Periodontal bacteria aggravate experimental autoimmune myocarditis in mice

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Ashigaki N, Suzuki J, Ogawa M, Watanabe R, Aoyama N, Kobayashi N, Hanatani T, Sekinishi A, Zempo H, Tada Y, Takamura C, Wakayama K, Hirata Y, Nagai R, Izumi Y, Isobe M. Periodontal bacteria aggravate experimental autoimmune myocarditis in mice. Am J Physiol Heart Circ Physiol 304: H740–H748, 2013. First published December 21, 2012; doi:10.1152/ajpheart.00634.2012.—Periodontitis is one of the most common infections in humans. This disease is known to contribute to systemic inflammation (40). Recent studies suggest that oral infection, especially periodontitis, is associated with several types of systemic diseases, such as infectious endocarditis and cardiovascular disease (32). Porphyromonas gingivalis (P.g.) is a major periodontal pathogen. P.g. cells occasionally gain entry to the human circulatory system, and the resulting transient bacteremia may induce life-threatening infective endocarditis, disseminated intravascular coagulation, immune thrombocytopenia purpura, and even an increased risk of stroke or myocardial infection (36). However, little is known about the relationship between P.g. infection and periodontitis and myocarditis. Thus we used EAM to examined this relationship.

MATERIALS AND METHODS

Animals. The male BALB/c A Jcl mice (7 wk, 20–25 g) were purchased from CLEA Japan (Tokyo, Japan). They were given a standard diet and water-maintained in compliance with the animal welfare guidelines of the Institute of Experimental Animal, Tokyo Medical and Dental University and the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health. This study was also approved by the Animal Care and Use Committee of the Tokyo Medical and Dental University. We divided the mice into the P.g.-injected EAM group (n = 16) and the PBS-injected EAM group (n = 12). Murine hearts were harvested 21 days after the induction of EAM.

The mouse subcutaneous chamber model. We used a modification of the subcutaneous chamber model as previously described (15). Chambers (10-mm length, 5.0-mm diameter), which were constructed from coils of stainless wire, were implanted subcutaneously in the back of each mouse. After the healing period, the chambers were used as a biological compartment for inducing inflammation.

Induction of EAM. α-Myosin heavy chain peptide (Ac-RSLKLMATLFEQVANK-0361) was synthesized at a high purity by Applied Biochemistry Co., Ltd. (Tokyo, Japan) in phosphate-buffered saline at a concentration of 1 mg/ml. The peptide was emulsified with an equal volume of complete Freund adjuvant (Difco Lot 0097536) to a final concentration of 1.0 mg/ml. The chambers were then injected with 0.1 ml of the emulsion (0.1 ml/mouse), which yielded an immunizing dose of 100-μg cardiac myosin per mouse. The day of myosin infection was defined as day 0 (5).

Bacterial growth. P.g., strain ATCC A7A1-28, was grown on blood agar plates in an anaerobic chamber with 85% N2-5% H2-10% CO2. After incubation at 37°C for 2–3 days, the bacterial cells were harvested and used for infection.
Porphyromonas gingivalis (P.g.) or PBS injection was performed on days 0, 7, and 14. Cardiac myosin heavy chain (MHC) peptide injection was performed on days 0 and 7.

incubated into a peptone yeast extract for 1 wk of incubation under the same conditions. The bacterial concentrations were standardized to $10^7$ colony-forming units (CFU)/ml, and the subcutaneous injections were performed once a week for 3 wk from day 0. Figure 1 shows the time schedule of this study.

Anti-P.g. IgG by ELISA. The levels of anti-P.g. IgG in the plasma were determined by an ELISA as previously described (28). The plasma samples were obtained before coil implantation and at death of the P.g.-injected or the PBS-injected EAM group.

Anticardiac myosin autoantibody by ELISA. The levels of anticardiac myosin antibodies in the serum were determined by an ELISA as previously described (35). The serum samples were obtained 21 days after immunization.

