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Clarithromycin suppresses the periodontal bacteria-accelerated abdominal aortic aneurysms in mice

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Background and Objective: Although clarithromycin (CAM) has many biological functions, including regulation of MMPs, little is known about its effect on abdominal aortic aneurysms. Periodontopathic bacteria have been reported to be associated with several kinds of circulatory diseases. The purpose of this study was therefore to clarify the effect of CAM on periodontopathic bacteria-accelerated abdominal aortic aneurysms.

Material and Methods: Abdominal aortic aneurysm was produced in mice by the peri-aortic application of 0.25 M $CaCl_2$. The mice were inoculated once per week with live *Porphyromonas gingivalis*, which is one of the major periodontopathic bacteria. Test mice ($n = 8$) were given a daily oral dose of CAM, while control mice ($n = 13$) were not.

Results: Four weeks after the operation, the *P. gingivalis*-injected and CAM-treated mice showed a significant decrease in the aortic diameter in comparison with the mice only injected with *P. gingivalis*. Histopathologically, the samples obtained from the *P. gingivalis*-injected and CAM-treated mice showed less elastic degradation. Moreover, the plasma MMP-2 concentration of the CAM-treated mice decreased significantly.

Conclusion: These findings suggest that CAM administration is useful to suppress periodontal bacteria-accelerated abdominal aortic aneurysms via MMP regulation.

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Abdominal aortic aneurysm (AAA) is a common and life-threatening disorder (1,2), and inflammation appears to play a critical role in AAA development and progression (3). An increase of MMPs has been demonstrated in human aneurysm tissue specimens (4,5). In periodontal diseases, which are characterized by gingival inflammation and the loss of periodontal support tissue, MMPs also play important roles (6). Periodontopathic bacteria generate host immunological inflammatory responses, thus resulting in the secretion of cytokines and MMPs (7), and eventually leading to the extracellular matrix destruction of the periodontal tissues (8).

Recent studies suggest that oral infection, such as gingivitis and periodontitis, is associated with several kinds of systemic diseases, such as infectious endocarditis and cardiovascular disease (9,10). Epidemiological studies showed that periodontal disease significantly increased the risk of cardiovascular diseases (11–14).

Furthermore, animal studies demonstrated an association between the prevalence of periodontal pathogens, bacterial products, periodontitis and the incidence of cardiovascular events (15). Bacteremia has been reported after periodontal treatment and daily routine oral hygiene procedures (16). Periodontopathic pathogens, especially *Porphyromonas gingivalis*, which is one of the most common pathogens related to periodontitis, were detected in a large proportion of specimens of diseased arteries from AAA patients (17). Periodontal bacteria have been found in diseased vessel walls, although the contribution of these bacteria to the acceleration of vascular diseases remains unknown (18). Our previous study demonstrated that infection with periodontal bacteria could induce the development of AAAs in mice (19). We discovered that *P. gingivalis*, but not *Aggregatibacter actinomycetemcomitans*, could accelerate or even initiate the progression of experimental AAA. A significant increase was observed in the aortic diameter of *P. gingivalis*-injected mice compared with the vehicle-treated control mice ($p < 0.05$). Immunohistochemical analysis found a significantly higher number of CD8-positive and monocyte/macrophage marker (MOMA2)-positive cells in the aneurysmal samples of *P. gingivalis*-injected mice. The concentration of MMP-2 in the aorta was also higher in *P. gingivalis*-injected mice.

Clarithromycin (CAM) is known to be a potent antibiotic in microbial infections. Recently, multiple biological effects of CAM have been reported, such as alteration of inflammatory factors (20,21). We recently revealed that CAM suppressed cardiac rejection (22) and myocarditis (23). In both studies, it was shown that CAM could suppress MMP expression and activity. Although long-term doxycycline treatment for MMP inhibition has been reported, little is known about the effect of CAM on MMP regulation and AAA induced by periodontopathic bacteria. On this basis, the purpose of the present study was to clarify whether CAM was effective in the development of periodontopathic bacteria-accelerated AAA and to compare the effectiveness of CAM in AAA suppression with doxycycline.

