Receptor for Advanced Glycation End Products is Required for HMGB1/S100A4/NF-κβ Interaction In Porphyromonas gingivalis Induced Gingival Inflammation

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Abstract: High-mobility group box 1 (HMGB1) and the receptor for advanced glycation end products (RAGE) are thought to play key roles in the progression of chronic inflammatory diseases. We recently showed that HMGB1 and RAGE might be involved in the progression of human gingival inflammation as novel inflammatory mediators. The molecular mechanisms governing the ability of RAGE to induce ligand-specific responses in gingival inflammation are still unknown. We identified HMGB1/S100A4/NF-κβ pathway, a global regulator of inflammation, as a major RAGE-responsive molecule induced by Porphyromonas gingivalis (P. gingivalis) in rat gingiva. Consistent with the observation that RAGE can be activated by multiple ligands, the elevated expression of RAGE-mediated HMGB1 and S100A4 expression in gingival inflammation. Phosphorylation of NF-κβ and IL-1β immunoreactivity was stronger in P. gingivalis challenged gingiva compared to the control. Quantitative real-time RT-PCR confirmed the increased expression of HMGB1, RAGE, and IL-1β mRNA in P. gingivalis challenged gingiva. Our results indicate that RAGE is a major target gene shared by HMGB1 and S100A4 and the coordinated action of RAGE and NF-κβ leads to the induction of gingival inflammation.

Key words: HMGB1, RAGE, S100A4, NF-κβ, Gingiva, P. gingivalis

Introduction

A unique receptor of the immunoglobulin superfamily of cell surface molecules known as the receptor for advanced glycation end products (RAGE) has been implicated in chronic inflammatory conditions such as Sjögren’s syndrome, diabetes, and rheumatoid arthritis1-3). RAGE signaling stimulates a host of pro-inflammatory events. Normally, these appear to play an important role in acute inflammation. In contrast, when responding to persistent elevations of endogenous ligands, RAGE signaling promotes chronic inflammation. RAGE is expressed on a variety of cell types such as endothelial and smooth muscle cells and it binds to multiple families of ligands, namely advanced glycation end products (AGEs), the S100/calgranulin family, and high-mobility group box 1 (HMGB1)4.

HMGB1 is one of the most abundant nuclear non-histone proteins5). HMGB1 acts as a DNA chaperone that facilitates the rate-limiting DNA distortion during nucleosome sliding and regulates transcription6). In contrast, HMGB1 acts as a cytokine when released into the extracellular milieu upon tissue injury or inflammation7). In oral cavity, Porphyromonas gingivalis (P. gingivalis) induced HMGB1 secretion from human gingival fibroblasts which might contribute to periodontal tissue destruction8). The active secretion of HMGB1 has been demonstrated in monocytes/macrophages under certain conditions, such as stimulation with lipopolysaccharides (LPS), tumor necrosis factor-α (TNF-α), and interleukin-1α (IL-1β)9, 10). Thus, HMGB1 triggers tissue repair and immunological defense programs.

One of the key targets of RAGE in the cells is the transcription factor, nuclear factor kappa beta (NF-κβ), a critical factor in the transduction of a variety of inflammatory and pro- or anti-apoptotic signals in the cells, depending on the time course, site, and chronicity of the stimulus. RAGE-ligand interaction induces sustained activation of the pro-inflammatory transcription factor NF-κβ11). An important consequence of RAGE-dependent activation of NF-κβ is the upregulation of RAGE itself12).
Activation of NF-κB is marked by the phosphorylation of its subunits and their nuclear translocation\(^\text{13}\). S100A4, RAGE, and activated NF-κB have been found to be increased at inflammation sites and share several downstream targets\(^\text{14-16}\). Based on our data and published reports, we questioned whether there is a possibility of convergence of HMGB1, S100A4 and RAGE-mediated signaling to NF-κB in gingival inflammation.

While a number of different RAGE ligands have been identified, the S100 protein family, in particular, has been implicated in the regulation of inflammatory RAGE signaling\(^\text{17}\). S100A4 acts as a potent cytokine via RAGE, stimulating expression of RAGE itself while generating oxidative stress,
synthesis and secretion of pro-inflammatory cytokines, and chemotaxis. RAGE is an important component of the inflammation-associated pathway. This prompted us to investigate if S100A4 requires RAGE for its inflammation-promoting function.

Although numerous published studies are focused on the role of RAGE and its ligands during inflammation, very little has been done to determine its status, role, and mechanism of action during the development of disease. The current study is the first to investigate the role of HMGB1/S100A4/RAGE during gingival inflammation in an experimental rat model and the underlying mechanism. By employing *P. gingivalis*-induced rat gingivitis model, we provide evidence that the HMGB1 and S100A4 protein play an inflammation-promoting role in the development of gingivitis by regulating the function of NF-κB/RAGE molecule. Taken together, we showed that RAGE plays a critical role during gingivitis development and could serve as a molecular target for novel therapies to treat inflammatory disease.