Histopathology. Hearts were harvested immediately after the mice were killed. We obtained three transverse sections per heart for histological examination. Slices were stained with hematoxylin and eosin. The areas of myocardium affected by cell infiltration were determined as infiltrated areas. The affected area ratio (affected/entire area expressed as a percentage) was calculated as previously described (38). We stained heart samples with Mallory staining to evaluate the differences in the fibrotic area. The affected area ratio was calculated as described previously (20).

Echocardiogram. We performed a transthoracic echocardiography 21 days after immunization. An echocardiography machine with a 7.5-MHz transducer (Toshiba, Tokyo, Japan) was used for left anterior descending artery (LAD) echocardiography recording. We carried out echocardiography under anesthesia (3.6% trichloroacetaldehyde). The M-mode echocardiogram was performed at the papillary muscle level, and fractional shortening (FS), diastolic interventricular septum thickness (IVSTd), and diastolic posterior wall thickness (LVPWd) were calculated.

Lung weight. We measured body weight and lung weight 21 days after EAM operation. We calculated lung weight-to-body weight ratio.

Immunohistochemistry. Frozen sections were incubated with primary antibodies against CD4, CD8, or CD11b (monoclonal antibodies; Pharmingen, San Diego, CA) and F4/80 (monoclonal antibodies; Abcam, Cambridge, United Kingdom) for 24 h at 4°C. Incubations with a Histofine Simple Stain Kit as secondary antibodies (Nichirei, Tokyo) were carried out at room temperature for 30 min. Cell numbers were determined by counting all of the fields.

Real-time RT-PCR. Total RNA was isolated from each individual heart with the TRIzol reagent (Invitrogen/Life Technologies, San Diego, CA) after homogenization with a Polytron homogenizer. Real-time PCR was used to determine mRNA expression of IL-6 (assay ID: Mm00446190_m1), IL-10 (assay ID: Mm00439616_m1), IFN-γ (assay ID: Mm00801778_m1), matrix metalloproteinase-2 (MMP-2; assay ID: Mm00439498_m1), MMP-9 (assay ID: Mm00601631_m1), monocyte chemoattractant protein-1 (MCP-1; assay ID: Mm00441242_m1), and IL-17A (assay ID: Mm00439618_m1) in the heart. The cDNA was run in duplicates. To account for differences in cDNA preparation and cDNA amplification efficiency, mRNA expression of each of the target genes was normalized by 18s rRNA (4308329). Quantitative data were calculated using the comparative cycle threshold (ΔΔCT) method.

Serum level of cytokines by ELISA. The serum levels of IL-6, IL-10, IFN-γ, and MCP-1 were determined using an ELISA (ELISA MAX Deluxe Sets; BioLegend, San Diego, CA). ELISA was performed according to the manufacturer’s instructions. The serum samples were obtained from the P.g.-injected EAM, the PBS-injected EAM group, and the P.g.-injected non-EAM group 3 h after the injection on days 7, 14, and 21.

Zymography. Equal amounts of protein samples were adjusted to 30 μl with 2× sample buffer (0.5 M Tris-HCl, pH 7.6, 2.0 ml of glycerol, 10% SDS, 0.1% bromophenol blue) and incubated for 10 min at room temperature before loading to zymogram gel [lower gel: 10% SDS, 1.5 M Tris-HCl, pH 8.8, 40% acrylamide, 20 mg/ml gelatin, 11.7 ml of water, 10% ammonium persulfate (APS), 30 μl of $N\_N\_N\_N$-tetramethylethylenediamine (TEMED); upper gel: 10% SDS, 0.4 M Tris-HCl, pH 6.8, 40% acrylamide, 12.9 ml of water, 10% APS, 20 μl of TEMED]. Gels were run at 200 V for 5 h. The gels were incubated in 2.5% Triton X-100 for renaturation for 30 min. They were then equilibrated in fresh developing buffer (50 mmol/l Tris-HCl, 0.2 mmol/l NaCl, 5 mmol/l CaCl₂, 0.02% Brij 35) and incubated at 37°C for 48 h. Gels were stained with 0.5% Coomassie blue G (40% methanol, 10% acetic acid) for 2 h and destained with methanol-acetic acid-water (5:1:4) for 30 min. Areas of protease activity appear as clear bands against a dark blue background where the protease has digested the gelatin substrate.