Material and methods

The murine subcutaneous chamber model

Male C57BL/6 mice (8–10 wk old, weighing 20–25 g) were obtained from Japan Clea, Co. (Tokyo, Japan). We used a modification of the subcutaneous chamber model as previously described (24,25). Mice were anesthetized with 3.6% chloral hydrate (intraperitoneal administration, 0.1 mL/10 g body weight). Chambers (length 10 mm, diameter 5.0 mm) constructed from coils of stainless-steel wire were implanted subcutaneously in the back of each mouse. This investigation conforms to the Guide for the Care and Use of Laboratory Animals in the Tokyo Medical and Dental University.

Bacterial growth and immunization

Porphyromonas gingivalis, strain ATCC A7A1-28, was grown on blood agar plates in an anaerobic chamber with 85% N_2, 5% H_2 and 10% CO_2. After incubation at 37°C for 2–3 d, the bacterial cells were inoculated into a peptone yeast extract with brain–heart infusion for 2 d incubation in the same conditions. The bacterial concentrations were standardized to 10^8 colony-forming units (CFU)/mL. Fourteen days before AAA induction, the mice were immunized by subcutaneous injections of heat-killed (at 80°C for 10 min) *P. gingivalis* (0.1 mL of 10^8 CFU/mL). The levels of anti-*P. gingivalis*-specific IgG in the plasma were determined by an ELISA, as previously described (26). The plasma samples were obtained before coil implantation and when the mice were killed with overdose application of anesthetic agent.

Aneurysm induction and infection

The mice were anesthetized with 3.6% chloral hydrate (intraperitoneal administration, 0.1 mL/10 g body weight) and then underwent a laparotomy. Aneurysm induction by a 15 min application of 0.25 M $CaCl_2$ was performed as described previously (3). The diameter of the abdominal aorta was measured before application and immediately postmortem. The subcutaneous injections of live *P. gingivalis* (0.1 mL of 10^8 CFU/mL) were performed once per week. Four weeks after $CaCl_2$ application, samples of the aorta were obtained for pathological analysis. Figure 1 shows the time schedule of this study.

Reagents

Clarithromycin was kindly provided by Taisho Toyama Pharmaceutical Co., Ltd (Tokyo, Japan). In the test group ($n = 8$), the mice were dosed with CAM (100 mg/kg/d) orally once a day. In the control group ($n = 13$), CAM administration was not performed. We selected the dose based on our previous papers (22,23). Doxycycline is known to be an effective inhibitor of MMPs. Long-term doxycycline treatment for AAA prevention via MMP inhibition has been reported (27). In order to know the comparative effect of CAM, we also used doxycycline (Sigma-Aldrich, St Louis, MO, USA) in this study. The doxycycline group of mice ($n = 8$) were dosed with doxycycline (30 mg/kg/d) orally once a day.

Histopathology

A histopathological analysis was performed as described previously (22). The aorta samples were obtained from the area where the maximal aortic expansion was observed when the mice were killed. The sections were stained with Elastica van Gieson. We used a modification of the average aortic wall architecture score, as previously described (28). 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.

