Altered Expression of Toll-like Receptors in Human Oral Epithelium in Oral Lichenoid Reactions

Abdelhakim Salem, DDS,*† Rabeia Mustafa, DDS,† Dyah Listyarifah, MD,† Ahmed Al-Samadi, PhD,† Goncalo Barreto, PhD,* ‡ Dan Nordström, PhD,*§ and Kari K. Eklund, PhD*¶

INTRODUCTION

Oral lichenoid reactions (OLRs), which are sometimes referred to as oral lichenoid lesions, are common chronic inflammatory lesions that affect buccal, gingival, and lingual mucosa. Clinically, OLR patients commonly present with unilateral lesions, although bilateral distribution has been documented. Typically, lesions appear as striations, papules, erosions, or as atrophic lesions in oral mucosa, and patients experience pain or discomfort.1,2 Historically, OLRs exhibit diffuse inflammation extending deep in the lamina propria below degenerated basal cell layers. Several cell types including T lymphocytes, basophils, eosinophils, and dendritic cells (DCs) are increased in OLRs.1-3 Unlike the idiopathic oral lichen planus (OLP), the etiology of OLRs has been linked to the allergens used in dental restorative materials, such as dental amalgam and composite restorations.4-8 Principally, surgical revision and filling removal are the main current methods to cure OLRs. This approach may however increase the risk of inducing harmful effects on the dental pulp and increase the costs for the patients.9,10 It would therefore be important to find alternative therapeutic agents that could interfere with the inflammatory cascade in OLR, and thus limit the need for surgical interventions.

Healthy oral mucosa acts as a shield, under normal conditions, by exhibiting potent immune responses mediated through a variety of pattern recognition receptors. Toll-like receptors (TLRs) are well-characterized members of pattern recognition receptors that are continually surveying their environments and recognizing conserved microbe-associated and danger-associated molecular patterns (MAMPs/DAMPs). Once activated by specific ligands, TLRs are capable of initiating inflammatory response by inducing robust inflammatory cytokines, chemokines, proteases, and subsequent local damage of host tissue.11,12 Despite the crucial role of TLRs in mediating protection against microbial infections, it is also believed that TLRs could mediate harmful effects in many inflammatory diseases, such as contact dermatitis and atherosclerosis.13,14 So far, 10 TLRs, and many of their ligands, have been identified in human oral epithelial cells. Moreover, the differential expression of TLRs in such cells has been studied in several oral mucosal diseases, including OLP, recurrent aphtous ulcers, and oral squamous cell carcinoma.15-19 However, the expression and the potential role of TLRs in the initiation and pathogenesis of OLRs have not been elucidated so far.
MATERIALS AND METHODS

Patients and Ethical Approval
This study was approved by the Ethics Committee of the Hospital District of Helsinki and Uusimaa (42/13/03/01/2013). Twenty-five patients (mean age, 56 years; range, 22–84 years) were examined and diagnosed with OLRs based on history, clinical, and histopathological examination. OLR tissue biopsies were collected from healthy control donors (mean age, 26 years; range, 12–51 years). The samples were fixed in 10% neutral-buffered formalin and embedded in paraffin (formalin-fixed, paraffin-embedded samples; FFPE). All participants have given their written informed consents before participating in the research.