Blood analysis. Blood was obtained from an orbit vein with heparinized microtubes on days 7, 14, and 21 after the EAM induction under

Fig. 1. Time schedule. Porphyromonas gingivalis (P.g.) or PBS injection was performed on days 0, 7, and 14. Cardiac myosin heavy chain (MHC) peptide injection was performed on days 0 and 7.

Fig. 2. IgG titer. A: the effects of the injection of P.g. on the plasma levels of anti-P.g. antibodies were determined. *P < 0.05. PBS-injected experimental autoimmune myocarditis (EAM) group, n = 8; P.g.-injected EAM group, n = 8. B shows the levels of anticardiac myosin autoantibody. **P < 0.05 vs. PBS-injected non-EAM group; #P < 0.05 vs. P.g.-injected non-EAM group; ##P < 0.05 vs. P.g.-injected EAM group; ###P < 0.05 vs. PBS-injected EAM group; n = 4; P.g.-injected non-EAM group, n = 4; PBS-injected EAM group, n = 4; P.g.-injected EAM group, n = 4.
anesthesia (3.6% trichloroacetaldehyde monohydrate). White blood cell counts and fractions were measured by Mitsubishi Chemical Medience.

Statistical analyses. Results were expressed as means ± SE. We used Student’s t-test to compare the two groups. We used the Mann-Whitney U test to obtain results of quantitative RT-PCR to compare the two groups. Differences in data between multiple groups were analyzed by one-way ANOVA followed by Scheffé’s test. P < 0.05 was considered statistical significance.

RESULTS

Quantification of antibacterial antibodies. The repeated bacterial injections elevated the plasma level of anti-P.g. IgG significantly. The PBS-injected group had no effect on the levels of anti-P.g. antibodies (Fig. 2A).

Quantification of ant cardiac myosin autoantibody. The PBS-injected EAM group and the P.g.-injected EAM group showed significantly elevated levels of ant cardiac myosin autoantibody (Fig. 2B).

Histopathology. Hearts in the P.g.-injected EAM group showed severe inflammatory cell infiltration. In contrast, moderate inflammatory lesion was observed in hearts in the PBS-injected EAM group. The affected areas were significantly enhanced in the P.g.-injected EAM group compared with the PBS-injected EAM group. On the other hand, hearts in the PBS- or P.g.-injected non-EAM group showed no inflammatory cell infiltration (Fig. 3A). Moreover, we showed four serial images of each heart (Fig. 3B). The area ratio was significantly enhanced in the P.g.-injected EAM group compared with the PBS-injected EAM group. The fibrotic area was not detected in the PBS-injected non-EAM group and P.g.-injected non-EAM group (Fig. 3C).

Echocardiogram. We examined evaluation of cardiac function. However, there was no statistical difference between the P.g.-injected EAM group and the PBS-injected EAM group in cardiac function of FS, IVSTd, and LVPWd (Fig. 4).

Lung weight and lung weight-to-body weight ratio. We measured the lung weight and calculated lung weight-to-body weight ratio 21 days after EAM operation. The lung weight and lung weight-to-body weight ratio were significantly increased in the P.g.-injected EAM group compared with the PBS-injected EAM group (Fig. 5).

Immunohistochemical findings. We detected CD4-, CD8-, and CD11b-positive infiltrating cells in both groups. We counted CD4-, CD8-, CD11b-, and F4/80-positive infiltrating cells. The immunohistochemistry of infiltrating CD4- and CD8-positive cells were comparable between the P.g.-injected EAM group and the PBS-injected EAM group (Fig. 6, A and B). The infiltrating CD11b-positive cells and F4/80-positive cells were higher in the P.g.-injected EAM group than in the PBS-injected EAM group (Fig. 6, C and D).

