Journal of
PERIODONTAL RESEARCH

J Periodont Res 2011; 46: 176–183

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2010.01326.x

# *Porphyromonas gingivalis* promotes murine abdominal aortic aneurysms via matrix metalloproteinase-2 induction

**N. Aoyama[1], J.Suzuki[2,3], D. Wang[2,4], M. Ogawa[2,3], N. Kobayashi[1], T. Hanatani[1], Y. Takeuchi[1], Y. Izumi[1,5], M. Isobe[2]**

[1]Section of Periodontology, Department of Hard Tissue Engineering, Graduate School of Medicine and Dentistry, Tokyo Medical and Dental University, Tokyo, Japan, [2]Department of Cardiovascular Medicine, Tokyo Medical and Dental University, Tokyo, Japan, [3]Department of Advanced Clinical Science and Therapeutics, University of Tokyo, Tokyo, Japan, [4]Department of Periodontics and Oral Medicine, Beijing Stomatological Hospital, Capital Medical University, Beijing, China and [5]Global Center of Excellence Program, International Research Center for Molecular Science in Tooth and Bone Diseases, Tokyo Medical and Dental University, Tokyo, Japan

*Aoyama N, Suzuki J, Wang D, Ogawa M, Kobayashi N, Hanatani T, Takeuchi Y, Izumi Y, Isobe M.* Porphyromonas gingivalis *promotes murine abdominal aortic aneurysms via matrix metalloproteinase-2 induction. J Periodont Res 2011; 46: 176–183. © 2010 John Wiley & Sons A/S*

*Background and Objective:* Abdominal aortic aneurysm (AAA) is a common and lethal disorder, and MMPs are highly expressed in AAA lesions. Large numbers of periodontopathic bacteria have been reported to be present in specimens obtained from the aortic walls of patients with an AAA. The purpose of this study was to analyze the influence of periodontopathic bacteria on AAA dilatation.

*Material and Methods:* AAAs were produced in mice by the periaortic application of 0.25 M $CaCl_2$, and NaCl was used as a control. The mice were inoculated once weekly with live *Porphyromonas gingivalis*, live *Aggregatibacter actinomycetemcomitans* or vehicle.

*Results:* Four weeks after the periaortic application of either $CaCl_2$ or NaCl, a significant increase was observed in the aortic diameter of *P. gingivalis*-challenged mice compared with the vehicle control mice ($p < 0.05$), whereas there was no statistically significant increase in the aortic diameter of the *A. actinomycetemcomitans*-challenged mice. Immunohistochemical analysis found significantly higher numbers of CD8-positive and MOMA2-positive cells and significantly higher levels of MMP-2 in the aneurysmal samples of *P. gingivalis*-challenged mice compared with control mice. Live *P. gingivalis* promoted a significant proliferation of splenocytes in comparison with *P. gingivalis*-lipopolysaccharide and live *A. actinomycetemcomitans* ($p < 0.05$).

*Conclusion:* These findings demonstrate that challenge with *P. gingivalis*, but not with *A. actinomycetemcomitans*, can accelerate, or even initiate, the progression of experimental AAA through the increased expression of MMPs.

Mitsuaki Isobe, MD, PhD, Department of Cardiovascular Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8549, Japan
Tel: 81-3-5803-5951
Fax: 81-3-5803-0238
e-mail: isobemi.cvm@tmd.ac.jp

Key words: animal model; matrix metalloproteinase; inflammation; *Porphyromonas gingivalis*

Accepted for publication September 29, 2010

Abdominal aortic aneurysm (AAA) is a common and lethal disorder (1,2). Inflammation, which is associated with a disruption of the orderly lamellar structure of the aortic media, appears to play a critical role in the development and progression of AAA (3). Evaluations of human AAA tissue showed high levels of inflammatory infiltrates in both the media and the adventitia (4,5). The normal lamellar structure, with flattening and fragmentation of elastin fibers, is associated with aortic expansion. An increased expression of MMPs has been observed in human aneurysm tissue specimens (6,7).

In periodontal diseases, MMPs also play key roles. Periodontitis is characterized by gingival inflammation and the loss of periodontal support tissue

(8). Periodontopathic bacteria generate host immunological inflammatory responses, resulting in the secretion of cytokines and MMPs (9) and eventually leading to the destruction of extracellular matrix in periodontal tissues (10). Recent studies suggest that oral infection, especially periodontitis, is associated with several types of systemic diseases, such as infectious endocarditis and cardiovascular disease (11,12). Transient bacteremia caused by professional dental procedures and daily tooth brushing, which can result in the dissemination of oral bacteria into the bloodstream (13), may initiate systemic inflammatory reactions (14). Several studies have reported the detection of periodontal bacteria in cardiovascular specimens (15,16). Periodontopathic bacteria, especially *Porphyromonas gingivalis*, which is one of the most common pathogens related to periodontitis, were present in a high percentage of specimens of diseased arteries from AAA patients and were also found throughout the whole aneurysmal wall (15). The detection of *Aggregatibacter actinomycetemcomitans*, which is another major periodontopathic pathogen, has been reported in atheromatous specimens obtained during carotid endarterectomies (16).

The $CaCl_2$-induced AAA model is popular for the experimental development of AAA (17). The application of $CaCl_2$ promotes inflammatory responses, followed by structural disruption of the aortic walls. This destruction leads to the development of luminal dilatation and AAA progression (18). This $CaCl_2$-induced AAA model depends on aortic inflammation (19).

Recent epidemiological reports suggest periodontal disease to be an important risk factor for cardiovascular disease (20). Periodontopathic bacteria may play a role in the development of AAAs, but the effect of these bacteria on the aneurysmal wall has not yet been established. The purpose of the present investigation was to determine the effect of periodontal microorganisms on AAA in the mouse model. The results of this study revealed, for the first time, that periodontopathic pathogens induced AAA dilatation with a high expression of MMPs.

