic sinonasal inflammation was additional exclusion criterion in the controls. All patients applied intranasal corticosteroids for at least 3 months prior the investigation and all the asthmatic patients used inhalative steroids. All of the participating subjects gave their signed informed consent and the study was approved by the national (Health Science Council, Scientific and Ethics Board) and local Review Boards.

Histopathology

Tissue samples were fixed in buffered formalin and the paraffin-embedded blocks cut in 4μm slices and stained with hematoxylin-eosin. Semi-quantitative evaluation was performed by two pathologists with the use of light microscopy and a scoring system (0-3) as follows:

epithelium (0:normal, 1:erosion, 2:metaplasia); intraepithelial lymphocytosis (IEL, 0:not visible, 1:slight); mucous secretion (0:not visible, 1:yes /goblet cell, mucinous salivary gland/); basal membrane (0:normal, 1:thick); stroma (0:normal, 1:edema, 2:fibrosis); inflammatory cells (0:not visible, 1:granulocyte, 2:lymphocyte/plasma cell); eosinophil cells (0:not visible, 1:significant [x/1 HPF /high power field/]; mast cells (0:not visible, 1: significant [x/1 HPF].

Gene expression analysis

Gene expression analysis (n=55) was performed to measure the mRNA expression of all 10 TLRs, CAMP, LYZ, LTF, human β-defensins 1-4 (DEFBs) and FOXP3, T-bet (TBX21), GATA-3 transcription factors.

Total RNA was extracted from tissue samples by using the TRIzol reagent method (Invitrogen, Carlsbad, CA, USA), as described by the manufacturer. The quantity and quality of RNA isolates were checked by spectrophotometry, and the isolates were purified with the SV Total RNA Isolation System (Promega, Madison, WI, USA). 250 ng of total RNA was reverse-transcribed with the RT^2 PCR First Strand Kit (QIAGEN SABiosciences) according to the manufacturer's instructions. Reverse transcription (RT) was followed by amplification with an iCycler machine (iCycler IQ Real Time PCR, BioRad Laboratories, Hercules, CA, USA). For mRNA detection the RT^2 Custom PCR SuperArray Kit (QIAGEN SABiosciences) was used as described by the manufacturer. The kit contained the 20 genes of interest with 5 housekeeping genes, internal and external RT and PCR controls.

Immunohistochemistry

We used tissue microarray techniques (TMA) for immunostaining. The staining protocol used the EnVision™ FLEX kit on Dako Autostainer Plus (Dako, Denmark). The sections were incubated as primary antibody by Lysozyme Ab-1 (Labvision, USA) 1:200; TLR2 (Ab6100, Abcam, UK) 1:50; TLR3 (ab13915, Abcam, UK) 1:200; TLR4 (ab22048, Abcam,

UK) 1:200; T-bet (H-210) (Santa Cruz, USA) 1:200; GATA-3 (HG3-31) (Santa Cruz, USA) 1:50; Beta 2 defensin (ab63982, Abcam, UK) 1:1000; TLR5 (H-127) (Santa Cruz, USA)1:50; Lactoferrin (H-65) (Santa Cruz, USA)1:50; Cathelicidin (ab64892, Abcam UK) 1:500. Semi-quantitative evaluation was performed, the density of the immunosignal was scored (0-3) by two independent specialists. The scoring was based on the intensity and range of the signals: no signal positive cells:0; randomly 1-1 positive cells:1; several positive cells:2; many cells with intensive signal:3.

Statistical analysis

For statistical evaluation of the relative mRNA gene expressions of the samples, the web-based RT^2 Profiler PCR Array Data Analysis Version 3.5 software was used. Quality control was performed using this software. Fold changes in gene expression between control and different groups of patients were calculated using the ΔΔCt method in this PCR data analysis software. Distribution of the samples was tested with the Kolmogorov-Smirnov test. Analysis of the p values was performed with the non-parametric Kruskall-Wallis ANOVA test of the replicate 2^(- Delta Ct) values for each gene in the control and patient groups. The level of significance was accepted at p values less than 0.05. The Mann-Whitney U and Student-t-test were performed to compare paired sets of immunohistochemical data. Fisher exact test and Bonferroni correction was applied in the assessment of the demographic data. The level of statistical significance was taken at a $p < 0.05$.

