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The role of HMGB1 signalling axis via RAGE and TLR4 in the immunopathology of oral lichen planus—a potential drug target?

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Running title: HMGB1 signalling in oral lichen planus

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Abstract Page

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Abstract High mobility group box 1 (HMGB1) is an extremely-conserved DNA-binding protein that stabilizes nucleosomes and facilitates gene transcription in mammalian cells. When released extracellularly, HMGB1 becomes an alarmin that can mediate systemic diseases. HMGB1 signals via two main receptors: receptor for advanced glycation end products (RAGE) and toll-like receptor-4 (TLR4). We hypothesized that HMGB1 expression is increased in oral lichen planus (OLP) compared with healthy controls. Therefore, HMGB1 and its receptors were mapped in tissue biopsies from 25 OLP patients and 20 healthy controls by immunostaining and ImageJ analysis. HMGB1 was induced in oral keratinocytes in all OLP patients. The band-like cell infiltrate in OLP revealed intense RAGE staining. Likewise, TLR4 was overexpressed throughout OLP mucosa which co-localized with HMGB1. In conclusion, we suggest that OLP could partly be HMGB1-mediated condition by creating a proinflammatory loop cycle via RAGE- and TLR4-signalling axis, which may contribute to the disease chronicity.

Key Words: HMGB1; Receptor for advanced glycation end products; TLR4; Oral lichen planus; Oral pathology.

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The high mobility group box 1 protein (HMGB1) is an evolutionary ancient, non-histone, chromosomal protein that regulates many crucial processes, such as gene transcription, DNA repair, and extracellular signalling (1). HMGB1 is composed of 215 amino acids that are folded into two homologous DNA-binding units (designated as box-A and box-B) and a C-terminal tail (2, 3). Interestingly, HMGB1 functions depend on its location in the host tissue, as follows: a) the nuclear HMGB1 promotes the assembly of DNA-binding proteins and gene transcription; b) the extracellular HMGB1, when released upon cell damage, is serving as a danger-associated molecular pattern (DAMP), which induces cytokine production, and mediates inflammation (3). Indeed, HMGB1 effect is mediated via multiple pattern recognition receptors (PRRs), most importantly the receptor for advanced glycation end-products (RAGE), and toll-like receptor 4 (TLR4). In fact, the upregulation of HMGB1 and its receptors has been linked with many inflammatory and malignant disorders (4, 5). It was also shown that oral epithelial cells can produce considerable amounts of HMGB1 when challenged with TNF-α in a proinflammatory loop cycle (6).

Oral lichen planus (OLP) is one of the most common oral inflammatory diseases, which has also been considered as a risk factor for oral cancer (7, 8). Histopathologically, OLP is characterized by a liquefactive degeneration of the epithelial basal cells, deranged basement membrane, and an extensive subepithelial inflammatory cell infiltrate (9). Etiologically, several reports suggested the involvement of alarmin-mediated processes in OLP pathogenesis, which render the oral epithelium susceptible to the host immune responses (10). In spite of the ongoing efforts, there is no clear evidence of any specific intervention in managing OLP or relieving its clinical signs (11, 12). Although HMGB1 mediates the inflammatory processes in many diseases and the interest toward medications that can limit its effects is swiftly growing, its role in OLP remains obscure. Therefore, we tested the hypothesis that HMGB1 is increased in OLP and plays a proinflammatory role via its two main receptors (RAGE and TLR4).

Materials and methods

Patients and samples

Surgical biopsies obtained from 25 OLP patients (mean age 56 years, range 22–84) and 20 healthy control individuals (mean age 26 years, range 18–51) were enrolled in this study. OLP patients were 17 females (68%) and 8 men (32%), which represented a typical age and sex distribution of OLP patients (Table 1). The patient samples were retrieved from OLP patients who have been consecutively biopsied and diagnosed at the pathology department. A total number of thirty-four patients were biopsied during the period (04.12.2012-26.02.2013). However, nine cases were excluded from the study for not meeting the inclusion criteria. The patients were selected according to the following inclusion criteria: 1) clinical and histological diagnosis of OLP lesions according to the WHO recommendation and its 2009 modification; 2) patients had no diagnosed systemic diseases at the time of sample biopsy; 3) patients have to be off of corticosteroids for at least one year at the time of biopsy; 4) patients does not present non-characteristic features. The normal controls were collected from the buccal mucosa during regular check-ups (n=4) or when extracting the third molars (n=16). The harvested biopsies were collected and processed as previously reported (9). This study was approved by the Ethics Committee of the Hospital District of Helsinki and Uusimaa (42/13/03/01/2013). All participants provided written informed consent prior to participating in the study.