## Material and methods

### The murine subcutaneous chamber model

We used a modification of the subcutaneous chamber model, as previously described (21,22). Male C57BL/6 mice (8–10 wk, 20–25 g) were obtained from Japan Clea, Co. (Tokyo, Japan). Chambers (length 10 mm, diameter 5 mm), which was constructed from coils of stainless-steel wire, was implanted subcutaneously into the back of each mouse. After the healing period, the chambers were used as a biological compartment for inducing inflammation. This investigation conformed to the Guide for the Care and Use of Laboratory Animals of the Tokyo Medical and Dental University.

### Bacterial growth

*P. gingivalis* (ATCC A7A1-28) was grown on blood agar plates in an anaerobic chamber (containing an atmosphere of 85% $N_2$, 5% $H_2$, 10% $CO_2$). After incubation at 37°C for 2–3 d, the bacterial cells were inoculated into liquid medium of peptone yeast extract and incubated for a further week under the same culture conditions. *A. actinomycetemcomitans* strain Y4 was obtained by an initial culture on trypticase soy agar plates supplemented with horse serum, bacitracin and vancomycin for 5–7 d, followed by incubation for 3–5 d at 5% $CO_2$ in air at 37°C. The purity of the cultures was checked by phase-contrast microscopy. Bacterial cells were transferred to peptone yeast extract for 1–2 d. The bacterial concentrations were standardized to $10^8$ colony-forming units (CFUs)/mL.

### Immunization

The bacteria were heat-killed by incubation at 80°C for 10 min. Fourteen days before induction of the AAA by the application of either $CaCl_2$ or NaCl, the mice were immunized by the subcutaneous injection of heat-killed *P. gingivalis* or *A. actinomycetemcomitans* (0.1 mL of $10^8$ CFU/mL). The levels of anti-*P. gingivalis*- or anti-*A. actinomycetemcomitans*-specific IgG in the plasma were determined using an ELISA, as previously described (23), before coil implantation and after the *P. gingivalis*- or *A. actinomycetemcomitans*-infected or vehicle control mice were killed.

### Aneurysm induction

Aneurysm induction was performed as described previously (3). The mice were anesthetized with 3.6% chloral hydrate (intraperitoneal administration, 0.1 mL/10 g body weight) and then underwent a laparotomy. The diameter of the aorta was measured in triplicate midway between the renal artery origin and iliac artery bifurcation. After taking baseline measurements, either 0.25 M $CaCl_2$ or 0.9% NaCl (control) was applied to the external surface of the aorta. The mice were divided into six groups, $CaCl_2$ was applied and then inoculated with live *P. gingivalis* (0.1 mL of $10^8$ CFU/mL) ($n = 10$), live *A. actinomycetemcomitans* (0.1 mL of $10^8$ CFU/mL) ($n = 9$) or vehicle containing diluted phosphate-buffered saline (PBS) (0.1 mL) ($n = 10$), or NaCl was applied and then inoculated with live *P. gingivalis* (0.1 mL of $10^8$ CFU/mL) ($n = 10$), live A. actinomycetemcomitans (0.1 mL of $10^8$ CFU/mL) ($n = 8$) or vehicle (0.1 mL) ($n = 13$). The subcutaneous injections were performed once per week for 4 wk. Four weeks later the mice underwent a laparotomy and dissection. The measurements were repeated at the same location in the midinfrarenal aorta.

### Histopathology and immunohistochemistry

Histopathological analyses were performed as described previously (24). The sections were stained with hematoxylin and eosin and with Elastica van Gieson. We used a modification of the average aortic wall architecture score, as previously described (25). The score was assessed by three observers who were blinded to both injection and treatment. The elastic fiber integrity was assessed in four representative areas on a scale from 0 to 3, with

scoring as follows: 0, completely intact with wavy organization; 1, mild disorganization without fragmentation; 2, local degradation and fragmentation; and 3, extensive fragmentation and degradation.

The sections (four samples in each group) were incubated with primary antibodies against CD4, CD8, MOMA2 (BD Biosciences Pharmingen, San Diego, CA, USA), MMP-2 or MMP-9 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4°C for 12 h. Antibody–horseradish peroxidase conjugate was detected using the Histofine Simplestain kit (Nichirei Corporation, Tokyo, Japan) according to the manufacturer's instructions. The enzyme activity was detected with 3-amino-9-ethylcarbazole. Immunohistochemical staining was quantified and graded as previously described (26): 0, no staining; 1, very mild staining; 2, mild staining; 3, moderate staining; and 4, abundant positive staining.

### Quantification of MMP-2 and TIMP-1

The plasma levels of MMP-2 and TIMP-1 were determined using an ELISA (Quantikine ELISA kit; R&D Systems, Minneapolis, MN, USA). Murine blood was collected, a 38% solution of aqueous citric acid was added and the blood was centrifuged at 1000 *g* within 15 min of collection. The plasma was stored at −80°C. ELISA was performed according to the manufacturer's instructions.

### Proliferation assay

Splenocytes were isolated from native mice and stimulated with *P. gingivalis* lipopolysaccharide (LPS) (1 μg/mL) (Invivogen, San Diego, CA, USA) from *P. gingivalis* ATCC 33277 (27), live *P. gingivalis* ($10^7$ CFU/mL) or live *A. actinomycetemcomitans* ($10^7$ CFU/mL). Forty-eight hours after stimulation with *P. gingivalis*-LPS or live bacteria, the samples were centrifuged and the supernatants were collected and stored. A proliferation assay was performed as previously described (28). The level of MMP-2 in the supernatants was measured using an ELISA, as described above.

### Statistical analysis

Every value was expressed as the mean ± SE. The paired Student's *t*-test was used to compare the original and final diameters. The unpaired Student's *t*-test was used to compare the values of two groups and plasma MMP-2 and TIMP-1 levels. An analysis of variance (ANOVA) was used to compare the values between the groups. Statistical significance was accepted at a $p < 0.05$.

