

Research Article

The Expression of TLR-2 and TLR-4 Protein in the Epithelial Cells of the Oral Mucosal Patients with Recurrent Aphthous Stomatitis (RAS)

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ABSTRACT

Background: Toll-Like Receptors (TLRs) play an important role in the human immune system. The objective of this study was to disclose or discover RAS using etiopathogenetic molecular approach by observing the TLRs. Particular objective of this study was to understand the expression of toll-like receptors 2 (TLRs-2) and TLR-4 in epithelial cells of oral mucosa, and to investigate the role of toll-like receptors in the innate immunity. Methods: Human oral epithelial cells were obtained by scraping the oral mucosal from 40 patients with recurrent aphthous stomatitis and 10 healthy adult volunteers. The epithelial cells are made into smears. Immunohistochemistry was performed for identification of TLR-2 and TLR-4 protein using monoclonal antibodies anti-TLR-2 and TLR-4. Result: TLR2 and TLR4 protein were expressed in the oral mucosal epithelial cells especially in surface and nuclear cells. The expression of the TLR-2 in patients with minor RAU was 41,02%, while major RAS is expressed 43,58%. RAS patients with positiv TLR-4 was 48,71% in major RAS, while in minor RAS TLR-4 was expressed 38,46%. Conclusion: This study is the first to establish the presence of both TLR-2 and TLR-4 protein on epithelial cells of oral mucosa, and their expression can be up-regulated in infectious conditions. These results show that TLR-2 and TLR-4 may play a important role in local host defense of oral mucosal.

Keywords : Toll-like receptors, Recurrent Aphthous Stomatitis, oral mucosal epithelium. Expression TLR-2 and TLR-4.

INTRODUCTION

Toll-Like Receptors (TLRs) are vital components playing important role in pathogen recognition and natural immunity. TLRs recognize microbial structure and transmit this information into the cell, culminating in an inflammatory cytokine response and in co-stimulatory molecule expression involve in induction of adaptive immunity. Failure of the receptor to recognize pathogen may result in recurrent infection in hosts¹.

Oral mucosal cells such as epithelial cells are thought to act as physical barrier against the invasion of pathogenic organism, but they have an ability to produce inflammatory cytokines and express adhesion molecules. Oral epithelial cells are refractory to many bacterial components although they express TLR/MyD88². Epithelium of oral mucosal is the first line of defense against invading pathogens^{2,3}.

TLR-4, along with CD14 and other adaptor molecules, recognize pathogen-associated molecular patterns such as lipopolysaccharides (LPS) from gram negative enteric bacteria. TLR-2 along with TLR1/TLR6, recognize gram positive peptidoglycans (eg. From oral commensal). One recent study indicates that both TLR-2 and TLR-4 positive cells infiltrate the oral mucosa in mucosal health and disease., but very little is understood about the overall expression pattern of PRRs in the human oral mucosal health and in Recurrent Aphthous Stomatitis and how they regulate local immune responsiveness^{1,4}.

Recurrent Aphthous Stomatitis (RAS) or Recurrent Aphthous Ulcerative (RAU) are the most common oral disease characterized by repeated development of painful ulcers. The etiology of RAS is still unknown. Many local and systemic factors such as viral or bacterial infection, food hypersensitivity, genetic factors, and systemic disease could be involved in the pathogenesis of RAS. Previous study have also suggested that this inflammatory disease is a result of abnormal immune response directed towards the oral mucosal.

The Innate immune system also plays a role in several human diseases. The primary function of Toll Like receptors (TLRs) is to recognized pathogens. TLRs can also specifically recognize pathogen-associated molecular patters (PAMPs), transduce signal into cells and initiate complex signal cascade leading to activation of the transcriptional factors such as nuclear factor kappa β (NF- κ β) and inteferon regulatory factors⁵⁻⁷.

The objective of this study was to disclose or discover RAS using etiopathogenetic molecular approach by observing the TLRs. Particular objective of this study was to prove the presence of toll-like receptors-2 and TLR-4 at the surface of epithelial cell membrane and macrophage and understood the role of TLRs in the innate immunity of oral mucosa.

