Toll-Like Receptor-2 Plays a Fundamental Role in Periodontal Bacteria-Accelerated Abdominal Aortic Aneurysms

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**Background:** Periodontopathic bacteria are detected at a high rate in specimens obtained from the aortic walls of patients with abdominal aortic aneurysm (AAA) and are involved in AAA development. The purpose of this study was to clarify the role of Toll-like receptors (TLRs), which are key receptors of virulence factors of many periodontal bacteria, on periodontopathic bacteria-accelerated AAA progression.

**Methods and Results:** AAA was produced by peri-aortic application of 0.25 mol/L CaCl$_2$, with NaCl used as a control. The mice were inoculated with live *Porphyromonas* (*P.*) *gingivalis* or vehicle once weekly. At 4 weeks after the application of CaCl$_2$, the aortic diameter of the *P. gingivalis*-infected wild-type mice showed a significant increase in comparison with vehicle control mice (*P*<0.05). The *P. gingivalis*-infected TLR-2 deficient mice showed no statistical increase in aortic diameter over the same period. The aortic diameter of the *P. gingivalis*-infected TLR-4 deficient mice statistically increased. Immunohistochemically, the levels of matrix metalloproteinase-2 and -9 in the aneurysmal samples from wild-type mice were higher than in TLR-2 deficient mice.

**Conclusions:** *P. gingivalis* accelerated the progression of experimental AAA through TLR-2 signaling. (Circ J 2013; 77: 1565–1573)

**Key Words:** Aneurysm; Inflammation; Matrix metalloproteinase; *Porphyromonas gingivalis*; Toll-like receptor

Abdominal aortic aneurysm (AAA) is a common and lethal disorder and inflammation appears to play a fundamental role in its development. The expression of matrix metalloproteinases (MMPs) increases in human aneurysm tissue specimens, and MMPs play a critical role in the progression of AAAs.

Periodontal disease is a chronic inflammatory disease characterized by gingival inflammation and the loss of periodontal support tissue, and is one of the most common chronic infectious diseases in humans. Periodontopathic bacteria induce host immunological inflammatory responses, resulting in the secretion of cytokines and MMPs. Recent studies suggest that chronic infectious diseases, including oral infection, are associated with an increased risk of cardiovascular diseases. Periodontopathic pathogens, especially *Porphyromonas* (*P.*) *gingivalis*, which is one of the most common bacteria related to periodontitis, were present in a high percentage of diseased artery specimens from AAA patients. Toll-like receptors (TLRs) are the most extensively studied innate receptors and their roles in innate and adaptive immunity are well documented. TLRs are a group of pattern-recognition molecules that mediate the innate host response to microbes, and are selectively upregulated after infection. TLR-2 and TLR-4 have been identified as the principal signaling receptors for bacterial cell wall components, and it has been demonstrated that *P. gingivalis* stimulates TLR-2 rather than TLR-4. Our hypothesis is that stimulation of TLRs by oral pathogens accelerates circulative diseases such as AAA.

Recent studies imply that periodontal disease may be a risk factor for circulative diseases, including AAAs. However, the...
relationship between periodontal bacterial infection and the development of aortic aneurysms has not yet been proven. The purpose of the present investigation was to analyze the role of TLRs in the progression of aneurysms with periodontal bacterial infection.

**Methods**

**Murine Subcutaneous Chamber Model**
The male TLR-2–/– mice and TLR-4–/– mice were obtained from Oriental Yeast Co (Tokyo, Japan) and wild-type (WT) mice (C57BL/6) were obtained from Japan Clea Co (Tokyo, Japan). The mice were anesthetized with 3.6% chloral hydrate (intraperitoneal IP administration, 0.1 ml/10 g body weight) and then chambers (length 10 mm, diameter 5.0 mm), which were constructed from coils of stainless wire, were implanted subcutaneously in the back of each mouse (8–10 weeks old, 20–25 g) as previously described. This investigation conformed to the Guide for the Care and Use of Laboratory Animals in the Tokyo Medical and Dental University.

