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Plasminogen interaction and activation on *Streptococcus mutans* surface

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A number of pathogenic microorganisms have been previously shown to bind plasminogen. The subsequent activation of plasminogen into plasmin can contribute to their virulence. In this study, we have shown that *Streptococcus mutans* is able to bind both human plasminogen and plasmin. Binding of plasminogen to *S. mutans* was inhibited by L-lysine and ε-aminocaproic acid, indicating that binding is mediated via lysine-binding sites of plasminogen. *S. mutans* enhanced the activation of plasminogen by tissue plasminogen activator but not by urokinase. This enhancement turned out to be dependent on cell concentration. Zymogram analysis showed that the plasmin activity acquired after plasminogen binding and activation is the most important proteolytic activity in the strain tested. These results suggest a mechanism involving acquisition of a host protease that might contribute to the infective process of this microorganism.

Key words: plasminogen; plasminogen activation; *Streptococcus mutans*

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Streptococcus mutans is part of the human oral flora and can be an oral pathogen. The presence of *S. mutans* in the dental plaque has been consistently linked with the formation of human dental caries (19). This organism has been also associated with bacterial endocarditis (2). Several virulence factors of *S. mutans* have been defined and include adhesins, exoenzymes involved in extracellular sucrose metabolism and proteases (1). Proteases contributing to virulence in *S. mutans* have been implicated in the degradation of host structural protein and in the bacterial nutrition.

An increasing number of pathogens, including group A, C, and G streptococci, are able to bind plasminogen, the zymogenic form of the broad-spectrum protease plasmin (4, 5, 17). The binding of plasminogen is mediated principally by lysine binding sites (kringle domains) in the plasminogen molecule. Plasminogen is found in plasma and other biological fluids such as gingival crevicular fluid (16). After plasminogen binding and activation by either a host or its own plasminogen activator, the pathogen acquires a protease activity which does not stimulate the host immune response. This activity could replace or supplement the pathogen's proteases. In the case of the pathogens *Yersinia pestis*, *Borrelia burgdorferi, Borrelia crocidurae*, and group A streptococci, the interaction with plasminogen has been demonstrated to be implicated in the invasiveness within the host (11, 12, 18, 22). The plasminogen binding by *Fusobacterium nucleatum*, an oral bacterium, has been implicated in the invasion and tissue destruction during periodontitis (7).

S. mutans binds some host proteins such as fibronectin and other components of the extracellular matrix (3). This binding has been suggested to have a role in pathogenesis, specifically in endocarditis. Since plasminogen could also play a role in the pathogenesis of *S. mutans*, this work explores the plasminogen binding capacity of *S. mutans* and its subsequent activation in plasmin by plasminogen activators.

Material and methods

Bacteria and growth conditions

The type strain *S. mutans* 660 from Venezuelan Center of Microorganism Collection (CVCM) was used in this study. This strain was characterized by the API 20 Strep system (Biomerieux, Lyon, France) and by polymerase chain reaction as described elsewhere (24). Bacteria were grown in brain heart infusion broth at 37°C under aerobic conditions supplemented with 5% CO_2. Turbidity of the bacterial suspension was measured by spectrophotometry at 660 nm. A standard curve relating the culture turbidity and bacterial cell numbers was established. Three times PBS (phosphate-buffered saline, pH 7.4) washed bacteria harvested from the

stationary phase of the growth curve was used in all experiments.

Proteins

Human plasminogen was purified to homogeneity from fresh human plasma as described previously (8). Plasmin was generated by incubating 10 μM plasminogen with 20 nM streptokinase (kabikinase from Pharmacia, Upjohn, Uppsalla, Sweden) in PBS for 2 h at 25°C. Tissue plasminogen activator (t-PA) and urokinase were obtained from Chromogenix (Milan, Italy) and Choay Laboratory (Paris, France), respectively.