Immunohistochemical staining

Tissue sections were deparaffinized in xylene and rehydrated in ethanol multi-concentration series. The antigen retrieval was performed in Micromed T/T Mega Microwave (HACKER Instruments & Industries Inc., Winnsboro, SC, USA). To block non-specific staining, sections were incubated in 1% H_2O_2 at RT for 10 min and then incubated in 10% normal horse serum (Vector Laboratories, Burlingame, CA, USA) for 1 hour at RT. Sections were then incubated overnight at +4ºC in: 1) 1 µg/ml mouse anti-human monoclonal HMGB1 antibody (Abnova Corp., Taipei City, Taiwan); 2) 1 µg/ml mouse anti-human monoclonal RAGE antibody (EMD Millipore, Billerica, MA, US). Biotin-

conjugated secondary anti-mouse antibodies (Vector Laboratories; 1:200) were applied for 1 hour at RT. ABC complex (Vector Laboratories; 1:200) was applied for 1 hour at RT. Finally, 3,3′-diaminobenzidine-tetrahydrochloride-dihydrate was applied for 10 min at RT. Counterstaining of the slides was performed using hematoxylin and mounted in Mountex (HistoLab, Gothenburg, Sweden).

Immunofluorescence staining

The procedure was performed as described in our recent report (14). Briefly, slides were incubated overnight at +4°C in mouse anti-human monoclonal HMGB1 antibody, and rabbit polyclonal antibody raised against human TLR4 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA). The slides were then incubated for 1 hour in secondary antibodies conjugated with Alexa Fluor® 488 for HMGB1 or Alexa Fluor® 568 for TLR4 (1:200, Invitrogen, Carlsbad, CA, USA). Diamidino-2-phenylindole (DAPI) was applied for 10 min at RT. Slides were mounted using ProLong® Gold Antifade Mountant (Thermo Fisher Scientific, MA, US).

Imaging and image analysis

Leica DM6000 microscope with Leica DFC365-FX camera (Leica Microsystems, Wetzlar, Germany) were used. The analysis of the staining intensity was performed by the ImageJ software (version 1.47; National Institute of Health, Bethesda, MD, US). Three different areas per tissue section of OLP samples (n=25) and controls (n=20) were analysed at field magnification of 40x. A stop filter type was used with a hue value between 0 and 161. The relative staining intensity of each antigen was determined by applying the inverse mean grey value.

Statistical analysis

SPSS software programme version 21.0 (IBM SPSS Statistics, SPSS Inc., Chicago, IL, USA) was used to analyze the data. The paired Student's t-test and the One-way ANOVA and post-hoc Tukey's test were used where appropriate. Data are presented as means ± SD. Differences were considered statistically significant with $P \leq 0.05$.

Results

HMGB1 expression was mostly confined to the basal cell layers in the normal oral epithelium, and it was localized intracellularly and within the nuclei (Fig. 1A). In contrast, HMGB1 staining was intense in OLP throughout the epithelial layers in the intra- and extracellular spaces (Fig. 1B). Additionally, HMGB1 was intensely positive in the inflammatory cell infiltrate in the subepithelial lamina propria in OLP compared with few positive cells in the normal controls. The overall HMGB1 staining was strongly induced in OLP compared with the normal controls (Paired t-test; $P = 0.01$; Fig. 1C). The upper and middle epithelial layers, in addition to the lamina propria, revealed an upregulated expression of HMGB1 in OLP (One-way ANOVA, $P = 0.01$, 0.001, and 0.01, respectively), while the expression was somewhat similar at the basal layers in both OLP and controls (Fig. 1D).

