Original Article
Relationship of TLR2, TLR4 and tissue remodeling in chronic rhinosinusitis

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Abstract: In order to explore the role of innate immunity in the remodeling of CRS (chronic rhinosinusitis), we investigated the correlation between TLR2, TLR4 and remodeling involved cytokines and histopathological features. Immunohistochemical staining was applied to detect the expression of TLR2, TLR4 and TGF-β1. Masson staining was used for observing the collagen deposition. The other histopathologic features of remodeling were observed by hematoxylin and eosin (HE) staining. Nasal epithelial cell culture was used to elucidate the effect of TLR2, TLR4 agonists and inhibitors on the expression of TGF-β1 and MMP-9. The association study showed that the significantly higher expression of TLR2, TLR4, TGF-β1 and collagen appeared in CRSsNP (chronic rhinosinusitis without nasal polyps) patients compared with CRSwNP (chronic rhinosinusitis with nasal polyps) patients. In CRSsNP, patients with a severe epithelial damage (score 3) had a significantly higher expression of TLR2 than patients with mild epithelial damage (score ≤ 2) (P < 0.05). Moreover the expression of TLR2 correlated negatively with squamous hyperplasia in CRSsNP, and positively with gland hyperplasia in CRSwNP. The expression of TLR2 and TLR4 was closely related to neutrophil infiltration in CRSsNP (P < 0.01). TGF-β1 was downregulated by TLR2 agonist in CRSwNP and upregulated by TLR4 agonist in CRSsNP (P < 0.05). MMP-9 was upregulated by TLR4 agonist in CRSwNP (P < 0.05). TLR2 and TLR4 had close relationship with TGF-β1 and the histologic features of remodeling, especially collagen deposition and neutrophil infiltration in CRSsNP. The innate immunity could influence the histologic characteristics and involved cytokines through TLR2 and TLR4 in the remodeling of CRS.

Keywords: TLR2, TLR4, TGF-β1, tissue remodeling, chronic rhinosinusitis

Introduction

Remodeling is defined as a dynamic process leading to transient or permanent changes in tissue architecture, which results in both extracellular matrix production and degradation. Remodeling involves with the normal reconstruction process as well as the pathological reconstruction with formation of pathological tissue [1]. The mucosal remodeling was first described in the lower airway in asthmatic patients [1], and has been extensively studied. The upper airway has the similar remodeling phenomenon and it has been proved that remodeling is also present in chronic rhinosinusitis (CRS). Moreover different patterns of CRS are characterized by different remodeling features.

According to the inflammatory and remodeling features, chronic rhinosinusitis can be divided into two major subgroups: chronic rhinosinusitis with nasal polyps (CRSwNP) and chronic rhinosinusitis without nasal polyps (CRSsNP). In Caucasians, CRSwNP is characterized by a predominant Th2-skewed eosinophilic inflammation with high levels of IL-5, ECP and eotaxin, and high levels of local IgE [2, 3, 5]. However, in Asian CRSwNP, it was observed as a Th1/Th17 skewed neutrophilic inflammation [4]. Typical remodeling features in CRSwNP are characterized by the pseudocysts formation consisting of albumin accumulation and oedema formation, the lack of collagen within the extracellular matrix and the downregulation of TGF-β1 expression. In contrast, CRSsNP is characterized by a mainly Th1-skewed neutrophilic inflammation with high levels of IFN-γ and upregulation of TGF-β1 expression with subsequent excessive collagen deposition and fibrosis formation [2, 3, 5, 12].
The description of mucosal remodeling in CRS may imply an irreversible mucosal disease similar to the asthma. But studies have not offered more precise criteria for the entity of the irreversible remodeling mucosa in CRS. More research about remodeling should be focused on the potential implications for the medical and surgical management of CRS [7].

CRS is a heterogenous inflammation with multifactorial pathogenesis, such as histopathology, inflammatory cell and T-cell patterns, tissue remodeling, eicosanoid and IgE production, microorganisms, and epithelial barrier malfunction [6]. No single pathogen, molecule or cell is the cause of the disease.