Quantitative RT-PCR. We examined heart IL-6, IL-10, MMP-2, MMP-9, MCP-1, IFN-γ, and IL-17A mRNA levels using quantitative RT-PCR. MMP-9, MCP-1, and IFN-γ levels were significantly enhanced in the P.g.-injected EAM group compared with the PBS-injected EAM group. IL-6, IL-10, MMP-2, and IL-17A mRNA levels were comparable between the two groups (Fig. 7).

Serum level of cytokines by ELISA. We measured serum cytokine levels using ELISA. On day 7, serum levels of IL-6 were significantly enhanced in the P.g.-injected EAM group compared with the PBS-injected EAM group. On days 7 and 21, serum levels of IL-6 were significantly enhanced in the P.g.-injected EAM group compared with the PBS-injected EAM group.
has not yet been elucidated. In this study, we investigated the influence of periodontal pathogens on acute myocarditis using a murine EAM model. Our results demonstrate that periodontal bacteria significantly deteriorate EAM in mice. It is known that high serum antibody levels from major periodontal pathogens are associated with heart disease (42).

We confirmed that anti-P.g. IgG titer indicated that infection to the mice actually occurred and MCP-1, MMP-9, and IFN-γ mRNA levels significantly increased with P.g. injection. Inflammation associated with immunization recruits CD11b-positive depends on MCP-1 (2). Great amounts of inflammatory cells migrating into the myocardium are considered to be the decisive process of pathogenesis in myocarditis (6, 23). Indeed, it has been reported that MCP-1 was upregulated in myocarditis (13). Moreover, some reports said that the local host response to periodontal bacteria consists of monocytes/macrophages and neutrophils (41). Periodontopathic bacteria generated host immunological inflammatory responses, which resulted in the secretion of cytokines and MMPs (43). MMPs have an important role in cell migration and are involved in the migratory capabilities of inflammatory cells such as T cells (30). MMP-9 is especially important in inflammation as gelatinase has been shown to have a critical role in the process of infiltration in inflamed tissues (4). Our experiments showed that MMP-9 mRNA expression was significantly enhanced in the P.g.-injected EAM group. However, MMP-9 enzymatic activity was stable. In previous reports, correlation between mRNA and protein levels was insufficient to predict protein expression levels from quantitative mRNA data (19). We assumed that MMP-9 mRNA expression might promote inflammatory response. We detected inflammatory cell infiltration in the hearts of both groups. In this study, we detected CD4-, CD8-, and CD11b-positive infiltrating cells in heart. In some reports, there is a decrease in CD4-positive cells and an increased of CD8 in the infected human peripheral blood sample of periodontitis subjects compared with those of healthy subjects (25, 34, 45). P.g. markedly induced monocyte migration and significantly enhanced production of the proinflammatory cytokines (41). In this study, it was suggested that P.g. effects systemic inflammation, which deteriorated inflammation of the hearts. The altered T helper 1 (Th1)-Th2 cell balance regulates the clinical course of EAM (39). Previous studies also reported that production of proinflammatory cytokines affected development of EAM (14). Moreover, the excessive production of a number of inflammatory cytokines is suggested to play a crucial role in the pathogenesis of chronic periodontitis (12). Thus we examined the production of Th1 and Th2 cytokines. MCP-1 affects both Th1 and Th2 cells (18), and it has been reported that Th2 cells express IL-6 and IL-10 (1). The MCP-1 mRNA levels were positively correlated with IL-6 mRNA levels in the same lesion of EAM (27). Reports also assert that the serum IL-6 level has increased in experimental periodontitis in mice (29). Indeed, our experiments demonstrated that serum IL-6 levels were significantly increased in P.g.-injected EAM group. Evidence from studies on animal models of periodontal disease endorse a pivotal role of IL-10 in downregulating destructive inflammatory responses (44). During the inflammatory phase of EAM, proinflammatory cytokines including IFN-γ are produced (39). In addition, IFN-γ was considered to be essential for the development of EAM (10). We found that the injection of P.g. increased the