## Results

### Quantification of antibacterial antibodies

The plasma levels of anti-*P. gingivalis* or anti-*A. actinomycetemcomitans* IgG are shown in Fig. 1A and 1B. Repeated stimulation with bacteria, through subcutaneous injections, significantly increased the plasma level of anti-*P. gingivalis* IgG and of anti-*A. actinomycetemcomitans* IgG. The injection of PBS did not stimulate the production of either anti-*P. gingivalis* or anti-*A. actinomycetemcomitans* IgG.

### Diameter of abdominal aorta in uninfected mice and in mice infected with bacteria

Uninfected mice showed a significant increase in the aortic diameter after treatment with $CaCl_2$ (Fig. 2). There was no difference in the aortic diameter of the mice in any group before and after treatment with NaCl. There was a

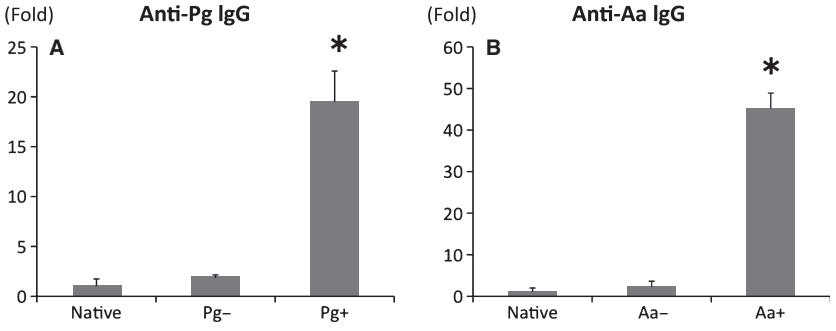


*Fig. 1.* The effects of injection of *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* on the plasma levels of anti-*P. gingivalis* (A) or anti-*A. actinomycetemcomitans* (B) IgG, respectively, were determined. The plasma samples were obtained before coil implantation (Native) and after control (Pg- or Aa-), *P. gingivalis*-infected (Pg+) or *A. actinomycetemcomitans*-infected mice (Aa+) were killed. *$p < 0.05$ vs. Native.

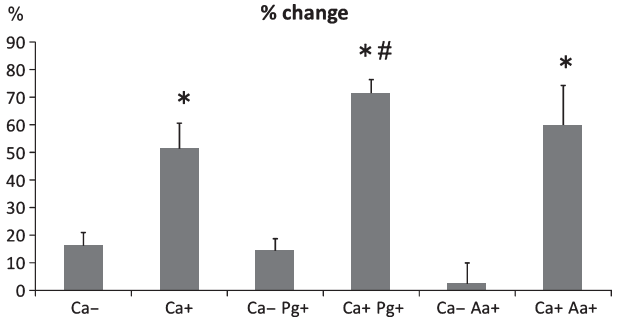


*Fig. 2.* Aortic diameter measurements in *Porphyromonas gingivalis*- or *Aggregatibacter actinomycetemcomitans*-infected and control mice after treatment with NaCl or $CaCl_2$. The aortic diameters were measured before the application of $CaCl_2$ or NaCl and after the mice were killed. The percentage increase in the aortic diameter after death, relative to the original diameter, is shown. Aa+, *A. actinomycetemcomitans* infected; Aa−, no infection with *A. actinomycetemcomitans*; Ca+, $CaCl_2$ treatment; Ca−, NaCl treatment; Pg+, *P. gingivalis* infected; Pg−, no infection with *P. gingivalis*. *$p < 0.05$ vs. Ca−. $p < 0.05$ vs. Ca+.

significant increase in the aortic diameter of *P. gingivalis*-infected mice after $CaCl_2$ treatment and also in comparison with unchallenged mice. However, there was no significant increase in the aortic diameter of *A. actinomycetemcomitans*-infected mice in comparison with unchallenged mice after $CaCl_2$ treatment.

A histopathological analysis of aortic sections from these mice showed the fragmentation of medial elastic lamellae (Fig. 3A and 3B). The *P. gingivalis*-infected + $CaCl_2$-treated mice showed the greatest destruction (score 2.3 ± 0.3) of aortic tissue among the groups (Fig. 3C). Surprisingly, severe destruction (score 2.3 ± 0.2) of aortic tissue was also found in *P. gingivalis*-infected and NaCl-treated mice.

### Immunohistochemistry

The number of CD8-positive cells was higher in the samples infected with *P. gingivalis* than in the samples from uninfected mice, but there was no difference between samples from mice treated with $CaCl_2$ and those treated with NaCl (Fig. 4A). No statistical difference in the expression of CD4, MOMA2 and MMP-9 was observed in the four groups (NaCl + PBS, $CaCl_2$ + PBS, NaCl + *P. gingivalis*, and $CaCl_2$ + *P. gingivalis*) except for MOMA2 between the *P. gingivalis* + NaCl group and the uninfected $CaCl_2$-treated group. As shown in Fig. 4B and 4C, MMP-2 was highly expressed in the aortic sample of the *P. gingivalis*-infected mice in comparison with the control and *A. actinomycetemcomitans*-infected mice. In addition, no difference in the expression of MMP-2 between the $CaCl_2$- and NaCl-treated groups was observed. Because the expression of MMP-2 was stronger in the aortic sample from *P. gingivalis*-infected mice, we therefore analysed MMP-2 in more detail.

### Concentrations of MMP-2 and TIMP-1 in plasma

Figure 5 shows the concentrations of MMP-2 (A) and TIMP-1 (B) in the plasma of the untreated and *P. gingivalis*-infected mice at death. The TIMP-1 level in *P. gingivalis*-infected $CaCl_2$-treated mice increased in comparison with that of the uninfected, NaCl-treated mice.

