Measurements of eicosanoid and inflammatory cytokines in rats with spontaneous periodontal disease

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When periodontal disease develops, many inflammatory cells, mainly lymphocytes, are observed infiltrating the lesion. Chemokines such as MCP-1 and IL-8, which are produced by gingival epithelial cells undergoing stimulation by bacterial cell components from plaque bacteria through the toll-like receptor-2, are involved in the migration of these immunocompetent cells to the lesion site. It has been confirmed that the expression of these inflammatory cytokines is elevated in the gingival tissue and gingival sulcus exudates of periodontal disease patients. We investigated the dynamics of the inflammatory cytokines IL-1β, IL-6, TNF-α, IFN-γ, MCP-1 and anti-inflammatory cytokine IL-10 in rats with spontaneous periodontal disease (ODUS/Odu), an animal model of periodontal disease developed and maintained in our laboratory. In this study, we measured the plasma levels of the eicosanoid PGE₂ and inflammatory cytokines cytokine-induced neutrophil chemoattractant-1 (CINC-1), and the anti-inflammatory cytokine IL-4 in ODUS/Odu. Plasma cytokine levels were measured in these animals and in control rats (Res) at 0 (five weeks after birth), 1, 3, 6, 9 and 12 months after the start of the experiment using an enzyme-linked immunosorbent assay (ELISA) kit. The plasma levels of the eicosanoid PGE₂ and inflammatory cytokine CINC-1 were greater in ODUS/Odu than in Res throughout the experimental period. However, the plasma levels of the anti-inflammatory cytokine IL-4 was lower in ODUS/Odu than in Res. These results, similar to those obtained in patients with periodontal disease, suggest that the ODUS/Odu are a useful animal model of human periodontal disease. (J Osaka Dent Univ 2011; 45: 167-173)

Key words: Eicosanoid; Inflammatory cytokine; Periodontal disease

INTRODUCTION

Although periodontal disease is a chronic inflammatory disease caused by periodontal bacteria, its development and progression involve host immune responses, and the pathology of periodontal disease is characterized by desquamation of adhered gingiva, periodontal pocket formation, and alveolar bone resorption. Studies have progressed, and it is now widely known that Porphyromonas gingivalis (P. gingivalis) and Actinomyces actinomycetemcomitans (A. actinomycetemcomitans) are the pathogens of adult periodontitis and localized juvenile periodontitis, respectively.

It has recently been clarified that the influence of periodontal disease is not limited to the oral cavity, and that it is associated with systemic diseases; it induces several systemic diseases such as diabetes⁴ and coronary arterial heart disease.⁵,⁶ However, there are several difficult problems in investigating the cause and course of periodontal disease and establishing treatment and preventive methods. Firstly, local and systemic factors are involved in the cause of periodontal disease in a complex way. Secondly, the development of periodontal disease is mainly due to the injurious property of dental plaques adhering to the teeth and gingival sulcus, and, thus, both dental plaques and the host should always be considered. Thirdly, the development and application of the most appropriate animal ex-
experimental model are necessary to elucidate periodontal disease.

Although experimental induction of alveolar bone resorption by creating inflammation in periodontal tissue has been reported,7-9 the Osaka Dental University Pharmacology Department succeeded in developing rats with spontaneous periodontal disease (ODUS/Odu) by administering only commercial powder diet and tap water without artificial manipulation. The rats have been passaged and maintained, and we have presented their usefulness as an experimental animal model for studies on periodontal disease in many reports on the disease.10-23

As described above, although inflammatory reactions are induced by dental plaque-derived bacterial stimulation, dental plaques are present outside the gingiva, for which elimination by immunocytes is insufficient. Subsequently, there is an excess amplification of the interaction between periodontal tissue cells and immunocytes, leading to protraction of inflammation, and the persistent induction inflammatory cytokines. The excess production of cytokines is thought associated with the promotion of tissue destruction.24-26 Enhanced expression of these inflammatory cytokines in gingival tissue and exudate in the gingival sulcus has been confirmed in periodontal disease patients. Thus, we measured PGE2, which is an eicosanoid with vascular permeability-promoting and immunosuppressive actions detected in gingival tissue, CINC-1, which is an inflammatory cytokine, and IL-4, which is an anti-inflammatory cytokine, in an animal model of periodontal disease, ODUS/Odu.

MATERIALS AND METHODS

Experimental animals

The experimental animals used in this study were ODUS/Odu, which were originally derived from the Wistar-Kyoto strain that was first bred in our laboratory in 1972. Experiments were started when the rats were 5 weeks old. ODUS/Odu exhibit pronounced plaque formation in the mandibular incisors and develop both gingivitis and periodontal pockets after being fed a commercially available powdered diet (MF powdered diet; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. Animals that do not form plaque and gingivitis under the same dietary conditions are known as ODU plaque-resistant rats (Res). ODUS/Odu are useful as an animal model of periodontal disease, and have now reached the 119th generation. Measurements were initiated at 5 weeks after birth (time 0 for start of the experiment), and at 1, 3, 6, 9, and 12 months thereafter in 9 each of male ODUS/Odu and Res. Periodontal pockets were also examined in the ODUS/Odu group using a WHO-standard probe. This animal study was approved by the Osaka Dental University Animal Research Committee (approval number 09-02031), and performed in accordance with the guidelines related to animal experiments.