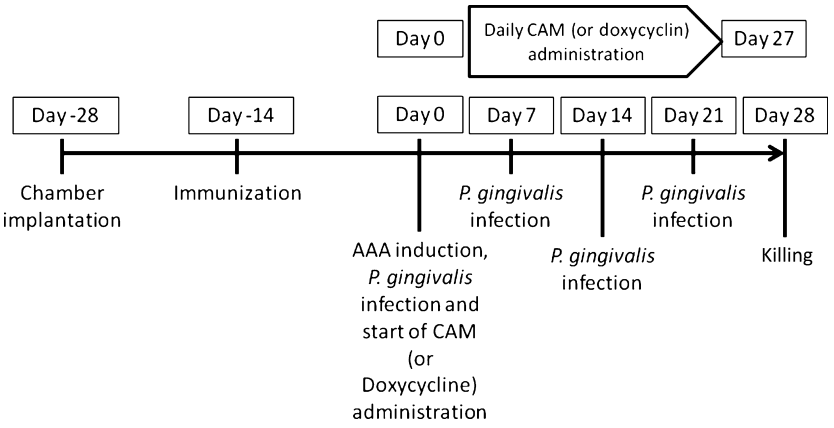

Fig. 1. Time schedule of this study. Live *Porphyromonas gingivalis* was injected immediately after $CaCl_2$ treatment and on days 7, 14 and 21 after $CaCl_2$ treatment. Clarithromycin (CAM) or doxycycline was administered immediately after $CaCl_2$ treatment and *P. gingivalis* infection. Clarithromycin or doxycycline administration continued daily for 27 d after $CaCl_2$ treatment.

Quantification of MMP-2, MMP-9 and TIMP-1 in the plasma using ELISA

The plasma levels of MMP-2, MMP-9 and TIMP-1 were determined by ELISA with a Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA). Murine plasma was collected before coil implantation and immediately postmortem, and ELISA was performed according to the manufacturer's instructions.

Microbiological analysis of chamber exudates

The chamber exudates were collected on days 1, 3 and 6 after *P. gingivalis* injection. We performed the anaerobic culture of the samples in the same way as described above (subsection 'Bacterial growth and immunization'). The concentration of live *P. gingivalis* was calculated.

Statistical analysis

All data are expressed as mean values ± SEM. Pearson's chi-squared goodness-of-fit test was used to determine whether data were normally distributed or not. The intragroup differences of anti-*P. gingivalis* antibodies (Fig. 2) and the diameters (Table 1) were analysed using Student's paired *t*-test, because the data were normally distributed. The diametric difference between the three groups (Table 1) was analysed using one-way analysis of variance combined with Bonferroni test, because the data were normally distributed. Intergroup MMP and TIMP levels after treatment (Table 2) were analysed using Student's unpaired *t*-test, because the data were normally distributed. The intergroup difference of elastic degradation scores (Fig. 4) was analysed using the Mann–Whitney *U*-test, because the data were not a continuous variable. A value of $p < 0.05$ was considered to be statistically significant. Statistical analysis was performed using StatView software (SAS Institute Inc., Cary, NC, USA).

Results

Diameter of abdominal aorta

The repeated bacterial injections significantly increased the plasma concentration of anti-*P. gingivalis* IgG in both groups (Fig. 2). Table 1 shows the diameter of abdominal aortas before and after treatment. The aortic diameter of *P. gingivalis*-infected mice showed a significant increase after the $CaCl_2$ treatment compared with pretreatment ($p < 0.05$). In contrast, CAM administration inhibited the progressive effect of $CaCl_2$ treatment and *P. gingivalis* infection. A statistical difference of aortic diameter was not demonstrated between before and after treatment in the CAM group. In comparison with the doxycycline group, we discovered the effect of CAM was comparable (Table 1). The aortic diameter of CAM-treated mice increased 1.16 ± 0.05-fold and that of doxycycline-treated mice increased 1.16 ± 0.05-fold, while that of control mice increased 1.51 ± 0.06-fold.

To calculate the number of live *P. gingivalis* in the chamber, we obtained the chamber exudates after *P. gingivalis* injection and cultured these samples. The live *P. gingivalis* in the chamber of the CAM-treated mice showed no statistical difference at each time point of measurement in comparison with the control group (data not shown).

Histopathological analysis

A histopathological analysis of the aortic sections from the mice was performed (Fig. 3). Fragmentation of the medial elastic lamellae was shown in $CaCl_2$-treated and *P. gingivalis*-infected mice. Degradation and the flattening of the elastic lamellae were inhibited by CAM administration. The average aortic wall architecture score was measured (Fig. 4). Degradation of the aortic wall was greater in the group infected with *P. gingivalis* only than in the *P. gingivalis*-infected and CAM-treated group ($p < 0.05$).

Plasma levels of MMP-2, MMP-9 and TIMP-1

Table 2 shows the concentrations of MMP-2, MMP-9 and TIMP-1 in the plasma of the control and CAM-treated mice before coil implantation and postmortem. The MMP-2 level of *P. gingivalis*-infected and CAM-treated mice was significantly reduced in comparison with the mice infected with *P. gingivalis* only. The MMP-9 and TIMP-1 levels did not change with the administration of CAM.