### Effect of *P. gingivalis*-LPS, *P. gingivalis* and *A. actinomycetemcomitans* on the proliferation of splenocytes

We investigated whether stimulation with *P. gingivalis*-LPS, *P. gingivalis* or *A. actinomycetemcomitans* could induce the proliferation of splenocytes. The results presented in Fig. 6A show that *P. gingivalis*-LPS, live *P. gingivalis* and live *A. actinomycetemcomitans* stimulated splenocyte proliferation ($p < 0.05$), and that proliferation was

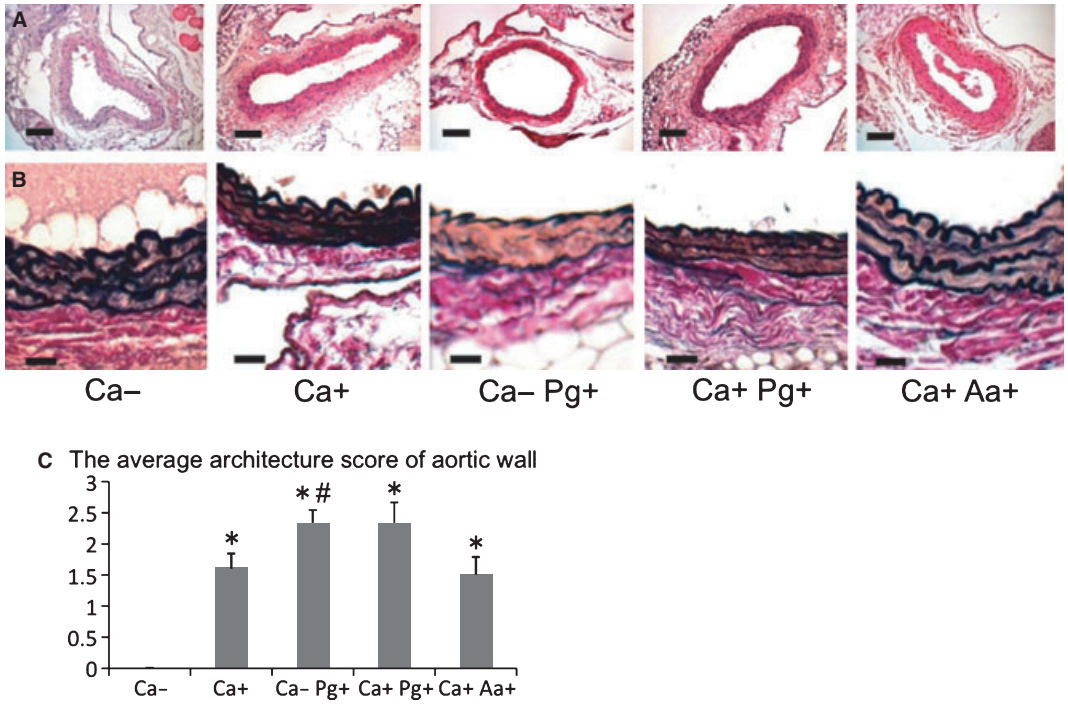


*Fig. 3.* Histological changes of the aorta. Representative aortic specimens are shown after (A) hematoxylin and eosin (HE) staining and (B) Elastica van Gieson (EvG) staining. Scale bars = 100 μm (A) or 10 μm (B). (C) The average architecture score of the aortic wall was determined as described in the Material and methods. Aa+, *Aggregatibacter actinomycetemcomitans* infected; Ca+, $CaCl_2$ treatment; Ca−, NaCl treatment; Pg+, *Porphyromonas gingivalis* infected. *$p < 0.05$ vs. Ca−. $p < 0.05$ vs. Ca+.

