Local and peripheral cytokines profiling on Porphyromonas gingivalis induced rat periodontitis model

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Received December 11, 2015; Accepted March 5, 2016; Epub March 15, 2016; Published March 30, 2016

Abstract: Periodontitis is a chronic bacterial infection. The host immune response greatly influences the extent and severity of this disease. Up-to-date, reliable experimental model to elucidate the pathogenesis of periodontitis remains unavailable. Here, we established a ligature-induced periodontitis rat model with inflamed gingiva, periodontal attachment and alveolar bone loss. Using this model, we further elucidated the dynamic changes of multiple cytokines during periodontitis development. All cytokines including IL-1β, IL-6, IL-10, IL-18 and TNF-α as well as IgG levels were found to be significantly increased in the gingiva tissue after four weeks of ligature. Interestingly, IL-1β, IL-10, IL-18 and TNF-α continued to increase 8 weeks after induction of ligature, suggesting a prolonged inflammation of the model, which is similar to the chronic inflammation induced by periodontitis in human. In addition, increases in the TRL-4 but not TRL-2 expression indicating that Toll-like receptor may play a role in the recognition of pathogen and the secretion of chemokines during the formation of periodontitis.

Keywords: Porphyromonas gingivalis, periodontitis, animal model, cytokine profiling

Introduction

Dental diseases remain major public health concerns due to their high prevalence in general population. While epidemiological studies showed that certain countries like Sweden and Switzerland have low prevalence of periodontal disease [1, 2], the prevalence rate of periodontitis can reach as high as 70.9% in 35-44-year-old adults and 87.4% in 75-84-year-old seniors in developed countries like Germany despite the advanced healthcare system [3]. Similarly, according to the National Health Service, it is estimated that more than 50% of the US adult population is affected by periodontal disease to certain degree and approximately 15% of UK population has been diagnosed with severe periodontitis.

Periodontitis is the most common form of dental disease. It is a chronic inflammatory disease that results in the breakdown of tooth-supporting tissues, ultimately leading to resorption of the alveolar bone. Multitude of pathogens are known to have a role in the development of chronic periodontitis, but the gram-negative, anaerobic bacterium Porphyromonas gingivalis is considered to be one of the key pathogens in periodontitis [4]. This bacterium releases copious amounts of outer membrane vesicles containing LPS, which can penetrate periodontal tissue and thus participates in destruction associated with activation of the inflammatory and innate immune response. The inflammatory and immune processes that develop in the periodontal tissues in response to the long-term presence of the subgingival biofilm are protective by intent but result in considerable tissue damage. This may be referred to as bystander damage, denoting that the host response is responsible for the tissue damage.

Therefore, it is important to elucidate the key mediators involve in orchestrating the host immune response during periodontitis. Among the mediators, cytokines play a fundamental role in inflammation and represent key players in periodontal disease [5]. Cytokines, transiently produce by the tissues, bind with their respective receptors at low concentration, to initiate...
intracellular signaling cascades that result in phenotypic changes [6, 7]. The cytokines can regulate their own expression either in an autocrine or paracrine fashion and have pleiotropic effects (i.e., multiple biologic activities) on a large number of cell types.

Resident cells in the periodontium, including fibroblasts and epithelial cells, can secrete large amount of cytokines [8]. The cytokines can signal, broadcast, and amplify the immune responses during infection. However, chronic inflammation with excessive production of cytokines results in tissue damage of the periodontium. For example, cytokines lead to connective tissue and alveolar bone destruction through the induction of proteolytic enzymes in the fibroblasts and osteoclasts that break down structural components in the connective tissues [9].

There is significant overlap and redundancy between the function of individual cytokines. Cytokines do not act in isolation, but rather in flexible and complex networks that involved both pro-inflammatory and anti-inflammatory effects to bring together both innate and acquired immunity aspects [10]. Cytokines play a key role at all stages of the immune response in periodontal disease. IL-1β and TNF-α are among the most studied (and probably the most important) cytokines in periodontal. Both cytokines play a role in the initiation, regulation, and perpetuation of innate immune responses in the periodontium, resulting in vascular changes and migration of effector cells such as neutrophils into the periodontium as part of a normal immune response to the presence of sub-gingival bacteria [11].

Since cytokines are the key factors that affect the host’s immune response by promoting the development of periodontitis, investigating the dynamic changes of the interleukines and cytokines during progression of periodontitis will assist future development for preventive, diagnostic and therapeutic options of this disease. The objective of this study is to profiling various local and peripheral inflammatory mediators during periodontitis by establishment of a relevant, replicable and stable in vivo animal model.