Discussion

We have previously reported that periodontal bacterial infection promoted the progression of AAA (19). The results of that study showed that

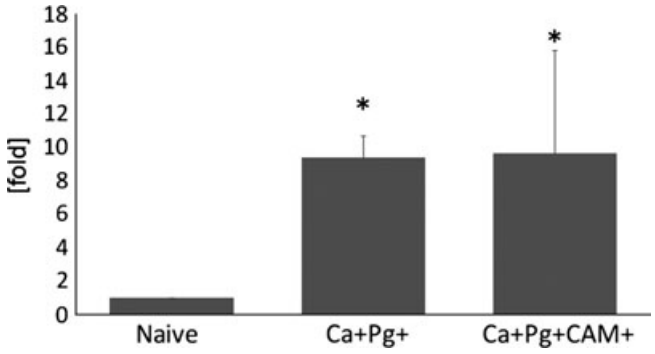

Fig. 2. Anti-*P. gingivalis* antibody. The effect of the injection of bacteria on the plasma levels of anti-*P. gingivalis* antibody was determined. The plasma samples were obtained before coil implantation (naive) and at postmortem of *P. gingivalis*-infected mice with (Ca+ Pg+ - CAM+) and without CAM administration (Ca+ Pg+). *$p < 0.05$ vs. naive.

Table 1. Diameter of abdominal aorta (in millimeters; means ± SEM)

	Ca+ Pg+	Ca+ Pg+ CAM+	Ca+ Pg+ DOX+
Before	0.53 ± 0.03	0.57 ± 0.03	0.50 ± 0.03
After	0.80 ± 0.03*	0.66 ± 0.03*†	0.58 ± 0.02*†

Abbreviations: Ca+, $CaCl_2$ treated; CAM+, CAM administered; DOX+, doxycycline administered; and Pg+, *P. gingivalis* injected.
*$p < 0.05$ vs. before the treatment.
†$p < 0.05$ vs. the control group.

Table 2. Plasma MMP and TIMP levels (in nanograms per milliliter; means ± SEM)

	Naive	Ca+ Pg+	Ca+ Pg+ CAM+
MMP-2	169.9 ± 7.8	136.4 ± 19.1	83.9 ± 10.8*
MMP-9	12.3 ± 7.1	84.3 ± 17.2	79.1 ± 14.8
TIMP-1	0.7 ± 0.1	2.3 ± 0.3	1.7 ± 0.7

Abbreviations as in Table 1.
*$p < 0.05$ vs. Ca+ Pg+ group.

repeated injection with *P. gingivalis* accelerated the progression of AAA in a murine model. A significant increase in the concentration of MMP-2 in aorta samples from *P. gingivalis*-infected mice was also demonstrated. This means that *P. gingivalis* infection induced an inflammatory reaction in the aorta and increased the level of MMP-2. This could be the reason why elastic fiber degradation and AAA progression occurred after *P. gingivalis* infection. Although periodontal treatment is the best way to decrease the burden of bacteria, some pharmacological approaches should be found. In this report, we demonstrated that CAM inhibited the periodontal pathogen-accelerated experimental AAA progression and MMP expression. We found that the aortic diameter of the *P. gingivalis*-infected and CAM-treated mice increased slightly, while the *P. gingivalis*-infected mice showed a greater increase in the aortic diameter after $CaCl_2$ treatment (Table 1). Figure 2 shows that anti-*P. gingivalis* antibody increased after *P. gingivalis* infection regardless of CAM administration. Moreover, the volume of living *P. gingivalis* in the CAM-treated group was not statistically different from that in group not treated with CAM (data not shown). These observations mean that CAM would be effective for the prevention of AAA development separately from antimicrobial activity. Clarithromycin might be useful as an anti-MMP agent.