**Confirmation of Absence of TLR-2 and TLR-4 Expression**
To confirm the absence of TLR-2 expression in TLR-2–/– mice and TLR-4 expression in TLR-4–/– mice, we checked gene expressions in 3 samples of TLR-2–/– mice and 3 samples of TLR-4–/– mice by polymerase chain reaction (PCR) according to the instruction of the knockout mice provider. For detection of the mutated allele in TLR-2–/– mice, we used the following primers: 5′-TTG GAT AAG TCT GAT AGC CTT GCC TCC-3′ and 5′-ATC GCC TTC TAT CGC CTT GTC GAG GAG-3′. The amplified products were approximately 900 base pairs. For detection of the mutated allele in TLR-4–/– mice, we used the following primers: 5′-TGT TGC CCT TCA GTC ACA GAG ACT CTG-3′ and 5′-TGT TGG GCT GTT TGT TCG GAT CCG TCG-3′. The amplified products were approximately 1,200 base pairs. Cycling temperatures were: 35 cycles of 94°C for 30 s; 30 s to 67°C; 67°C for 1 min; 74°C for 1 min; then 74°C for 10 min prior to cooling to 4°C.

**Bacterial Growth and Immunization**
The P. gingivalis strain ATCC A711-28 was grown as described previously. The bacterial concentration was standardized to 10⁶ colony-forming units (CFU)/ml. At 14 days after AAA induction by applying either CaCl₂ or NaCl, the mice were immunized by the subcutaneous injections of heat-killed (80°C for 10 min) P. gingivalis (0.1 ml of 10⁶ CFU/ml). Plasma samples were obtained before coil implantation and at the death of P. gingivalis-infected or vehicle control mice.

**Aneurysm Induction**
Aneurysm induction by 0.25 mol/L CaCl₂ was performed as described previously; 0.9% NaCl was applied as control. The mice were anesthetized with 3.6% chloral hydrate (0.1 ml/10 g body weight IP) and then underwent a laparotomy. The diameter of the aorta midway between the renal artery origin and iliac artery bifurcation was measured in triplicate. The mice were divided into 4 groups: application of CaCl₂ or NaCl and inoculation with live P. gingivalis (0.1 ml of 10⁶ CFU/ml), or vehicle containing diluted medium, phosphate-buffered saline (PBS) (0.1 ml) (n=10–14 in each group). The subcutaneous injections of bacteria were performed once weekly for 4 weeks. The mice then underwent another laparotomy and dissection. The measurements were repeated at the same location in the mid-infrarenal aorta.

**Histopathology and Immunohistochemistry**
Histopathologic analysis was performed as described previously. The sections were stained with elastica van Gieson staining. Immunohistochemical analysis was performed as described previously. The sections were incubated with a primary antibody against MMP-2 and MMP-9 (Santa Cruz Biotechnology, Inc, Santa Cruz, California, USA). Immunohistochemical staining was quantified and graded as previously described: 0, no staining; 1, very mild staining; 2, mild staining; 3, moderate staining; and 4, abundant positive staining (n=4–6 in each group).

**PCR**
Aortic samples were collected from each mouse during the bacterial challenge regimen. Total DNA was collected using a QiaAmp kit (Qiagen K.K., Tokyo, Japan), and the P. gingivalis 16S gene was detected by PCR as described previously (n=4 in each group).

**Western Blotting**
Western blot analysis was performed as described previously. The polyvinylidene difluoride membrane was incubated with primary antibodies against MMP-2 and β-actin (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA). The membrane enhanced the chemiluminescence reagent (Pierce Biotechnology Inc, Rockford, IL, USA). Enhanced chemiluminescence was detected with an LAS-1000 (Fujifilm Corporation, Tokyo, Japan). The data were obtained from 3 independent experiments (n=4 in each group).

**Quantification of MMP-2, -9, Tissue Inhibitor of Metalloproteinase (TIMP)-1 and High-Sensitivity C-Reactive Protein (hsCRP) in the Plasma Using ELISA**
The plasma levels of MMP-2, MMP-9, TIMP-1 and hsCRP were determined by an ELISA with an immunooassay kit (Quantikine ELISA kit, R&D Systems, Minneapolis, MN, USA). Murine blood was collected and within 15 min of col-
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Figure 2. All plasma samples were obtained at time of death. The level of anti- Porphyromonas (P.) gingivalis antibody in plasma was determined using ELISA. *P<0.05 between uninfected (Pg–) and infected (Pg+) groups. #P<0.05 between P. gingivalis-infected groups.

Figure 3. Aortic diameter measured before CaCl₂ or NaCl application and at time of death. (A) Wild-type (WT) mice, (B) TLR-2 knockout mice, (C) TLR-4 knockout mice. Ca–, NaCl-treated; Ca+, CaCl₂-treated; Pg–, uninfected, Pg+, Porphyromonas gingivalis-infected group. *P<0.05 compared with diameter before the treatment. #P<0.05 between groups. TLR, toll-like receptor.

