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Host Defense of Oral Mucosa and the Molecular Mechanism of Oral Mucosal Signal Transduction Diseases

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Abstract: It is speculated more that 400 bacterial species reside in the oral cavity, and the integrity of oral mucosa is maintained in such an environment. Oral mucosal cells such as epithelial cells and fibroblasts express a wide variety of receptors and produce many immune regulators i.e., cytokines, chemokines, proteases, and chemical mediators and actively participate in host defenses by interacting with immune cells either directly or indirectly. Furthermore, it was evident that saliva contains many immune regulatory factors. Thus, a triangle consisting of oral mucosal cells, immune cells and saliva formed by connecting with the immune regulator is considered important to maintaining the integrity of oral mucosa, and dysfunction (disorder) of the triangle leads to onset of oral mucosal diseases and inflammation such as oral lichen planus, pemphigus, Sjögren syndrome, oral cancer, leukoplakia, and periodontitis. We consider oral mucosal diseases to be "oral mucosal signal transduction diseases" and are investigated the molecular mechanism involved in disease onset. To overcome "oral mucosal signal transduction diseases", we attempted to clarify the underlying molecular mechanisms and develop appropriate therapeutic strategies. This review focuses on our recent study regarding 1) regulation of innate immune responses by host and parasitic proteases in oral mucosa, 2) cross-talk between oral mucosal cells and immune cells, 3) activation of oral mucosal cells by proteases, and 4) regulation of oral mucosal responses by salivary components.

Introduction

It is speculated more that 400 bacterial species reside in the oral cavity, and the integrity of oral mucosa is maintained in such an environment. Oral mucosal cells such as epithelial cells and fibroblasts express a wide variety of receptors and produce many immune regulators i.e., cytokines, chemokines, proteases, and chemical mediators and actively participate in host defenses by interacting with immune cells either directly or indirectly. Furthermore, it was evident that saliva contains many immune regulatory factors. Thus, a triangle consisting of oral mucosal cells, immune cells and saliva formed by connecting with the immune regulator is considered important to maintaining the integrity of oral mucosa, and dysfunction (disorder) of the triangle leads to onset of oral mucosal diseases and inflammation such as oral lichen planus, pemphigus, Sjögren syndrome, oral cancer, leukoplakia, and periodontitis (Fig. 1). We consider the oral mucosal diseases to be "oral mucosal signal transduction diseases" and are investigating the molecular mechanism involved in disease onset. To overcome "oral mucosal signal transduction diseases",

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we are attempting to clarify the underlying molecular mechanisms and develop appropriate therapeutic strategies. This paper will review our recent study on the issues.

**Regulation of Innate Immune Responses by Host and Parasitic Proteases in Oral Mucosa**

An oral chronic inflammation, i.e., periodontitis is one of the major diseases afflicting mankind and caused by a bacterial infection leading to gingival inflammation, destruction of periodontal tissues, loss of alveolar bone, and culminating in tooth loss\(^2\). *Porphyromonas gingivalis* has been implicated as a principal bacterium not only in chronic periodontitis but also in aggressive periodontitis\(^2\). *P. gingivalis* possesses a number of putative virulence factors such as LPS, fimbriae, toxic products of metabolism and proteinases, all of which enable this anaerobe to cause the disease either directly or indirectly by activation of host cells to release inflammatory mediators\(^3\).

It is now clear that all of the trypsin-like proteinase activity of *P. gingivalis* is due to two cysteine proteinases\(^4\). Two types of cysteine proteinases specific for Arg-X (50 and 95 kDa) and Lys-X (105 kDa) bonds have been purified and characterized and are referred to as arginine-specific gingipain (Rgp) and lysine-specific gingipain (Kgp), respectively. The 95-kDa high molecular mass Rgp (HRgpA) differs from the 50-kDa Rgp (RgpB) in that the protein noncovalently complexes with the hemagglutinin adhesin domain in the same manner as Kgp\(^4\). It has been shown that gingipains play a critical role in the onset of inflammation through enhancement of vascular permeability by activation of the kalikrein kinin pathway, dysregulation of plasma clot formation, activation of complement components, and modification of neutrophil function. Gingipains are also indispensable for the expression of *P. gingivalis* fimbriae, an important cell surface structure of this bacterium for adhesion and colonization, through normal processing of immature fimbritlin\(^3\).