Materials and methods

Reagents

All antibodies in this study were purchased from Boster Biological Technology (Wuhan, China), Streptavidin biotin complex peroxidase kit and ELISA kit were obtained from Boster Biological Technology (Wuhan, China).

Bacterial culture

_Porphyromonas gingivalis_ W83 was obtained from the Department of Oral Biology at China Medical University. _P. gingivalis_ W83 was cultured in freshly prepared brain heart infusion (BHI) agar plate (Difco Laboratories, MI) and supplemented with 5% sterile defibrinated sheep blood, 1% hemin, and 0.1% menadione in a chamber under anaerobic conditions of 80% N₂, 10% H₂ and 10% CO₂ [12].

Animals

The animals were handled in accordance with the guiding principles in the care and use of animals, approved by the council of China Medical University. All mice were pre-fed with Azithromycin water for 4 days in order to eradicate endogenous microbial.

Eighteen six-week-old male Sprague Dawley (SD) rats (200 to 250 g) were randomly divided into three groups. The control group (_n_ =6) remained untreated, and the treatment groups (4 weeks group and 8 weeks groups) were subjected to a 0.2 mm wire placement around the bilateral maxillary first molars prior infected with 1 × 10⁹ CFU/mL (1.5 mL) _P. gingivalis_ W83 by oral delivery for 10 consecutive days. Animals were euthanized by cervical dislocation. The blood and gingival tissues were harvested for further analysis.

X-ray imaging

The alveolar bone resorption was evaluated with PLAN-MECA X-ray imaging system (Helsinki, Finland). The images were acquired at 63 kV, 8 mA and 0.02 s exposure time and analyzed with DIGIMED viewer system (Madrid Spain).

ELISA

Whole blood was collected by cardiac puncture. Plasma was reserved for cytokine measurement by ELISA. ELISA kits, for IL-1β, IL-6, IL-10, IL-8, IgG and TNF-α were obtained from Boster Biological Technology (Wuhan, China) and cytokine measurement was performed according to the manufacturer’s instruction.
Immunohistochemistry

After 4 and 8 weeks of ligature, maxilla and gingival tissue were harvested. The tissues were fixed in 10% formaldehyde for 3 days and then dehydrated in 70% ethanol for 24 hours before tissue processing. The tissues were embedded in paraffin and sectioned with the thickness of 5 µm. Sections were placed on polyglycine-coated slides (Dako, US) and stored at 4 degree until further usage.

Immunohistochemistry was performed as described elsewhere [12]. Briefly, the slides were subjected to gradient hydration steps in 100%, 95% and 80% ethanol. Antigen retrieval was performed in citrate buffer bath between 90 and 95 degree. Slides were blocked with H2O2 for endogenous peroxidase activity for 10 minutes and followed by horse serum incubation for 20 minutes. 100 µL of primary antibodies were added to the slides and incubated overnight. Slides were incubated with universal secondary antibody for 30 minutes, followed by substrate addition and color development (Boster Biological Technology). The slides were then counter stained by hematoxylin and mounted for microscopy viewing. The entire process was performed at room temperature and after each step the slides were rinsed in PBS three times.

The staining results were quantified by meta-morph imaging microscopic image analysis system (UIC, USA). Positive rate was recorded by means ± standard deviations (SD).

Stereomicroscope

Alveolar bone loss was measured by stereomicroscopy. Animals were sacrificed by cervical dislocation and mandibular jaws were removed.
The specimens were dehydrated by a serial increase in the concentration of ethanol followed by immersion in 1% potassium hydroxide for 2 days after soft tissues removal and fixation in 10% formalin. Specimens were stored in glycerin and followed by alizarin red staining. Examination of the specimens was performed on stereomicroscope with digital camera (Leica MZ FLIII, Leica, Wetzlar, Germany).

Statistical analysis

All experiments were performed in triplicate and repeated at least three times. Data were expressed as means ± standard deviations (SD). Independent samples t-test was used for two-group comparison between treatment and control groups, and ANOVA was used for multi-group comparison (SPSS Inc., IL). P-value <0.05 (*) was considered as statistically significant.

