Changes of plasma cytokine levels in ODUS/Odu

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Periodontal disease is an inflammatory condition caused by periodontal bacteria. Its development and progression are related not only to bacterial pathogenicity, but also to the host response in the inflamed periodontal tissue. Recent studies have clarified the relationship between periodontal and systemic diseases. In this study, we measured the plasma levels of the proinflammatory cytokines interferon gamma (IFN-γ) and monocyte chemoattractant protein-1 (MCP-1), and the anti-inflammatory cytokine interleukin-10 (IL-10) in rats with spontaneous periodontal disease (ODUS/Odu), an animal model of periodontal disease developed and maintained in our laboratory. These rats are prone to marked plaque formation on the mandibular incisors, and develop gingivitis and periodontal pockets.

Plasma cytokine levels were measured in ODUS/Odu and control rats (Res) at 0 (5 weeks of 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 proinflammatory cytokines IFN-γ and MCP-1 were significantly greater in ODUS/Odu than in Res throughout the experimental period (p<0.001). However, the plasma level of the anti-inflammatory cytokine IL-10 was significantly lower in ODUS/Odu than in Res throughout the experimental period (p<0.001). 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 2009; 43: 175–181)

Key words: Inflammation; Cytokine; Periodontal disease

INTRODUCTION

Periodontal disease is a chronic inflammatory disease caused by periodontal bacteria in dental plaque. According to Socransky et al., the four factors leading to the onset of periodontal disease are a change in the individual’s sensitivity, proliferation of pathogenic microorganisms, alteration of microflora, and an environment favoring disease development. They speculated that the active phase of periodontal disease begins when all four factors are present. Since the destruction of periodontal tissue begins in the active phase, it is important to assess the status of disease activity in order to treat and prevent periodontal disease. Many studies have recently been conducted to biochemically evaluate the status of periodontal disease by measuring inflammatory cytokines and other chemical mediators of inflammation.

Studies have shown that periodontal disease not only affects the oral cavity, but also has been implicated in several systemic diseases such as coronary heart disease and diabetes. However, the local and systemic factors involved in the pathogenesis of periodontal disease are interrelated in a very complex manner. The disease is mainly caused by the harmful effects of dental plaque adhering to the tooth and gingival sulcus. Therefore, to establish methods for the prevention and treatment of periodontal disease by elucidating its cause and progress, it is necessary to consider the issue from the viewpoint of both dental plaque and the host response. In addition, investigations of periodontal disease involve the difficult problem of developing
and applying an optimal experimental animal model.

Previous studies reported the artificial induction of periodontal inflammation and alveolar bone resorption. However, without using artificial means, our laboratory succeeded in developing rats with spontaneous periodontal disease (ODUS/Odu) by feeding the animals a commercial powder diet and water. We maintained them for several generations, and published many reports showing that these rats serve as a useful experimental animal model to study periodontal disease.

As described above, the bacteria of dental plaque stimulate an inflammatory response in the adjacent periodontal tissue. Since dental plaque cannot be fully eliminated by immunocompetent cells, the interaction between periodontal tissue cells and immunocompetent cells is excessively amplified, and the inflammatory response becomes prolonged. As a result, it is said that inflammatory cytokine production is continuously induced, and that the excessive production of cytokines promotes periodontal tissue destruction. 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.

Goodson et al. reported that PGE$_2$ levels in gingival tissue were greater in severe periodontal disease patients than in normal healthy subjects. Offenbacher et al. noted that PGE$_2$ levels in gingival sulcus exudates were significantly greater in periodontitis patients with attachment loss than in gingivitis patients. When we investigated the dynamics of the inflammatory cytokines interleukin-1$\beta$ (IL-1$\beta$), interleukin-6 (IL-6), and tumor necrosis factor-$\alpha$ (TNF-$\alpha$), we found that the plasma levels of all three were significantly greater in ODUS/Odu than in the controls throughout the experimental period. These findings were similar to those in humans. In this study, we compared the dynamics of the inflammatory cytokines interferon gamma (IFN-$\gamma$) and monocyte chemotactant protein-1 (MCP-1), and the anti-inflammatory cytokine interleukin-10 (IL-10) in ODUS/Odu with that in control rats (Res).

