Porphyromonas gingivalis Accelerates Neointimal Formation after Arterial Injury

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Abstract

Background: Inflammation plays a key role in neointimal hyperplasia after an arterial injury. Chronic infectious disorders, such as periodontitis, are associated with an increased risk of cardiovascular diseases. However, the effects of a periodontal infection on vascular remodeling have not been examined. We assess the hypothesis that periodontal infection could promote neointimal formation after an arterial injury. Methods: Mice were implanted with subcutaneous chambers (n = 41). Two weeks after implantation, the femoral arteries were injured, and Porphyromonas gingivalis (n = 21) or phosphate-buffered saline (n = 20) was injected into the chamber. The murine femoral arteries were obtained for the histopathological analysis. The expression level of mRNA in the femoral arteries was analyzed using quantitative reverse transcriptase polymerase chain reaction (n = 19–20). Results: The intima/media thickness ratio in the P. gingivalis infected group was found to be significantly increased in comparison to the non-infected group. The expression of matrix metalloproteinase-2 mRNA was significantly increased in the P. gingivalis infected group compared to the non-infected group. Conclusion: These findings demonstrate that P. gingivalis injection can promote neointimal formation after an arterial injury. Periodontitis may be a critical factor in the development of restenosis after arterial intervention.
CVD [3]. Several studies have demonstrated that the presence of oral bacteria in atherosclerotic plaques is implicated in the pathogenesis of CVD, and that atherosclerosis and CVD are both accelerated by periodontal disease [4, 5].

Severe generalized periodontitis causes a chronic systemic inflammatory response and changes in serum cholesterol and systolic blood pressure [6]. The patients with bacteremia after dental extraction [7, 8], dental scaling [9] and endodontic treatment [10, 11] were studied by means of lysis filtration of blood samples, with subsequent aerobic and anaerobic incubation. Recent evidence suggests that periodontal disease may play a significant role in the process of atherosclerosis [12], and DNA from Porphyromonas gingivalis, a major periodontal pathogen, has been detected in carotid atherosclerotic plaques [4] and atherosclerotic vessels [5]. Periodontal pathogens, which are present in atherosclerotic plaques, may play a role in the development and progression of atherosclerosis, leading to CVD [1, 4, 13].

Balloon angioplasty and stent implantation are the most widely used techniques for treating coronary artery stenosis. However, mechanical injury to the vascular lumen occurs during angioplasty and causes neointimal hyperplasia and vascular remodeling, thus often resulting in restenosis of the culprit lesion [14]. Although the pathogenic mechanism has not been completely resolved, an accumulating body of evidence suggests that the inflammatory response plays a key role in these processes.

Matrix metalloproteinases (MMPs) have been suggested to affect the chemotaxis of inflammatory cells through multiple mechanisms [15]. Many CVDs, such as myocardial infarction [16, 17], atherosclerosis [18] and arteriosclerosis [19, 20], involve the upregulation of MMPs by inflammatory cells and vascular smooth muscle cells. In addition, the activation of MMPs predicts the progression of vascular remodeling [21]. We hypothesized that P. gingivalis infection would contribute to the development of neointimal hyperplasia after percutaneous coronary intervention. Our laboratory previously demonstrated that infection with P. gingivalis could accelerate the progression of experimental abdominal aortic aneurysm by increasing the expression of MMP-2 [22]. The purpose of the present study was to analyze the expression of MMP-2 and other factors following P. gingivalis infection and to examine the impact of the infection on neointimal formation after an arterial injury.

Material and Methods

Animals and Study Protocol
Male mice (C57BL/6, aged 7 weeks, 20–25 g) were obtained from Japan Clea Co., Tokyo, Japan, for this study. The experimental procedures described were approved by the Animal Welfare Committee and performed in accordance with the Animal Care Standards of Tokyo Medical and Dental University.

Bacterial Preparation
P. gingivalis, strain ATCC A7A1-28, was cultivated on blood agar plates in an anaerobic chamber with 85% N₂, 5% H₂, 10% CO₂. After incubation at 37°C for 2–3 days, the bacterial cells were inoculated into a peptone yeast extract and incubated for another week. The bacterial concentration was standardized to 10⁸ colony-forming units/ml.

Chamber Model
Coil-shaped subcutaneous chambers were prepared from 0.5-mm stainless-steel wire and surgically implanted into the subcutaneous tissue of the back region of each mouse (n = 41). Fourteen days elapsed before the chambers were inoculated with P. gingivalis (n = 21). During this period, the outer incision healed completely, and the chambers became encapsulated by a thin vascularized layer of fibrous connective tissue. The chambers gradually filled with approximately 0.5 ml of light-colored transudate. Fourteen days after implantation, mice were inoculated with a 0.1-ml suspension of P. gingivalis in phosphate-buffered saline (PBS). The non-infected group was inoculated with phosphate-buffered saline only (n = 20). Mice were sacrificed 7 or 14 days after arterial injury, and the plasma was separated from blood obtained from an orbita plexus venosus puncture.

