Periodontal Pathogen *Aggregatibacter actinomycetemcomitans* Deteriorates Pressure Overload-Induced Myocardial Hypertrophy in Mice

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SUMMARY

Although a relationship between periodontitis and myocardial hypertrophy has been reported, the precise mechanism has not been clarified. The purpose of this study was to investigate the association between periodontal infection and myocardial hypertrophy. Transverse aortic constriction (TAC) was performed. Mice were injected with *Aggregatibacter actinomycetemcomitans* (A.a.) (0.1 mL of 10⁸ CFU/mL) in the infected group and PBS in the control group. Echocardiography, histopathology, and immunohistochemistry were performed. Echocardiography indicated that left ventricular fractional shortening had decreased in the infected group compared to the control group on day 28. Heart to body weight ratio increased in the infected group compared to the control group. Histopathologically, A.a.-infected mice showed markedly enhanced cardiac hypertrophy, fibrosis and arteriosclerosis 4 weeks after TAC operation. Immunohistochemistry revealed that expression of MMP-2 in the interstitial tissue was enhanced in the infected group. These results suggested that the periodontal pathogen caused a deterioration of pressure overload-induced myocardial hypertrophy through MMP activation. (Int Heart J 2012; 53: 324-330)

Key words: Ventricular remodeling, Bacteria

Myocardial hypertrophy is an adaptive response to compensate for pressure overload caused by hypertension and aortic stenosis.¹⁾ This remodeling process consists of hypertrophic changes in cardiac myocytes and abnormalities of the extracellular matrix (ECM) network, which are both responsible for changes in systolic and diastolic function.²⁾ Matrix metalloproteinases (MMPs) represent an important biologic system within the myocardium designed to maintain the microenvironment of the ECM and play a significant role in cardiac remodeling.³⁾ MMP-2 and -9 expression was reported to be enhanced in pressure-overloaded cardiac hypertrophy.⁴⁾ The myocardial mRNA levels of tissue inhibitor of matrix metalloproteinase (TIMP)-1 and -2 were also significantly elevated.⁵⁾ Brain natriuretic peptide (BNP) is known to be a marker for volume and pressure overload⁶⁾ and its level increased in documented left ventricular diastolic dysfunction.⁷⁾

Periodontitis is a chronic inflammatory disease caused by gram-negative anaerobic bacteria, that results in bone resorption, destruction of the connective tissue, and loss of teeth.⁸⁾ Inflammatory cells secrete cytokines and matrix-degrading pro-

teases, such as tumor necrosis factor (TNF)-alpha, interleukin (IL)-1beta, IL-6, IL-8, prostaglandin E2, and MMPs which play a significant role in periodontal destruction.^{9,10)} MMPs, particularly MMP-2, MMP-9, and MMP-13 are involved in the gingival ECM destruction of periodontitis.¹¹⁾ Higher concentrations of MMP-9 were detected in blood samples of periodontitis patients.¹²⁾ Recently, researchers have indicated that there may be a connection between periodontitis and cardiac hypertrophy,^{3,13)} however, no study has clarified this relationship. Thus, the purpose of this study was to investigate the association between periodontal disease and cardiac hypertrophy.

METHODS

Animals: The male C57BL/6 mice (8-10 weeks, 20-25 g) were obtained from Japan Clea, Co. (Tokyo). Chambers (length 10 mm, diameter 5.0 mm), which were constructed from coils of stainless wire, were implanted subcutaneously into the backs of the mice. After the healing period, the cham-

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bers were used as a biological compartment for inducing inflammation. This investigation conformed to the Guide for the Care and Use of Laboratory Animals of Tokyo Medical and Dental University.

Bacterial growth: *Aggregatibacter actinomycetemcomitans* (A.a.) is a gram-negative facultative anaerobic species that is known to be a major cause of aggressive periodontitis. A.a., strain Y4, was obtained by culturing on TSBV agar plates and incubating in 5% CO₂ in air at 37°C for 3 to 5 days. The purity of each culture was checked by phase-contrast microscopy and bacterial cells were transferred to peptone yeast extract for one to two days. The bacterial concentrations were standardized to 10⁸ colony forming units (CFU)/mL.