Statistical Analysis

Measurements of the aortic diameter and each value are expressed as the mean±standard error of mean. Analysis of variance combined with Tukey-Kramer test was used to compare the values of the groups (Figures 2, 3, 6). The paired Student’s t-test was used to compare the original and final aortic diam-

lection 38% aqueous solution of citric acid was added before centrifugation at 1,000×g. The plasma was stored in at –80°C. ELISA was performed according to the manufacturer’s instructions (n=6 in each group).
The aortic diameters of mice before and after treatment are shown in Figure 3. Uninfected WT mice showed a significant increase in aortic diameter after CaCl2 treatment (Figure 3A). The aortic diameter of the mice in all groups showed no statistical difference before and after NaCl treatment. The aortic diameter of the \textit{P. gingivalis}-infected and CaCl2-treated WT mice showed statistical difference in comparison with uninfected CaCl2-treated WT mice (P<0.05). This confirms the fact that \textit{P. gingivalis} infection accelerates development of AAA.

In TLR-2–/– mice, neither CaCl2 treatment nor \textit{P. gingivalis} infection induced progression of AAA (Figure 3B). On the other hand, the aortic diameter of the \textit{P. gingivalis}-infected and CaCl2-treated TLR-4–/– mice statistically increased, although only CaCl2 treatment did not induce progression of AAA in the TLR-4–/– mice (Figure 3C).

### Diameter of Abdominal Aorta

The aortic diameters of mice before and after treatment are shown in Figure 3. Uninfected WT mice showed a significant increase in aortic diameter after CaCl2 treatment (Figure 3A). The aortic diameter of the mice in all groups showed no statistical difference before and after NaCl treatment. The aortic diameter of the \textit{P. gingivalis}-infected and CaCl2-treated WT mice showed statistical difference in comparison with uninfected CaCl2-treated WT mice (P<0.05). This confirms the fact that \textit{P. gingivalis} infection accelerates development of AAA.

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### Histopathologic Analysis

The histopathologic analysis of aortic sections from these mice showed fragmentation of medial elastic lamellae (Figure 4). The \textit{P. gingivalis}-infected and CaCl2-treated WT mice showed degradation and fragmentation of elastic fibers compared with the uninfected CaCl2-treated WT mice. The elastic fibers in \textit{P. gingivalis}-infected and CaCl2-treated TLR-2–/– mice were undamaged, but in the \textit{P. gingivalis}-infected and CaCl2-treated TLR-4–/– mice they were mildly degraded.
Figure 5. (A) Aortic sections from the CaCl$_2$-treated and Porphyromonas (P.) gingivalis-infected groups were incubated with matrix metalloproteinase (MMP)-2. Scale bars=50 μm. Arrows indicate high expression of MMP-2. (B) The average MMP-2 stained score of the aortic wall was determined (described in the Methods). (C) Aortic sections from the CaCl$_2$-treated and P. gingivalis-infected groups incubated with MMP-9. Scale bars=50 μm. Arrows indicate high expression of MMP-9. (D) The average MMP-9 stained score of the aortic wall was determined (described in the Methods).
treated WT mice, which means that *P. gingivalis* infection leads to MMP-2 upregulation in the aorta. However, a very low level of MMP-2 in *P. gingivalis*-infected and CaCl₂-treated TLR-2−/− mice was observed (Figure 5B). In *P. gingivalis*-infected and CaCl₂-treated TLR-4−/− mice, we observed a

Immunohistochemistry

Aortic samples of each group were also analyzed immunohistochemically (Figure 5). Consistent with previous results,²² the MMP-2 level in the *P. gingivalis*-infected and CaCl₂-treated WT mice increased compared with uninfected CaCl₂-treated WT mice, which means that *P. gingivalis* infection leads to MMP-2 upregulation in the aorta. However, a very low level of MMP-2 in *P. gingivalis*-infected and CaCl₂-treated TLR-2−/− mice was observed (Figure 5B). In *P. gingivalis*-infected and CaCl₂-treated TLR-4−/− mice, we observed a

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**Figure 6.** Expression of MMP-2/β-actin in aorta analyzed by western blotting. Pg−, uninfected group; Pg+, infected group; MMP, matrix metalloproteinase.