An increase of MMPs has been demonstrated in human aneurysm tissue specimens (4,5). Both MMP-9 and MMP-2 are required and work in concert to produce AAA (3). The MMPs have been shown to play a pivotal role in aneurysm development in the AAA induction model (29). Therefore, regulation of MMPs could be a main strategy to suppress AAA development. In contrast, in periodontitis patients, inflammatory markers, such as interleukin-1β, prostaglandin E_2 and tumor necrosis factor-α (TNF-α), are increased (30). It has been shown that periodontopathic bacteria generate host immunological inflammatory responses, thus resulting in the secretion of cytokines and MMPs (7). In human studies, it has already been demonstrated that periodontal infection results in bacteremia (31,32), endothelial dysfunction (33) and systemic inflammation (30). Several experiments indicate that *P. gingivalis* infection can lead to MMP production in an inflammatory reaction (34–36). These reports indicate that periodontal infection may represent a favorable background for circulatory diseases; however, the underlying mechanism has not yet been clarified. Although a number of recent epidemiological studies suggest that periodontitis is one of the key risk factors for the onset of cardiovascular diseases (18), epidemiological research cannot identify the cause. Metastatic pathways may be responsible for the consequences of periodontal infections for cardiovascular diseases, such as spread of infection resulting from a transient bacteremia, injury by circulating microbial toxins, and excess inflammation arising from an immune response to oral bacteria (37).

Clarithromycin is known to be a potent antibiotic against microbial infections. However, it has been reported that CAM has many other biological effects, such as alteration of inflammatory markers (20,21). Clarithromycin has been reported to be able to inhibit MMP expression and gelatinolytic activities, which results in inflammatory cell infiltration (38). In the present study, CAM-treated mice showed lower MMP levels in plasma samples (Table 2). Although we expected an anti-MMP effect rather than bactericidal effect of CAM, we

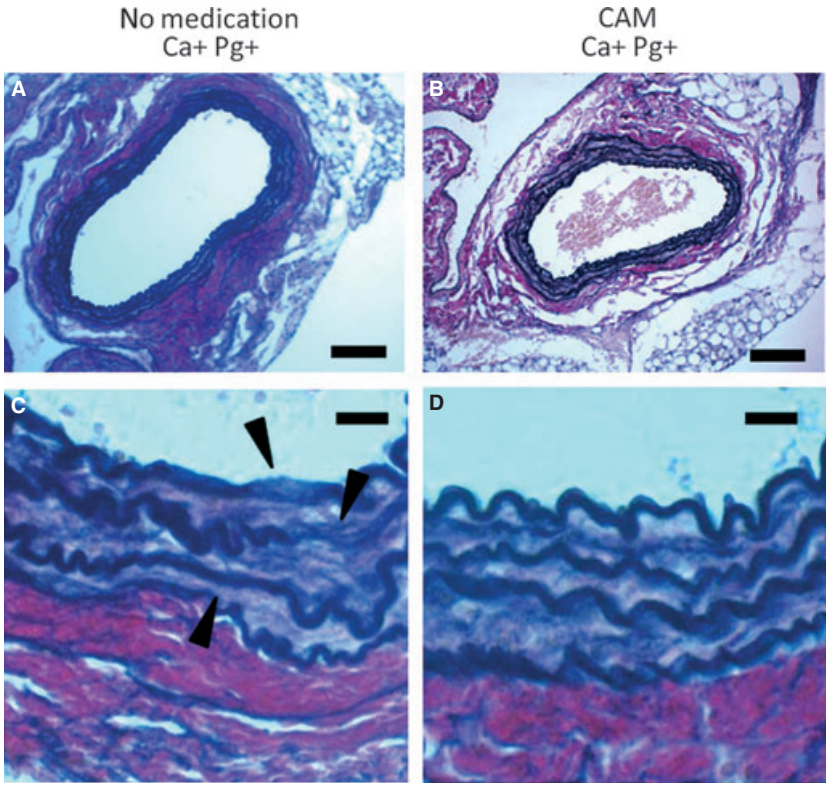

Fig. 3. Histopathological analysis. Representative aortic specimens with Elastica van Gieson staining are shown. (A) and (C) show aortic specimens of $CaCl_2$-treated and *P. gingivalis*-infected mice without medication. (B) and (D) show aortic specimens of $CaCl_2$-treated and *P. gingivalis*-infected mice with CAM. Scale bars represent 100 (A and B) or 10 μm (C and D). Arrowheads show the fragmentation and the flattening of elastic fiber.