CD14, mainly expressed on monocytes, functions as a major receptor for lipopolysaccharide (LPS)\(^6\). CD14 also exists in serum as a soluble protein (soluble CD14), and a complex of soluble CD14 and LPS activates CD14-negative cells, such as endothelial and epithelial cells. Furthermore, CD14 acts as a receptor for pathogen-associated molecular patterns (PAMPs) including LPS. As CD14 is a 55 kDa glycosylphosphatidylinositol-anchored membrane protein that lacks transmembrane and cytoplasmic domains, CD14 itself does not elicit intracellular signaling. The Toll-like receptors (TLRs) of vertebrates, homologues of *Drosophila* Toll, have been implicated in the innate immune response in vertebrates\(^7\), and it has recently been shown that 10 members of TLRs were identified, and PAMPs are recognized specifically by the respective TLR on the cell surface of hosts, e.g. TLR4 and MD-2 mediate the entry of LPS signal into the cells of human and mouse\(^8\).

Activation of monocytes through CD14 TLR by LPS and other PAMPs leads to the production of numerous inflammatory mediators, and the activated monocytes macrophages and the released mediators function as the first line of defense against infectious bacteria. Chronic periodontitis is characterized by the phases of chronic and sometimes active destruction of periodontal tissues with marked inflammation followed by periods of remission, which leads to the hypothesis that *P. gingivalis* could evade immune surveillance by monocytes by cleaving receptor(s) for PAMPs on monocytes using the bacterial proteinases.
and survive in periodontal tissues. We examined this hypothesis and showed gingipains preferentially cleave human monocyte CD14 but not TLR4, and as a consequence, inhibit a CD14-dependent monocyte activation pathway triggered by LPS\(^9\). These results suggest that gingipains cleave monocyte CD14, resulting in attenuation of the cellular recognition of bacteria, and as a consequence sustain chronic inflammation (Fig. 2). We showed that human gingival fibroblasts (HGF) heterogeneously express CD14 and the CD14-expressed fibroblasts secrete interleukin-8 (IL-8) in response to LPS in a CD14-dependent manner\(^10,11\). Gingipains also cleaved CD14 on HGF and reduced LPS-induced IL-8 production by HGF\(^12\). The findings further support the hypothesis that gingipains are involved in immune evasion by the bacterium in periodontal tissues. Furthermore, human leukocyte elastase (HLE) cleaves CD14 on HGF and inhibits a CD14-dependent cell activation\(^13\), suggesting that activated neutrophils have a potential negative feedback mechanism for HGF function at the site of inflammation, particularly in periodontal tissues (Fig. 2).

In periodontitis, gingival epithelial cells express IL-8, a chemoattractant and activator of neutrophils, and express intercellular adhesion molecule-1 (ICAM-1, CD54), which mediates neutrophil-epithelial cell interaction\(^14\). ICAM-1 expression is restricted to the junctional or sulcular epithelium\(^15-17\), and levels of the expression increase from basal cells toward the surface of the junctional epithelium\(^18\). ICAM-1 is a key molecule in neutrophil-epithelium adhesion through its recognition of \(\beta\)2 integrin counter receptors, CD11a/CD18 and CD11b/CD18, on neutrophils\(^19\). We showed that purified gingipains cleaved ICAM-1 on oral epithelial cells, consequently inhibiting neutrophil-oral epithelial cell interaction\(^20\), indicating that gingipains cleave ICAM-1 on oral epithelial cells, consequently disrupt neutrophil-oral epithelial cell interaction, and are also involved in immune evasion by the bacterium in periodontal tissues (Fig. 2).