EXPERIMENTAL METHOD

Samples

Inform consent approved by the Research Ethics Committee was obtained from all the participants. The criteria for exclusion were the presence of systemic disease (eg. anemia or cyclic neutropenia available by haemogram, or history of bowel disease, Diabetes mellitus, HIV infection, or Bechet's disease). Tissue samples were obtained from Twenty patients diagnosed with major RAU and twenty minor RAU (24 women and 16 man) and who had experienced at least one episode each month within the last year were subjected to biopsy of the aphthae (half ulcer and half epithelium) from lip or buccal mucosa during an acute episode (within 72 hour of the onset of the ulcers). Ten healthy volunteers (seven women and three man; mean age 45 years; range 20-55) who were checked to be RAU free were also subjected to a biopsy of their clinically normal buccal or lip mucosa. All specimen were divided into two parts: one being frozen and store at -70°C for subsequent RNA extraction and the other fixed in 10% buffered formalin and submitted to histopathologic examination to support clinical diagnosis. Five ml of venous blood were drawn from the anterior arm, anti-coagulated with heparin and diluted 1:1 with Hank's liquid. Then the mixture was gently added to a centrifuge tube containing 5 ml of lymphocyte separating liquid and centrifuge at 700r/min for 20 minute. The cells in gray middle layer were collected and then washed twice with PBS. The deposit contained about 25% monocytes and 75% lymphocytes.

Immunohistochemistry

For immunohistochemistry single immunoenzyme staining was performed by the biotin-streptavidin-peroxidase method with the antibodies (from Biosciences) and the specificity of the antibodies was confirmed by replacing each with the respective isotype control. (To quantitate the infiltration of tissue by TLR positive cells, light microscopy images were acquired with a Nikon Eclipse E600 microscope equipped with a color high resolution charge-coupled device CCD camera.). Scrapped specimen oral epithelial biopsy in oral mucosal and fixed on to object glass with alcohol 90% (15 minutes), and incubated in refrigerator or directly blocked with bovine serum albumin 1% (BSA 1%) for 15 minutes then incubated in CO₂ at the temperature 37°C for 45 minutes. After being washed PBS, sample is reacted with monoclonal antibody TLR anti TLR-2 and TLR-4, re-incubated in CO₂ incubator at 37°C for one hour. After being washed PBS, the sample was analyzed using immunofluorescence microscope by magnifying with 40x.

RESULT

A study has been conducted to 20 and 20 patients with major and minor RAU, respectively, and to 10 non-RAU patients as control in order to identify the presence of protein like receptors (TLR) in epithelial cells and macrophage of patients with Recurrent Aphthous Stomatitis (RAS).

In this study, it was found that TLR is expressed at the surface of epithelial cell membrane of oral mucosa and

macrophage in both major and minor RAS patients. TLR is not expressed specifically in non-RAS patients.

The patients with positive TLRs-2 were 41.02%, found among minor RAS patients, while in major RAS TLRs-2 is expressed 43.58% (Fig.2).

RAS patients with positive Toll-Like Receptors-4 (TLRs-4) was 48.71%, found in major RAS patients, while in minor RAU TLRs-4 was expressed 38.46%. (See Fig.3)

Expression of TLR-2 determined by RT-PCR in oral epithelial mucosal in RAS major patients. Line M: marker; Line 1: sample patients RAU mayor. Line 2: sample negative (non RAU)

The results above showed indication that functional TLR expression by epithelial cells in oral mucosa had remarkable implication on natural immune response and disease pathogenesis

DISCUSSION

Toll-Like Receptors (TLRs) represent a class of transmembrane pattern recognition receptors essential for microbial recognition and control of innate immune responses. Commensal bacteria play an important role in maintaining tolerance and active stability of the oral mucosal epithelial barrier by suppressing oral mucosal inflammation. Epithelium of oral mucosal is the the first line of defense against invading pathogens⁸.

