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Activation of plasminogen by *Streptococcus mutans*

Micheala N. Jones, Robert G. Holt *

Department of Microbiology, Meharry Medical College, Nashville, TN 37208, USA

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Abstract

Streptococcus mutans, a member of the viridans streptococci, is the etiologic agent of dental caries and is also a causative agent of subacute infective endocarditis. The generation of proteolytic molecules, such as plasmin, may be important in the pathogenesis of endocarditis caused by *S. mutans*. In this study, we demonstrate that *S. mutans* cells have the ability to bind and activate plasminogen to plasmin. Incubation of *S. mutans* cells with plasminogen was found to be sufficient for the activation of plasminogen, which suggests the presence of an endogenously produced plasminogen activator. The plasmin activity generated by *S. mutans* cells was shown to be inhibited by ε-aminocaproic acid, lysine, aprotinin, and α_2-macroglobulin. We also show that *S. mutans* cells have the ability to bind and activate plasminogen from human plasma as well as human serum. The plasmin activity generated on the surface of *S. mutans* cells could degrade the extracellular matrix molecule, fibronectin.

Keywords: *Streptococcus mutans*; Plasminogen activation; Endocarditis

Plasminogen is a single chain glycoprotein of approximately 90 kDa present in the plasma and serum of mammals. The plasminogen molecule contains five characteristic kringle domains that mediate interactions with multiple ligands, such as fibrinogen, α_2-antiplasmin, and cellular plasminogen receptors [1,2]. Plasminogen is the zymogen for the major mediator of fibrinolysis in humans and is converted to the trypsin-like serine protease, plasmin, by the splitting of a single peptide bond [3]. Plasminogen can be converted into plasmin during proteolytic cleavage by two types of activators, tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). Bacterial enzymes such as streptokinase and staphylokinase can also activate plasminogen into plasmin by a nonproteolytic mechanism [4,5]. The activities of plasmin and its host activators are regulated extracellularly by a number of inhibitors including α_2-macroglobulin and α_2-antiplasmin [6]. Although plasmin has a well-established role in fibrin and basement membrane degradation, the proteolytic activity of plasmin is believed to play a role in the spread of bacterial infections by facilitating the digestion of extracellular matrix proteins [7,8]. Additionally, plasmin can catalyze the conversion of procollagenase to collagenase, which would further enhance extracellular matrix protein digestion [9]. A number of bacterial species, including group A streptococci and *Streptococcus pneumoniae*, capture plasminogen on their surface that is converted into plasmin activity [10–12].

Streptococcus mutans is a member of the viridans streptococci that colonize the oral cavity and is the etiologic agent of dental caries [13]. However, *S. mutans* is also a causative agent of infective endocarditis. *S. mutans* is primarily associated with subacute (chronic) infective endocarditis, which involves colonization of heart tissue that has preexisting endothelial damage [14]. While the mechanisms by which *S. mutans* cells colonize heart tissue are still not clear, it has been suggested that bacterial binding to components of the extracellular matrix, fibrin, and platelets is crucial in the development of endocarditis. The virulence determinants that are responsible for the

* Corresponding author. Fax: 1 615 327 6072.
E-mail address: rholt@mmc.edu (R.G. Holt).

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destruction of underlying cardiac tissue during the pathogenesis of endocarditis are not yet defined. During initial colonization by bacteria, extracellular matrix components of damaged tissue, which would not normally be exposed, may act as receptors for circulating bacteria. The exposed extracellular matrix proteins trigger the deposition of fibrin-platelet clots to which bacteria can bind with a strong affinity, resulting in the formation of vegetations that have the potential to protect the bacteria from host defense mechanisms [15]. In this study, we show that *S. mutans* cells have the ability to interact with plasminogen and generate plasmin activity.

Materials and methods

Bacteria and growth conditions. Streptococcus mutans M51 is a strain isolated from an endocarditis patient and has been shown to interact with several extracellular matrix proteins [16]. This strain was routinely grown anaerobically at 37 °C in brain heart infusion (BHI) media using the Gas-Pak Plus system.

