Forum Minireview

Roles of Oral Bacteria in Cardiovascular Diseases — From Molecular Mechanisms to Clinical Cases:***Porphyromonas gingivalis* Is the Important Role of Intimal Hyperplasia in the Aorta**Kazuya Hokamura¹ and Kazuo Umemura^{2,*}¹Molecular Imaging Frontier Research Center, ²Department of Pharmacology, Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan

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Abstract. It has been reported that DNA of oral bacterial species, such as *Porphyromonas gingivalis* and *Streptococcus mutans*, was detected frequently in specimens of arteriosclerotic vessels. However, the source of DNA, whether from live intact bacteria or a part of the bacteria, has not been identified yet. Moreover, there was no precise evidence concerning involvement of oral bacteria in the progression of arteriosclerosis. We tried to clarify the involvement of *P. gingivalis* on the mechanisms of development of aortic intimal hyperplasia. Intravenous administration of *P. gingivalis* dramatically induced intimal hyperplasia in the mouse model with photochemical impairment of the femoral artery. However there were no changes identified in the mice without aortic impairment, even with the *P. gingivalis* infection. Concomitantly, S100 calcium-binding protein A9 (S100A9) and the embryonic isoform of myosin heavy chain (SMemb), a proliferative phenotypic marker of smooth muscle cells, were significantly overexpressed on the surfaces of smooth muscle cells present in the injured blood vessels. Similarly, increased expressions of S100A9 and SMemb proteins were observed in aneurysmal specimens obtained from *P. gingivalis*-infected patients. We found that bacteremia induced by *P. gingivalis* leads to intimal hyperplasia associated with overexpressions of S100A9 and SMemb. Our results strongly suggest that oral-hematogenous spreading of *P. gingivalis* is a causative event in the development of aortic hyperplasia in periodontitis patients.

Keywords: periodontal pathogen, smooth muscle cell, atherosclerosis, aorta, oral bacteria

1. Introduction

Porphyromonas gingivalis is a Gram-negative anaerobic bacterium considered to be a periodontal pathogen that causes severe periodontitis. Several epidemiologic studies have strongly indicated the involvement of *P. gingivalis* in the development of systemic diseases, in particular atherosclerosis (1–3). However, there has been no definitive evidence or systematic explanation about the precise involvement of *P. gingivalis* in the mechanisms of development of such systemic diseases.

We have reported that the frequency of detection of

P. gingivalis in specimens from aneurysmal or atherosclerotic blood vessels from patients was markedly lower than that of other oral bacteria such as *Streptococcus mutans*, which is thought to be involved in the pathogenesis of infective endocarditis (2). Therefore, it remains unclear whether invasion of *P. gingivalis* into blood vessels is actually essential and sufficient to cause cardiovascular diseases. There are numerous questions about the involvement of oral bacteria in the development of systemic diseases. At the present moment, we cannot persuasively answer these questions because no systematic studies from an in vivo animal model to an in vitro cell culture system have been conducted. In this review, we have obtained the answer to the above questions and clarified the precise involvement of these bacteria in the mechanisms of development of occlusive vascular dis-

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eases such as blood vessel wall hyperplasia (4, 5).

2. Invasion of oral bacteria into blood vessels and intimal hyperplasia after endothelial cell injury

We investigated whether endothelial cell injury might trigger the invasion of bacteria into blood vessels using a photochemically induced thrombosis model (6). Hyperplasia was observed in the injured vessels at 28 days following the administration of *P. gingivalis* strain OMZ314 (injured vessel + *P. gingivalis* in Fig. 1). Neointima occupied most lesions in hyperplasia. In contrast, slight hyperplasia was observed in the injured vessels (injured vessel control), but not in the non-injured vessels at 28 days following the administration of *P. gingivalis* (non-injured vessel + *P. gingivalis*). In addition, slight or no hyperplasia was observed in the injured vessels following *S. mutans* strain TW871 administration. These results may indicate that endothelial injury in the presence of *P. gingivalis* in the blood flow is essential to cause intimal hyperplasia in the aorta.