% 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 114th 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 10 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 08–02301), 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 at 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 cytokines**

Blood plasma samples with heparin were collected from the animals, and the levels of IFN-$\gamma$, MCP-1, and IL-10 were measured using ELISA kits (Endogen® IFN-$\gamma$, MCP-1 and IL-10 ELISA Kits, respectively, Pierce Biotechnology Inc., IL, USA) as shown in Figs. 1–3.
1. Add 50 μL of sample diluent to each well. Add a duplicate 50 μL of standards or samples to each well.
2. Cover plate and incubate at room temperature (20–25 °C) for 1 hour.
3. Wash plate three times.
4. Add 100 μL of biotinylated antibody reagent to each well. Cover plate and incubate at room temperature for 30 minutes.
5. Wash plate three times.
6. Add 100 μL of prepared streptavidin-HRP solution to each well.
7. Cover plate and incubate at room temperature for 30 minutes.
8. Wash plate three times.
9. Add 100 μL of premixed TMB substrate solution to each well.
10. Develop the plate in the dark at room temperature for 30 minutes.
11. Stop reaction by adding 100 μL of stop solution to each well.
12. Measure absorbance on a plate reader at 450 nm.
13. Calculate results.

**Fig. 1** Procedure for measuring IFN-γ.

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**Fig. 3** Procedure for measuring IL-10.

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RESULTS

Pocket probing depths in ODUS/Odu during experimental period

The pocket probing depths increased at 1, 3, 6, 9, and 12 months. There was a positive correlation between the periodontal pocket probing depth and time (Fig. 4).

![Fig. 4](image-url) Pocket probing depths in ODUS/Odu during experimental period. The pocket probing depths increased at 1, 3, 6, 9, and 12 months. There was a positive correlation between the periodontal pocket probing depth and time during the experimental period.
Fig. 5 Changes of IFN-γ in the blood plasma. The level of IFN-γ 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 IFN-γ in the ODUS/Odu group increased with time from 3 to 12 months (n=10, *p<0.001).

Fig. 6 Changes of MCP-1 in the blood plasma. The level of MCP-1 in the blood plasma samples that had added heparin was also greater in the ODUS/Odu group than in the Res group throughout the experiment (p<0.001). Furthermore, the amount of MCP-1 in the ODUS/Odu group increased with time from 1 to 12 months (n=10, *p<0.001).

Fig. 7 Changes of IL-10 in the blood plasma. The level of IL-10 in the blood plasma samples that had added heparin was significantly greater in the Res group than in the ODUS/Odu group throughout the experiment (p<0.001). Moreover, the amount of IL-10 in the Res group increased with time from 3 to 12 months (n=10, *p<0.001).

**DISCUSSION**

Periodontal disease is caused by bacteria living in biofilms, called dental plaque.

Gram-negative anaerobic bacteria, typified by *P. gingivalis*, are frequently detected in dental plaque in the subgingival pockets of periodontitis patients. The endotoxin of these bacteria and their metabolites injures periodontal tissue, and cause chronic inflammation. These lesions are composed of various immunocompetent cells as well as gingival fibroblasts, gingival epithelial cells, and periodontal ligament cells, which comprise periodontal tissues. In single bacterial infections, the causative bacterium is usually eliminated by immune cells, eventually leading to the resolution of inflammation induced at the site of infection. However, in periodontal disease, the dental plaque becomes established and
forms a biofilm in the periodontal pocket. As a result, the causative factor is not effectively eliminated by the immune system, prolonging inflammation. The remaining causative factors continue to pathologically stimulate the periodontal tissue, and locally produced cytokines act on surrounding cells to induce further cytokine synthesis, leading to the amplification of cytokine production. Thus, cytokines, which play an essential role in homeostasis, are continuously produced in periodontal tissue because of the pathologic characteristics of periodontal disease, resulting in an imbalance in the normal cytokine network that leads to the progression of periodontal tissue destruction.

Since cytokines such as IL-1, IL-6, and TNF-α play an important role in the maintenance of bone tissue homeostasis, their overproduction in inflamed periodontal tissue results in continuous osteoclast activation, leading to alveolar bone resorption. In particular, IL-6 promotes the differentiation of B cells into antibody-producing cells. Thus, it has been suggested that IL-6 overproduction results in local antibody production, inducing autoantibody synthesis in periodontal tissue. As a result, the levels of IL-1, IL-6, and TNF-α, which are cytokines considered to be involved in periodontal tissue destruction, are greater in inflamed human periodontal tissue than in normal tissue.