Plasminogen activation by S. mutans. The ability of *S. mutans* to activate plasminogen to plasmin was detected utilizing BHI agar supplemented with skim milk and also using the chromogenic substrate, S2251 (D-Val-Leu-Lys-*p*-nitroanilide) [17,18]. In both assays, *S. mutans* cells from a 16 h overnight culture were washed three times in PBS and the optical density of the cell suspension was adjusted to 1.0 at 550 nm. In the milk plate assay, 50 μl volumes of *S. mutans* M51 cell suspension were incubated for 1 h in separate tubes in the presence of an equal volume of either plasminogen (3×10^{-2} U/ml in PBS) or plasmin (0.15 U/ml in PBS) along with tubes containing 50 μl M51 cell suspension diluted to 100 μl with PBS. For treatment of cells with urokinase, 50 μl urokinase (0.15 U/ml in PBS) was added to a set of tubes containing *S. mutans* cells that had been incubated with plasminogen. After a further incubation at 37 °C for 1 h, aliquots of samples of each tube along with equal volumes of BHI broth and PBS were then added to wells punched into BHI agar plates containing 1% skim milk and the plates were incubated overnight at 37 °C. In the assay utilizing S2251, 200 μl of cell suspension of *S. mutans* M51 was added to an equal volume of PBS or PBS containing either plasminogen (3×10^{-2} U/ml in PBS) or plasmin (0.15 U/ml in PBS) and the suspensions were incubated at 37 °C for 1 h with gentle shaking. Cells were then pelleted by centrifugation (12,000*g*, 5 min) and washed three times in PBS. M51 cells that had been incubated with plasmin were resuspended in 100 μl PBS and plasmin activity using S2251 was assayed as described below. Washed M51 cells that had been incubated in the presence of plasminogen were resuspended in either 200 μl PBS or PBS containing urokinase (0.15 U/ml in PBS) and incubated at 37 °C for an additional 1 h. Cells were pelleted by centrifugation, washed three times with PBS, and then resuspended in 100 μl PBS. The cell suspensions were incubated with S2251 solution (20 μl of a 2 mg/ml solution in PBS) for 1 h at 37 °C. Finally, bacteria were pelleted and the absorbance of the supernatant fluid was measured at 405 nm in a microplate reader. The activation experiments were performed at least three times with independent bacterial cultures and individual assays were performed in triplicate.

Inhibition of plasminogen activation. Inhibition assays were performed as described above for plasminogen activation of *S. mutans* with the exception that 100 μl of cells was incubated 1 h at room temperature with plasminogen in the presence of 100 μg/ml of either ε-aminocaproic acid (ε-ACA), lysine, aprotinin or α_2-macroglobulin. Assays where cells were incubated with either plasmin or plasminogen followed by urokinase were included as positive controls. M51 cells incubated alone were used as a negative control.

Activation of plasminogen in plasma or serum by S. mutans cells. Bacterial cells from an overnight culture were grown in BHI, washed with PBS, and incubated for 6 h at 37 °C in 500 μl PBS containing 20% human plasma or serum. Bacteria were harvested by centrifugation and washed twice with PBS to remove unbound components. Aliquots of 100 μl of the cells were incubated for 1 h at 37 °C with the chromogenic substrate S2251and the absorbance at 405 nm of supernatant fluids was determined as described above.

Degradation of fibronectin by plasminogen-coated S. mutans cells. Cultures of *S. mutans* cells were grown anaerobically in BHI at 37 °C, washed with PBS, and resuspended as described above. One hundred microliters of cell suspension was incubated for 2 h with an equal volume of plasminogen (3×10^{-1} U/ml in PBS). After two washes with PBS, the cells were resuspended in 25 μl of either PBS or PBS containing urokinase (0.75 U/ml in PBS) and incubated 1 h at 37 C. Cells were then washed once in PBS and suspended in 50 μl PBS. Forty microliters of untreated or urokinase-treated bacterial suspension was incubated for 16 h at 37 °C with either fibronectin (10 μl of a 1 mg/ml solution in PBS) alone or fibronectin in the presence of 100 μg/ml of either ε-ACA, lysine, aprotinin or α_2-macroglobulin. Bacteria were then removed from the mixtures by centrifugation and lysis buffer was added to an aliquot of the supernatant fluid. The mixtures were boiled for 10 min and subjected to electrophoresis in an 8% SDS–polyacrylamide gel. After electrophoresis, proteins were either silver-stained or electrophoretically transferred to a nitrocellulose membrane and probed with rabbit anti-human fibronectin antibody (Sigma Chemical).