3. Target molecules involved in the intimal hyperplasia induced by *P. gingivalis* administration after blood vessel injury

To investigate the target molecules that are involved in *P. gingivalis*-induced vascular disease, we performed an exhaustive analysis of gene alterations in injured blood vessels of mice using a DNA microarray. To reduce the confounding effect of false signals, we compared the results obtained under three different conditions and found significant alterations of 97 commonly shared genes. Genes encoding chemokines and calcium-binding proteins accounted for the majority of the gene alterations. To confirm the results, we performed immunohistochemical staining of potential target molecules, including C-C chemokine ligand (CCL) 6, CCL9, and S100 calcium-binding protein A9 (S100A9). Increased expression of CCL6 and CCL9 was observed in the injured blood vessels even at 6 h after the administration of *P. gingivalis*. However, the increased expression of CCL6 and CCL9 were diminished at 7 days after the bacterial administration. In contrast, increased expression of S100A9 was observed not only after (6 h), but also at 7 days after the administration of *P. gingivalis*. Furthermore, the expression of S100A9 was mainly observed in the proliferating smooth muscle cells (SMCs) at 7 days after the administration of the bacteria. S100A9 is a member of the S100 calcium-binding protein family, and it has been reported that the expression of S100A9 is related to cancer and inflammation (7). However, the biological roles and functional mechanisms remain unclear

(8). These results indicate that the prolonged expression of S100A9, but not other S100 proteins, both on the inflammatory cells and the SMCs may play an important role in the induction of intimal hyperplasia by *P. gingivalis*. Additionally, the expression of the embryonic isoform of myosin heavy chain (9), a phenotypic marker of the proliferative type of SMCs, was observed in the proliferating SMCs in the intimal hyperplasia region at 7

Fig. 1. Intimal hyperplasia of the aorta induced by oral bacteria after blood vessel injury. A: Typical microscopic photographs of intimal hyperplasia of the aorta induced by oral bacteria after blood vessel injury. Samples were collected from the mice at 28 days after administration of oral bacteria or vehicle. Tissue sections were stained with Masson's trichrome. B: Quantification of the intimal hyperplasia in the injured blood vessels at 28 days after administration of *P. gingivalis*, *S. mutans*, or vehicle (Control). Intimal hyperplasia was calculated as the intima/media ratio (I/M ratio) from maximum field prepared from each individual sample. Each column represents the mean $\pm$ S.E.M. * $P < 0.05$ vs. control. This figure was modified from Ref. 5 with permission.

days after the administration of *P. gingivalis*. These results indicate that SMCs in the neonatal hyperplasia area was transformed to the proliferative phenotype.

4. Interaction of *P. gingivalis* with plasma is essential for the stimulation of human aortic smooth muscle cell (hAOSMC) growth

To investigate the mechanisms of hyperplasia associated with the transformation of SMCs observed in vivo in our smooth muscle cell-dependent animal model, we used an in vitro cultured human aortic smooth muscle cell (hAOSMC) culture system. We applied the supernatant of plasma incubated with *P. gingivalis* strain OMZ314 to the hAOSMC culture system. Addition of the supernatant of plasma incubated with the *P. gingivalis* dramatically stimulated the growth of hAOSMCs in comparison with that in the plasma control (plasma alone). Interestingly, marked morphological changes were observed in the hAOSMCs treated with the supernatant of plasma incubated with *P. gingivalis* as compared to the morphology in the plasma-alone (plasma control) or no-treatment groups (Fig. 2). These results indicate that the transformation of the hAOSMCs from

the contractile to proliferative phenotype is induced by exposure of the cells to plasma with *P. gingivalis* treatment, which is consistent with the observations in the in vivo hyperplasia model.