Infection: A.a. (0.1 mL of 10⁸ CFU/mL) or vehicle containing phosphate-buffered saline (PBS) (0.1 mL) was injected into the chambers to induce inflammation every week from the day of operation. Plasma samples were obtained following the sacrifice of the mice and the levels of anti-A.a.-specific IgG antibodies in the plasma were determined by an enzyme-linked immunosorbent assay (ELISA).

Transverse aortic constriction: Transverse aortic constriction (TAC) was performed as described previously.¹⁴⁾ Briefly, the mice were anesthetized with 3.6% chloral hydrate (intraperitoneal administration, 0.1 mL/10 g body weight) and then a thoracotomy was performed via the second intercostal space at the central upper sternal border to display the transversal aorta. The ascending aorta was ligated with an overlying 27-gauge needle, which was removed soon after ligation. Sham-operated mice underwent a similar procedure without ligation of the aorta. We used non-TAC hearts without bacteria infection as native samples.

Measurements of blood pressure: Blood pressure was measured using a tail-cuff system (BP-98A, Softron Co., Tokyo) pre-operatively and 1 week after the TAC operation.¹⁵⁾

Echocardiography: The mice were anesthetized with 3.6% chloral hydrate (intraperitoneal administration, 0.1 mL/10 g body weight). Echocardiography was performed with ultrasound equipment (Nemio, Toshiba, Tokyo) in week 4 after the TAC operation. B and M-mode views were employed to measure the left ventricular (LV) dimensions. LV end-diastolic dimension (LVIDd) and end-systolic dimension (LVIDs), and fractional shortening (%FS = [(LVIDd-LVIDs)/LVIDd]*100) were obtained from the B and M-mode tracings at the level of the papillary muscle.^{14,16)}

Organ weight: Four weeks after the TAC operation, an echocardiographic study was performed under anesthesia with 3.6% chloral hydrate. A thoracotomy was then performed and the hearts were harvested and the heart weight was measured.¹⁴⁾

Heart dissection: The hearts were cut at the level of the atrial appendage and divided into two parts (atrial and ventricular parts). Ventricular parts were then isolated into 3 equal sections across the septum.

Histopathology: Formalin (10%)-fixed, paraffin-embedded hearts were sectioned (5 µm) and stained with hematoxylin-eosin (HE) and Mallory.¹⁷⁾ We obtained 3 transverse sections per heart for histological examination. To evaluate myocardial hypertrophy, we measured the thickness of the left ventricular wall at 5 randomly selected points. The wall thickness is the average of these 5 values. To quantify the fibrosis area, blue staining of collagen fibers in samples with Mallory staining

was traced using Image-Pro Express software. The ratio of fibrosis per whole myocardium area was calculated.

Immunohistochemistry: The sections were incubated with primary antibodies against MMP-2 and MMP-9 at 4°C for 12 hours.⁸⁾ Antibody-HRP conjugate was detected with a Histofine Simplestain Kit (Nichirei Corporation, Tokyo) according to the manufacturer's protocol. The enzyme activity was detected with 3-amino-9-ethylcarbazole.¹⁸⁾ Immunohistochemical staining was semiquantitatively graded as 0; no staining, 1; very mild staining, 2; mild staining, 3; moderate staining, and 4; abundant positive staining.¹⁹⁾

RNA isolation and RT-PCR: Total RNA was isolated according to the manufacturer's protocol by Trizol reagent (Invitrogen Life Technologies).¹⁶⁾ Complementary deoxyribonucleic acid was prepared with a reverse transcriptase-polymerase chain reaction (RT-PCR) kit. PCR was performed with a PCR-kit in the presence of oligo-primers for MMP-2 (Assay ID: Mm00439498 m1), MMP-9 (Assay ID: Mm00600163 m1), TIMP1 (Assay ID: Mm00441818 m1), TIMP2 (Assay ID: Mm00441825 m1), BNP (Assay ID: Mm1255770-g1), TGF-beta (Assay ID: Mm01178820 m1), collagen I (Assay ID: Mm01302043-g1), Myh7 (Assay ID: Mm01319006-g1), NPPA (Assay ID: Mm01255747-g1) and 18s rRNA as a control.^{20,21)} Quantitative data were calculated using the comparative CT ($\Delta\Delta CT$) method and the mRNA expression was normalized by native samples.