Recent studies have suggested that TGF-β acts as a master switch in the remodeling of CRSwNP and CRSsNP. TGF-β can also influence the balance between matrix metalloproteinases (MMPs) and their natural inhibitors—tissue inhibitors of metalloproteinase (TIMP), which possibly lead to the pathologic tissue remodeling in CRS. Moreover, TGF-β impacts the differentiation of T cells toward regulatory T (Treg) cells, leading to different inflammatory patterns in CRS [8]. The innate immune system may influence these factors involved in remodeling. Innate immunity is our first line of defense against foreign intruders in nasal sinuses. The innate immune system consists of phagocytizing cells such as macrophages, dendritic cells, and polymorphonuclear granulocytes, as well as cytokines, chemokines, and activation of the complement cascade [9-11]. Our research will focus on the evidence linking the innate immune system to remodeling in CRS.

Toll like receptors (TLRs) are the most important pattern recognition receptors (PPR) in the innate immune system. TLRs recognize pathogens and activate the signaling pathway to control the initiation, maintenance, modulation and termination of innate host defense. Both TLR2 and TLR4 are the most studied in the pathogenesis of CRS. In this study, we examined not only the expression of TLR2, TLR4 but also the histomorphological features and the expression of TGF-β1 involved in the remodeling in CRS. Furthermore we observed the influence of agonists and inhibitors of TLR2, TLR4 for the expression of TGF-β1 and MMP-9. We hope to reveal the potential relation of the innate immunity with the remodeling in the pathogenesis of CRS.

**Methods**

**Subjects**

All nasal tissues for experiments were obtained from inpatients in our department. 21 patients with CRSwNP, 15 patients with CRSsNP, and 13 controls were enrolled for the study. All patients with CRS were diagnosed by the European Position Paper on Rhinosinusitis and Nasal Polyps 2012. NP tissues in CRSwNP and diseased ethmoid sinus mucosa tissues in CRSsNP were collected during surgery. The control tissues were the inferior turbinate mucosa of control patients undergoing septoplasty because of anatomic variations and without sinus diseases. All the tissues were removed during the normal course of endoscopic surgery. Freshly obtained tissue was fixed in 10% formaldehyde solution for haematoxylin/eosin and immunohistochemical staining. Patients were excluded if they had used a course of antibiotics or systemic corticosteroids in the 4 weeks prior to the surgery. Patients with immune deficiencies and other genetic disorders such as cystic fibrosis or primary ciliary dyskinesia were also excluded. Each patient had a CT scan that was graded for a Lund-Mackay score. The visual analogue scale (VAS) was evaluated for all patients with CRS. Table 1 shows the demographic and clinical characteristics of the 3 groups of subjects.
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Figure 1. The expression of TLR2, TLR4, TGF-β1 and collagen in chronic rhinosinusitis and controls (CRSwNP: chronic rhinosinusitis with nasal polyps; CRSsNP: chronic rhinosinusitis without nasal polyps; *P < 0.05).
enrolled in the study. This study was approved by the ethics committee of our institution and written informed consent was obtained from all patients.

**Immunohistochemistry**

Paraffin-embedded tissue blocks were cut into 5-μm-thick sections, mounted on the slides and heated at 64°C for ten minutes. Serial sections from each block were deparaffinized, hydrated and treated with 3% hydrogen peroxide for 10 minutes. 10% goat serum was used to block the sections for 10 minutes. Each section was incubated by the primary antibodies respectively at 4°C overnight. Primary antibodies were anti-TLR2 (1:500), anti-TLR4 (1:100), anti-TGF-β (1:150). Negative controls consisted of PBS instead of primary antibodies. Species-matched secondary antibodies were incubated for 30 minutes. The slides were washed three times with PBS, and then were visualized by diaminobenzadine (DAB). Following by a final wash, the slides were mounted, coverslipped, and sealed. The number of positive cells per HP field was analyzed. To further identify the relationship between TLR2, TLR4 and TGF-β expression, immunohistochemical staining was conducted on consecutive serial sections. Ten fields per sample were randomly selected and