### Materials and Methods

#### In vivo experiment

Twelve male Sprague-Dawley rats (3 weeks old) were obtained from Nihon SLC, Shizuoka, Japan and housed in isolation cages throughout the experimental period. They were fed a standardized diet of hard briquettes and water, and maintained under a 12-h light/dark cycle at a temperature of 22 °C and relative humidity of 50 %. Rats were given sulfamethoxazole (1 mg/ml) and trimethoprim (200 μg/ml) in drinking water, ad libitum, for 4 days to reduce their native oral flora. This was followed by a 4-day antibiotic-free period. Rats were orally challenged with *Porphyromonas gingivalis* A TCC 33277 (laboratory stock), which was suspended in 5 % carboxymethylcellulose. Each rat received 0.5 ml (1 × 10⁹ cells/ml) of the suspension by oral gavage (3 times) at 48-hour interval. Control rats received carboxymethylcellulose only. The animals were sacrificed after 3 and 7 days of gingivitis and the upper jaws were excised. Formalin-fixed specimens were decalcified with 10% EDTA-2Na for 2 weeks and embedded in paraffin. The experimental procedures of this study were conducted under protocols approved by the Animal Care and Ethics Committee in accordance with Kanagawa Dental University guidelines.

#### Immunohistochemistry

The specimens were subjected to antigen retrieval with sodium citrate buffer pH 6.0 (abcam, Tokyo, Japan) treatment for 20 min. After blocking with endogenous peroxidase for 10 min and protein
block (DAKO, Tokyo, Japan) for 1 hour, the specimens were rinsed with phosphate-buffered saline (PBS). Anti-HMGB1 antibody (abcam), anti-RAGE antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-S100A4 antibody (abcam), anti-phospho NF-κβ antibody (Bioss, Inc., Woburn, MA) and anti-IL-1β antibody (Santa Cruz Biotechnology) were used as primary antibodies. After an overnight incubation at 4 °C, the specimens were rinsed with PBS and incubated at room temperature for 30 min with secondary antibody conjugated to peroxidase (Nichirei Biosciences, Tokyo, Japan). After rinsing with PBS, all specimens were color-developed with diaminobenzidine (DAB) solution (DAKO) and counterstained with hematoxylin. The immunostaining of all specimens was performed simultaneously to ensure the same antibody reaction and DAB exposure conditions.

RNA extraction and quantitative RT-PCR analysis
Total RNA was isolated from rat gingiva using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instruction and purified total RNA using RNeasy Mini Kit (QIAGEN, Tokyo, Japan). First-strand cDNA was synthesized from 1μg of total RNA using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA), according to the manufacturer’s protocol. One-hundredth aliquot of the cDNA was subjected to real-time RT-PCR using TaqMan Gene Expression Assays (Applied Biosystems) for HMGB1, RAGE, and IL-1β, and Pre-Developed TaqMan Assay Reagents (Applied Biosystems) for beta-actin as an internal control. Three independent measurements were averaged and relative gene expression levels were calculated as a ratio to beta-actin expression of each tissue.

Statistical analyses
Significant differences were analyzed by Fisher’s exact test. A p value of less than 0.05 (*) was considered significant, 0.01 (**) ; highly significant.

Results
P. gingivalis-induced gingival inflammation triggers activation of RAGE/NF-κβ signaling pathway
Histologically, enlarged blood vessels and marked infiltration of lymphocytes in subepithelial layer of the crevicular epithelium of rats challenged with P. gingivalis compared to the control at 3 days. Histological evaluation of the periodontal tissues did not revealed significant pathological changes compared to the control except the presence of slight lymphocyte infiltration in the epithelium at 7 days after P. gingivalis challenge (Fig. 1). Immunohistochemical analysis revealed a higher expression in HMGB1 and RAGE expression in P. gingivalis challenged gingival epithelium (3 days) compared to the control (Fig. 2). IL-1β, and S100A4 were weakly expressed in the gingiva of control animals. The strong reaction with anti-IL-1β and anti-S100A4 antibodies was found in P. gingivalis challenged gingival epithelium (Fig. 3). IL-1β and S100A4 expression in inflammatory cells were stronger than in the epithelium. Phosphorylation of NF-κβ was strongly expressed in P. gingivalis challenged gingival epithelium compared to the control (Fig. 3).

P. gingivalis stimulates RAGE mRNA in rat gingival tissues
To understand whether inflammatory gingival activation alters the expression levels of HMGB1, RAGE, and IL-1β, their expression was evaluated in P. gingivalis challenged rat gingival tissues by quantitative real-time RT-PCR. In line with the cytokine functions of HMGB1, the data demonstrates that P. gingivalis increased HMGB1, RAGE, and IL-1β expression in rat gingiva (Fig. 4).

