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Short communication

Contribution of cell surface protein antigen c of *Streptococcus mutans* to platelet aggregation

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Matsumoto-Nakano M, Tsuji M, Inagaki S, Fujita K, Nagayama K, Nomura R, Ooshima T. Contribution of cell surface protein antigen c of Streptococcus mutans *to platelet aggregation.*
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Introduction: *Streptococcus mutans* is considered to be one of the pathogens that cause infective endocarditis. The purpose of the present study was to examine the properties of *S. mutans* with regard to platelet aggregation by focusing on its high molecular protein antigen c (PAc).
Methods: The platelet aggregation properties of six clinical strains and one isogenic mutant strain of *S. mutans* were analysed using an aggregometer and confocal microscopy, as well as with an inhibition assay of platelet aggregation using anti-PAc serum.
Results: *S. mutans* strains with PAc expression induced platelet aggregation, while a PAc-deficient mutant and two clinical isolates with no PAc expression did not. When platelets were pretreated with higher amounts of anti-PAc serum, the platelet aggregation rate was reduced in a dose-dependent manner, indicating that PAc binds directly to platelets.
Conclusion: *S. mutans* PAc is involved in human platelet aggregation and may be one of the virulence factors in the pathogenesis of infective endocarditis.

Key words: platelet aggregation; protein antigen c; *Streptococcus mutans*

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Streptococcus mutans is occasionally isolated from the blood of patients with infective endocarditis (IE) (6, 12). Oral streptococci including *S. mutans* are known to enter the bloodstream following dental treatment such as tooth extraction, and even tooth brushing and flossing are considered to be culprits in transient bacteremia (21). It is generally thought that transient bacteremia does not lead to the onset of IE in healthy subjects, whereas individuals with certain heart disorders may have an elevated risk for the onset of IE following its development. The major pathogenic factors of IE are considered to be bacterial attachment to impaired endothelium and formation of vegetation, which is composed of platelets and fibrin.

The cell surface protein antigen c (PAc) of *S. mutans* is known to be involved in the initial adherence of the organism to tooth surfaces. PAc participates in adherence to tooth surfaces through its interaction with the salivary pellicle, and the gene coding PAc of *S. mutans* serotype *c* has been cloned and sequenced (18). Several studies have reported an association of PAc with the virulence of IE caused by *S. mutans*. PAc antibody titers were found to be elevated in IE patients compared with healthy subjects, whereas the same study found that PAc was not related to endocarditis virulence in rat models (20). On the other hand, PAc has been shown to contribute to the interactions of *S. mutans* cells with fibronectin, collagen type I, and fibrinogen (1).

Induction of platelet aggregation and formation of bacterial thrombotic vegetation are considered to be important in the pathogenesis of IE, as platelet aggregation proteins in *Streptococcus sanguinis* (7), and more recently surface proteins of *Streptococcus gordonii* and *Streptococcus mitis* (2, 13, 14), were found to be associated with adhesion to human platelets. As for *S. mutans*, serotype-specific polysaccharides on the cell surface have

Table 1. Streptococcus mutans strains used in this study

S. mutans	Serotype	PAc expression	Platelet aggregation property	Relevant characteristics	References
MT8148	*c*	+	+	Wild-type	(19)
PD	*c*	−	−	PAc-deficient mutant strain derived from MT8148; erythromycin resistant	(17)
NN2008	*c*	−	−	Oral isolate from a healthy 4-year-old girl	(17)
TW295	*k*	−	−	Blood isolate from a 59-year-old male with bacteremia following a tooth extraction procedure	(8)
TW871	*k*	+	+	Blood isolate from a 45-year-old male with IE complicated by subarachnoid hemorrhage	(8)
TW964	*f*	+	+	Blood isolate from a 72-year-old male with IE	(8)
TW1378	*e*	+	+	Blood isolate from a 59-year-old male with IE	(8)

IE, infective endocarditis; PAc, protein antigen c.

been reported to be related to platelet aggregation (4, 16).

In the present study, we investigated the association of *S. mutans* PAc with platelet aggregation using laboratory strains and clinical isolates obtained from IE patients.

Binding of *S. mutans* to human platelets

To determine the contribution of *S. mutans* to platelet aggregation, strain MT8148 (serotype *c*) (19) and a PAc-defective mutant strain (PD) (17) were used, as well as four *S. mutans* strains isolated from patients with IE or bacteremia (strains TW295, TW871, TW964, and TW1378) (8), and an oral isolate with no PAc expression (strain NN2008) (17) (Table 1). PD was constructed by insertional inactivation of the gene encoding PAc, as described previously (17). This study was approved by the Ethics Committee of Osaka University Graduate School of Dentistry.