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**Figure 2.** The immunohistochemical staining of TLR2, TLR4, TGF-β1 and Masson staining in chronic rhinosinusitis and controls (Magnification × 400). (CRSwNP: chronic rhinosinusitis with nasal polyps; CRSsNP: chronic rhinosinusitis without nasal polyps).
Figure 3. No significant correlation between the expression of TLR2, TLR4 and basal membrane thickness in chronic rhinosinusitis.
Figure 4. Relationship between the expression of TLR2, TLR4 and epithelial damage in chronic rhinosinusitis (*significant difference in the expression of TLR2 in CRSsNP, $P < 0.05$).
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Histologic analysis

Paraffin sections (5 μm) were stained with hematoxylin and eosin (HE) staining to observe the pathologic features of remodeling. And they were also stained with Masson dye to observe the collagen deposition. The remodeling features included the epithelium damage, basement membrane (BM) thickening, gland hyperplasia and squamous metaplasia. The epithelium damage was scored on a 4-point scale, with 0 representing intact epithelium, 1 representing absence of cilia, 2 representing erosion of upper cell layer and intact basal cell layer, 3 representing erosion of epithelium. BM thickening was also scored on a 4-point scale, with 0 representing no BM visible, 1 representing mild BM thickening (<10 μm), 2 representing moderate BM thickening (10-20 μm), 3 representing severe BM thickening (>20 μm). BM thickness in control tissues is less than 5 μm.

<table>
<thead>
<tr>
<th>Feature</th>
<th>CRSwNP</th>
<th>CRSsNP</th>
<th>CRSwNP</th>
<th>CRSsNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gland</td>
<td>-</td>
<td>3.5 (0.5-7.4)*</td>
<td>6 (16.7-33)</td>
<td>6 (3.5-10.5)</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>+</td>
<td>15 (8-16.5)*</td>
<td>25 (22.5-35.6)</td>
<td>8 (5.8-16)</td>
</tr>
<tr>
<td>Squamous</td>
<td>-</td>
<td>4.5 (2.7-9)</td>
<td>27.5 (25-36.9)*</td>
<td>4 (3.9-9.5)</td>
</tr>
<tr>
<td>Metaplasia</td>
<td>+</td>
<td>8 (2-10)</td>
<td>9 (6-16)*</td>
<td>8 (5.5-12)</td>
</tr>
</tbody>
</table>

*there was significant difference between these two groups, P < 0.01. (CRSwNP, chronic rhinosinusitis with nasal polyps; CRSsNP, chronic rhinosinusitis without nasal polyps; TLR, toll-like receptor.)

Statistical analysis

Data of immunohistochemical staining are expressed as medians and interquartile ranges, or in box-and-whisker plots. Data of inflammatory cells and ELISA are presented as the mean ± standard deviation (SD). When comparisons were made between groups, the Kruskal-Wallis H-test was used to assess significant intergroup variability. The Mann-Whitney U 2-tailed test was used for between-group comparison. The Spearman test was used to determine correlations, and significance was accepted for P < 0.05. Statistical analysis was performed with SPSS 13.0 (SPSS, Inc, Chicago).

Results

Immunohistochemistry for TLR2, TLR4 and TGF-β1

As Presented in Figure 1, the number of TLR2-positive and TLR4-positive cells was both sig-
significantly higher in CRSsNP than controls and CRSwNP. But there was no significant difference between CRSwNP and controls. The expression of TGF-β1 was significantly higher in CRSwNP and CRSsNP than controls. Moreover the level of TGF-β1 was significantly upregulated in CRSsNP compared with CRSwNP, which had the same expression tendency as TLR2 and TLR4. Representative sections of immunohistochemical staining in controls, CRSsNP, and CRSwNP are shown in Figure 2.