Results and discussion

Establishment of rat periodontitis model

Periodontitis is a disease caused by bacterial infection and exaggerated by the susceptibility of the respective host. Currently, no reliable experimental rat model is available to simulate the pathogenesis of human periodontal disease [13]. Such model is critical for downstream investigation of the host-microbe interaction and the development of new therapies. In this study, we established a ligature-induced periodontitis rat model by placing a sterilized 0.2 mm wire around the cervical part of the first molar followed by oral exposure of $1.0 \times 10^9$ CFU/ml P. gingivalis W83 for 5 days. After 4 weeks, this ligature model resulted in periodontitis formation (Figure 1A-G). Specifically, loss of connective tissue attachment and severe alveolar bone resorption occurred, characterized by an increase in the distance between the cemento-enamel junctions to alveolar bone crest (Figure 1D-I). Both X-ray and stereomicroscope imaging showed that no alveolar bone corruption was found in the vehicle control rat. Our results, in line with other groups’ studies [14], confirmed that ligature can induce a stable periodontal model. Moreover, monomicrobial infection effectively expedites the alveolar bone breakdown. Therefore, we exploited this model for the following functional studies.

Cytokines expression pattern on periodontitis

To investigate the P. gingivalis W83-induced immunologic response in a local and peripheral manners, IHC and ELISA were performed to
Cytokine changes on rat periodontitis model

After four weeks of ligature, all cytokines including IL-1β, IL-6, IL-10, IL-18, TNF-α and IgG were found to be significantly increased in the gingiva tissue (Figure 2). IL-1β, IL-10, IL-18 and TNF-α continued to increase 8 weeks after induction of ligature, suggesting a prolonged inflammation of the model, which is similar to the chronic inflammation induced by periodontitis in human.

Among the cytokines of interest, IL-18 is a highly inflammatory cytokine capable of induces interferon-γ [15, 16]. It stimulates T cell and NK-cell to produce IL-4, IL-5, IL-10 and IL-13. Similarly, IL-1β and TNF-α have been shown to be key molecules that contribute to the adhesion, chemotaxis and lysosomal release of neutrophil. The increases of these cytokines in both local gingival fluid and plasma in a time-dependent manner suggested that monomicrobial infection activates the host immune response and leads to the release of abundant pro-inflammatory mediators. Enhanced influx of immune cells and the resulting cytokines surge create a malicious inflammatory loop for the pathogenesis of periodontitis (Figure 3), thereby contributes to alveolar bone loss. Additionally, IL-18 enhances macrophage maturation to increase PGE2 production, which can ultimately promote alveolar bone loss [17, 18].

IL-6 is another inflammatory cytokine found to increase in gingival fluid during the development of periodontitis. It is secreted by a wide range of immune and non-immune cells including T-cell, macrophage, osteoblast, endothelial cells and fibroblast. Therefore, it has been used as a biomarker to monitor the severity of periodontitis [19-21]. Our model showed that the IL-6 level in the gingiva tissue increased significantly on week 4 and week 8 (Figure 2). We proposed that the local gingivitis stimulated by monomicrobial infection results in the secretion of IL-6, thereby recruits inflammatory cells. The infiltrating immune cells diminish tissue repair ability by damaging the periodontal tissue and inhibiting the growth of periodontal ligament cells. In parallel, IL-6 promotes the formation of osteoclast differentiation factors and matrix metalloproteinases, causing the formation of osteoclasts and the degradation of bone matrix. Moreover, IL-6 affects bone resorption by inhibiting the activity of alkaline phosphatase in osteoblast and thus reduces the formation of collagen. The IL-6 level in the plasma remains unchanged throughout the formation of periodontitis (Figure 3), suggesting that this local inflammation phenomenon may be sufficient to create a

**Figure 3.** Expression of inflammatory cytokines, anti-inflammatory cytokines and IgG in gingival tissue. (A) The protein concentration of IL-6, IL-18, IL-1β, TNF-α, IL-10 and (B) IgG with indicated treatments were measured by ELISA. Triplicates were performed for each biological sample. *, P<0.05.
Cytokine changes on rat periodontitis model

Elevated IgG level in the plasma reflects an activated state of immune system [22]. Periodontal lesion is also signified by increased B cells infiltration [22]. Indeed, we observed a significant increase of IgG in the plasma after 4 and 8 weeks of ligature, suggesting that the animals have mounted an adaptive immune response against the pathogen. Multiple immune cell types including macrophage, NK cells and T cells express FcR that binds to the Fc portion of antibody. The binding can elicit downstream inflammatory signaling pathway including NF-κB which in turn stimulate production of inflammatory cytokines like IL-1β, IL-6 and TNF-α secretion. Therefore, the elevated IgG level observed in the animals may play the role of promoting the local and peripheral inflammatory response in periodontitis [23].