RAGE was positively expressed throughout the normal oral epithelium with more tendency towards the uppermost and middle layers (Fig. 2A). Similarly, RAGE receptors were observed in all epithelial layers of OLP samples, but it was strongly expressed in the inflammatory cell infiltrate of the lamina propria region (One-way ANOVA, $P = 0.001$; Fig. 2BC). HMGB1-RAGE double staining results were not different between OLP and controls except in the subepithelial inflammatory cell region (data not shown).

TLR4 was detectable mainly in the basal layer of the normal oral mucosa, with a few positive cells in the middle layer (Fig. 3A). On the other hand, TLR4 staining was markedly increased among the superficial, the intermediate, and the basal cell layers, in addition to the subepithelial inflammatory cell infiltrate in OLP specimens (Fig. 3B). The double immunofluorescence staining revealed strong and consistent HMGB1-TLR4 signalling axis in OLP mucosa compared with the normal controls (Fig. 3CD). There were no noticeable differences in the findings between the reticular type group (n=14) and the group of other types (n=11).

Discussion

Oral epithelial cells are capable to initiate and orchestrate potent inflammatory responses by releasing various DAMPs, such as HMGB1, when they are exposed to pathogens or undergo necrosis (6). Extracellular HMGB1 can initiate innate immunity and potent pro-inflammatory cytokine production, followed by an adaptive immune response. We report that oral keratinocytes express ubiquitous nuclear HMGB1 at the "dividing" basal layers, where it acts as a transcriptional regulator of DNA. In OLP, HMGB1 expression was noticeably increased. Such dramatic change could be attributed to certain exogenous OLP-relevant stimuli of HMGB1, such as bacterial LPS, or by endogenous cytokines, such as TNF-α and IFN-γ (15, 6). HMGB1-carrying DNA breaks could passively leak out from the damaged basal keratinocytes in OLP. In fact, HMGB1 appears to be an active contributor to oral diseases. We previously showed that HMGB1 is highly increased in recurrent aphthous ulcer patients. Additionally, SUPIC et al. found that *HMGB1* polymorphism could be utilized as an indicator of oral cancer progression and could be associated with an increased oral cancer incidence in OLP patients (17). In this context, HMGB1 is induced in many head and neck cancers, where it is correlated with nodal metastasis and poor prognosis (18). HMGB1 was also upregulated in several systemic disorders such as lupus erythematosus, diabetes and rheumatoid arthritis (3).

HMGB1 can virtually bind with multiple members of PRR-family, such as RAGE and TLR4. We showed that oral epithelial cells express RAGE under the normal physiological conditions. Once released extracellularly, HMGB1 binds to $RAGE^+$ cells and induces immune cell migration to the "danger" site, where it can also enhance the later immunosuppressive capacity of regulatory T cells (Treg) (3, 5). It is therefore reasonable to assume that such strong $RAGE^+$ inflammatory cell infiltrate in OLP could possibly be associated with the inhibitory pathway to compensate the insufficient Treg population in OLP (7).

TLR4/MD-2 complex is a vital component for HMGB1-induced cytokine production and inflammation (3). Recently, we showed that TLR4 is upregulated in oral lichenoid lesions (19).

Noteworthy, anti-TLR4 antibodies has significantly reduced the HMGB1-mediated cytokine release by immune cells, such as macrophages (4). Such highly upregulated TLR4 status in OLP lesions may translate the involvement of HMGB1-TLR4 axis in OLP, which may contribute to the disease chronicity (19). However, the results should be interpreted with caution as TLR4 upregulation in OLP could also be ascribed to various DAMPs/PAMPs other than HMGB1 (e.g. S100A8, S100A9 and bacterial lipopolysaccharides) and thus further experiments using specific HMGB1 blockers are needed.

Based on our findings, we suggest the involvement of HMGB1-RAGE and HMGB1-TLR4 downstream signalling pathways in OLP, possibly by inducing the proinflammatory loop cycle, which constitutes a pathognomonic feature of OLP. However, we acknowledge some limitations in our report such as the lack of functional HMGB1 experiments; such experiments are warranted to be performed in the future. Additionally, the difference in age between the OLP patients and controls is another limitation in our study. This could partly be attributed to the fact that OLP is more common in adult population, while the normal samples were collected from relatively younger volunteers. However, it is unlikely that such gap would have a major impact on the findings, since HMGB1 expression was also low in other normal mucosal tissues (e.g. colon) obtained from both young and old age controls compared with overexpressed patient samples (20).