Results

Subjects and patients

Demographic data of the different CRS subgroups are displayed in Table 2. There was no statistical difference in respect of allergy and asthma between CRSsNP (allergy5/19; asthma 4/19) and ASA(-) CRSwNP (allergy 4/17; asthma 4/17) groups of patients. The incidence of allergy was also not different between ASA(+) (1/7) and ASA(-) CRSwNP (4/17)subjects. Asthma was significantly more frequent in aspirin intolerant CRSwNP (5/7) compared to ASA(-) CRSwNP (4/17, p=0.004) and CRSsNP (5/19, p=0.004). The incidence of previous endoscopic surgeries was higher in the CRSwNP (3.2$\pm$1.2,) group compared to the CRSsNP (0.19$\pm$0.1, p=0.001) and ASA(-) CRSwNP (1.59$\pm$0.4, p=0.005) groups of patients. Smoking was detected by one subject only in the ASA(-) CRSwNP group.

Histopathology

Histological examination revealed no relevant differences between CRSsNP and CRSwNP samples regarding the epithelium, basal membrane, inflammatory and mast cells. The number of goblet cells, mucinous salivary glands and fibrosis was increased in the CRSsNP samples, whereas intraepithelial lymphocytosis (IEL) was more frequently seen in CRSwNP. Remarkable tissue eosinophilia was found in both CRSwNP groups (ASA(-):16/17; ASA(+):6/7) as compared to CRSsNP (7/19) and controls (2/12, 5/HPF=2). The absolute eosinophil cell number (>25/HPF) was elevated in the aspirin intolerants. For the evaluation of the results semi-quantitative analysis was utilized.

Expressions of the TLR-genes

All the 10 examined TLRs were expressed in our CRS samples, TLR2 was the only gene which was significantly up-regulated (5.472) in the CRSsNP group compared to controls (p=0.025). TLR2 (6.994, p=0.005), TLR5 (2.057, p=0.004), TLR6 (2.902, p=0.038), TLR7 (5.219, p=0.000), TLR8 (3.345, p=0.000) and TLR9 (2.112, p=0.008) mRNA levels were

significantly increased in the CRSwNP group relative to the controls. TLR7 (2.853, p=0.027), TLR8 (2.117, p=0.008) and TLR9 (1.490, p=0.008) mRNA expressions exhibited significant elevation in CRSwNP compared to CRSsNP. If aspirin tolerant (ASA-) polyps were compared to controls TLR2 (6.651, p=0.018), TLR5 (2.100, p=0.008), TLR7 (4.000, p=0.005), TLR8 (3.620, p=0.001) and TLR9 (2.295, p=0.006) genes displayed significant increase of expression, which was found only in TLR7 gene (8.004, p=0.005) in the ASA(+) polyps. There was no statistical difference between the expressions profiles of the aspirin tolerant and intolerant subgroups (Table 3A-4A).

Gene expressions of the antimicrobial peptides

LTF (2.128, p=0.005), CAMP (18.415, p=0.036), DEFB1 (2.254, p=0.001) and DEFB4 (3.248, p=0.009) showed significant up-regulation in the CRSwNP group as compared to controls. The expression of LTF was increased in CRSsNP samples compared to controls (1.644, p=0.044) and the expression of DEFB1 in CRSwNP group was significant (2.362, p=0.036) relative to CRSsNP samples. In ASA(-) CRSwNP group the expressions of LTF (1.696, p=0.026), CAMP (23.237, p=0.039), DEFB1 (2.695, p=0.001) and DEFB4 (3.200, p=0.016) genes were significantly increased versus controls. None of the genes showed up-regulation in the ASA(+) CRSwNP subgroup compared to controls and aspirin tolerant CRSwNP subgroup (Table 3A-4A.).

Transcription factors

Increased FOXP3 expression could be detected in CRSwNP (2.041, p=0.001) and ASA(-) CRSwNP (2.137, p=0.001) samples versus controls. TBX21 (T-bet) but not GATA3 expression was found to be higher compared to controls in both CRSsNP (4.893, p=0.005) and CRSwNP (6.346, p=0.000) groups of patients. Statistical evaluation was accomplished in all gene expression studies by means of the Kruskall-Wallis ANOVA test.