Platelet aggregation assays were carried out using human platelets. First, blood was collected from a healthy human volunteer who had received no medication during the preceding 2 weeks; it was then mixed with a 3.13% sodium citrate solution at a final volume ratio of 1 : 9. Thereafter, the sample was centrifuged at 200 ***g*** for 10 min at 25°C. After centrifugation, the upper layer was collected as the platelet-rich plasma (PRP) layer. Platelet-poor plasma was obtained following centrifugation of the remaining blood sample at 800 ***g*** for 15 min at 25°C and used as a negative control. Platelet aggregation was analysed using an impedance method with an aggregometer (Whole-blood Aggregometer C540; Baxter Ltd., Tokyo, Japan). PRP preparations were prewarmed for 3 min before the addition of bacteria. All procedures were carried out at 37°C with shaking at 100 ***g***. In addition, PRP mixtures with the MT8148 or PD strain at the same concentrations as described above were fixed in formaldehyde, stained with a fluorescein isothiocyanate-conjugated CD9 monoclonal antibody (R&D Systems, Minneapolis, MN) for 1 h at 37°C to bind the platelets, and examined by confocal microscopy.

A representative image of aggregation of human PRP by MT8148 cells obtained by confocal microscopy is shown in Fig. 1. Clumps were identified in the mixture of MT8148 cells and platelets (Fig. 1A), whereas the bacterial cells and platelets were separated in the mixture of PD cells and platelets (Fig. 1B). Aggregation assays using the blood isolate TW295 and oral isolate NN2008, which had extremely low PAc expression, were also performed; however, platelet aggregation was not observed (data not shown).

Platelet aggregation has been reported to be induced by various species of viridans streptococci, indicating the existence of some common properties or structurally related components shared among species. PAc-like molecules are a family of major surface proteins that are expressed in nearly all oral streptococcal species (15), with several proteins with a high homology to *S. mutans* PAc reported, such as the 180-kDa immunodominant antigen of *Streptococcus oralis* (3), streptococcal surface proteins (SspA and SspB) of *S. gordonii* (5), and Pas protein of *Streptococcus intermedius* (22). Each of those organisms possesses multifunctional adhesion abilities and may be correlated with inflammatory disorders such as IE. In addition, *S. sanguinis* strain 133-79, a prototype of many blood culture isolates obtained from patients with IE, was reported to adhere to human platelets as well as saliva-coated hydroxyapatite using *in vitro* models of the salivary enamel pellicle (9, 11, 21). Taken together, these results indicate that PAc-like molecules of oral streptococci such as *S. mutans* may be important factors in platelet aggregation.

Inhibition of platelet aggregation by anti-PAc serum

An inhibition assay using anti-PAc serum was carried out as follows. PRP preparations were prewarmed for 3 min with

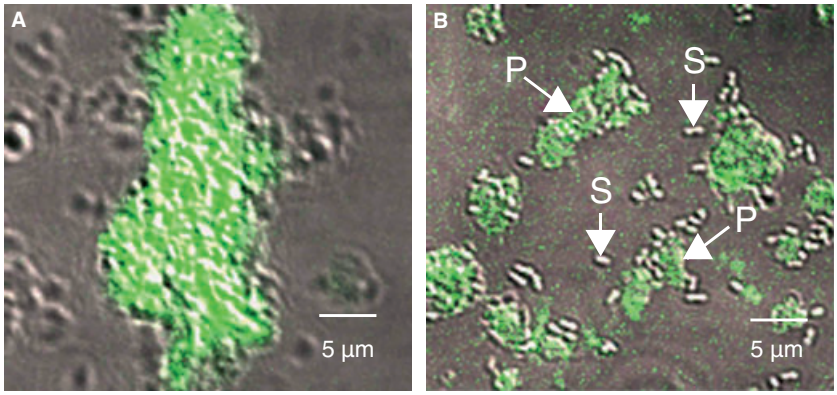

Fig. 1. Confocal microscopic images of interactions of *Streptococcus mutans* strains and human platelets. (A) strain MT8148, (B) strain PD. P, platelets; S, *S. mutans* cells.