**DISCUSSION**

Periodontal diseases are highly prevalent and can affect up to 90% of the worldwide population. In addition, periodontal inflammation could have a role in the initiation or progression of cardiovascular disease (40). We were focused on systemic inflammation in this study. The mouse subcutaneous chamber model is a model of local infection in a distant area, which was previously shown to mimic the sequence of localized inflammatory events observed in diseased human periodontal pockets (21, 26). Moreover, it was said that assessment of host responses to localized infections can be performed quite easily with the use of subcutaneous chambers implanted in mice (16). The influence of periodontal pathogens on acute myocarditis

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**A**

**B**

Lung Weight

Lung weight/body weight

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**Fig. 5.** A and B: the lung weight (A) and lung weight-to-body weight ratio (B) 21 days after EAM operation. The lung weight and lung weight-to-body weight ratio were significantly increased in the P.g.-injected EAM group ($n=5$) compared with the PBS-injected EAM group ($n=5$; $^*P<0.05$).
Fig. 6. Immunohistochemistry: CD4 (A), CD8 (B), CD11b (C), and F4/80 (D) staining are shown. We showed control IgG staining (c and d in A–D). The brownish stains exhibited positive cells. CD4- and CD8-positive cells were comparable between the P.g.-injected (n = 9) and the PBS-injected EAM group (n = 12; a, b, and e in A and B). CD11b-positive cells were higher in the P.g.-injected EAM group (n = 9) than in the PBS-injected EAM group (n = 12; a, b, and e in C). F4/80-positive cells were higher in the P.g.-injected EAM group (n = 5) than in the PBS-injected EAM group (n = 5; a, b, and e in D; *P < 0.05). Original magnification of all panels was ×200. Scale bar is 50 μm.
expression of IFN-γ and decreased IL-10. P.g. injection also increased production of Th1 cytokines and decreased production of Th2 cytokines. Thus the injection of P.g. significantly developed EAM. Therefore, it is reasonable to conclude P.g. injection significantly increased MCP-1, MMP-9, and IFN-γ mRNA levels (Fig. 7). Recent reports assert that Th17 cells play important roles in the pathogenesis of EAM (3, 46). The severity of chronic human periodontal disease is positively correlated with IL-17 levels (22, 31). P.g. leads to the generation of Th17-supporting cytokines such as IL-1β, IL-6, and IL-23 but not Th1-related IL-12. The ability of P.g. to drive inflammatory responses, particularly of a Th17 nature, may underlie tissue pathology in periodontitis (33). We assumed that P.g. infection might promote EAM via Th17 systems. Moreover, Toll-like receptors (TLR) are essential for protective immunity against infection, and inappropriate TLR responses contribute to acute and chronic inflammation as well as to systemic autoimmune diseases (24). Expression of TLR-4 on dendritic cells was reported to be required for initiation of EAM (17). Moreover, the development of disease following injection of myosin-loaded dendritic cells was found to involve TLR-4 stimulation (9). In this way, TLR system plays important roles for pathogenesis and progression of EAM. P.g. utilizes either TLR-2 or TLR-4 to mediate inflammatory signaling (7, 37). P.g. infection might promote EAM via TLR systems.

In summary, P.g. injection could deteriorate EAM in mice through CD11b-positive cells, cytokines, and MMP-9 expression. Infection with P.g. seems to be a potential risk factor for acute myocarditis. To our knowledge, this is the first report to demonstrate that a periodontal pathogen promoted the development of EAM. Further investigation is needed to elucidate whether myocarditis deteriorates in patients with P.g.-induced periodontitis.

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Fig. 10. Blood analysis: white blood cell counts and fractions data are shown. P.g.-injected non-EAM group, n = 4; PBS-injected EAM group, n = 4; P.g.-injected EAM group, n = 4; P.g.-injected non-EAM group, n = 4 vs. PBS-injected EAM group; #P < 0.05 vs. P.g.-injected non-EAM group.

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