Ligand binding assays

S. mutans (2×10^8 cells) was incubated for 90 min at room temperature in PBS−0.1% BSA and different concentrations of plasminogen (final volume 200 μl). After three washings with PBS, the cells were resuspended in PBS−0.1% BSA (80 μl) containing 5 nM streptokinase and incubated for another hour. The specific plasmin chromogenic substrate, 0.6 mM S2251 (Chromogenix, Milan, Italy) was added to the cell suspension in a final volume of 100 μl. The change in absorbance at 405 nm over time was then determined using a microtiter reader. In some cases plasminogen was incubated in the presence or absence of either L-lysine or ε-aminocaproic acid, a lysine analog. Plasmin was also tested as ligand and, in this case, the plasminogen activator was omitted.

Plasminogen activation assay

Kinetic measurement of plasminogen activation was performed by monitoring the amidolytic activity of generated plasmin in a test volume of 100 μl. Plasminogen (200 nM) was incubated in PBS with the chromogenic substrate S2251 (0.6 mM) in the presence of different cell concentrations. Activation of plasminogen was initiated by addition of 10 nM t-PA or 2.5 nM urokinase, unless otherwise indicated, and the absorbance at 405 nm was monitored at different times. To obtain an estimated value of activation velocity, the slope of plots A vs. time^2 was calculated (29).

Zymogram analysis

Cells which had been pretreated with 1 μM plasminogen, in the presence of 1% BSA, and washed three times with PBS, were submitted to SDS-PAGE under nonreducing conditions. The resolving gel was copolymerized with 0.2% casein and 15 μg/ml streptokinase. In some experiments, other protein substrates such as gelatin and BSA were used. Untreated cells were included as control. Following electrophoresis, gels were washed for 1 h in 2.5% Triton X-100 and rinsed twice with 50 mM Tris-HCl, pH 7.7. When the proteolytic activity was tested at pH 5, another incubation buffer was used (50 mM acetate buffer, pH 5). Proteolytic activity was detected as a translucent band in the staining gel with 0.1% Coomassie blue.

Results

The association of plasminogen with *S. mutans* was tested by measuring the bacteria-associated plasmin activity. Following plasminogen incubation, washings and incubation with plasminogen activator, the chromogenic plasmin substrate was added. As shown in Fig. 1A (bar 1) plasmin activity could be detected on the cell suspension, indicating bacteria–plasminogen binding. Incubation with plasminogen omitting the plasminogen activator (Fig. 1A, bar 2) did not produce any significant activity, indicating that *S. mutans* does not produce plasminogen activator. No plasmin activity was observed in the absence of plasminogen (Fig. 1A, bar 3). The same result was obtained in the presence of only plasminogen activator. Plasmin activity was also detected when plasmin was used instead of plasminogen (Fig. 1A, bar 4), indicating that *S. mutans* also has the ability to directly bind the active form. The plasminogen binding was in a dose-dependent manner (Fig. 1B). Both ε-aminocaproic acid and L-lysine were able to inhibit the plasminogen binding on *S. mutans* surface (Fig. 1C), indicating binding via lysine residues. The observed inhibition was around 75% with both inhibitors.

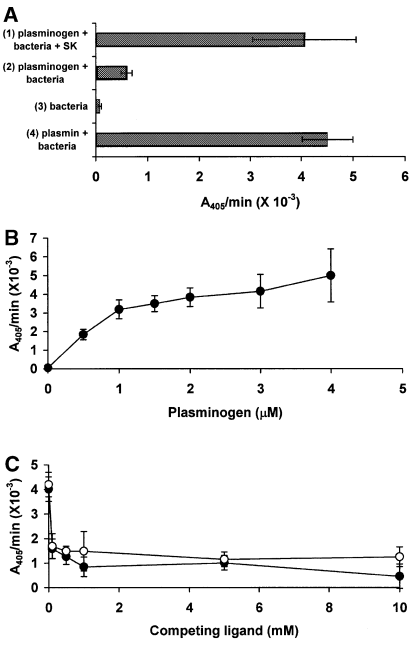

Fig. 1. Interaction of plasminogen with *S. mutans*. A, Bacteria were incubated with 2 μM of either plasminogen or plasmin. After washings, the associated plasminogen was activated with streptokinase (SK). Plasmin was detected using the chromogenic substrate S2251. B, Bacteria-associated plasmin activity after incubation with different plasminogen concentrations. Streptokinase was used as activator. C, 2 μM plasminogen was incubated with *S. mutans* as specified above in the presence of different concentrations of either L-lysine (○) or ε-aminocaproic acid (●). The mean values (± SD) of four different experiments are shown.