Indeed, if further studies could successfully translate HMGB1 experiments into pharmaceutical applications, the presented findings may impart a significance in the clinical setting. In such occasion, HMGB1-based medication could be used to ameliorate OLP symptoms or even to treat the disease. Of note, the actual mainstay treatment of OLP (i.e. corticosteroids) which ameliorate the inflammation, showed significant reduction of extracellular synovial HMGB1 in arthritis patients and thus suggesting a possible interference with HMGB1-signalling pathway (4). Indeed, targeting DAMP-PRRs pathways by local blockers or neutralizing antagonists that specifically attenuate HMGB1-mediated pathogenic responses without compromising the innate immunity remains a

challenge. Nevertheless, the novel selective HMGB1-inhibitors (e.g. the tetramer peptide P5779) have shown promising outcomes as therapeutic targets in many inflammatory diseases, which indicate that such approaches are feasible (4, 5, 21).

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Conflicts of interests – The authors declare no conflict of interests.

References

1. CZURA CJ, WANG H, TRACEY KJ. Dual roles for HMGB1: DNA binding and cytokine. *J Endotoxin Res* 2001; **7**: 315-321.
2. YANG H, WANG H, CHAVAN SS, ANDERSSON U. High Mobility Group Box Protein 1 (HMGB1): The Prototypical Endogenous Danger Molecule. *Mol Med* 2015; **21**: 6-12.
3. LOTZE MT, TRACEY KJ. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat Rev Immunol* 2005; **5**: 331-342.
4. ANDERSSON U, TRACEY KJ. HMGB1 is a therapeutic target for sterile inflammation and infection. *Annu Rev Immunol* 2011; **29**: 139-162.
5. SIMS GP, ROWE DC, RIETDIJK ST, HERBST R, COYLE AJ. HMGB1 and RAGE in inflammation and cancer. *Annu Rev Immunol* 2010; **28**: 367-388.
6. MORIMOTO Y, KAWAHARA KI, TANCHAROEN S, KIKUCHI K, MATSUYAMA T, HASHIGUCHI T, IZUMI Y, MARUYAMA I. Tumor necrosis factor-alpha stimulates gingival epithelial cells to release high mobility-group box 1. *J Periodontal Res* 2008; **43**: 76–83.

7. SUGERMAN PB, SAVAGE NW, WALSH LJ, ZHAO ZZ, ZHOU XJ, KHAN A, SEYMOUR GJ, BIGBY M. The pathogenesis of oral lichen planus. *Crit Rev Oral Biol Med* 2002; **13**: 350–365.
8. HALONEN P, JAKOBSSON M, HEIKINHEIMO O, RISKA A, GISSLER M, PUKKALA E. Cancer risk of Lichen planus: A cohort study of 13,100 women in Finland. *Int J Cancer* 2017. doi: 10.1002/ijc.31025. PMID: 28857160.
9. SALEM A, AL-SAMADI A, STEGAJEV V, STARK H, HÄYRINEN-IMMONEN R, AINOLA M, HIETANEN J, KONTTINEN YT. Histamine H_4 receptor in oral lichen planus. *Oral Dis* 2015; **21**: 378–385.
10. PAYERAS MR, CHERUBINI K, FIGUEIREDO MA, SALUM FG. Oral lichen planus: focus on etiopathogenesis. *Arch Oral Biol* 2013; **58**: 1057-1069.
11. SURESH SS, CHOKSHI K, DESAI S, MALU R, CHOKSHI A. Medical Management of Oral Lichen Planus: A Systematic Review. *J Clin Diagn Res* 2016; **10**: ZE10-5.
12. FARHI D, DUPIN N. Pathophysiology, etiologic factors, and clinical management of oral lichen planus, part I: facts and controversies. *Clin Dermatol* 2010; **28**: 100-108.
13. RAD M, HASHEMIPOOR MA, MOJTAHEDI A, ZAREI MR, CHAMANI G, KAKOEI S, IZADI N. Correlation between clinical and histopathologic diagnoses of oral lichen planus based on modified WHO diagnostic criteria. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2009; **107**: 796–800.
14. SALEM A, ALMAHMOUDI R, LISTYARIFAH D, SIPONEN M, MAANINKA K, AL-SAMADI A, SALO T, EKLUND KK. Histamine H_4 receptor signalling in tongue cancer and its potential role in oral carcinogenesis - a short report. *Cell Oncol* 2017. doi: 10.1007/s13402-017-0336-6. PMID: 28653289.
15. SALEM A, ROZOV S, AL-SAMADI A, STEGAJEV V, LISTYARIFAH D, KOURI VP, HAN X, NORDSTRÖM D, HAGSTRÖM J, EKLUND KK. Histamine metabolism and