In-gel zymography: In-gel zymography was performed using gelatin in an SDS gel as the substrate. They were separated by SDS-PAGE, and following the renaturation of the proteins with 2.5% Triton X-100, the gels were incubated for 22 hours in a zymogram developing buffer (Invitrogen). Gels were then stained with Coomassie Blue and degradation assessed densitometrically.

Statistical analysis: All data are expressed as the mean $\pm$ SEM. Between-group comparisons of the means were per-

Figure 1. ELISA. Plasma levels of anti-A.a. antibodies are shown. OD indicates optical density.

Figure 2. Echocardiogram. Representative M-mode echocardiographic images of the mice 4 weeks after TAC operation (A). Fractional shortening (%FS = [(LVIDD-LVIDs)/LVIDD]*100) values were obtained from the M-mode tracings (B).

Table. Echocardiography

	TAC + PBS (n = 5)	TAC + A.a. (n = 5)
IVSth (mm)	1.0	1.1
PWth (mm)	1.4	1.3
LVIDD (mm)	2.7	3.3
LVIDs (mm)	1.6	2.4

formed by ANOVA (Stat View, SAS Institute, Inc.). We used Student's *t*-test to compare the two groups. Differences were considered statistically significant at a value of $P < 0.05$.^{14,22)}

RESULTS

Anti-bacterial antibodies and bacterial gene detection: The plasma levels of anti-A.a. antibodies are shown in Figure 1. The plasma level of anti-A.a. IgG increased significantly. The injection of PBS had no effect on anti-A.a. antibodies (Figure 1).

Figure 3. Organ weight. Heart to body weight ratios. Heart to body weight ratios increased in mice 4 weeks after TAC operation. Heart to body weight ratios further increased in the infected group compared to the noninfected group.

Echocardiography: Echocardiographic measurements indicated that A.a.-infection decreased FS compared to the noninfected group 4 weeks after the TAC operation (Figure 2 and Table).

Blood pressure: Blood pressure was not changed one week after the TAC operation (data not shown).

Organ weight: Heart weight and heart to body weight ratios were increased in the mice 4 weeks after the TAC operation. Heart to body weight ratios further increased in the infected group compared to the sham group (Figure 3).

Histopathology: Transverse cross-sections of hearts from TAC and sham mice were evaluated. Myocardial hypertrophy, fibrosis, and arteriosclerosis were observed in mice 4 weeks after the operation. A.a.-infected mice showed additionally enhanced cardiac hypertrophy, fibrosis, and arteriosclerosis 4 weeks after the operation. Quantitative analysis revealed that the average thickness of the left ventricular wall increased in A.a.-infected mice compared to noninfected mice. The fibrosis area ratio also increased in A.a.-infected mice compared to noninfected mice (Figure 4).

Immunohistochemistry: MMP-2 and MMP-9 were expressed in the hearts of TAC mice from both groups. Quantitatively, the levels of MMP-2 were significantly elevated in the A.a.-infected mice compared to that of noninfected mice (Figure 5).

RT-PCR: The mRNA levels of MMP-2, MMP-9, TIMP1,

Figure 4. Histopathology. Representative histopathological findings with Mallory staining (A to C) and quantitative data (D and E). **A** shows myocardial hypertrophy (× 20), **B**, fibrosis (× 200) and **C**, arteriosclerosis (× 400). Myocardial hypertrophy, fibrosis, and arteriosclerosis were observed in mice 4 weeks after TAC operation. A.a.-infected mice showed additionally enhanced cardiac hypertrophy, fibrosis, and arteriosclerosis 4 weeks after TAC operation. Quantitative analysis revealed that the average thickness of the left ventricular wall increased in A.a.-infected mice compared to noninfected mice (**D**). The fibrosis area ratio also increased in A.a.-infected mice compared to noninfected mice (**E**).

TIMP2, BNP, TGF-beta, collagen I, Myh7, and NPPA were statistically comparable among the 3 groups. However, the mRNA levels of MMP-2, MMP-9, TIMP-1, TIMP-2, BNP, Myh7, and NPPA tended to be elevated in the A.a.-infected group compared with that of the noninfected group (Figure 6).