Immunohistochemical Staining
Pilot experiments were performed to confirm the optimal concentration for immunohistochemical staining for each TLR antibody. Tissue sections of 5 μm thickness from patients with OLRs (n = 25) and control samples (n = 15) were deparaffinized in xylene and rehydrated in a decreasing ethanol series. For antigen retrieval, the slides were incubated in 700 mL of 10 mM citrate buffer, pH 6.0, and microwaved in a microwave processing laboratory station (MicroMED VT Mega Histoprocessing Labstation; Milestone Srl, Sorisole, Italy) according to the manufacturer’s instructions. Slides were washed in 10 mM phosphate-buffered 140 nM saline (PBS; pH 7.4) and incubated at room temperature if not otherwise indicated in (1) 1% H2O2 for 10 minutes and washed in PBS; (2) 10% normal rabbit or goat serum (Vector Laboratories, Burlingame, CA) for blocking of nonspecific staining for 1 hour; (3) 1 μg/mL-1 rabbit anti-human TLR1 immunoglobulin G (IgG), 2 μg/mL-1 rabbit anti-human TLR2 IgG, 1.8 μg/mL-1 rabbit anti-human TLR3 IgG, 1.5 μg/mL-1 rabbit anti-human TLR4 IgG, 1 μg/mL-1 rabbit anti-human TLR5 IgG, 1 μg/mL-1 goat anti-human TLR6 IgG, 1 μg/mL-1 goat anti-human TLR7 IgG, 1 μg/mL-1 rabbit anti-human TLR8 IgG, 1 μg/mL-1 rabbit anti-human TLR9 IgG, or 2 μg/mL-1 goat anti-human TLR10 IgG (Santa Cruz Biotechnology, Santa Cruz, CA); (4) biotin-conjugated secondary antibodies (Vector Laboratories; 1:200) for 1 hour and washed in PBS; (5) avidin–biotin–peroxidase complex (Vector Laboratories; 1:200) for 1 hour and washed in PBS; (6) diaminobenzidine tetrahydrochloride for 10 minutes and washed in dH2O. Counterstaining of the slides was performed using hematoxylin and mounted in Mountex (Histolab, Gothenburg, Sweden). We used nonimmune normal rabbit or goat IgG as isotype controls (R&D Systems, Minneapolis, MN).

Microscopic Imaging
Fully automated Leica DM6000 microscope with Leica DFC365-FX digital camera (Leica Microsystems, Wetzlar, Germany) was used to image the stained samples.

Scoring of Samples
TLR level of expression was scored as 0–3 in the basa/ suprabasal, intermediate, and upper cell layers of oral epithelium. Samples were graded to negative staining (0), weakly positive (1), moderately positive (2), and strongly positive (3). Scoring was performed independently by 3 researchers, who were blinded to clinical data and outcomes.

Quantitative Real-Time Polymerase Chain Reaction
Maxwell 16 Low Elution Volume RNA-FFPE Kit (firmware 4.97; Promega Corp, WI) was used to isolate total RNA from control and OLR FFPE-samples (n = 15; n = 25, respectively). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed as described by Salem et al.20 SsoAdvanced Universal SYBR Green Supermix or iQ SYBR Green supermix were used (Bio-Rad Laboratories Inc.). Glyceraldehyde 3-phosphate dehydrogenase and ribosomal protein, large, P0 were used as housekeeping genes. Human primer sequences used in this study are listed in Table 1.

Statistical Analyses
Statistical analyses were performed using SPSS software program version 21.0 (IBM SPSS Statistics, SPSS Inc, Chicago, IL). Student t test was used to estimate the statistical significance between two-grouped data. P < 0.05 was regarded as statistically significant.

<table>
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<tr>
<th>TABLE 1. Human Primers Used for Real-Time PCR</th>
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<td><strong>Gene</strong></td>
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GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RPLP0, ribosomal protein, large, P0.
RESULTS

Immunohistochemical Staining

Healthy Controls

Intense cytoplasmic staining of all studied TLRs was observed in the basal and suprabasal cell layers of human oral epithelium in the healthy control group. In contrast, the intermediate oral epithelial cells up to the superficial cell layers were either negative or stained weakly for TLRs (Figs. 1, 2). This pattern of expression was more or less observed for all TLRs, except for TLR5, which exhibited moderately positive staining throughout intermediate layers (Table 2). TLR10 showed faint staining and inconsistent distribution in all epithelial layers (data not shown).

OLR Patients

The staining pattern of TLRs in OLR samples was clearly different compared with controls. In most cases,
immunostaining disclosed moderate-to-strong expression of TLRs that extended from the basal cell layer to the most superficial layers through the entire oral epithelium (Figs. 1, 2 and Table 2). However, TLR5 immunoreactivity was clearly reduced with weak-to-negative staining in OLR (Fig. 2), whereas TLR10 expression remained faint and inconsistent as seen in healthy controls (data not shown).