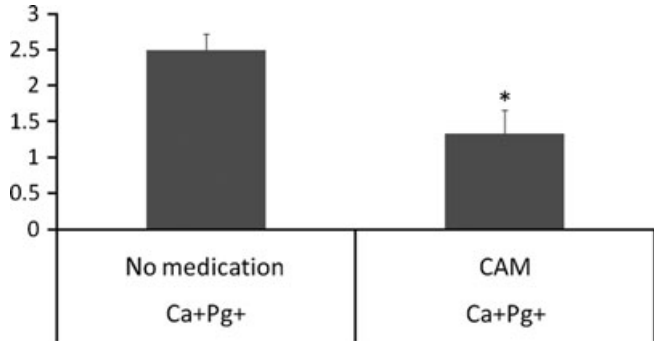

Fig. 4. Average aortic wall architecture score. The average aortic wall architecture score was measured as detailed in the Material and Methods section. The degradation of aortic wall was inhibited by CAM administration. $*p < 0.05$.

had no information regarding CAM dose as an anti-MMP agent in mice. Thus, the dose of 100 mg/kg/d, as used in the previous paper from our laboratory (22), was selected. From the results, we consider that the dose of CAM used in this study accomplished no or little bactericidal effect and had a potent anti-MMP effect. Some tetracycline antibiotics have already been studied because of their known inhibition of MMPs (39). It was demonstrated that doxycycline could suppress aortic wall MMP activity, elastin degradation and aneurysm development (40). In the $CaCl_2$ model, doxycycline showed dose-dependent inhibition of aneurysm expansion (41). Moreover, several clinical studies have suggested that doxycycline can inhibit MMPs in aneurysm tissue (42,43). In the present study, we observed that CAM and doxycycline effectively prevented AAA expansion. As there are many reports in which doxycycline inhibited AAA progression as described above, we only showed data regarding the aortic diameter of the doxycycline-treated group (Table 1). However, continued administration of the same class of agents can cause adverse effects, such as allergy. Although every agent has some cytotoxity, the cytotoxic activity of tetracycline is higher than CAM (44). Clarithromycin is superior to doxycycline because it is safer when taken for a long period. Long-term 14-member ring macrolide therapy has been established for the clinical treatment of diffuse panbronchiolitis (45). It is also reported that long-term CAM therapy is effective for the reduction of cardiovascular events (46–48), and that no serious adverse events were observed. Although long-term doxycycline treatment for MMP inhibition has been reported, in this regard, CAM is superior to tetracycline. Our study showed the possibility of CAM intake to inhibit the activation of MMPs and the progression of AAA. As CAM is broadly used as a popular antibiotic, a next stage of clinical trials using CAM could be performed with relative ease.

The mechanism by which CAM inhibited the release of MMP-2 in this study is very important. Many *in vitro* and *in vivo* studies have suggested that macrolides, including CAM, inhibit the release of proinflammatory cytokines, especially TNF- α (49–51). Proinflammatory cytokines, such as TNF- α, have been shown to induce the release of MMP-2 (52). Therefore, MMP-2 regulation by CAM could be induced via TNF-α inhibition. However, the underlying mechanisms of the anti-inflammatory effects of CAM are still unclear (49). Changes in local or plasma concentrations of MMPs or cytokines, such as TNF-α, could not fully explain the mechanism of *P. gingivalis*-induced AAA. Further investigation is needed to understand the mechanism of AAA progression with *P. gingivalis* infection.

Mechanical intervention is currently the only treatment shown to be effective in preventing AAA rupture and aneurysm-related death (43). There is an urgent need to seek new methods for medical management of small AAA. In conclusion, it was shown that MMP inhibition by CAM suppressed the periodontal bacteria-accelerated AAA in this study. Periodontal treatment combined with MMP regulation by CAM may prevent periodontal bacteria-accelerated AAA in clinical settings.

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