Immunohistochemistry

The semi-quantitative analysis of TLR2 (p=0.009), TLR3 (p=0.000), TLR5 (p=0.032) and CAMP (p=0.029) proteins revealed significantly higher expression versus controls in the mucosal samples of subjects with CRSsNP. In CRSwNP samples TLR2 (p=0.033, Fig. 1), TLR3 (p=0.027), TLR4 (p=0.002), LYZ (p=0.035, Fig. 2), LTF (p=0.012), DEFB2 (p=0.024) and GATA3 (p=0.038) expressions were found to be increased compared to controls. The expressions profile was similar in the aspirin tolerant (TLR3: p=0.013; TLR4: p=0.000; LYZ: p=0.032; HBD2: p=0.024) CRSwNP samples (Table 3B-4B). Localization of the TLR and AMP-positive cells revealed, that the staining was found mainly in the intraepithelial and subepithelial region and in inflammatory cells. TLR2, TLR3, TLR4 and lysozyme positive staining was observed dominantly in macrophages of ASA(-) and ASA(+) CRSwNP samples. The Mann-Whitney U and Student-t-test were performed to compare paired sets of immunohistochemical data.

Discussion

Up-regulation of TLR genes was found about twofold more frequently in the literature [9,11,14,18] than down-regulation (Table 2). The majority of these studies looked at a certain group of TLRs and AMPs and selection of CRS-subgroups was often missing. Our findings demonstrate clearly, that there is an apparent difference between the CRSsNP and CRSwNP subjects regarding the activation profiles of TLRs and AMPs (Figure 3). In our CRSwNP patients 6 out of 10 TLRs and 4 out of 7 AMPs showed significant mRNA up-regulation compared to healthy controls in contrast to the CRSsNP samples (1/10, 1/7). Both TLRs responsible for bacterial (TLR2, TLR5, TLR6) and viral recognition (TLR7, TLR8, TLR9) were found to be overexpressed in CRSwNP. The “activation boost” of almost all TLRs can perpetuate a chronic inflammatory state. The TLRs not only function as pathogen recognition receptors, but also can transmit “danger” signals (DAMPs) from the neighbouring damaged cells. The activation of TLRs may therefore play an important role in the maintenance of pathologically overwhelmed innate immune mechanisms in NP tissue.

It is noteworthy, that our results regarding the up-regulation of TLR5, TLR6, TLR8 and DEFB4 genes could not be matched with other results, because these genes have not been intensively studied so far. Interestingly up-regulation of TLR4, which is triggered by Gram negative bacterial LPS was not observed in our CRSsNP or CRSwNP subjects. The protein expressions of TLR2 and TLR4 were in good consistency with our mRNA findings in CRSwNP compared to controls and CRSsNP. The majority of the examined AMPs (3/4) were overexpressed on the immunostaining samples of CRSwNP compared to controls, which was less in CRSsNP (1/4). We found intensive TLR2, TLR3 and LYZ protein expressions in the macrophages of the mucosal and glandular epithelium in our CRSwNP subjects (Figure 1-2). It has been recently described, that macrophages - regardless of the atopic status - are extensively involved in the inflammatory mechanisms of CRSwNP, which may contribute to

the Th2 skewing in this subgroup [22]. These findings suggest, that TLRs may have important role in the activation mechanisms of macrophages.

Up-regulation of LTF, LYZ, DEFB1 and DEFB2 genes in CRSwNP has already been published previously [23, 24, 25, 26, 27] and it was also demonstrated, that defensins may enhance immunogenicity of the microbial antigens and may act as mucosal adjuvants [28]. Besides LTF, CAMP and DEFB1 we detected enhanced DEFB4 mRNA expression in our CRSwNP samples and this result may complete the findings already described by others. In the study conducted by Liu et al. [23] the authors reported that LTF was among the 5 most over-expressed genes out of 192 up-regulated genes studied with DNA microarray in CRSwNP samples relative to normal sinus tissue. Psaltis et al. [29] found, that decreased LTF expression correlates with biofilm formation. LTF was also shown to play an important role in eosinophil activation and de-granulation [30], as well as in NP formation via its ability to inhibit the growth of NP fibroblasts [31]. The pivotal role of LTF seems to be supported by our results, since besides it was expressed in CRSwNP on both mRNA and protein level, it is the only AMP which was found to be elevated in CRSsNP.