Wire Injury Model
In this study, we developed a modified arterial injury model [23]. Briefly, the femoral artery was looped and tied off with 6-0 silk sutures for temporary vascular control during the procedure. A transverse arteriotomy was made, and a flexible angioplasty guidewire was introduced and advanced 1 cm. Endothelial denudation injury of the artery was achieved by wire withdrawal injury [24].

Mice were divided into 2 groups; injured via arterial surgery and inoculated with live P. gingivalis (0.1 ml of 10⁸ colony-forming units/ml) or injured via arterial surgery and inoculated with vehicle-containing diluted medium (0.1 ml). The subcutaneous injections were performed once per week for 14 days. Fourteen days later, the mice underwent laparotomy and dissection [25]. Figure 1 shows the time schedule of this study.

Histological and Morphometrical Analyses
The histopathological analyses were performed as described previously [19]. The sections were stained with Elastica van Gieson (EvG). Complete transverse sections of arteries approximately 3 mm in length were obtained [24]. The persons who selected and measured the histological sections were blinded for the intervention. The thickness of the intima, media and lumen within a cross-section of the artery in the slides stained with EvG were calculated using the Image-Pro Express software program (Media Cybernetics, Silver Spring, Md., USA; n = 11 in both groups). We measured the neointimal and medial areas of at least 6 sections.
per artery. We excluded samples from the statistical analysis when they included massive thrombus formation.

**Immunohistochemistry**

For immunohistochemical staining, an anti-monocyte chemoattractant protein-1 (MCP-1) antibody and anti-MMP-2 antibody, both from Santa Cruz Biotechnology (Santa Cruz, Calif., USA), were used (n = 3–5). Immunohistochemical staining was quantified and investigators were blinded to the treatments of animals.

**Real-Time Reverse Transcription Polymerase Chain Reaction**

Reverse transcription polymerase chain reaction (RT-PCR) was used to determine the messenger ribonucleic acid (mRNA) expression of MCP-1 (assay ID: Mm00441242_m1; Applied Biosystems, Tokyo, Japan), MMP-2 (assay ID: Mm0043948_m1), MMP-3 (assay ID: Mm00440295_m1), MMP-9 (assay ID: Mm00600163_m1), tissue inhibitor of metalloproteinases-1 (TIMP-1; assay ID: Mm00441818_m1) and TIMP-2 (assay ID: Mm00441825_m1). To account for differences in cDNA preparation and cDNA amplification efficiency, the mRNA expression of the target gene was normalized by 18S rRNA. Quantitative data were calculated using the comparative CT (ΔΔCT) method (n = 19–20) [26].

**Detection of P. gingivalis**

Arteries were taken from each group of mice during the bacterial challenge. Total DNA was collected using a QiaAmp kit (QiaGen, Md., USA), and the P. gingivalis 16S gene was detected by PCR, as described previously (n = 3 in both groups) [27].

**Statistical Analysis**

All data are expressed as the mean ± SEM. The differences between the two groups were analyzed by Student’s t test. Differences with p values <0.05 were considered to be significant.

**Results**

**Quantification of Antibacterial Antibodies and Detection of Bacterial DNA**

The effects of the repeated injection of P. gingivalis or vehicle on the plasma levels of anti-P. gingivalis IgG were determined by ELISA 14 days after injury. As expected, the level of anti-P. gingivalis antibodies was significantly increased in the P. gingivalis infected mice compared to the non-infected mice. The effects of the repeated injection of P. gingivalis or vehicle on the plasma levels of anti-P. gingivalis IgG were determined by ELISA 14 days after injury. As expected, the level of anti-P. gingivalis antibodies was significantly increased in the P. gingivalis infected mice compared to the non-infected mice. The P. gingivalis IgG level of the non-infected group was 0.17 ± 0.05 (n = 6). The P. gingivalis IgG level of the infected group was 1.10 ± 0.20 (n = 6; p < 0.05; fig. 2). The systemic pressure and heart rate of the mice did not significantly change 2 weeks after arterial injury in both the non-infected and infected group (data not shown).

P. gingivalis 16S DNA was not detected in any of the samples from the injured arteries of any of the mice 14 days after injury (n = 3 in both groups, data not shown).

**Quantitative Analysis of Intimal and Medial Thickening after Wire Injury**

The areas of the vascular wall component were quantitatively analyzed in the arteries of the non-infected and
P. gingivalis infected mice 14 days after injury. In all of the vascular wall sections, the internal and external elastic laminae were identifiable by EvG staining. The histopathological analysis revealed that the intima/media thickness ratio in the P. gingivalis infected group (4.54 ± 0.94; n = 11) was significantly increased in comparison to the non-infected group (2.31 ± 0.35; n = 11; p < 0.05) (fig. 3). This result suggested that P. gingivalis infection promoted neointimal hyperplasia. The uninjured arteries did not change the structure of the artery in both the non-infected and the infected group (data not shown).