The plasminogen binding on *S. mutans* could have a functional effect and, thus, *S. mutans* could regulate the plasminogen activation. To test this hypothesis, we investigated whether *S. mutans* can affect plasminogen activation by physiological activators. *S. mutans* at different cell concentrations was added to a mixture of plasminogen and either t-PA or urokinase, and kinetic measurements of plasmin generation were monitored. As shown in Fig. 2A, the plasminogen activation by t-PA is enhanced by *S. mutans* in a cell concentration-dependent manner. The maximal enhancement was 4 ± 0.5-fold compared to plasminogen activation in the absence of cells. However, when urokinase was used, only small differences were seen (Fig. 2B). No plasmin activity was observed when the plasminogen activator was omitted (Fig. 2C), indicating that *S. mutans* is not capable of activating plasminogen on its own. Enhancement of t-PA-catalyzed plasminogen activation by *S. mutans* was seen at different t-PA concentrations (Fig. 3A). No enhancement of plasminogen activation was observed at the different urokinase concentration tested (Fig. 3B). These results indicate that *S. mutans* modulates the t-PA-catalyzed plasminogen activation and suggest that *S. mutans* may have an additional interaction with t-PA but not with urokinase. Both plasminogen and t-PA have lysine binding sites in their kringle modules (15) but this is not the case with urokinase.

The proteolytic activity acquired by *S. mutans* after plasminogen binding and activation by plasminogen activator could give this organism an additional proteolytic capacity. It has been reported that

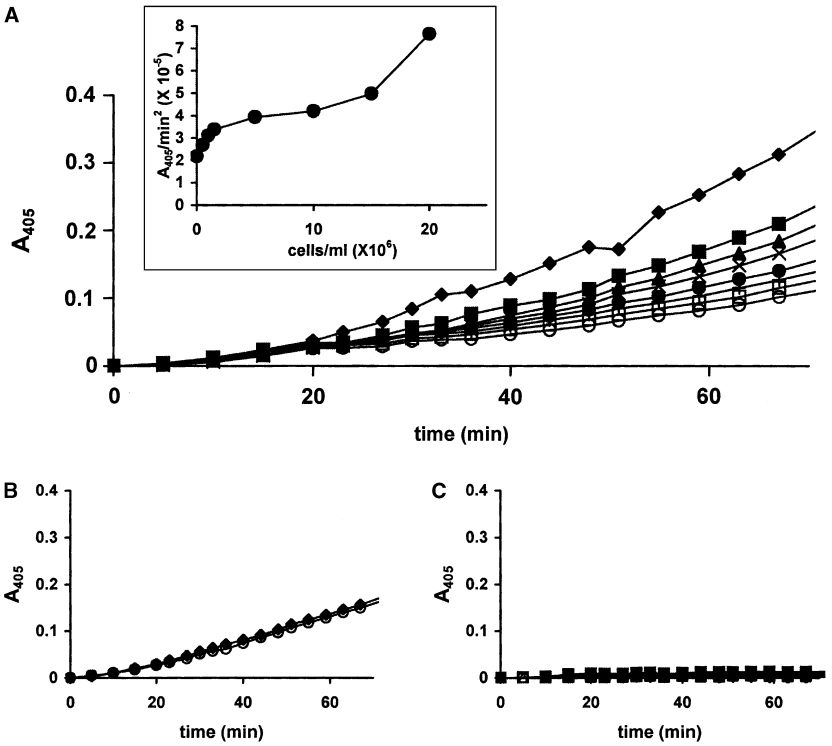

Fig. 2. Activation of plasminogen in the presence of *S. mutans*. 10 nM t-PA (A), 2.5 nM urokinase (B), and no plasminogen activator (C) was added to a mixture, in a final volume of 100 µl containing 200 nM plasminogen, 0.6 mM S2251 and different concentrations of cells: (◆) 20×10^6, (■) 15×10^6, (▲) 10×10^6, (X) 5×10^6, (●) 2×10^6, (+) 1×10^6, (□) 0.5×10^6, (○) 0 cells. In (B) only 20×10^6 (◆) and 0 (○) cells are shown. The absorbance at 405 nm at different times was determined. Inset in (A) shows velocity of activation determined from plots of A_{405} vs. min^2. The data shown are from a single experiment representative of four experiments that were performed.