With one exception none of the TLRs and AMPs were overexpressed in ASA(+) CRSwNP compared to controls and ASA(-) CRSwNP subjects. Moreover, the expression profiles of CRSwNP and ASA(-) CRSwNP patients exhibited remarkable similarities, which suggests, that the characteristics of the CRSwNP group are independent from aspirin intolerance. This might be surprising in the light of that, patients with aspirin-exacerbated respiratory disease (AERD) make up a clinically more severe subgroup with more frequent asthma exacerbations, recurrences of nasal polyps, increased tissue colonization rate of Staphylococcus aureus, elevated polyclonal tissue IgE and eosinophilia [32]. According to our genetic study [21] this subset of patients may also be different in their genetic predisposing factors as well. Lack of up-regulated innate immunity in ASA(+) CRSwNP may be a specific feature in AERD.

Moreover, our results seem to be free of the effects of allergy, smoking and systemic corticosteroid application, because the incidence of these factors was not significantly different between the patient groups and systemic steroid usage was excluded.

It has been suggested earlier that the dysfunctions of the mucosal innate immune system can lead to alterations in the down-stream regulation of the adaptive immunity [7, 33]. In order to find out whether alterations in the TLR and AMP activation have an effect on the T cell response associated transcription factors we measured FOXP3, TBX21 (T-bet) and GATA3 expressions in our samples. Enhancement of TBX21 gene expression in CRSsNP and CRSwNP, up-regulation of FOXP3 expression in CRSwNP together with elevated GATA3 protein expression in CRSwNP samples compared to controls reflects increased activation of these transcription factors. It can be assumed, that this indicates more intensive downstream-signalling from the innate to the adaptive immunity. This presumption needs further support, since all the information gathered from mixed mucosal samples - containing epithelial cells, fibroblasts, immune and inflammatory cells - have limitations.

In contrast to the CRSsNP group the expression boost of the TLRs and AMPs in CRSwNP indicate a wide-ranged activation of the innate immune system in this subgroup of patients. It remains the question under debate, whether the wide-ranged over-expressions in CRSwNP or the missing up-regulation of genes in CRSsNP should be considered as primary or more relevant. Both, dysfunctional or insufficient up-regulation of the innate immune system in CRSsNP and/or an over-regulation in CRSwNP patients may lead to persistent mucosal inflammation and improper signalling to the adaptive system. It is also not clear whether the enhanced TLR-mediated signalling or the overexpression of TLR-genes has to be considered as a key feature. Regarding the constant microbial load to the upper respiratory tract the normal and balanced functions of the innate immune system seem to be essential in order to maintain the individual sinus microbiome and mucosal health in the sinuses [5]. TLRs are

considered as appropriate targets to be addressed via therapeutic agents [9], but it seems to be essential whether TLR agonists or antagonist should be taken into consideration in the future treatment options of CRS. More sophisticated genetic investigations may give further important viewpoints [21, 34] to understand the pathogenesis of CRS.

Conclusion

We found significantly increased expressions of 6 out of 10 TLR and 4 out of 7 AMP genes in CRSwNP, which was notably different from the findings in CRSsNP (1/10, 1/7). The distinct activation patterns of the innate immune system may support the concept, that CRSsNP and CRSwNP are different endotypes of CRS. Associated factors such as allergy, asthma, smoking, aspirin intolerance and systemic steroid application had no effect on the results. Lack of up-regulated innate immunity in ASA(+) CRSwNP may be a specific feature in AERD. The enhanced expression of proteins in macrophages of CRSwNP samples support the role of these cells in TLR activation. Further studies are needed to elucidate the genetic background of TLR and AMP activation mechanisms in the different groups of CRS patients.

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