Preparation of samples

A blood plasma sample with heparin collected from each animal was examined at the initiation of the experiment and after 1, 3, 6, 9, and 12 months thereafter. The blood plasma samples were stored at 2–8°C, and assaying was performed within 24 hours after collection. The samples were aliquoted, and frozen at −80°C for long-term storage.

Measurement of eicosanoid and cytokines

Blood plasma samples with heparin were collected

1. Add duplicate 100 μL of standards or samples to each well.
2. Add 50 μL of assay buffer into the non solution blank (NSB) wells.
3. Add 50 μL conjugate into each well, except for the total activity (TA) and blank wells.
4. Add 50 μL of the antibody solution to each well, except for the blank, TA and NSB wells.
5. Cover plate and incubate at room temperature (20–25°C) for 2 hours.
6. Wash plate three times.
7. Add 5 μL of the blue conjugate to the TA wells.
8. Add 200 μL of the pNpp substrate solution to each well.
9. Cover plate and incubate at room temperature for 45 minutes without shaking.
10. Add 50 μL of the stop solution to every well.
11. Measure absorbance on a plate reader at 405 nm.
12. Calculate results.

Fig. 1 Procedure for measuring PGE2.
1. Add 50 µL of sample diluent to each well.
2. Add 50 µL of standards, control or samples to each well in duplicate.
3. Mix by gently tapping the plate frame for 1 minute.
4. Cover with the adhesive strip provided and incubate at room temperature (20–25°C) for 2 hours.
5. Wash plate five times with wash buffer.
6. Add 100 µL of rat CINC-1 conjugate to each well. Cover with a new adhesive strip and incubate at room temperature for 2 hours.
7. Wash plate five times with wash buffer.
8. Add 100 µL of substrate solution to each well.
9. Develop the plate in the dark at room temperature for 30 minutes.
10. Stop reaction by adding 100 µL of stop solution to each well. Gently tap the plate to ensure thorough mixing.
11. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm.
12. Calculate results.

Fig. 2 Procedure for measuring CINC-1.

from the animals, and the levels of prostaglandin E₂ (PGE₂: Prostaglandin E₂ Correlate™ Kit; Assay designs Inc., Ann Arbor, MI, USA), cytokine-induced neutrophil chemoattractant-1 (CINC-1: Quantikine®; R&D Systems Inc., Minneapolis, MN, USA), and interleukin-4 (IL-4: Quantikine®; R&D Systems Inc.) were measured using ELISA kits as shown in Figs. 1–3.

RESULTS

Periodontal pocket probing depths increased in ODUS/Odu during the experimental period. There was a positive correlation between the periodontal pocket probing depth and time (Fig. 4).

Determination of PGE₂ and cytokines in blood plasma

The level of PGE₂ in the blood plasma samples was significantly greater in the ODUS/Odu group than in the Res group at all experimental time points (p<0.001). Furthermore, the amount of PGE₂ in the ODUS/Odu group increased with time between 3 and 12 months (Fig. 5). The level of CINC-1 in the blood plasma samples containing heparin was also greater in the ODUS/Odu group than in the Res group at all experimental times (p<0.001). Furthermore, the amount of CINC-1 in the ODUS/Odu group increased with time between 3 and 12 months (Fig. 6). The level of IL-4 in the blood plasma samples containing heparin was significantly greater in the Res group than in the
ODUS/Odu group at all times (p < 0.001). Moreover, the amount of IL-4 in the Res group increased with time between 1 and 12 months (Fig. 7).

**DISCUSSION**

The prevention and treatment of periodontal disease, a chronic disease with a characteristic pathology, are important to maintain and retain the teeth for life and to maintain homeostasis of the body. Frequent detection of specific bacterial flora associated with periodontal disease in gingival plaque has been reported in long-term epidemiological surveys of more than 30 years in periodontal disease patients, suggesting that periodontal disease is a bacterial infectious disease. Studies have progressed, and it is now widely known that *P. gingivalis* and *A. actinomycetemcomitans* are the pathogens of adult periodontitis and localized juvenile periodontitis, respectively. To develop preventive and treatment methods of this disease, it is necessary to elucidate the mechanism of periodontal tissue destruction by these pathogens.

*P. gingivalis* and *A. actinomycetemcomitans* adhere to gingival epithelium in periodontal tissue, form colonies, and invade the tissue. However, these periodontal pathogens have been shown to produce no exotoxin directly inducing alveolar bone resorption, the characteristic of this disease. Accordingly, it is thought that metabolic products (such as proteolytic enzymes) and bacterial cell components of periodontal pathogens irritate periodontal tissue in the processes of adhering to the tissue, forming colonies, and invading the tissue. In addition, it is thought that these metabolic products promote inflammatory cytokine production. Through diverse physiological activities of various cytokines (IL-1α, IL-1β, TNF-α, IL-4, IL-8, and IFN-γ), pathological conditions, such as gingival inflammation and alveolar bone resorption, may be established.