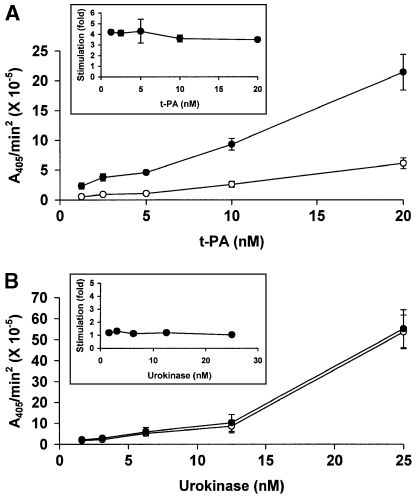

Fig. 3. Activation of plasminogen at different plasminogen activator concentration. Plasminogen 200 nM was incubated with 20×10^6 (●) or 0 (○) cells and activated with different concentrations of t-PA (A) or urokinase (B). Plasmin was detected using the chromogenic substrate S2251. The absorbance at 405 nm at different times was measured and velocity of activation determined from plots of A_{405} vs. min^2. Stimulation of plasminogen activation with the corresponding plasminogen activator (velocity with cells/velocity without cells) is shown in the insets. The mean values (± SD) of three different experiments are shown.

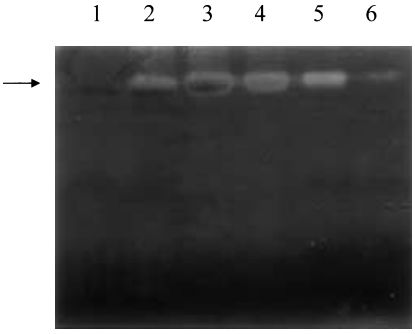

Fig. 4. SDS-PAGE zymogram. *S. mutans* (~ 1.5×10^6 cells) pretreated with plasminogen and washed were submitted to electrophoresis, under nonreducing conditions, onto 10% polyacrylamide gel containing 0.2% casein and 15 µg/ml streptokinase. Lane 1: *S. mutans* without treatment. Lane 2: Pretreated *S. mutans*. Lanes 3, 4, 5 and 6: 100, 50, 25, 10, and 1 pmol plasminogen, respectively. Arrow shows plasmin activity.

S. mutans has proteolytic activity on its surface (14). Moreover, several predicted surface or extracellular proteases were identified in the genome of *S. mutans* strain UA159 (1). In order to study the relative importance of the acquired plasmin activity with regard to its own proteolytic activity, extracts of cells which had been incubated with plasminogen and washed extensively were analyzed by zymography. Figure 4 shows the analysis with zymography using casein as substrate. Cells pretreated with plasminogen yielded a proteolytic activity corresponding to activated plasminogen. Cells without treatment did not reveal any activity. Since endogenous proteolytic activity in *S. mutans* has been reported in zymography, the same experiment was performed using gelatin and BSA as substrate. No endogenous proteolytic activity was observed using these substrates at any pH studied or in gels when performed under native conditions (data not shown). These results indicate that plasmin activity is the most important proteolytic activity detected in zymography under the conditions tested.

Discussion

Plasminogen and its active form plasmin have been principally studied regarding their function in fibrinolysis. However, these molecules are involved in other functions such as tumor cell invasion, inflammation, and neurodevelopment (6, 28). Plasminogen receptors have been identified on a variety of peripheral blood cells, fibroblasts, endothelial cells, and malignant cells (23). The interaction of these cells with plasminogen has been related, in some cases, to its migration behavior. The association of plasminogen and plasmin with an increasing number of bacteria is also well documented and this association offers a highly potential proteolytic system that could contribute to their virulence (4, 5). This study shows that *S. mutans* is also able to bind plasminogen and plasmin. This binding is saturable and dependent on plasminogen concentration. The interaction with plasminogen was decreased by lysine and a lysine analog, ε-aminocaproic acid, inferring interaction by lysine sites on the cell surface. Lysine binding sites are found on the kringle domains of the plasmin(ogen) molecule and are also responsible for the interaction of plasminogen with cells (23). The observed inhibition was only 75% with 10 mM ε-aminocaproic or lysine, suggesting that a fraction of binding is due to lysine independent interaction. Several plasmin(ogen) receptors have been