5. Knockdown of S100A9 attenuates increase in hAOSMC growth induced by exposure to plasma plus *P. gingivalis*

To investigate whether S100A9 is one of the potential targets in the induction of transformation and stimulation of the proliferative activity of the SMCs, we designed small interference RNAs (siRNAs) for S100A9 and applied them to the hAOSMC culture system. All of the three siRNA sequences effectively suppressed the expression of the S100A9 protein that was induced following exposure of the cells to the supernatant of plasma incubated with *P. gingivalis*. Under our experimental condition, all of the siRNA sequences for S100A9 also inhibited the increased cell growth of the hAOSMCs treated with the supernatant of plasma incubated with *P. gingivalis*. In addition, transformation of hAOSMCs from the contractile to a proliferative phenotype induced by the supernatant of plasma incubated with *P. gingivalis*

Fig. 2. Interaction of plasma with *P. gingivalis* for growth of human aortic smooth muscle cells. A: Effect of each group on the cell growth of hAOSMCs. The cell growth rate was calculated by fold increase of plasma control. Each column represents the mean $\pm$ S.E.M. from 7 – 12 independent experiments. B: Typical photographs of hAOSMCs at 72 h after treatment with the supernatant of plasma + *P. gingivalis* or plasma. Marked transformation was observed in hAOSMCs treated with the supernatant of plasma + *P. gingivalis*. This figure was modified from Ref. 4 with permission.

Fig. 3. Upregulation of the S100A9 caused by *P. gingivalis* infection is proposed to be an important event in the development of intimal hyperplasia.

was also inhibited by the siRNA sequences. These results suggest that upregulation of S100A9 in the SMCs induced by the plasma with *P. gingivalis* treatment is involved in the mechanisms underlying the transformation and proliferation of SMCs involved in the hyperplasia in vivo.

6. Increased expression of S100A9 protein in the cardiovascular lesions of patients orally infected with *P. gingivalis*

We investigated whether the increased expression of S100A9 that was induced by *P. gingivalis* in both in vitro and in vivo studies may also be observed in vascular tissue specimens obtained from the patients with cardiovascular diseases. Because the frequency of detection of *P. gingivalis* in specimens from aneurysmal or atherosclerotic blood vessels from patients was relatively low (2), as "*P. gingivalis*-positive" samples in this study, we used samples from subjects in which *P. gingivalis* was detected only in dental plaque but not in the atheromatous specimen. Strong expression of the S100A9 protein was detected in the tissue sections processed from aneurysmal blood vessels of patients who were *P. gingivalis*-positive but not in those from the blood vessels of patients who were *P. gingivalis*-negative. These observations suggest that upregulation of S100A9 by *P. gingivalis* is involved in the important events in the development of cardiovascular lesions, such as aneurysm, aortic hyperplasia, and atherosclerosis, induced by *P. gingivalis*.

7. Concluding remarks

Many previous reports have suggested the involvement of *P. gingivalis* in the development of various systemic diseases such as cardiovascular diseases (1–3). However, there have been no definitive evidences or system-

atic explanations of why and how *P. gingivalis* could be involved in the development of such vascular diseases.

Our animal experimental data indicate that bacteremia induced by *P. gingivalis* can lead to aortic intimal hyperplasia, and endothelial injury is essential for lesion formation. In contrast, the etiology of disease caused by *S. mutans* was not demonstrated in this model.

According to the results of DNA microarray, the results indicate that upregulation of S100A9 in the SMCs by *P. gingivalis* may be the important factor involved in the induction of intimal hyperplasia in the aorta.

Exposure of the hAOSMCs to plasma plus *P. gingivalis* treatment induced transformation and increased the growth of the hAOSMCs. These results reflected the phenomena observed in vivo in the animal models and clearly indicated that proliferation of SMCs was induced by the supernatant of plasma plus *P. gingivalis* in vitro. Knockdown of S100A9 protein by specific siRNA dramatically diminished the increase in growth of the hAOSMCs induced by the treatment with plasma plus *P. gingivalis*, indicating that S100A9 is the potential target in the induction of hyperplasia.

We also confirmed that the strong expression of S100A9 proteins was detected in tissue sections processed from aneurysmal blood vessels of patients who were *P. gingivalis*-positive. The results suggest that upregulation of S100A9 by *P. gingivalis* may be one of the important events in the development of cardiovascular lesions such as aneurysm, aortic hyperplasia, and atherosclerosis.

In conclusion, we clearly showed that upregulation of the S100A9 by *P. gingivalis* is the important event in the development of intimal hyperplasia in the aorta (Fig. 3). Our data presented here may open up a new field about the investigation of oral bacteria-induced systemic diseases.

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