transport are deranged in human keratinocytes in oral lichen planus. *Br J Dermatol* 2017; **176**: 1213-1223.

16. AL-SAMADI A, DROZD A, SALEM A, HIETANEN J, HÄYRINEN-IMMONEN R, KONTTINEN YT. Epithelial Cell Apoptosis in Recurrent Aphthous Ulcers. *J Dent Res* 2015; **94**: 928-935.
17. SUPIC G, KOZOMARA R, ZELJIC K, STANIMIROVIC D, MAGIC M, SURBATOVIC M, JOVIC N, MAGIC Z. HMGB1 genetic polymorphisms in oral squamous cell carcinoma and oral lichen planus patients. *Oral Dis* 2015; **21**: 536-543.
18. NGUYEN A, BHAVSAR S, RILEY E, CAPONETTI G, AGRAWAL D. Clinical Value of High Mobility Group Box 1 and the Receptor for Advanced Glycation End-products in Head and Neck Cancer: A Systematic Review. *Int Arch Otorhinolaryngol* 2016; **20**: 382-389.
19. SALEM A, MUSTAFA R, LISTYARIFAH D, AL-SAMADI A, BARRETO G, NORDSTRÖM D, EKLUND KK. Altered Expression of Toll-like Receptors in Human Oral Epithelium in Oral Lichenoid Reactions. *Am J Dermatopathol* 2016. doi:10.1097/DAD.0000000000000807. PMID: 28027081.
20. LEE H, SONG M, SHIN N, SHIN CH, MIN BS, KIM HS, YOO JS, KIM H. Diagnostic significance of serum HMGB1 in colorectal carcinomas. PLoS One 2012; 7: e34318.
21. VENEREAU E, DE LEO F, MEZZAPELLE R, CARECCIA G, MUSCO G, BIANCHI ME. HMGB1 as biomarker and drug target. *Pharmacol Res* 2016; **111**: 534-544.

Figure legends

Fig. 1. (A) HMGB1 expression in normal oral mucosa was localized to the basal cell layers (*arrows*). (B) HMGB1 was observed in the nuclei, cytoplasm and epithelial intercellular spaces in oral lichen planus (OLP). (C) Overall HMGB1-immunoexpression was increased in OLP compared with the normal controls. (D) HMGB1 expression was induced in the upper and middle oral epithelial cell layers in addition to the inflammatory cell infiltrate in OLP. Scale bar = 100 µm.

Fig. 2. (A) Moderate expression of RAGE was seen in normal oral epithelial cells. (B) RAGE expression was induced in oral lichen planus (OLP) in the band-like inflammatory cell infiltrate. (C) The staining intensity of RAGE was noticeably induced in the lamina propria of OLP than in the normal control. Scale bar = 100 µm.

Fig. 3. (A) TLR4 was expressed in the basal cell layers in the normal oral mucosa with very faint existence in the middle epithelial layers. (B) TLR4 was strongly induced in all the epithelial layers and the subepithelial inflammatory cell infiltrate in oral lichen planus (OLP). (C) HMGB1-TLR4 double immunofluorescence staining revealed weak expression in the normal mucosa (*white arrows*). (D) Strong co-localization between HMGB1 and TLR4 was seen in OLP lesion. Blue: DAPI, Green: HMGB1, Red: TLR4. Scale bar= 100 µm.