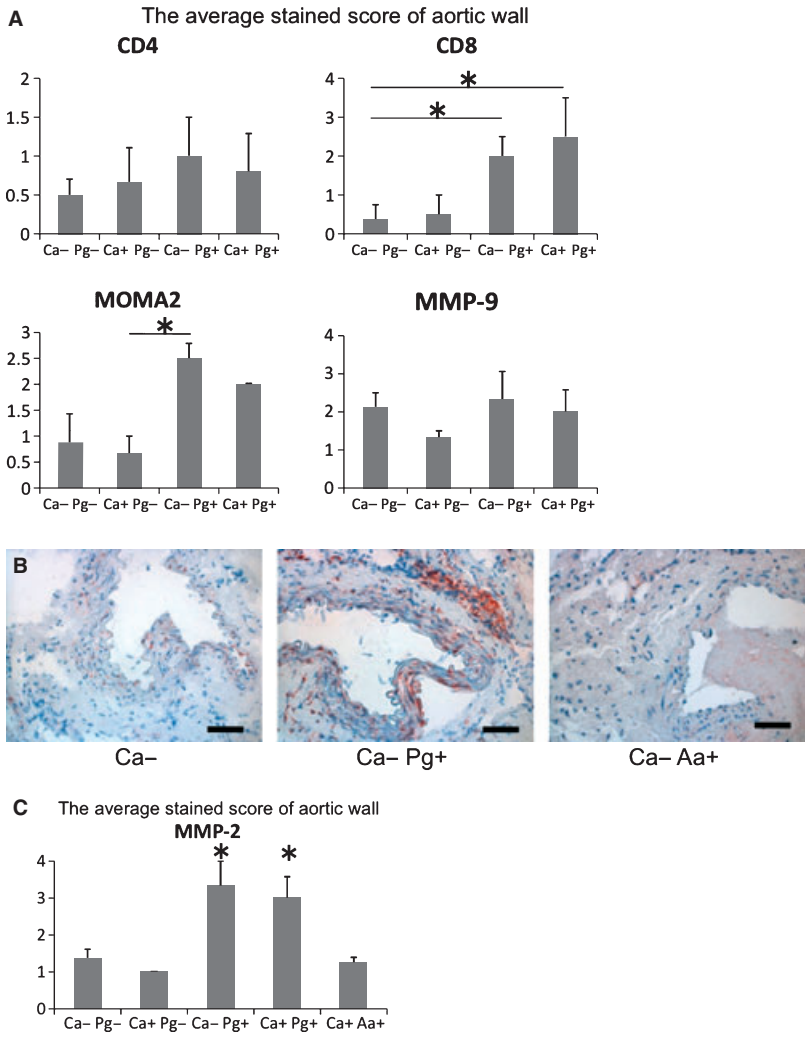


*Fig. 4.* Immunohistochemistry. (A) The sections were incubated with CD4, CD8, MOMA2 and MMP-9, and the average staining score of the aortic wall was determined as described in the Material and methods. (B) MMP-2-stained sections. Scale bars = 50 μm. (C) The average stained score of the aortic wall incubated with MMP-2. Aa+, *Aggregatibacter actinomycetemcomitans* infected; Ca+, $CaCl_2$ treatment; Ca–, NaCl treatment; Pg+, *Porphyromonas gingivalis* infected; Pg–, no infection with *P. gingivalis*. *$p < 0.05$ vs. Ca-Pg-.

higher after stimulation with live *P. gingivalis* than after stimulation with *P. gingivalis*-LPS or with *A. actinomycetemcomitans* ($p < 0.05$).

### MMP-2 induction by *P. gingivalis*-LPS, *P. gingivalis* and *A. actinomycetemcomitans*

After stimulation of splenocytes with *P. gingivalis*-LPS, *P. gingivalis* or *A. actinomycetemcomitans*, the supernatants were collected and the concentration of MMP-2 was measured using ELISA. The concentration of MMP-2 showed a dramatic increase after stimulation with *P. gingivalis* ($p < 0.05$) (Fig. 6B).

## Discussion

The results of this study showed that repeated challenge with *P. gingivalis* accelerated the progression of AAA in a murine model. It is therefore suggested that periodontitis may be a major risk factor for AAA development.

Periodontal disease is a common condition worldwide. A strong association between dental disease and coronary heart diseases has been demonstrated (29,30), and periodontal disease has been reported to be a significant independent risk factor for peripheral vascular disease (31). In periodontitis patients, markers of inflammation are increased (14), indicating that systemic inflammation can be caused by periodontal infection. Salvi & Lang (9) showed that periodontopathic bacteria generated host immunological inflammatory responses, which resulted in the secretion of cytokines and MMPs. Moreover, periodontal disease occasionally results in bacteremia, and periodontopathic bacteria attach to vascular endothelial cells, especially those with intimal injury, such as atherosclerotic or aneurysmal changes (32). In patients with periodontitis, subgingival plaque organisms can be introduced into the bloodstream many times a day through chewing and toothbrushing (33). However, it remains to be elucidated whether or not systemic bacteremia causes an acceleration of cardiovascular disorders.

In the present study, increased levels of anti-*P. gingivalis* or anti-*A. actinomycetemcomitans* IgG were found in plasma samples from *P. gingivalis*- and *A. actinomycetemcomitans*-infected mice, respectively (Fig. 1). This means that an infection with periodontal pathogens clearly occurred in our model. There was no increase in the aortic diameter of *A. actinomycetemcomitans*-infected mice, but a greater increase in the aortic diameter of *P. gingivalis*-infected mice after $CaCl_2$ treatment than in the uninfected mice was found (Fig. 2). To our knowledge, this is the first report to demonstrate that a periodontal pathogen promotes the development of AAA. There was a statistical difference in the elastic degradation score between the presence and absence of *P. gingivalis* infection in NaCl groups (Fig. 3C). This observation indicates that *P. gingivalis* infection can induce extracellular matrix degradation. However, we did not observe a statistical difference in elastic destruction between *P. gingivalis* + $CaCl_2$ and $CaCl_2$-only treatment groups. This may be because $CaCl_2$ treatment induced some matrix degradation in the present study. We next analyzed *P. gingivalis*-challenged mice in several

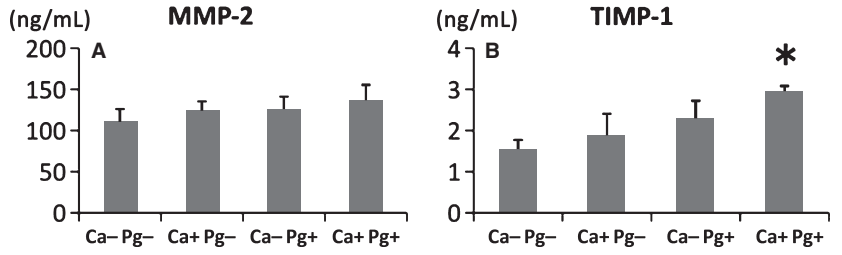


*Fig. 5.* MMP-2 and TIMP-1 levels in plasma. All plasma samples were obtained after the mice were killed. MMP-2 (A) and TIMP-1 (B) levels in plasma were determined. Ca +, $CaCl_2$ treatment; Ca–, NaCl treatment; Pg +, *Porphyromonas gingivalis* infected; Pg–, no infection with *P. gingivalis*. *$p < 0.05$ vs. Ca-Pg-.