**Histologic characters of remodeling**

Masson staining was used for the detection of collagen fibers in the study. The collagen fibers were stained blue and the background is stained red. The total collagen amount in the ECM was found significantly higher in CRSsNP (median percentage of area, 50.0) than CRSwNP (median percentage of area, 30.0) and controls (median percentage of area, 37.5). There was no significant difference between CRSwNP and controls (Figures 1, 2). The expression of collagen and TGF-β1 were both increased within their respective samples.

BM thickening was detected in 95.2% (20/21) of CRSwNP and all CRSsNP. 19.0% (4/21) of CRSwNP and 60% (9/15) of CRSsNP was in 3-points BM thickening. There was no significant difference and correlation between the expression of TLR2, TLR4 and the BM thickening in CRSwNP and CRSsNP respectively (Figure 3). 95.2% (20/21) of CRSwNP and 86.7% (13/15) of CRSsNP demonstrated epithelial damage. And 38.1% (8/21) of CRSwNP and 60% (9/15) of CRSsNP had the epithelium completely eroded away (3 points) down to the level of the basement membrane. The expression of TLR2 had correlation with epithelial damage in CRSsNP. There was significant difference between severe epithelial damage (3 points) and mild epithelial damage (≤ 2 points) with respect to the expression of TLR2 in CRSsNP (P < 0.05) (Figure 4).

The presence of gland hyperplasia was observed in 33.3% (7/21) of CRSwNP and 80% (12/15) of CRSsNP. Squamous metaplasia showed in 66.7% (14/21) of CRSwNP and 33.3% (5/15) of CRSsNP. The expression of TLR2 had significant difference not only between positive gland hyperplasia and negative gland hyperplasia in CRSwNP (r = 0.553, P < 0.01), but also between positive squamous metaplasia and negative squamous metaplasia in CRSsNP (r = -0.74, P < 0.01) (Table 2; Figure 5).

Neutrophils were expressed more highly in CRSsNP than in CRSwNP and controls (P < 0.05). Eosinophil numbers were higher in CRSwNP than in CRSsNP and controls (P < 0.05). There is no significant difference between CRSsNP and controls for eosinophils (Figure 6A). The expression of TLR2 had close relation with neutrophil infiltration in CRSsNP (r = 0.868, P < 0.01) (Figure 6B). But there is no significant correlation between the expression of TLR2 and eosinophils in CRSwNP (r = 0.195, P > 0.05). The expression of TLR4 is closely related to neutrophil infiltration in CRSsNP (r = 0.853, P < 0.01) (Figure 6C). But there is no significant correlation between the expression of TLR4 and eosinophils in CRSwNP (r = 0.007, P > 0.05).

**Effect of agonists and inhibitors of TLR2, TLR4 on the expression of TGF-β1 and MMP-9**

Cultured epithelial cells from CRSwNP, CRSsNP and controls were stimulated by agonists of TLR2, TLR4 and inhibited by inhibitors of TLR2, TLR4 respectively. The results showed that the expression of TGF-β1 was significantly lower in CRSwNP than in CRSsNP and in controls (P < 0.05) when stimulated with TLR2 agonist. But the expression of TGF-β1 was significantly higher in CRSwNP than in CRSsNP and controls (P < 0.05) when inhibited with TLR2 inhibitor (Figure 7). The levels of TGF-β1 were upregulated significantly by TLR4 agonist and inhibited significantly by TLR4 inhibitor in CRSsNP than in CRSwNP and controls (P < 0.05) (Figure 7). The expression of MMP-9 was upregulated significantly by TLR4 agonist in CRSwNP than in controls (P < 0.05) (Figure 7). With the inhibition of TLR4 inhibitor, the expression level of MMP-9 was higher in CRSwNP than in controls (P < 0.05) (Figure 7).