**Determination of Gene Expression by qRT-PCR**

The qRT-PCR analysis showed that all 10 TLR genes were expressed in human oral epithelium. Six genes [TLR1 \( P = 0.02 \), TLR2 \( P = 0.009 \), TLR4 \( P = 0.04 \), TLR7, TLR8 \( P = 0.009 \) and TLR9 \( P = 0.02 \)] were upregulated in human oral epithelium in OLR patients compared with healthy controls. However, 3 genes [TLR3, TLR5, TLR6 \( P = 0.03 \)] were downregulated in OLR patients. TLR10 gene expression remained nearly unchanged in both sample groups. The differential expressions of TLR genes are shown in Figures 3 and 4.

**DISCUSSION**

In the present study, we compared the expression of TLRs in healthy human oral epithelium and OLR patients as determined by immunostaining and qRT-PCR. We hereby confirm the previously reported data that human oral epithelium is equipped with TLR+ units.15–19

Based on our findings, basal and suprabasal cell layers revealed robust positive staining of several TLRs in healthy oral epithelium, rendering oral epithelial cells readily responsive to PAMPs/DAMPs. Accordingly, if such molecules penetrated deep to basal epithelial layers, they would encounter the TLR+ units and would induce potent inflammatory responses, production of proteases, and the eventual damage of surrounding tissue.21 However, both intermediate and superficial oral epithelial layers were, by and large, weakly positive or exhibited negative staining for TLRs (TLR−/−) and thus would be less responsive to PAMPs/DAMPs (Table 2). Protection against invaders, in such layers, can be attributed to the tight epithelial junctions (occluding junctions), which restrain PAMPs/DAMPs and other allergens from crossing the intercellular spaces.17,22 Although immunologic tolerance is probably because of multiple complex mechanisms, such TLR+/− zone could confer protection to epithelial cells from inadvertent TLR-mediated responses mounted against oral microbiota.23–25

The integrity of oral epithelium, owing to trauma and other factors, is disrupted in OLP and OLRs, which thus paves the way for PAMPs/DAMPs and hapten to diffuse into deeper epithelial layers.2,26 This could partly explain our findings that TLR architecture in OLR was, for most receptors, upregulated and extended from basal to superficial layers to combat the invading allergens. Immunostaining results showed that TLR1, TLR2, TLR4, TLR7, TLR8, and TLR9 were clearly upregulated in OLRs, which was further confirmed through gene expression analysis. Such local upregulation of TLRs has apparently resulted in diminished passive/tolerant TLR+/− zone and induction of subsequent inflammatory responses. This is evidently relevant to the inflammatory nature of the lichenoid reactions.1–3

Noteworthy, TLR4, in particular, has shown to be able to ligate certain metal sensitizers (eg, Nickel), trigger inflammatory responses, and thus induce production of potent proinflammatory cytokines and chemokines.27–29 Interestingly, nickel, zinc, copper, cobalt-chromium alloys, and other metals, which are also identified as “contact sensitizers,” have been widely used in dental implants and fillings.30,31 It is therefore interesting to find that TLR4 is highly upregulated in oral epithelium of OLR patients at both gene expression and protein levels. Principally, OLRs result as a response of the host tissue to chronic contact with allergens (eg, Amalgam fillings), in which most patients show improvement after removal of sensitizing material.7 In fact, this is compatible with type IV (delayed type) hypersensitivity reactions where TLRs are believed to play a fundamental role in recognizing allergenic molecules and orchestrating inflammatory processes.3,32,33

In OLR patients, allergic reaction is likely a response to low-grade, repeated, exposures to low–molecular weight molecules that do not initiate, at first, immune responses and thus patients remain asymptomatic. This period of repeated exposures to hapten (eg, metal ions) is called “sensitizing phase” and may naturally take several weeks to months before activating TLRs.34 Once TLRs are activated, they induce production of several proinflammatory cytokines such as interleukin-1β, which activates locally resident DCs. Subsequently, activated DCs migrate to the regional lymph nodes where they present antigens to naive and memory TLR+ T lymphocytes. In this regard, it is interesting that our findings disclosed a band-like infiltrate of subepithelial TLR+ peripheral blood mononuclear cells (PBMCs) in OLRs. The TLR immunoreactivity was intensely positive in these cells for TLR2, TLR3, TLR4, and TLR6, which may indicate their