**Cross-talk between Oral Mucosal Cells and Immune Cells**

IL-2 is a principal growth factor that induces the proliferation and differentiation of T cells and natural killer (NK) cells\(^21\) and shares many biological activities and the use of the IL-2 receptor (R) \(\beta\) chain and common \(\gamma\)-chain (\(\gamma\)c) with IL-15\(^21,22\). IL-2R and IL-15R have their own \(\alpha\) chain and the heterotrimers form fully functional high affinity IL-2R and IL-15R complexes. These subunits can be expressed individually or in various combinations, resulting in distinct receptor complexes that bind IL-2 and IL-15 with different affinities. Activation through IL-2R\(\beta\) and \(\gamma\)c is directly associated with phosphorylation of Janus tyrosine kinase (Jak) 1 and Jak3, resulting in signal transducer and activator of transcription (STAT)
3 and STAT5 phosphorylation, respectively\textsuperscript{23}. It has been shown that human monocytes also bear functional IL-2R\textbeta and \gamma c\textsuperscript{24}, human fibroblasts from adult bone marrow, embryonic skin and lung express IL-2R\alpha and IL-2R\beta\textsuperscript{25–27}, and fibroblast-like synoviocytes express functional IL-2R\beta and \gamma c\textsuperscript{28}. In addition, we have recently shown that normal HGF express functional IL-2R\beta and \gamma c and that IL-2 stimulation induced production of monocyte chemoattractant protein-1(MCP-1) and expression of ICAM-1 by HGF, resulting in the augmentation of ICAM-1-mediated neutrophil adhesion, and that IL-2-induced MCP-1 production was significantly inhibited by pretreatment with anti-IL-15 neutralizing antibody (Ab)\textsuperscript{29}. These observations suggest that IL-2 has a wide variety of biological activities in cells other than the lymphoid cell population, and that IL-15 expressed by HGF sustains IL-2-mediated signaling in HGF.

There are two isoforms of IL-15, one with a 48aa leading peptide and one with a 21aa leader peptide\textsuperscript{22}. The 48aa–IL-15 is targeted to the secretory pathway with a low secretion potential, whereas the 21aa–IL-15 appears to be restricted to the cytoplasm and nucleus. Recent reports showed that IL-15 is constitutively expressed on fibroblasts from human spleen and skin and also on human monocytes and leukemic progenitors without any IL-15 secretion. However, it is still unclear which of the isoforms are expressed on the cell surface.

Oral inflammation is characterized by the infiltration of T and B cells as well as neutrophils into inflamed oral tissues, resulting in direct and indirect destruction of the tissue\textsuperscript{30}. These observations led us to investigate 1) whether HGF from normal and inflamed regions differentially express IL-2R and IL-15R subunits and respond to IL-2 and IL-15 and 2) the role of endogenous IL-15 in IL-2-mediated signaling. We showed that normal HGF expressed IL-2R\beta and \gamma c but not IL-2R\alpha or IL-15R\alpha, whereas inflamed HGF expressed IL-2R\alpha, IL-15R\alpha, IL-2R\beta and \gamma c at mRNA and protein levels\textsuperscript{31}. Exogenous IL-2 and IL-15 induced production of MCP-1 but not IL-8 in normal HGF, and induced the production of both chemokines in inflamed HGF. Both HGF constitutively transcribed 48aa–IL-15 isoform, and the isoform was not actively secreted but rather existed as a membrane-bound form. Pretreatment with anti-IL-15 neutralizing mAb for 24 h completely inhibited the production of MCP-1 induced by IL-2 and IL-15 and IL-2-induced phosphorylation of Jak1 and 3 in HGF. The pretreatment and RNA interference targeted to IL-15 mRNA resulted in total inhibition of the IL-2R\beta and \gamma c expression at mRNA and protein levels. Furthermore, excess amount of IL-2 restored the inhibitory effect of anti-IL-15, inhibition of nuclear factor (NF)-\kappa B abrogated the expression of IL-2R\beta and \gamma c, and IL-2-induced nuclear translocation of NF-\kappa B was completely inhibited by the RNA interference in HGF. These results suggest that endogenous membrane-bound IL-15 sustains recruitment of IL-2R\beta and \gamma c through activation of NF-\kappa B in HGF (Fig. 3), that the activation of HGF by IL-2, which is released by activated T cells, may play an important role in innate immunity against pathogens by controlling Th1 and Th2 responses at the inflamed site, and that the endogenous membrane-bound IL-15 on HGF may
participate in the development, survival, and activation of NK and T cells in the oral mucosa.