**Discussion**

The features of tissue remodeling in different types of CRS have been confirmed in Caucasians and Asians. The link between remodeling and inflammation in CRS has also been discussed more extensively. Several factors have been implicated in remodeling, such as TGF-β, matrix metalloproteinases (MMPs), platelet-
derived growth factor (PDGF) and fibrinolytic components [8]. The role of adaptive immunity in remodeling has also been recently indicated [2, 12]. But the role of innate immunity in remodeling has not been involved in CRS.

TLRs have been at the forefront of research into innate immunity. And they are the best described family of receptors to detect microorganisms in the environment. TLRs are the important regulators of pathophysiological processes in CRS but have been scarce involved in studies relevant to remodeling. The role of TLRs in myocardial remodeling has been concerned recently [13]. The recent study also showed that nasal polyp fibroblasts can produce macrophage inflammatory protein-3α (MIP-3α) in stimulation with TLR2-5 agonists [14].

In order to determine the relationship of TLRs with remodeling in CRS, we observed the expression of TLR2, TLR4 and the correlation with the key cytokines and histopathologic features in remodeling. Dong Z et al [15] first observed that TLR2 and TLR4 mRNA had significantly higher expression in CRS. Sun Y and Zhou B et al [16] demonstrated that higher levels of TLR2 but not TLR4 were associated with bacterial biofilms in CRS. In the present study, we found that the expression of TLR-2 and TLR-4 significantly increased in CRS.

Figure 5. The significant correlation between the expression of TLR2 and gland hyperplasia in CRSwNP, squamous metaplasia in CRSsNP.

TLRs in the epithelial cells of nasal mucosa could be activated by pathogens, which results in the production and secretion of diffusible chemotactic molecules and cytokines, upregulation of cell surface adhesion molecules and enhanced expression of antimicrobial peptides [19]. But the role of TLRs in remodeling has not been involved in CRS. TLR2 recognizes the broadest microbial components including components of both gram-positive and gram-negative bacteria, in addition to components from fungi, parasites, and viruses. TLR4 recognizes...
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Figure 6. A: Neutrophil and eosinophil infiltration in CRSwNP, CRSsNP and controls (*significant difference in expression levels); B: The significant correlation between the expression of TLR2 and neutrophils in CRSsNP; C: The significant correlation between the expression of TLR4 and neutrophils in CRSsNP.
Figure 7. The effects of TLR2, TLR4 agonists and inhibitors on the expression of TGF-β1 and MMP-9. CRSwNP: chronic rhinosinusitis with nasal polyps (n = 3); CRSsNP: chronic rhinosinusitis without nasal polyps (n = 3); Controls (n = 3); *significant difference in expression levels contrasted with controls.
lipopolysaccharide (LPS) which is the cell wall component of gram-negative bacteria [13]. In order to observe the relationship of TLR and cytokines involved in remodeling, we examined the effect of agonists and inhibitors of TLR2 and TLR4 for the expression of TGF-β1 and MMP-9. Pam3CSK4, TLR2 agonist is a synthetic triacylated lipopeptide (LP) that is the bacterial cell wall component recognized by TLR2. LPS-EK, TLR4 agonist is the purified lipopolysaccharide (LPS), which is the principal component of Gram negative bacteria that activates predominantly TLR4. OxPAPC is generated by the oxidation of 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine (PAPC), which can inhibit the signaling induced by bacterial LP and LPS, thus blocking the signaling of TLR2 and TLR4. Our results demonstrated that TLR4 agonist upregulated the expression of TGF-β1 in CRSsNP while TLR2 agonist downregulated the expression of TGF-β1 in CRSwNP. TLR2 and TLR4 could play different roles in the regulation of TGF-β1 in CRSwNP and CRSsNP. But they produced the same results that TGF-β1 had lower expression in CRSwNP and higher expression in CRSsNP.