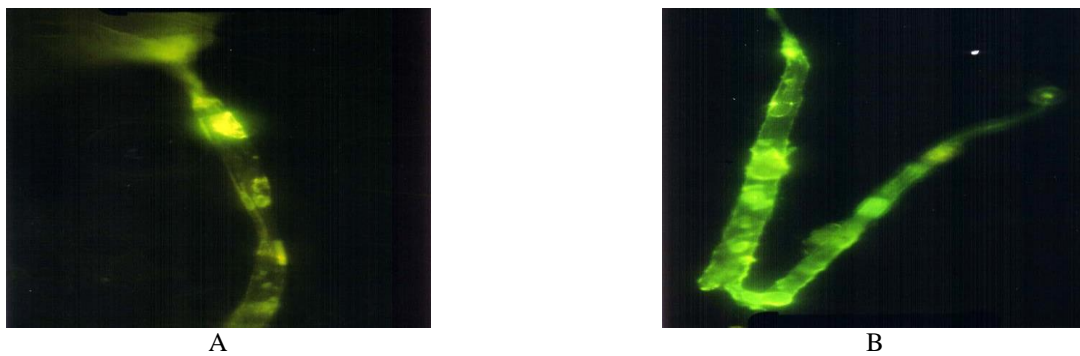
TLRs can specifically recognize pathogen-associated molecular patterns (PAMPS), transduce signals into cells and initiate complex signal cascades leading to activation of the transcriptional factors such as nuclear factor kappa B (NF- κ B) and interferon regulatory factor (IRF). Subsequently, the inflammatory mediators such as IL-1a/b, Il-6,8 and TNF- α are synthesized and released to active neutrophils and lymphocytes. This result in the initiation of innate and adaptive immune responses. Deficiency of TLRs may result in corresponding pathogen recognition failure and susceptibility to certain pathological microbes. Hyper-expression of TLRs in infectious tissue may promote excessive inflammation⁹.

Molecule of pathogen can expressed some PAMPs (Pathogen Associated Molecular Pattern), it were recognized of different TLRs, that heredity redundancy on immune system for blocking of microbial infection. PAMPs come from positive gram organism (peptidoglycan and lipoprotein), it is recognized TLR-2, the other member subfamily of TLR-2 is TLR-1 and TLR-6. TLR-2 combined with TLR-1 and TLR-6, that have as signal response to many kind of microbial pathogen as well as mycobacterium. TLR-2 signal is needed for fragmentation of *M. tuberculosis* in macrophage. TLR-2 in mouse is very important role because the individual more sensitive to *M tuberculosis* infection if they have decrease of TLR-2. On the other hand that TLR-2 also can bind components of herpes virus, which binds in turn to CD14 at the cell surface¹⁰.

RAS in Dental Hospital of Faculty Dentistry at Airlangga University more 43,7% is Found in mayor RAS. This phenomenon is very important role in Oral Medicine because the signaling through TLR-s occurs through a well described pathway in which receptor binding



A B
Figure 1: (A) Mayor RAS patients, (B) Minor RAS patients.



A B
Figure 2: Immunohistochemical detection of Toll-Like Receptors in mayor and minor RAS patients with scrapped oral epithelial biopsy. TLR-2 Expression on cytoplasmic and surface of epithelial cell membrane. A. Major RAS patients. B. Minor RAS patients.



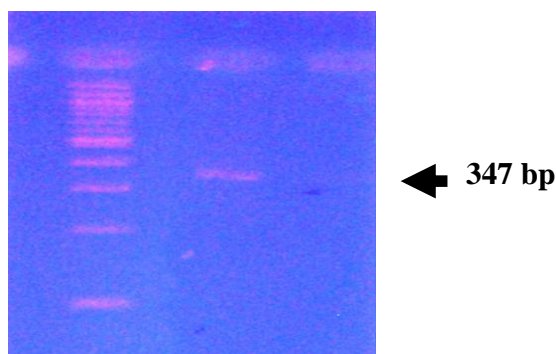
A B
Figure 3: (A) TLR-4 expression in this minor RAS case is not well distributed in all cells, either at the cell membrane surface or in the cytoplasm. (B) TLR-4 Expression in major RAS was found at epithelial surface and macrophage cell surface.

generates a signal through an adaptor molecule, MyD88, that leads to intracellular associated with IL-1 receptor-associated kinase.