IL-10 is an anti-inflammatory factor that plays an inhibitory role in the osteoclasts-induced alveolar bone loss [24, 25]. It is secreted by Th2 cell, macrophage, mesenchymal cells and activated B cells. Our result showed that the production of IL-10 in the gingival tissue and plasma was significantly higher in the ligature-induced periodontitis rat as compared with the vehicle control group (Figure 2). The observed increase may serve as a counter mechanism of the host immune system to prevent extensive tissue damage due to the secretion of pro-inflammatory cytokines and IL-10 from regulatory immune cells to escape eradication by host immune cells.

The alveolar bone loss in our model did not extend beyond 4 weeks after ligature (Figure 1). We propose that pro-inflammatory mediators contribute to the initial phase of periodontitis via innate immune cells including macrophage, neutrophils and NK cells. Upon prolonged host-microbe interaction, adaptive immune response develops and an antibody response is mounted, evidenced by the increased IgG levels in plasma even 8 weeks after the ligature. In parallel, to prevent excessive tissue damage, the anti-inflammatory mechanism is triggered by producing large quantity of IL-10 to dampen the activity of the pro-inflammatory mediators and chemokines. Studies have shown that monocytes and lymphocytes attraction, together with the periodontal ligament and fibroblast secrete IL-10 to inhibit IL-6 signaling pathways and prevent further alveolar bone loss. Together with the antigen presenting cells, such as monocytes, macrophage and dendritic cells, IL-10 inhibits NK cells and T cells from producing INF-γ and promotes tissue repairing. Therefore, the alveolar bone loss may be partially rescued by the enhanced level of IL-10 between week 4 and week 8.

Interplay and synergy of different cytokines secreted during periodontitis development can considerably magnify the severity and the extent of bone breakdown. IL-1β, TNF-α, IL-6, IL-11, IL-17 and PGE2 secreted by the macrophage, lymphocytes and fibroblast, can activate the RANK/RANKL/OPG pathway to modulate the osteoclast activity [22]. Others have shown that IL-6 and IL-18 levels are correlated positively and contribute to the severity of periodontitis [19-21]. Since IL-10 is a counter measure of the inflammatory cytokines, We asked the question whether the ratio of IL-1β and IL-10 may be indicative of the periodontitis progression. In this case, The IL-1β and IL-10 ratio remained unchanged in our periodontitis model. Yet, our results showed that the IL-1β, TNF-α and IL-6 levels correlated positively with each other (Table 1). The underlying mechanism is due to the master regulatory role of IL-1β. It synergizes with TNF-α to enhance the secretion of PGE2, and increases the production of IL-6 by interacting with p38 MAPK and NF-κB signaling pathway. Ultimately,
Cytokine changes on rat periodontitis model

the whole local inflammatory loop may be further reinforced via the positive feedback back by IL-1β.

Lastly, we used IHC to examine the expression of TLR-2 and TRL-4 in the gingival tissues because Toll-like receptor plays a major role in the recognition of pathogen and the secretion of chemokines [26, 27]. Results showed that TLR-4 was detected at week 4 after ligature and the expression continued to increase until week 8 (Figure 4). By contrast, the expression of TRL-2 remained unchanged throughout the course of periodontitis (Figure 4). While the mechanism of TLR in the development of periodontitis is still largely unknown, studies have shown that P. gingivalis components can act as an agonist for TLR-4 via its lipopolysaccharide component [28, 29]. Gingival fibroblasts have been shown to increase production of IL-1, IL-6 and IL-8 upon exposure to P. gingivalis LPS which binds to the TLR-4 expressing on fibroblast [30]. Therefore, the increase of TLR-4, but not TLR-2, may act as a key promoter in the potentiation of periodontitis induced by P. gingivalis.

Conclusion

The current ligature-induced periodontitis model successfully induced both local and peripheral immune response in animal and recapitulates the clinical scenario. We demonstrated that pro-inflammatory molecules increase throughout the development of periodontitis. In particular, IL-1, TNF-α and IL-6 showed a positive correlation in their respective levels, while the IL-1β and IL-10 ratio remained unaffected in our model. The results reveal the importance of host immune response, in particular the balance between the pro-inflammatory and anti-inflammatory cytokines during the course of periodontitis. Further investigation is required to understand the exact role of each cytokine and how they regulate and contribute to the host immune response system in order to develop effective counter measures that target specific cytokines.

Acknowledgements

This study was supported by the Natural Science Foundation of China (81470745) and Technology Plan Projects in Liaoning Province (2013225090).

Disclosure of conflict of interest

None.

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