characterized in pathogenic bacteria. Examples are enolase and glyceraldehyde 3-phosphate dehydrogenase of group A streptococci (17). Both are glycolytic enzymes but they can also be localized at the cell surface acting as receptors. Since the genome analysis of *S. mutans* UA159 (1) shows that there is a complete glycolytic pathway, both enolase and glyceraldehyde 3-phosphate dehydrogenase could be involved in the interaction with the main component of the plasminogen–plasmin system. Moreover, SMU.360, a protein similar to glyceraldehyde 3-phosphate dehydrogenase and *Streptococcus pyogenes* plasmin receptor, was also found in this genome (1). This plasmin receptor is specific for plasmin and has low affinity for plasminogen (20). In our study, the strain analyzed was able to bind both plasminogen and plasmin. It is not possible to establish from our results whether both ligands have the same binding sites on thecell. If the protein SMU.360 is responsible for the binding of plasmin, another receptor should be implicated in the plasminogen binding. The identification of receptors for plasminogen and plasmin remain to be identified.

When plasminogen interacts with the *S. mutans* surface, plasmin formation by t-PA is markedly enhanced compared with the reaction in solution. This enhancement effect was not observed with urokinase. It is known that the simultaneous colocalization of plasminogen with plasminogen activators on cell surface is required for enhancement of plasminogen activation in a template mechanism in which a localized raised reactant concentration in a cell-associated compartment is produced (25). This surface interaction between plasminogen and t-PA has been investigated extensively with fibrin as biological surface (10). Thus, the enhancement of plasminogen activation by t-PA on the surface of *S. mutans* suggests that t-PA also interacts with this surface. The enhancement effect was not observed with urokinase at any plasminogen activator concentration tested. Enhancement of plasminogen activation with urokinase has been observed in cells having specific urokinase receptor (9). Thus, one may suggest that *S. mutans* is devoid at least of this type of receptor.

The binding of plasminogen and its subsequent activation by t-PA results in arming the *S. mutans* surface with the proteolytic activity of plasmin. In our experimental conditions, this plasmin activity was the only proteolytic activity of *S. mutans* detected in zymography under different conditions. Endogenous surface proteolytic activity has been reported previously in *S. mutans* but it was very variable, depending on the *S. mutans* strain (14). Moreover, Svensäter et al. (27) have reported proteolytic activity in *S. mutans* that varies depending on culture condition. They also found that some strains had no proteolytic activity. The strain in the present study might have no endogenous proteolytic activity, or the activity could not be expressed under our culture conditions.

The interaction of *S. mutans* with components of the plasminogen–plasmin system raises the possibility that plasmin activity acquired by this organism may play a role as a virulence factor. Plasmin acts directly on the extracellular matrix by cleaving noncollagenous proteins and also by activating a whole range of other pro form of enzymes, among them the matrix metalloproteinases. Plasmin is also able to degrade complement components and this function could participate in pathogenesis. Thus, this plasmin activity could contribute to the ability to cause host damage and could protect the bacterium from possible host defense. Nutrition could be also another function for this acquired proteolytic activity. However, for plasmin activity to be generated, a plasminogen activator must activate plasminogen. Several studies have demonstrated that t-PA is present in gingival crevicular fluid at significantly greater concentrations than in plasma and is the predominant plasminogen activator with urokinase (16). This latter is present in a 10–15-fold lower concentration than t-PA (16). Another source of plasminogen activator could arise from other oral bacteria such as *Porphyromonas gingivalis,* which produces a serine-protease that activates plasminogen (13).

Finally, plasminogen might also be implicated in the binding of *S. mutans* to platelets in the pathogenesis of infective endocarditis. It has been suggested that platelets on the surface of damaged valves may facilitate the initiation of infection by serving as a binding site for circulating microorganisms (26). Since platelets bind plasminogen (21), this molecule might serve as a bridge for the bacteria. However, the physiological role of plasminogen binding on *S. mutans* and its subsequent activation by a plasminogen activator remains to be elucidated.

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