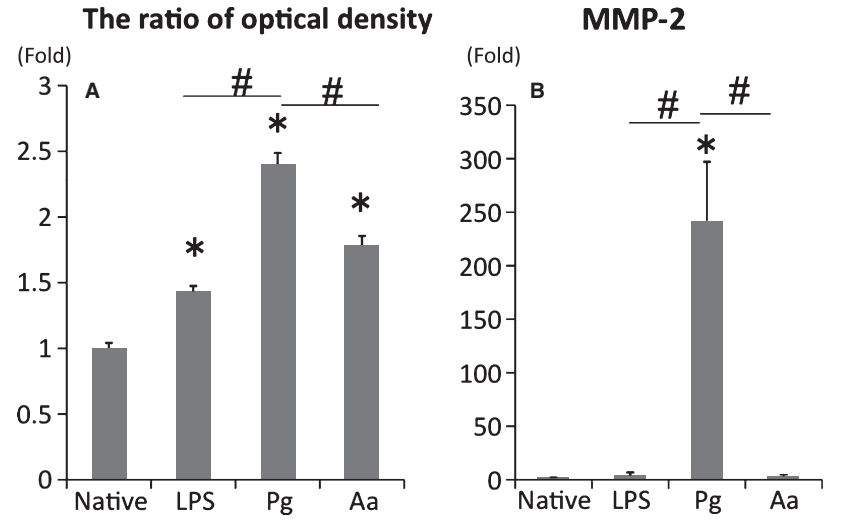


*Fig. 6.* Proliferation assay and level of MMP-2 derived from stimulated splenocytes. Forty-eight hours after the stimulation of splenocytes with *Porphyromonas gingivalis* lipopolysaccharide (LPS), *P. gingivalis* (Pg) and *Aggregatibacter actinomycetemcomitans* (Aa), the optical density (A) and the level of MMP-2 in the supernatant (B) of each sample were measured as described in the Material and methods. *Significant difference compared with Native or significant between-group difference. ($p < 0.05$).

ways. *P. gingivalis* was not detected in any aneurysm tissue specimens from uninfected mice, but it was detected in the aneurysm tissue specimens from one of the four *P. gingivalis*-infected mice in this study (data not shown). In human evaluations, large numbers of periodontopathic bacteria were found in both oral and arterial samples from AAA patients (15,34,35).

Because our model with *P. gingivalis*-infected and $CaCl_2$-induced AAA is original, we used immunohistochemical analysis to screen for several markers of inflammatory cells. We observed an increased infiltrate of MOMA2-positive and CD8-positive cells in the *P. gingivalis* infection groups (Fig. 4A). Macrophages are major cells for inducing MMPs (3), and the relationship between T cells and MMPs has already been reported. Inhibition of MMPs by doxycycline resulted in a reduction of AAA development and fewer $CD8^+$ cells infiltrating into the aortic region (36). MMP-2 and MMP-9 activities were reported to be decreased in $CD8^+$-deficient mice (37). Furthermore, it was shown that MMP-2 and MMP-9 mediated the migration of T cells (38). Therefore, the presence of MMPs may relate to $CD8^+$ cells in several ways.

We also demonstrated a significant increase in the concentration of MMP-2 in aorta samples from *P. gingivalis*-infected mice (Fig. 4B and 4C). MMP has been shown to play a pivotal role in aneurysm development in the AAA induction model (3,39). AAA is characterized with elastic degradation of the aortic wall and MMP is important in destruction of the extracellular matrix (40). In the same $CaCl_2$ model, MMP-2 or MMP-9 knockout mice showed no statistical increase in the aortic diameter after the chemical induction of AAA (3). In our model, if MMP activity was blocked, the effect of *P. gingivalis* infection and $CaCl_2$ treatment would be depressed. Our study showed that the presence of MMP-9 and the increase of MMP-2 stimulated by infection with *P. gingivalis* resulted in the development of AAA.

In studies of humans, patients with periodontitis were found to have higher levels of MMPs and TIMPs in blood compared to patients with no periodontitis (41,42). Grayson *et al.* (43) showed that gingipain, a protease induced by *P. gingivalis*, was capable of activating MMP-2, and supernatant from *P. gingivalis* can also activate MMP-2 (44). These experiments indicate that *P. gingivalis* infection can lead to the production of MMP in an inflammatory reaction. *P. gingivalis* A7A1-28, which was used in our study, has been reported to strongly stimulate the production of gingipain (45). Because gingipain acts as an etiologic factor (43), it may have been important in promoting inflammation in our study. By contrast, the potential of *A. actinomycetemcomitans* to stimulate an increase in MMPs remains controversial (46,47). No increase of plasma MMP-2 or TIMP-1 was, however, observed in this study.

In the present study, *P. gingivalis*-LPS, live *P. gingivalis* and live *A. actinomycetemcomitans* were able to stimulate the proliferation of spleen cells (Fig. 6A). The stimulatory effect of *P. gingivalis* was greater than that of *A. actinomycetemcomitans*, which may also be a reasonable explanation for the differences in the results observed in our mouse model study. The results shown in Fig. 6B indicate that *P. gingivalis* is a strong promoter of MMP-2 expression and that the effect of *A. actinomycetemcomitans* is much weaker. Moreover, splenocytes reacted

differently to whole *P. gingivalis* than to *P. gingivalis*-LPS. Although LPS is one of the major virulence factors of *P. gingivalis* (48), whole *P. gingivalis* has multiple virulence factors, such as fimbriae (49) and gingipain (43). These other factors might have affected the induction of MMPs in our study.

The mechanism of AAA development by periodontal disease remains highly controversial. In human studies, it has already been demonstrated that periodontal infection results in bacteremia (32,33), endothelial dysfunction (50) and systemic inflammation (14). In this study, the aorta was particularly targeted by immunoreactive cells, such as T lymphocytes and macrophages, because of the initiation of inflammation as a result of treatment with $CaCl_2$. Destruction of aortic tissue was found even in NaCl-treated and *P. gingivalis*-infected mice, although the expansion of aorta was not observed in this group, which means that *P. gingivalis* infection has a strong influence on systemic inflammation and the up-regulation of MMPs. Periodontal disease can be a risk factor for cardiovascular disease because of the increased expression of MMPs.

In conclusion, infection with periodontal pathogens plays an important role in AAA development via the increase in expression of MMPs from infiltrating inflammatory cells. Further investigations are needed to elucidate the pathophysiology of periodontitis and AAA.