Immunohistochemistry

The P. gingivalis infected group (29.0 ± 8.80; n = 5) showed more MCP-1-positive cells in the neointimal area compared to the non-infected group (4.6 ± 1.86; n = 5; p < 0.05) 14 days after injury (fig. 4a, b). Moreover, the P. gingivalis infected group (63.8 ± 19.1; n = 4) showed a significant increase in the number of MMP-2-positive cells in the adventitial area compared to the non-infected group (5.3 ± 2.1; n = 3; p < 0.05) 14 days after injury (fig. 4c, d).

Reverse Transcription PCR

The arteries from the P. gingivalis infected group showed significantly increased levels of MMP-2, MMP-3 and TIMP-1 mRNA compared to the arteries from the non-infected group on day 7 after injury (fig. 5). However, there were no significant differences in the MCP-1, MMP-9 or TIMP-2 mRNA levels in the arteries of the P. gingivalis infected and the non-infected group (fig. 5).

Discussion

Periodontitis has been implicated as a risk factor for various systemic diseases. Many researchers have shown that periodontal pathogens contributed to the pathogenesis of peripheral artery diseases. For example, Li et al. [28] reported that oral infection with P. gingivalis accelerated early atherosclerosis in apolipoprotein E knockout mice, whereas no lesions were seen in wild-type mice with P. gingivalis infections. However, there have been limited studies on whether oral pathogens deteriorate the development of vascular diseases [29].

The present study was based on the hypothesis that distant subcutaneous challenge with live P. gingivalis would contribute to the development of neointimal hyperplasia in mice after wire injury of femoral arteries. We used a model of localized distant infection by means of a subcutaneous chamber, which was previously shown to mimic the sequence of localized inflammatory events ob-
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Our histopathological analysis revealed that the intima/media thickness ratio in the *P. gingivalis* infected group was significantly increased in comparison to the non-infected group. There are many virulence factors of *P. gingivalis* such as lipopolysaccharides, fimbriae [33], gingipains, proteases [34] and hemagglutinin [35]. Previous studies indicated that *P. gingivalis* lipopolysaccharides trigger inflammatory pathways through the production of cytokines [36] and chemokines [37].

MCP-1 is an important factor involved in neointimal formation. Upregulation of MCP-1 gene expression after coronary angioplasty is known to result in the recruitment of monocytes and tissue macrophages to the arterial wall, leading to neointimal hyperplasia [38]. Additionally, treatment with a neutralizing MCP-1 antibody resulted in a significant reduction in neointimal formation in a rat model of carotid injury [39]. A previous report showed that MCP-1 expression was upregulated in *P. gingivalis* infected human umbilical vein endothelial cells [40]. Another study showed that *P. gingivalis* induced MCP-1 in an ex vivo human whole-blood model [41]. In the present study, our immunohistochemical results showed that the expression of MCP-1 in the neointimal area was enhanced in the *P. gingivalis* infected group compared to the non-infected group. Therefore, MCP-1 might play a critical role in the promotion of neointimal hyperplasia by *P. gingivalis* infection.
MMPs induced by *P. gingivalis* infection promotes neointimal hyperplasia.

In the present study, we showed that the infection with *P. gingivalis* enhanced neointimal formation after arterial injury in mice in association with the upregulation of inflammatory factors. Further studies will be needed to confirm our results, but periodontal treatment may become indispensable prior to balloon angioplasty and stent implantation if the existence of periodontitis promotes neointimal formation after arterial injury.

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In the injured arteries of the *P. gingivalis* infected group, the mRNA expression of MMP-2 and MMP-3 was increased compared to the injured arteries of the non-infected group. MMP-2 and MMP-3 were shown to be required for smooth muscle cell migration [42, 43]. It was also demonstrated that MMP-2 activity and protein expression increased in the injured arterial wall [44]. MMP-2 [45] and MMP-3 knockout mice [43] showed reduced neointimal hyperplasia after carotid artery ligation. In clinical studies, patients with periodontitis were found to have higher levels of MMPs and TIMPs in the blood compared to healthy control [46, 47]. We previously demonstrated that infection with *P. gingivalis* could accelerate the progression of experimental abdominal aortic aneurysm by increasing the expression of MMP-2 [22]. Another report indicated that sites with high gingival crevicular fluid levels of MMP-3 and TIMP-1 are at significantly greater risk for progression of periodontitis [48]. These findings suggest that the overexpression of MMPs induced by *P. gingivalis* infection promotes neointimal hyperplasia.

**Fig. 5.** Results of the RT-PCR analysis. The *P. gingivalis* infected group had elevated arterial mRNA levels of MMP-2, MMP-3 and TIMP-1 compared to the non-infection group. However, the mRNA levels of MMP-9, MCP-1 and TIMP-2 were not significantly different in the arteries from the *P. gingivalis* infected group and the non-infected group. Results are expressed as the mean ± SEM. *p < 0.05.
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References


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