We previously reported that the plasma levels of IL-1, IL-6, and TNF-α were significantly greater in ODUS/Odu than in Res throughout the experimental period. When periodontal disease develops, many inflammatory cells, mainly lymphocytes, are observed infiltrating the lesion. It is believed that chemokines like 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. Therefore, in this study, we measured the plasma levels of the inflammatory cytokines IFN-γ and MCP-1, and anti-inflammatory cytokine IL-10 in ODUS/Odu and Res at 0, 1, 3, 6, 9, and 12 months after the start of the experiment, and compared the results. IFN-α, -β, -γ, and -ω have been identified according to differences in their molecular structure and antigenicity. IFN-α is produced mainly by non T cells and NK cells upon stimulation by virus-infected cells or other cells, while IFN-β is produced by fibroblasts and epithelial cells in response to virus or nucleic acid stimulation. The IFN-α and -β genes are located on chromosome 9, both cytokines show an amino acid sequence homology of more than 30%, and they share a cell surface receptor. Therefore, they are considered to belong to the same family. On the other hand, IFN-γ measured in this study is produced by T and NK cells in response to antigen stimulation, has no amino acid sequence homology with IFN-α or IFN-β, and, unlike them, is known to bind to a unique receptor. IFN is thought to exhibit no direct antiviral effect. However, it binds to cells to stimulate the production of antiviral proteins, thereby conferring resistance to viruses and inhibiting their replication. In addition, IFN is known to have diverse biological activities, such as the inhibition of cell proliferation, regulation of the immune response, antitumor activity, and regulation of differentiation and development. In the present study, the plasma level of IFN-γ was greater in ODUS/Odu than in Res throughout the experimental period, suggesting that IFN-γ is an inflammatory cytokine.

MCP-1, an endogenous leukocyte chemoattractant, is a basic peptide with a marked heparin-binding affinity, consisting of 92–99 amino acids. It was isolated after IL-8 as a new factor. In the periodontal inflammatory infiltrate, chemokine MCP-1 is synthesized by a variety of cells and is responsible for monocytic infiltration of the periodontal lesion. Pradeep et al. compared the plasma level of MCP-1 in periodontal disease patients and healthy subjects, and reported that the level increased in proportion to disease severity, and decreased after treatment. Hanazawa et al. observed a high level of MCP-1 gene expression in the gingival tissues of all adult periodontal patients tested, but not of healthy subjects. We found that the plasma MCP-1 level was significantly greater in ODUS/Odu, an animal model of periodontal disease, than in control.
rats (Res) throughout the experimental period, in agreement with previous studies.

IL-10 is an acid-sensitive protein with a 35- to 40-kD homodimer. It is identified as a factor produced by Th2 cells. It inhibits cytokine production, and has attracted attention as an inhibitory cytokine. For this reason, IL-10 was originally called cytokine synthesis inhibitory factor (CSIF). However, its name was later changed to IL-10 when it was found to exhibit diverse biological activities in addition to cytokine synthesis inhibitory activity. Regulatory T cells (T1 or Tr1 cells) have been identified that produce IL-10 and transforming growth factor-β (TGF-β). This makes these regulatory T cells an important immune cell population. The bioactivities of IL-10 are broadly classified as inhibitory or stimulatory. It exhibits mainly inhibitory activity on Th cells and monocytes/macrophages, and stimulatory activity on B cells, thymocytes, mast cells, and cytotoxic T lymphocytes (CTL).

The measurement of serum IL-10 levels in patients with systemic lupus erythematosus (SLE), a human autoimmune disease, using a specific immunoenzymatic assay showed that the IL-10 level was significantly greater in active than in inactive SLE patients, and that it had a significant negative correlation with serum complement levels, an indicator of clinical activity.

Kuhn et al. reported that IL-10 knockout mice developed inflammatory bowel disease (IBD), which was improved by IL-10 administration. The serum IL-10 level was significantly lower in IBD patients than in healthy subjects, particularly in the active phase. During the active phase, although IL-1, IL-6, IL-8, and TNF-α were elevated in the local intestinal mucosa, IL-10 was decreased. The existence of SOCS3 showing inhibitory activity against IL-10-activated STAT3 was confirmed in 2001. In addition, studies have reported that IL-10 therapy suppressed the postoperative recurrence of Crohn's disease, and increased IFN-γ levels. In the present study, plasma IL-10 levels were lower in ODUS than in Res throughout the experimental period, indicating that IL-10 functions as an anti-inflammatory cytokine.

The above findings indicate that IFN-γ and MCP-1 function as inflammatory cytokines, and that IL-10 acts as an anti-inflammatory cytokine in the plasma of ODUS/Odu. Further studies will be conducted to determine the levels of IL-8 and PGE₂, which are reportedly elevated in human periodontal disease.

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