## Acknowledgements

We thank Ms Noriko Tamura and Ms Yasuko Matsuda for their excellent technical assistance. This work was supported by a Grant-in-Aid for Scientific Research (B) (No. 21390553), Ministry of Education, Culture, Sports, Science and Technology of Japan and National Nature Science Foundation of China (No. 30901678).

## References

1. Ernst CB. Abdominal aortic aneurysm. *N Engl J Med* 1993;**328:**1167–1172.
2. Alcorn HG, Wolfson SK Jr, Sutton-Tyrrell K, Kuller LH, O'Leary D. Risk factors for abdominal aortic aneurysms in older adults enrolled in The Cardiovascular Health Study. *Arterioscler Thromb Vasc Biol* 1996;**16:**963–970.
3. Longo GM, Xiong W, Greiner TC, Zhao Y, Fiotti N, Baxter BT. Matrix metalloproteinases 2 and 9 work in concert to produce aortic aneurysms. *J Clin Invest* 2002;**110:**625–632.
4. Bobryshev YV, Lord RS, Parsson H. Immunophenotypic analysis of the aortic aneurysm wall suggests that vascular dendritic cells are involved in immune responses. *Cardiovasc Surg* 1998;**6:**240–249.
5. Pearce WH, Koch AE. Cellular components and features of immune response in abdominal aortic aneurysms. *Ann N Y Acad Sci* 1996;**800:**175–185.
6. Freestone T, Turner RJ, Coady A, Higman DJ, Greenhalgh RM, Powell JT. Inflammation and matrix metalloproteinases in the enlarging abdominal aortic aneurysm. *Arterioscler Thromb Vasc Biol* 1995;**15:**1145–1151.
7. Davis V, Persidskaia R, Baca-Regen L *et al*. Matrix metalloproteinase-2 production and its binding to the matrix are increased in abdominal aortic aneurysms. *Arterioscler Thromb Vasc Biol* 1998;**18:**1625–1633.
8. Choi BK, Jung JH, Suh HY *et al*. Activation of matrix metalloproteinase-2 by a novel oral spirochetal species Treponema lecithinolyticum. *J Periodontol* 2001;**72:** 1594–1600.
9. Salvi GE, Lang NP. Host response modulation in the management of periodontal diseases. *J Clin Periodontol* 2005;**32**(suppl 6)**:**108–129.
10. Ashley RA. Clinical trials of a matrix metalloproteinase inhibitor in human periodontal disease. SDD Clinical Research Team. *Ann N Y Acad Sci* 1999;**878:**335–346.
11. Moreillon P, Que YA. Infective endocarditis. *Lancet* 2004;**363:**139–149.
12. Li X, Kolltveit KM, Tronstad L, Olsen I. Systemic diseases caused by oral infection. *Clin Microbiol Rev* 2000;**13:**547–558.
13. Seymour RA, Lowry R, Whitworth JM, Martin MV. Infective endocarditis, dentistry and antibiotic prophylaxis; time for a rethink? *Br Dent J* 2000;**189:**610–616.
14. Beck J, Garcia R, Heiss G, Vokonas PS, Offenbacher S. Periodontal disease and cardiovascular disease. *J Periodontol* 1996;**67:**1123–1137.
15. Kurihara N, Inoue Y, Iwai T, Umeda M, Huang Y, Ishikawa I. Detection and localization of periodontopathic bacteria in abdominal aortic aneurysms. *Eur J Vasc Endovasc Surg* 2004;**28:**553–558.
16. Haraszthy VI, Zambon JJ, Trevisan M, Zeid M, Genco RJ. Identification of periodontal pathogens in atheromatous plaques. *J Periodontol* 2000;**71:**1554–1560.
17. Tanaka A, Hasegawa T, Chen Z, Okita Y, Okada K. A novel rat model of abdominal aortic aneurysm using a combination of intraluminal elastase infusion and extraluminal calcium chloride exposure. *J Vasc Surg* 2009;**50:**1423–1432.
18. Daugherty A, Cassis LA. Mouse models of abdominal aortic aneurysms. *Arterioscler Thromb Vasc Biol* 2004;**24:**429–434.
19. Manning MW, Cassi LA, Huang J, Szilvassy SJ, Daugherty A. Abdominal aortic aneurysms: fresh insights from a novel animal model of the disease. *Vasc Med* 2002;**7:**45–54.
20. Dietrich T, Jimenez M, Krall Kaye EA, Vokonas PS, Garcia RI. Age-dependent associations between chronic periodontitis/edentulism and risk of coronary heart disease. *Circulation* 2008;**117:**1668–1674.
21. Genco CA, Arko RJ. Animal chamber models for study of host-parasite interactions. *Methods Enzymol* 1994;**235:**120–140.
22. Houri-Haddad Y, Itzchaki O, Ben-Nathan D, Shapira L. The effect of chronic emotional stress on the humoral immune response to *Porphyromonas gingivalis* in mice. *J Periodontal Res* 2003;**38:**204–209.
23. Kojima T, Yano K, Ishikawa I. Relationship between serum antibody levels and subgingival colonization of *Porphyromonas gingivalis* in patients with various types of periodontitis. *J Periodontol* 1997;**68:**618–625.
24. Ogawa M, Suzuki J, Hishikari K, Takayama K, Tanaka H, Isobe M. Clarithromycin attenuates acute and chronic rejection via matrix metalloproteinase suppression in murine cardiac transplantation. *J Am Coll Cardiol* 2008;**51:**1977–1985.
25. Chung AW, Yang HH, Radomski MW, van Breemen C. Long-term doxycycline is more effective than atenolol to prevent thoracic aortic aneurysm in marfan syndrome through the inhibition of matrix metalloproteinase-2 and -9. *Circ Res* 2008;**102:**e73–e85.
26. Kuula H, Salo T, Pirila E *et al*. Local and systemic responses in matrix metalloproteinase 8-deficient mice during *Porphyromonas gingivalis*-induced periodontitis. *Infect Immun* 2009;**77:**850–859.
27. Darveau RP, Pham TT, Lemley K *et al*. *Porphyromonas gingivalis* lipopolysaccharide contains multiple lipid A species that functionally interact with both toll-like receptors 2 and 4. *Infect Immun* 2004; **72:**5041–5051.
28. Ogawa M, Suzuki J, Kosuge H, Takayama K, Nagai R, Isobe M. The mechanism of anti-inflammatory effects of prostaglandin E2 receptor 4 activation in murine cardiac transplantation. *Transplantation* 2009;**87:** 1645–1653.
29. Mattila KJ, Nieminen MS, Valtonen VV *et al*. Association between dental health