**Activation of Oral Mucosal Cells by Proteases**

IL-18 was originally identified as an interferon-γ (IFN-γ) inducing factor\(^\text{32}\) and is a multifunctional regulator of innate and acquired immune responses through its activation of both Th1 and Th2 responses\(^\text{33}\). IL-18 has also been suggested to be a potent proinflammatory cytokine that regulates autoimmune and inflammatory diseases\(^\text{33-35}\). IL-18 is intracellularly produced as an inactive precursor form and secreted as an active cytokine after cleavage by caspase-1, originally designated IL-1β converting enzyme. Furthermore, IL-18 is identified by immune cells (activated macrophages, dendritic cells, Kupffer cells) and non-immune cells (keratinocytes, osteoblasts, adrenal cortex cells, epithelial cells, microglial cells, and synovial fibroblasts). This wide range of distribution implies that it plays physiological roles and acts as a component of immune regulation.

Protease-activated receptor (PAR) family members are G protein-coupled receptors which undergo proteolytic cleavage of the N terminus, thereby exposing tethered ligands and permitting autoactivation of its receptor function so that it can initiate multiple signaling cascades\(^\text{36}\). PAR-1, -3 and -4 are mainly activated by thrombin, whereas PAR-2 is activated by a number of proteases such as trypsin, mast cell tryptase, coagulation factors \(\text{VIIa and Xa}\), but not by thrombin. PAR-2 was identified by Nystedt, et al.\(^\text{37}\) and is expressed in a wide variety of tissues, including the gastrointestinal and respiratory tracts, pancreas, kidney, muscles, ovary, and skin, but it is not expressed in platelets\(^\text{36,37}\). Also, recent studies suggest that PAR-2 regulates several pathophysiological processes, including growth, development, inflammation, and tissue repair. Mice that lack PAR-2 gene exhibit diminished ear swelling and infiltration of inflammatory cells in a model of allergic dermatitis\(^\text{38}\) and are immune to a form of adjuvant-induced arthritis\(^\text{39}\).

Neutrophil-derived serine proteinases, HLE (EC 3.4.21.37), cathepsin G (Cat G : EC 3.4.21.20) and proteinase 3 (PR3 : EC 3.4.21.76) are stored in the azurophilic granules of neutrophils as active enzymes. The major physiological function of the proteinases is commonly thought to be the intralysosomal degradation of engulfed cell debris or microorganisms\(^\text{40}\). It has become evident that HLE, Cat G and PR3 play crucial roles in extracellular proteolytic processes at sites of inflammation.

Recently, we found that human oral epithelial cells constitutively express a precursor form of IL-18, and that PR3 induces the secretion of bioactive IL-18 from cells in combination with LPS after priming with IFN-γ\(^\text{41}\). Subsequently, we showed that PR3 activates cells through the PAR-2 pathway\(^\text{42}\), providing new insight into the possible involvement of a neutrophil protease in the induction of bioactive IL-18 in oral inflammation (Fig. 4). In addition, HLE and Cat G also activate secretory leukocyte protease-lacking nonepithelial cells such as human gingival fibroblasts via the PAR-2 pathway\(^\text{43}\). Human epithelial cells express secretory leukocyte protease inhibitor, which inhibits neutrophil serine proteinases including HLE and Cat G but not PR3, suggesting that neutrophil serine proteinases have an equal ability to activate nonepithelial cells through PAR-2. In vivo study confirmed that neutrophil recruitment and PAR-2 activation by neutrophil serine proteases are critical for induction of serum IL-18 and tumor necrosis factor-α (TNF-α) and IL-18-dependent liver injury in mice treated with heat-killed *Propionibacterium acnes* and LPS (Ikawa, et al., submitted for publication).