Discussion
In our previous study, we demonstrated the accumulation of HMGB1 in gingival tissues of chronic gingivitis and chronic periodontitis patients with the increased production of pro-inflammatory factors[19]. HMGB1 appears to have distinct functions in cellular systems. Intra-tracheal injection of HMGB1 led to the acute lung injury and triggered the inflammation by necrosis cells[20]. Anti-HMGB1 antibody decreased the severity of acute lung injury and the release of pro-inflammatory factors such as IL-1β and TNF-α[21, 22]. Extracellular HMGB1 plays critical roles in the progression of inflammatory diseases[10]. Macrophages in the connective tissue express HMGB1[23], suggesting that HMGB1 sustains inflammation and promotes disease progression. Previous studies reported the release of HMGB1 from gingival epithelial cells by TNF-α[24] and from gingival fibroblasts by LPS[25]. These results suggested that the continued release of HMGB1 can act, at least in part, as an important amplification signal for progressive periodontal destruction.

Since HMGB1 potentiates the action of LPS[26], it could exaggerate the outcome of bacterial insult. HMGB1 also stimulated the release of iNOS from human gingival cells via receptors including RAGE[27]. Several studies have reported RAGE reaction in the epithelial cells, including gingival epithelial cells[28], eye epithelial cells[29], lung epithelial cells[30], and skin epithelial cells[31]. Numerous migrating inflammatory cells seen in P. gingivalis group showed a positive RAGE reaction. These results suggest an important role of epithelial cells in the inflammatory and destructive processes taking place in the gingival tissues. Thus, one of the biological roles of RAGE expression in the gingival cells may be to facilitate the migration of the inflammatory cells. Lalla et al. [31] demonstrated accelerated periodontal breakdown, associated with increased AGE deposition and expression of vascular and monocyte RAGE in a murine model of P. gingivalis-induced periodontal disease. RAGE is activated by multiple ligands including S100 proteins, AGEs, HMGB1, amyloid-β, and
S100 proteins are reported to exhibit an affinity of binding to RAGE and participate in signal transduction. A potent source of S100 protein is activated monocytes, and released S100 can act in a paracrine manner to induce monocyte chemotaxis. This may explain the accumulation of S100 proteins at sites of inflammation where recruited monocytes are activated by RAGE ligands and other inflammatory stimuli (e.g. cytokines), resulting in the generation of S100. These findings are strong arguments in favor of the involvement of S100-RAGE interactions in the onset, amplification and maintenance of vascular inflammation.

The HMGB1-S100-RAGE interaction displays an interesting and complex pharmacology. Therefore, the role of HMGB1, S100A4, and RAGE in gingivitis has attracted more and more attention. Consistent with these previous studies, we found that the expression of HMGB1, S100A4, and RAGE were up-regulated in the inflamed gingival tissues of an experimental rat gingivitis model. Immunohistochemical staining revealed that HMGB1 is exclusively localized in the cytoplasm in the inflamed epithelium. Cytoplasmic HMGB1 is destined for release to the extracellular space, either actively or passively, when the cells receive an appropriate second signal. The reaction with anti-RAGE antibody was very weak in the healthy gingiva and very strong reaction was observed in the gingiva of *P. gingivalis*-treated group, especially in the inflammatory cells.

Our results demonstrated that the high expression of HMGB1 was accompanied by increased expression of IL-1β, suggesting that HMGB1 and IL-1β form a positive feedback loop to promote inflammation in chronic gingivitis. Amongst various cytokines, IL-1β promoted the release of HMGB1 from activated astrocytes through ERK/MAP kinase signaling. HMGB1 mRNA and protein were also produced by macrophages followed by increased NF-κB binding activity. The interaction of ligands with RAGE was reported to trigger the activation of NF-κB. NF-κB translocates from cytoplasm to cell nucleus to activate the transcription of other target genes, serves as one of the main downstream effectors in RAGE-mediated signal transduction.

It is well established that NF-κB is involved in the process of inflammation. Based on our current observation that S100A4 and NF-κB phosphorylation were induced in inflamed gingival tissues, we speculate that the sustained activation of NF-κB within inflammation-laden tissues could be due to the activation of S100A4. The current study showed that the S100A4 protein mediates its inflammatory effects on gingival tissues through RAGE. Previous studies showed that RAGE induces NF-κB. This study provides evidence that the induction of NF-κB by RAGE may be regulated by S100A4. Taken together, these data established the significance of the HMGB1/S100A4/RAGE/NF-κB molecular circuitry in gingival inflammation.

In conclusion, we identified that *P. gingivalis*-induced NF-κB phosphorylation and up-regulation of the RAGE ligands, HMGB1 and S100A4, are associated with gingival inflammation. This study provides important evidence for future utilization of recombinant RAGE for chronic inflammatory diseases.

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