Since Goodson et al. demonstrated the involvement of prostaglandin (PG) in the pathology of periodontal disease in 1974, a close association of PG with the development and progression of periodon-
ental diseases has been reported. PG detected in gingival tissue includes PGE₂, PGD₂, PGI₂, 6-keto-PGF₁α (PGI₂ metabolite), and thromboxane B₂ (thromboxane A₂ metabolite). The tissue becomes inflammatory and production of these PGs is promoted. Cells producing PG in periodontal tissue include fibroblasts, periodontal ligament cells, osteoblasts, monocytes/macrophages, polymorphonuclear leukocytes, and lymphocytes. These cells are thought to produce PG during inflammation in response to stimulation by inflammatory cytokines, such as interleukin-1β, interleukin-6, and tumor necrosis factor-α (TNF-α), as well as lipopolysaccharide (LPS). Regarding the physiological actions of PG, PGE₂ has been shown to promote vascular permeability, induce fever and pain, suppress immune responses, regulate IL-1 and collagenase production, and induce bone resorption. In addition, PGI₂ has been shown to promote vascular permeability. For these reasons, we measured PGE₂.

The plasma PGE₂ level was significantly greater in ODUS/Odu than in Res (p<0.001), the level tended to increase with the progression of the experimental period from 3 months to 6, 9, and 12 months, and these findings were consistent with those in previous reports. It is thought that reactive oxygen and protease produced by polymorphonuclear leukocytes originally as a host defense response against bacterial infection may injure host tissues and cells. These host cells include macrophages and fibroblasts which excessively produce inflammation-related substances, such as inflammatory cytokines (IL-1, IL-6, and TNF-α) and eicosanoid (PGE₂), inducing tissue destruction. IL-1 and TNF-α have been shown to act on macrophages and fibroblasts and promote production and activation of matrix metalloproteinase (MMP). IL-1 and TNF-α are also involved in connective tissue destruction. IL-1, IL-6, TNF-α, and PGE₂ have been shown to induce expression of receptor activator of NF-κB ligand (RANKL) in osteoblasts inducing osteoclasts and bone resorption. They are also involved in the pathogenesis of periodontal disease.

Host cells, such as macrophages and fibroblasts, regulate host responses by simultaneously producing anti-inflammatory cytokines, such as IL-4, IL-10, and IL-13, and anti-inflammatory-related substances, such as MMP activity-inhibitory tissue inhibitor of metalloproteinase (TIMP). Therefore, host responses in periodontal disease are regulated by a balance between inflammation-related and anti-inflammatory-related molecules. When inflammation-related molecules are produced in excess, tissue destruction progresses. Conversely, excess production of anti-inflammatory-related molecules inhibits the progression of periodontal disease. Using ODUS/Odu and Res, an animal model of periodontal disease developed and maintained as an inbred strain at our laboratory, we investigated the plasma cytokine dynamics in heparinized plasma collected at the initiation of the experiment (5 weeks after birth) and at 1, 3, 6, 9 and 12 months. We also measured and compared an inflammatory cytokine, CINC-1, and an anti-inflammatory cytokine, IL-4. These are mostly produced by monocytes and macrophage-lineage cells. IL-1 and TNF-α are also termed “alarm cytokines” because they are produced in the very early phase of inflammation. In addition, cells comprising intercellular interstitium, fibroblasts and vascular endothelial cells, can produce these cytokines. When IL-1 and TNF-α are produced, cytokines including IL-6 and IL-8 are subsequently produced, forming pathological conditions specific to individual inflammatory stimulations, and forming a cytokine cascade. IL-1 destroys alveolar bone because it has physiological activities promoting bone resorption and prostaglandin synthesis, and enhanced MMP production and activity lead to extracellular matrix destruction.

The plasma CINC-1 level was greater in ODUS/Odu than in Res throughout the experimental period. CINC-1 was identified as a neutrophil-chemotactic protein in rat renal NRK-52 E epithelial cells, and its physiological activity is similar to that of IL-8, being known as an IL-8-like chemokine. Thus, it is reasonable that ODUS/Odu would develop a significantly high level of periodontitis. IL-4 was originally discovered as a substance with B-cell growth-promoting activity and was termed B
cell growth factor (SCGF) or B cell stimulatory factor-1 (BSF-1). However, it was finally named IL-4 when cDNA was isolated in 1986. Later studies clarified that IL-4 shows diverse actions on not only B cells, but also broad blood cells, and it is now understood to be a multifunctional cytokine. It induces IgG and IgE production in B cells and T-cell proliferation, and inhibits inflammatory cytokine production in monocytes/macrophages. The plasma IL-4 level was significantly lower in ODUS/Odu than in Res throughout the experimental period (p<0.001), and tended to increase during the experimental period from 1 month to 3, 6, 9 and 12 months.

The above findings indicate that PGE_2 as eicosanoid and CINC-1 function as inflammatory cytokines, and that IL-4 acts as an anti-inflammatory cytokine in the plasma of ODUS/Odu. We are planning to analyze the association of the anti-inflammatory action of endogenous adenosine with periodontal disease using ODUS/Odu.

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