Anti-neutrophil cytoplasmic Abs (ANCA) were first described in 1982 by Davies, et al.\(^\text{44}\) in patients with necrotizing glomerulonephritis. ANCA is an autoantibody directed against the enzymes located in the primary granules of neutrophils and lysosomes of monocytes. PR3 is a major target antigen of ANCA in Wegener’s granulomatosis, a debilitating autoimmune disease characterized by necrotizing vasculitis\(^\text{45,46}\). Since then, ANCA have been detected in relation to a wide range of inflammatory, infectious, and neoplastic conditions\(^\text{47-50}\). Novo, et al.\(^\text{51,52}\) described a high rate of occurrence of ANCA in serum of patients with periodontal disease. Recently, human renal tubular epithelial cells were reported to express PR3\(^\text{53,54}\). In
contrast, Brouwer, et al.\textsuperscript{55} reported that PR3 detected in renal tissue was originally derived from neutrophils, and renal cells merely took up the released PR3. Therefore, it is still controversial whether or not PR3 is produced by nonhematopoietic cells\textsuperscript{56,57}. We showed that proinflammatory cytokines induce PR3 as membrane-bound and secretory forms in human oral epithelial cells and antibodies to PR3 activate the cells through PAR-2\textsuperscript{58}. These results suggest that oral epithelial cells express functional PR3 in the inflamed sites and respond to anti-PR3 Abs detected in diseased sera, and that these mechanisms may actively participate in the oral inflammatory process (Fig. 4).

### Regulation of Oral Mucosal Responses by Salivary Components

Saliva, a complex mix of fluids from major (parotid, submandibular, and sublingual) and minor salivary glands, is a most valuable oral fluid that is critical to the preservation and maintenance of oral health such as oral mucous and teeth\textsuperscript{59}. Saliva contains a number of antimicrobial agents, secretory immunoglobulin A, proteins (glycoproteins, statherins, agglutinins, histidine-rich proteins, and proline-rich proteins), mucins, lactoferrin, enzymes (lysozyme and peroxidase), and antimicrobial peptides\textsuperscript{60,61}. The concerted action of these agents is thought to provide a multi-
functional protective network against microorganisms. In addition, we have recently shown that major salivary glands constitutively express CD14 and a soluble form of CD14 is secreted in saliva\(^6\). The concentration of CD14 in parotid (a serous gland) saliva was comparable to that in normal serum and 10-fold the amount in whole saliva. In contrast to the case for serum, levels of LPS-binding protein (the serum protein that accelerates the binding of LPS to CD14\(^6\)) in whole and parotid saliva were below the detectable limit.

The physiological function of saliva CD14 remains unclear, and the evidence that monocytes phagocytose gram-negative bacteria in a CD14-dependent mechanism\(^63\) led us to investigate whether saliva CD14 is involved in the invasion and activation of oral epithelial cells by periodontopathic bacteria. The present study showed that saliva CD14 augmented the invasion of oral epithelial cells in culture by live Actinobacillus actinomycetemcomitans and as a consequence, up-regulated production of IL-8 by the epithelial cells\(^6\). These results suggest that saliva CD14 promoted the invasion of oral epithelial cells by A. actinomycetemcomitans and consequently augmented the production of IL-8 playing an important role in innate immunity in the oral cavity.

**Conclusion**

This article reviewed our recent studies regarding host defense mechanism of oral mucosa. In the cytokine cascade, IL-18 in synergy with IL-12 induces IFN-\(\gamma\), and IL-18 induces, directly or indirectly via IFN-\(\gamma\), production of effector cytokines such as TNF-\(\alpha\)\(^33\)\(^-\)\(^35\). Mucosal mast cells may play an important role not only in allergy but also in oral mucosal inflammation, since the cells express tryptase and TNF-\(\alpha\) as well as histamine. It is reported that mast cell tryptase is an agonist of PAR-\(2\)\(^36\). Thus, many proteases and their inhibitors may actively be involved in the cytokine cascade and mucosal immunity (Fig. 5). Furthermore, other undefined immune regulator may be expressed in salivary glands and secreted in saliva. More work is required for full understanding of host defense mechanism of oral mucosa.

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