| Table 2. Scoring Results of TLR Staining in Healthy Controls and Patients With OLR |
|-------------------------------|------------|---------------|---------------|
| TLR                            | Basal/Suprabasal Layers | Intermediate Layers | Superficial Layers |
| TLR1                           | Control | OLR | Control | OLR | Control | OLR |
| TLR2                           | 3   | 3  | 1   | 2  | 0   | 2   |
| TLR3                           | 3   | 3  | 1   | 3  | 0   | 3   |
| TLR4                           | 3   | 3  | 1   | 3  | 0   | 3   |
| TLR5                           | 3   | 3  | 1   | 3  | 0   | 3   |
| TLR6                           | 3   | 3  | 2   | 2  | 0   | 1   |
| TLR7                           | 3   | 3  | 1   | 3  | 0   | 3   |
| TLR8                           | 3   | 3  | 0   | 2  | 0   | 2   |
| TLR9                           | 3   | 3  | 1   | 3  | 0   | 3   |
| TLR10                          | 1   | 1  | 0   | 1  | 0   | 1   |

TLR staining intensity was graded in the basal/suprabasal, intermediate, and upper cell layers of oral epithelium as follows: negative staining as (0), weakly positive as (1), moderately positive as (2), and strongly positive as (3). Scoring was performed by 3 independent researchers who were blinded to the samples data.
involvement in the TLR-mediated pathogenesis (Figs. 1, 2). A similar observation of enhanced expression of TLR2 and TLR4 on PBMCs was previously reported in OLP.15,35 These TLR+ PBMCs, such as T lymphocytes, monocytes, plasma cells, and also polymorphonuclear leukocytes, are attracted to lamina propria where the antigen was first encountered forming there a diffuse subepithelial lymphocytic infiltrate. Later, the effector lymphocytes release robust proinflammatory cytokines and cytotoxins that induce apoptosis in affected oral epithelial cells. Collectively, these events, as
long as the exposure to sensitizers continues, contribute to the development of chronic inflammation and inflammatory loop cycle—that is seen in OLR patients.34–37

Indeed, the transcriptional expression of TLRs genes is regulated following the engagement of TLR with specific ligands.38 A wide range of inflammatory, environmental, and bacterial factors (eg, tumor necrosis factor α, interferon-γ, and bacterial lipopolysaccharides) have been implicated in TLR signaling and thus leading to negative-feedback loop and altered expression of TLR genes.38,39

We found that TLR5 was downregulated on both receptor and gene levels in OLR (Figs. 2, 4 and Table 2). Although it is not clearly understood why, this finding appears in line with a recent report showing that TLR5 is downregulated in oral keratinocytes of OLP patients.15 As a plausible explanation, TLR5 might be downregulated in response to persistent exposure to bacterial components like flagellin through the compromised junctional barriers in OLR patients.40,41 Additionally, this could in part explain the downregulated genes of TLR3 and TLR6 as outcome of the complex negative-feedback regulation on the transcriptional level.15,38 However, TLR10 showed almost no changes between healthy and OLR groups, which may suggest that it probably has a limited role in the pathogenesis of OLR. However, this study has some limitations, including the small size sample and the difficulty to distinguish between cytoplasmic and membrane-associated TLRs staining.

In summary, the outermost layers of oral epithelium confer physical protection through its occluding junctions and TLR−/− zone, whereas the deeper basal cells use their TLRs arsenal to provide immunologic backup. In OLRs, when low–molecular weight haptens, such as “contact sensitizers,” penetrate deep in to the epithelium, these cells respond by recruiting TLR+ leukocytes to the affected lamina propria forming diffuse inflammatory band. Inflammation in OLR is soon downregulated after the sensitizing factor is barred (eg, removal of the metallic restoration), leading to a swift recovery.7

These findings, taking together, suggest a fundamental role of TLRs in the initiation and perpetuation of OLRs, which could be significant, in particular, in the clinical practice. Interestingly, Lawrence et al42 showed, in a recent report, that monoclonal anti-TLR4 antibody has inhibitory effects on cobalt-mediated inflammatory responses in an in vitro cell model. Targeting TLRs by specific antibodies, or small molecular inhibitors, could therefore interfere with their signaling cascades and prevent subsequent inflammatory events making it a potentially promising, cost-effective, remedy for OLRs compared with the costly surgical revisions.

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