Although TLR2 agonist and inhibitor had no effect on the expression of MMP-9 in our study, TLR-4 agonist upregulated the expression level of MMP-9 in CRSwNP. Matrix metalloproteinases (MMPs) are a family of zinc-dependent and calcium-dependent endopeptidases that are known to be important to degrade the extracellular matrix [2]. Several studies have shown that MMP-9 is upregulated in CRSwNP [20-22], resulting from the relative low expression of TGF-β1. TGF-β1 induces the release of TIMP-1 and inhibits the proteolytic activity of MMP-9 [21, 23]. So upregulation of TGF-β1 in CRSsNP and MMP-9 in CRSwNP with stimulation of TLR4 agonist could explain the different remodeling pattern between CRSwNP and CRSsNP.

Our study showed the expression of TGF-β1 and collagen deposition was both higher in CRSsNP than in CRSwNP, which indicated the excessive tissue repair and fibrosis formation in CRSsNP. The more severe basement membrane thickening in CRSsNP than in CRSwNP also confirmed the influence of TGF-β1 for ECM production. These results are in accordance with the former studies [5, 12]. In order to explore the relationship of TLRs and morphologic features in remodeling, we compared the expression of TLR2, TLR4 with different histopathologic manifestations in CRS remodeling. In CRSsNP, patients with a severe epithelial damage (score 3) had a significantly higher expression of TLR2 than patients with mild epithelial damage (score ≤ 2). The severe damaged epithelium might provide more opportunities for the colonizing bacteria to invade the nasal and paranasal tissues and establish the infection. This could lead to the release of pathogen-associated ligands that trigger the innate immunity. Higher expression of TLR2 might be the result for the severe epithelial damage in CRSsNP. Moreover the expression of TLR2 correlated negatively with squamous hyperplasia in CRSsNP, and positively with gland hyperplasia in CRSwNP. Further studies are needed to unravel the underlying meaning of this correlation.

We observed that neutrophils were dominated in CRSsNP and eosinophils infiltrated highly in CRSwNP, which is in accordance with the former research about the inflammation of CRS. We also discussed the correlation between the expression of TLR2, TLR4 and the inflammatory infiltration in CRSwNP and CRSsNP. The expression of TLR2 and TLR4 had close relationship with neutrophil infiltration in CRSsNP. The stimulation of TLR2 and TLR4 contributes to Th1 response and decreases Th2 response in CRS [24], and CRSsNP is a Th1 skewed neutrophilic inflammation. So we could deduce that TLR2 and TLR4 have the important role in the pathogenesis of CRSsNP.

TLRs activation leads to the production of cytokines and antimicrobial molecules, which in turn activate cellular immune components such as macrophages. Activated TLRs on dendritic cells induce cell maturation, and the activated dendritic cells stimulate T-cell expansion and differentiation. TLRs also induce the expression of costimulatory molecules necessary for sustained activation of adaptive immunity [13]. The inflammatory cytokine signaling induced by TLRs can be the Th1, Th2, or type I interferon (IFN)-γ cytokine profile.

Previously, CRS was believed to be a disorder of the adaptive immune system, but recent research suggests that changes in the adaptive immunity are secondary to disorders in the innate immune system. So we could speculate that TLRs may have relationship with the impor-
tant factors involved in remodeling. The innate immune system may influence all factors involved with the remodeling process. Our study has shown that TLR2 and TLR4 mainly played the important role in the remodeling of CRSsNP. Higher expression levels of TLR2 and TLR4 coincided with the upregulated TGF-β1 and collagen deposition in CRSsNP compared with CRSwNP. TLR2 was highly related to epithelial damage and squamous metaplasia in the remodeling features of CRSsNP rather than CRSwNP. Furthermore TLR2 and TLR4 were correlated with neutrophil infiltration in Th1-biased inflammation of CRSsNP, but did not relate with eosinophils in Th2-biased inflammation of CRSwNP. In order to determine more firmly whether TLRs are important for remodeling in CRS, more evidence is needed from the TLRs signaling pathway and the effect of TLRs agonists for nasal epithelium and other immune cells. The role of innate immunity in the remodeling of CRS needs more attention, which will contribute to understand the pathophysiological mechanisms of remodeling and provide evidence in the future to improve the current medical and surgical management.

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Disclosure of conflict of interest

None.

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