In-gel zymography: The activity of MMP-2 increased in the non-infected group compared to the sham group. However, statistically A.a. infection did not further increase activation. MMP-9 was not detected in all groups (data not shown).

Figure 5. Immunohistochemistry. Representative immunohistochemical findings (A) and quantitative results (B). MMP-2 and MMP-9 were expressed in hearts of mice from both groups. Quantitatively, levels of MMP-2 were significantly elevated in the A.a.-infected mice compared to that of noninfected mice.

DISCUSSION

The present study revealed that periodontal pathogens deteriorated pressure overload-induced myocardial hypertrophy, interstitial fibrosis, perivascular fibrosis, and impaired cardiac function. Periodontal pathogens increased the expression of MMP-2 in the interstitial myocardium of the hearts. These findings suggest that MMPs are involved in cardiac inflammatory response after periodontal pathogen infection.

A relationship between periodontal disease and heart disease has been demonstrated.²³⁾ In patients with periodontitis, inflammatory markers increase causing systemic inflammation. Many papers also reported that bacteremia is caused by invasive dental treatment, such as tooth brushing, periodontal treatment, and tooth extraction.^{24,25)} High serum antibody levels to major periodontal pathogens are associated with heart disease.²⁶⁾ The detailed pathophysiological relationship between periodontal disease and heart disease still needs to be elucidated.

Pressure overload-induced myocardial hypertrophy is caused by changes in cardiac myocytes and abnormalities in the ECM network, which are both responsible for changes in systolic and diastolic function.²⁾ Progressive LV dilatation and ECM degradation are associated with increased MMP activity, particularly MMP-1, MMP-2, MMP-9, and/or MMP-13.²⁷⁾ It is well recognized that periodontal pathogens increase the ac-

Figure 6. RT-PCR. Quantitative data of RT-PCR. The mRNA levels of MMP-2, MMP-9, TIMP1, TIMP2, BNP, TGF-beta, collagen I, Myh7, and NPPA were statistically comparable among the 3 groups. However, mRNA levels of MMP-2, MMP-9, TIMP-1, TIMP-2, BNP, Myh7, and NPPA tended to be elevated in the A.a.-infected group compared with that of the noninfected group.

tivity of MMPs. Significantly elevated levels of MMP-8 and -9 were found in subjects with advanced periodontitis.²⁸⁾ The activation of MMP-2 was also induced by the LPS of A.a.²⁹⁾ MMP-2 is involved in physiological tissue remodeling and pathological ECM degradation in the pathogenesis of perio-

dontal diseases.¹⁰⁾ In this study, we revealed that this periodontal pathogen enhances MMP-2 which might result in pressure overload-induced myocardial hypertrophy.

It was reported that MMP-2 independently induced ventricular hypertrophy. Bergman, *et al* reported that MMP-2 transgenic hearts demonstrated myocyte hypertrophy, breakdown of Z-band registration, lysis of myofilaments, disruption of sarcomere and mitochondrial architecture, and cardiac fibroblast proliferation. Thus, they concluded that expression of MMP-2 is sufficient to induce severe ventricular remodeling in the absence of superimposed injury.³⁰⁾ These results may support our data that a periodontal pathogen-induced MMP-2 deteriorated myocardial hypertrophy in TAC hearts.

This study has a limitation. We used a murine model of periodontal bacteria infection with a coil chamber implantation. This model is known to represent chronic bacteremia with periodontitis as previously described.³¹⁻³³⁾ Although this model has been widely used as a chronic periodontitis model, it does not directly represent clinical periodontitis. Other murine periodontal bacteria infection models have been reported; an intravenous injection model represents an acute periodontitis and an intragingival injection model represents local infection. Because these models were not suitable to test the systemic and chronic adverse effects, we employed the coil chamber implantation model.

Our recent paper demonstrated that a periodontal pathogen promoted murine abdominal aortic aneurysms via MMP-2 induction.³¹⁾ We also revealed that a pharmacological inactivation of MMPs resulted in the prevention of abdominal aortic aneurysm development.³²⁾ Thus, MMPs are key factors connecting periodontitis and cardiovascular remodeling; an intervention might be effective to prevent the progression. Further study is needed to elucidate the mechanism and clinical implication between periodontitis and cardiovascular diseases.

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