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**Figure 7.** Plasma samples obtained at time of death. MMP-2 (A), MMP-9 (B), TIMP-1 (C) and hsCRP (D) levels in plasma were determined using ELISA. *P<0.05 between uninfected (Pg−) and infected (Pg+) groups. hsCRP, high-sensitivity C-reactive protein; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase.
The tendency of MMP-9 expression was similar to that of MMP-2. MMP-9 was highly expressed in aortas from *P. gingivalis*-infected and CaCl$_2$-treated WT mice, although a very low level of MMP-9 in *P. gingivalis*-infected and CaCl$_2$-treated TLR-2$^{-/-}$ mice was observed (Figure 5D).

**MMP-2 Level in Aortic Samples**

Figure 6 shows the level of MMP-2 protein in aortic samples by western blotting. MMP-2 in the aorta increased in *P. gingivalis*-infected WT mice, although the difference was not statistically significant. In TLR-2$^{-/-}$ and TLR-4$^{-/-}$ mice, MMP-2 levels did not increase after treatment and infection.

**Presence of Bacteria in the Aorta**

The PCR method was used to detect *P. gingivalis* in aortic samples. Samples obtained from *P. gingivalis*-infected WT, TLR-2$^{-/-}$ and TLR-4$^{-/-}$ mice were negative for *P. gingivalis* transcripts (data not shown).

**Levels of MMP-2 and -9, TIMP-1 and hsCRP in Plasma**

Figure 7 shows the concentrations of MMP-2, MMP-9, TIMP-1 and hsCRP in the plasma of uninfected and *P. gingivalis*-infected mice at the time of death. CaCl$_2$ treatment did not affect the plasma levels of MMPs, TIMP-1 and hsCRP, which is why we assessed the combined plasma levels of the NaCl- and CaCl$_2$-treated groups. In TLR-4$^{-/-}$ mice, the plasma MMP-9 level in *P. gingivalis*-infected mice increased in comparison with uninfected control mice. Apart from this, there was no increase of MMPs, TIMP-1 or hsCRP in plasma after *P. gingivalis* infection or CaCl$_2$ treatment.

**Discussion**

A strong association between periodontal disease and cardiovascular diseases has been widely demonstrated. Periodontal disease has been reported as a significant independent risk factor for vascular diseases, because it results in bacteremia and the periodontopathic bacteria attach to vascular endothelial cells, especially those with intimal injury. It has been demonstrated that periodontopathic bacteria generated host immunological inflammatory responses, resulting in the secretion of cytokines and MMPs. In contrast, the involvement of periodontal disease with vascular disorders has not been clearly elucidated.

TLRs recognize microbial components known as pathogen-associated molecular patterns. Recognition triggers the release of inflammatory cytokines for host defense. TLR-2 and -4 have a common signaling pathway, which involves recruitment of the Toll/IL-1 receptor domain-containing adapter protein and MyD88, and downstream signaling events activate nuclear factor $\kappa$B, leading to the induction of inflammatory cytokines. In this way, recognition by TLRs induces an inflammatory reaction followed by MMP activation. However, TLR-2 and TLR-4 have other signaling pathways, making it difficult to clarify the mechanism of TLR recognition. Many periodontal bacteria, such as *P. gingivalis*, stimulate TLR-2, whereas the lipopolysaccharide of Gram-negative bacteria is generally recognized by TLR-4. In this study, TLR-2 deficient mice with *P. gingivalis* infection showed less development of AAA than WT mice, which means that *P. gingivalis* recognition by TLR-2 induces an inflammatory reaction and promotes AAA progression. Because a smaller effect of TLR-4 knockout was observed in our study, TLR-2 is important with *P. gingivalis* infection for the promotion of inflammation. However, in TLR-4 knockout mice, aorta expansion was slightly inhibited after CaCl$_2$-treatment and *P. gingivalis* infection. Spiller et al showed that surface TLR-2 expression and sensitivity for TLR-2 ligands were enhanced in a TLR-4-dependent manner; thus, TLR-4 knockout may suppress TLR-2 signaling. In the TLR-2 deficient and TLR-4 deficient mice, aorta expansion was slight after CaCl$_2$ treatment without infection (Figure 3). This result leads to a hypothesis that TLR-2 and TLR-4 play a role in AAA development, at least in this CaCl$_2$ model. To our knowledge, there are no reports regarding the relationship between TLRs and AAA, although some studies have shown the involvement of TLRs in vascular diseases. TLR-2 mediated diet-associated atherosclerosis, and TLR-2-mediated inflammation and matrix degradation has been demonstrated in atherosclerosis. In this way, TLR deficiency influenced AAA development via inflammation and matrix degradation in the present study.