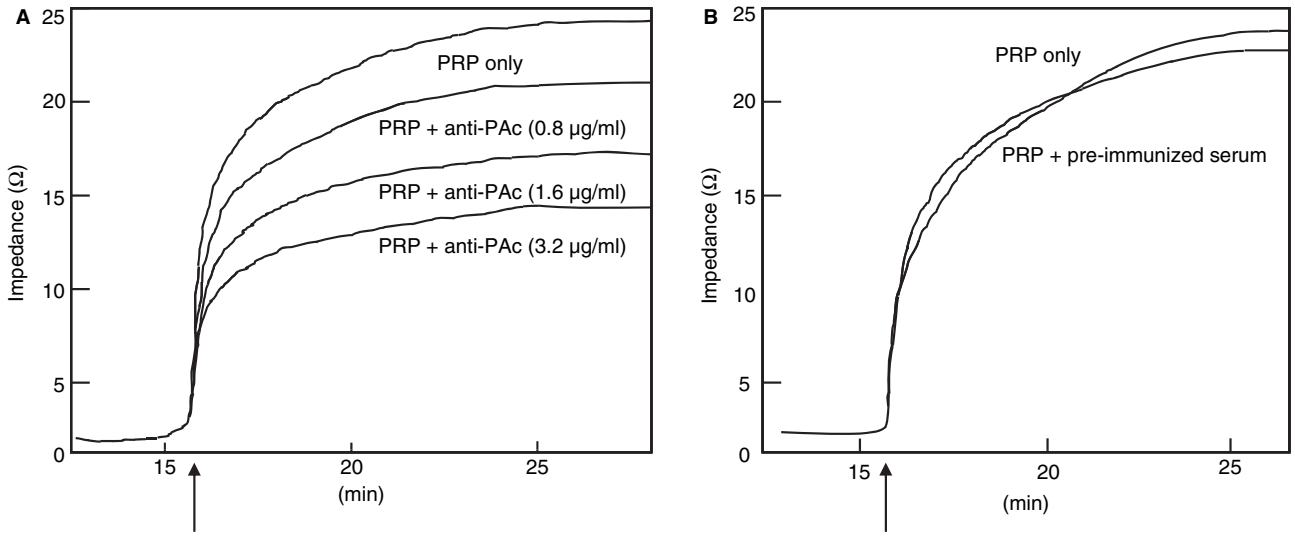

Fig. 2. Dose-dependent inhibitory effects of anti-protein antigen c (PAc) serum toward platelet aggregation caused by *Streptococcus mutans*. Arrow indicates the start time of platelet aggregation. (A) MT8148 was pretreated with anti-PAc serum, (B) MT8148 was pretreated with preimmunized serum.

anti-PAc serum (final concentration: 0.8–3.2 μg/ml), then bacteria were added to the mixture. Preimmunized serum (10) was used as a control in this assay. Figure 2 presents an image from the platelet aggregation assay, in which higher impedance indicates higher aggregation properties. Aggregation was initiated approximately 16 min after the start of incubation of the mixture of MT8148 cells and platelets, and the impedance value reached 24 Ω after approximately 25 min of incubation. In contrast, the impedance values were reduced when MT8148 cells were mixed with platelets pretreated with anti-PAc serum. Although the time required for aggregation was not significantly different among the strains, the maximum impedance values decreased as the concentration of anti-PAc serum increased. Aggregation was not inhibited when various amounts of anti-GTF serum were incubated with MT8148 cells (data not shown). Therefore, we speculated that PAc binds to platelets directly, which was also supported by direct observations of bacterial cells and platelets using confocal microscopy (Fig. 1).

Previously, we isolated and characterized four different *S. mutans* strains from IE patients (TW295, TW871, TW964, and TW1378), of which two (TW295 and TW871) were found to have lost glucose polymers in their serotype-specific polysaccharides (8). Chia et al. (4) also reported that serotype-specific polysaccharides of *S. mutans* were involved in the adherence of bacteria to both human and rabbit platelets, and were capable of triggering platelet aggregation in the presence of plasma. In the present study, TW871 (serotype *k*), TW964 (serotype *f*), and TW1378 (serotype *e*) strongly induced platelet aggregation, whereas TW295 (serotype *k*) had no such activity. Additional investigations are needed to clarify the relationships between platelet aggregation and serotype-specific polysaccharides. However, it is important to note that TW295, which had no expression of PAc and no platelet aggregation activity, was isolated following a tooth extraction procedure in a patient with bacteremia, who was reported to have no complications from IE. This phenomenon may have been derived from the non-aggregation properties of TW295. Together, our results support the notion that PAc plays an important role in the induction of IE, while platelet aggregation by *S. mutans* is a multifactorial function that involves other surface proteins in addition to PAc.

In summary, the present results suggest that *S. mutans* PAc is one of the surface antigens possibly associated with platelet aggregation and also indicate that most *S. mutans* oral strains with PAc expression induce vegetation formation. Additional studies are required to define the function of PAc in the induction of IE with regard to platelet aggregation.

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