and acute myocardial infarction. *BMJ* 1989;**298:**779–781.
30. Sakurai K, Wang D, Suzuki J *et al*. High incidence of *Actinobacillus actinomycetemcomitans* infection in acute coronary syndrome. *Int Heart J* 2007;**48:**663–675.
31. Mendez MV, Scott T, LaMorte W, Vokonas P, Menzoian JO, Garcia R. An association between periodontal disease and peripheral vascular disease. *Am J Surg* 1998;**176:**153–157.
32. Chiu B. Multiple infections in carotid atherosclerotic plaques. *Am Heart J* 1999; **138:**S534–S536.
33. Dorn BR, Dunn WA Jr, Progulske-Fox A. Invasion of human coronary artery cells by periodontal pathogens. *Infect Immun* 1999;**67:**5792–5798.
34. Martin MC, Andres MT, Fierro JF, Mendez FJ. Endarteritis and mycotic aortic aneurysm caused by an oral strain of *Actinobacillus actinomycetemcomitans*. *Eur J Clin Microbiol Infect Dis* 1998;**17:** 104–107.
35. Okuda K, Ishihara K, Nakagawa T, Hirayama A, Inayama Y. Detection of Treponema denticola in atherosclerotic lesions. *J Clin Microbiol* 2001;**39:**1114–1117.
36. Lindeman JH, Abdul-Hussien H, van Bockel JH, Wolterbeek R, Kleemann R. Clinical trial of doxycycline for matrix metalloproteinase-9 inhibition in patients with an abdominal aneurysm: doxycycline selectively depletes aortic wall neutrophils and cytotoxic T cells. *Circulation* 2009; **119:**2209–2216.
37. Borchers MT, Wesselkamper SC, Harris NL *et al*. CD8+ T cells contribute to macrophage accumulation and airspace enlargement following repeated irritant exposure. *Exp Mol Pathol* 2007;**83:**301–310.
38. Leppert D, Waubant E, Galardy R, Bunnett NW, Hauser SL. T cell gelatinases mediate basement membrane transmigration in vitro. *J Immunol* 1995;**154:**4379–4389.
39. Pyo R, Lee JK, Shipley JM *et al*. Targeted gene disruption of matrix metalloproteinase-9 (gelatinase B) suppresses development of experimental abdominal aortic aneurysms. *J Clin Invest* 2000;**105:**1641–1649.
40. Aziz F, Kuivaniemi H. Role of matrix metalloproteinase inhibitors in preventing abdominal aortic aneurysm. *Ann Vasc Surg* 2007;**21:**392–401.
41. Ramseier CA, Kinney JS, Herr AE *et al*. Identification of pathogen and host-response markers correlated with periodontal disease. *J Periodontol* 2009;**80:**436–446.
42. Soder PO, Meurman JH, Jogestrand T, Nowak J, Soder B. Matrix metalloproteinase-9 and tissue inhibitor of matrix metalloproteinase-1 in blood as markers for early atherosclerosis in subjects with chronic periodontitis. *J Periodontal Res* 2009;**44:**452–458.
43. Grayson R, Douglas CW, Heath J, Rawlinson A, Evans GS. Activation of human matrix metalloproteinase 2 by gingival crevicular fluid and *Porphyromonas gingivalis*. *J Clin Periodontol* 2003;**30:**542–550.
44. Pattamapun K, Tiranathanagul S, Yongchaitrakul T, Kuwatanasuchat J, Pavasant P. Activation of MMP-2 by *Porphyromonas gingivalis* in human periodontal ligament cells. *J Periodontal Res* 2003;**38:**115–121.
45. Kesavalu L, Holt SC, Ebersole JL. In vitro environmental regulation of *Porphyromonas gingivalis* growth and virulence. *Oral Microbiol Immunol* 2003;**18:**226–233.
46. Soder B, Airila Mansson S, Soder PO, Kari K, Meurman J. Levels of matrix metalloproteinases-8 and -9 with simultaneous presence of periodontal pathogens in gingival crevicular fluid as well as matrix metalloproteinase-9 and cholesterol in blood. *J Periodontal Res* 2006;**41:** 411–417.
47. Tuomainen AM, Jauhiainen M, Kovanen PT, Metso J, Paju S, Pussinen PJ. *Aggregatibacter actinomycetemcomitans* induces MMP-9 expression and proatherogenic lipoprotein profile in apoE-deficient mice. *Microb Pathog* 2008;**44:**111–117.
48. Zhou Q, Amar S. Identification of signaling pathways in macrophage exposed to *Porphyromonas gingivalis* or to its purified cell wall components. *J Immunol* 2007;**179:**7777–7790.
49. Holt SC, Kesavalu L, Walker S, Genco CA. Virulence factors of *Porphyromonas gingivalis*. *Periodontol 2000* 1999;**20:**168–238.
50. Tonetti MS, D'Aiuto F, Nibali L *et al*. Treatment of periodontitis and endothelial function. *N Engl J Med* 2007;**356:** 911–920.