The importance of TLR-2 and the effect of periodontal infection in circulative diseases have been reported recently. Increased TLR-2 expression after *P. gingivalis* stimulation was demonstrated. Excessive stimulation of TLR-2 was also shown after infection with Gram-negative bacteria, especially *P. gingivalis*. A genetic deficiency of TLR-2 reduces not only pathogen-induced but also diet-associated atherosclerosis. Invasive *P. gingivalis* increased periodontal destruction and accelerated atherosclerotic plaque formation. Although impaired *P. gingivalis* clearance in TLR-2 deficient mice has been reported, other investigators have shown rapid clearance of *P. gingivalis* in TLR-2 deficient mice. The role of TLR-2 in the progression of infection might depend on the particular cell types present at the infected site. In the present study, we assessed the presence and clearance of *P. gingivalis* in WT, TLR-2 deficient and TLR-4 deficient mice; however, *P. gingivalis* was not detected in any samples from these mice by PCR method. A recent review discussed the pathogenic mechanisms of vascular disease progression by periodontal infection. Several pathophysiologic pathways linking periodontal infection and vascular disease involve not only direct but also indirect interactions. Vascular infection via bacteremia is a direct mechanism, but systemic inflammation and mimicry are proposed to be indirect factors. Therefore, the precise mechanism of how periodontal infection accelerates vascular disease has not been proven. Considering the fundamental role of TLR-2 in the inflammatory response and infection clearance, further research regarding the effect of periodontal pathogens on circulative diseases through TLR recognition is needed.

Inflammation appears to play a fundamental role in the development and progression of AAA. It is associated with a disruption of the orderly lamellar structure of the aortic media. Human AAA specimens show high levels of inflammatory infiltrates in both the media and the adventitia. Increased expression of MMPs has been demonstrated in human aneurysm samples. MMPs, particularly MMP-2 and MMP-9, are critical factors for AAA initiation and development; experimental AAA was not induced in MMP-2- or MMP-9-deficient mice. In the present study, aortic expansion and fiber degradation accompanied high MMP-2 expression, which was confirmed by immunohistochemical (Figure 5) and western blot (Figure 6) analyses. The aortic dilatation of WT mice was the largest in the 3 groups after CaCl$_2$ treatment and *P. gingivalis* infection, and MMP-2 expression was high in the WT mice. On the other hand, the aortic expansion of TLR-2 deficient mice after CaCl$_2$ treatment and *P. gingivalis* infection was inhibited, with lower expression of MMP-2. Aortic samples...
were too small, which might be why there was no statistically significant difference between groups with western blotting. Circulating MMPs and TIMP-1 levels were comparable among the groups, except for the \textit{P. gingivalis}-infected TLR-4-deficient mice. Circulating hsCRP levels were also the same in each group of mice. Although an increased level of systemic MMP-9 in TLR-4-deficient mice after treatment and infection cannot fully explain the phenomenon of AAA development, the level of systemic MMP-9 may be a key factor in understanding the mechanism of periodontal infection. Further research into systemic factors at several time points is needed, because the mechanism underlying the relationship between periodontitis and circulative diseases is now being studied by many researchers worldwide.

**Study Limitations**

The limitation of the present study is the difficulty of creating a chronic infection model with periodontal bacteria. Periodontal bacteria are normally present in the oral cavity. The reason we used the subcutaneous chamber model was to induce the condition of chronic infection with periodontal pathogens. Although there is a model of orally administrated periodontal bacteria, the results from that model are not consistent. In another model, the method of intravenous bacterial infection can be used, but many researchers use an extremely high concentration of periodontal bacteria to create the infection in comparison with natural condition in humans. Therefore, we chose a steady and realistic model of periodontal bacterial infection. Immunizing with heat-killed bacteria before AAA induction is used to induce a similar condition in humans. Periodontitis patients have high levels of antibodies to periodontopathic bacteria. Because it is not proven which of the direct bacterial burden or increased inflammatory reaction enhances the acceleration of circulative disorders, we chose to use the model with immunization.

**Conclusions**

The mechanism of AAA acceleration by periodontal disease remains highly controversial, but it has been discovered that systemic inflammation initiated by TLR recognition of periodontal bacteria is important. Because elective AAA surgery has many risks, the prevention of AAA development is a fundamental clinical approach. Periodontal treatment may be an option to prevent AAA expansion. In conclusion, in systemic inflammatory reactions TLR-2 plays a fundamental role in periodontal bacteria-induced AAA. Therefore, TLR-2 blockade may be an effective option for preventing the development of AAA in periodontitis patients.

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**Disclosures**

Conflict of Interest: None declared.

**References**

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