TLR4 can mediated LPS as signaling from bacteria of gram negative that activated macrophage through TLR-2. Base on the type of agent of microbial and their PAMPs that the TLRs can recognize direct to plasma membrane as well as TLR-4 or direct in the phagosome like TLR-2. TLR response can appear in different cell exp. Epithelial gut cell using LPS that can associated with TLR4 in golgy complex nor in plasma membrane. This statement

like with TLR-s which expressed on mouth epithelial cell. However, on the basis of studies with TLR4 is similar but not identical to the signaling pathway activated by other TLRs. Also, the activation of cytokine production by TLRs plays an important role in recruiting other components of innate host defense against molecule pathogen as well¹⁰.

We show here that TLR-2 and TLR-4 cells are present in oral epithelial mucosal cells RAS mayor and minor patients (Fig 2 and Fig 3). The presentation of TLR-2 expression in major and minor RAS patients could be



Detection of the expression of Toll-Like Receptors-2 by RT-PCR analysis.

related with the presence of bacterial infection/LPS. Immunohistochemistry, Toll-Like Receptors-2 (TLRs-2) is an inflammation signaling in oral mucosa induced by gram-positive bacteria. Bacterial molecules that can induce TLRs-2 expression are outer membrane and polysaccharides. These molecules apparently had important role in RAS patients visiting dental out patients clinic. Therefore, in oral cavity, the presence of bacterial infection should be noticed although the bacteria are not the predominant cause of RAS^{11,12}.

RAU patients with positive Toll-Like Receptors-4 (TLRs-4) was 48.71%, found in major RAU patients, while in minor RAU TLRs-4 was expressed 38.46%. TLR-4 could be detected at the surface of cells and cytoplasm from oral mucosal epithelium in either major or minor RAU patients. In major RAU patients, the TLRs-4 expression was more predominant at the surface of epithelial cell and macrophage. This seemed reasonable since the oral mucosal epithelium of patients with ulcerative oral mucosa can respond the presence of bacterial endotoxin by activating TLRs-2 and TLRs-4. TNF-alpha is an inflammatory cytokine produced through TLR activation in its response against bacteria¹⁰.

TLRs with transmembrane protein and cell surface receptor and intra cytoplasm signal area may likely play more important role in this intracellular signaling. TLR-4 blocking by neutralizing antibody anti-TLR-4 apparently inhibited TNF-alpha after LPS administration¹⁰. A previous study using immunohistochemistry also shows that TLR2 and TLR4 cells numbers increase in the inflamed mucosal ulcers. Although level of transcript were not quantitated.

The predominant TLRs expressed in RAS patients were TLR-2, and -4. If related with the result of previous studies in its clinical implications, the causing agents or inducers were not only one type, but also accumulation of molecules in oral mucosa that played mutual role as predominant inducer. It has been proved immunohistochemistry that TLRs can be detected at the surface of oral mucosal epithelium and cytoplasm of RAU patients. TLR localization has been widely related with immune cells and inflammation.

Expression of TLR-2 and TLR-4 determined by RT-PCR in oral mucosal epithelial cells of RAS major patients. Our result showed that most oral mucosal epithelial cells

specimens expressed TLR-2 a TLR-4 weakly. This indicates that TLR-2 and TLR-4 might induce the occurrence of RAS which could be detected by expression TLR whether locally and sistemically. In conclusion that TLR expression at the surface of oral mucosal epithelial cells and macrophage in RAS patients, either major or minor. TLRs-2 and TLRs-4 has been expressed at the surface of cell membrane and macrophage in minor and major RAS. TLRs was more predominantly expressed in major RAS compared to minor RAS. Functional TLR expression by oral mucosal epithelial cells had higher implications towards natural immune response and disease pathogenesis. It is suggested to undertake molecular characterization to determine specific TLR against specific disease agents, so that it will be easy to identify the causing agent, with the result that RAS disease management can be established comprehensively.

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