Results

Activation of plasminogen by S. mutans cells

Initially, we used a qualitative assay to determine if *S. mutans* cells had the ability to activate plasminogen. This methodology is based on the premise that once plasminogen is activated to the serine protease, plasmin, it then has the ability to cleave the protein, casein, which is a major constituent of skim milk which was incorporated into BHI agar growth media [17]. When *S. mutans* cells pre-incubated with plasminogen were put into a well cut into BHI agar containing skim milk, a clearing of the area around the well was observed (Fig. 1). This result indicates that *S. mutans* does activate plasminogen and that *S. mutans* cells produce an endogenous plasminogen activator since incubation of *S. mutans* cells alone with plasminogen resulted in the production of plasmin activity. *S. mutans* cells incubated with plasmin or plasminogen and urokinase also exhibited clearings around wells (positive controls) while BHI media alone or BHI media containing *S. mutans* cells failed to produce a clearing around wells (negative controls). Results in Fig. 2 further suggest that *S. mutans* activates plasminogen and that *S. mutans* expresses an endogenous plasminogen activator. In these experiments, a quantitative assay was used to measure plasminogen activation based on the ability of plasmin to cleave the chromogenic substrate, S2251 [18]. Plasmin cleaves S2251 yielding a colorimetric change that can be detected as a change in the absorbance at 405 nm. Results from experiments using S2251 again demonstrated that

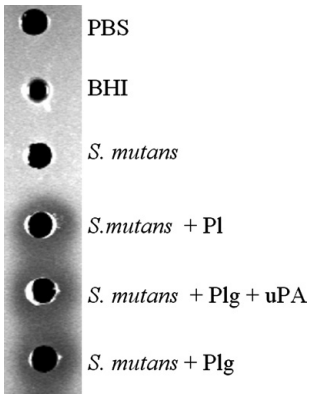

Fig. 1. Activation of plasminogen by *S. mutans* cells. *S. mutans* cells were incubated with either plasmin (Pl), plasminogen (Plg), or plasminogen followed by urokinase (uPA). Wells were cut into BHI agar media containing 1% skim milk. The cell suspensions were then added to wells and incubated overnight. Plasminogen activation is indicated by the caseinolytic zones around wells.

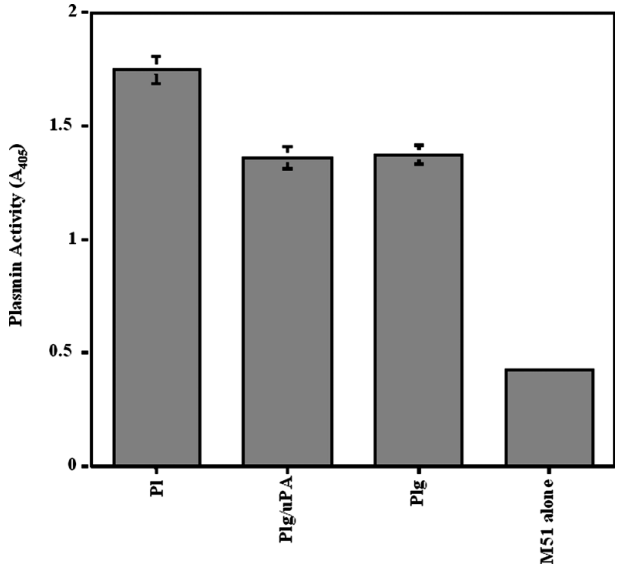

Fig. 2. Cell-associated plasmin activity following incubation of *S. mutans* cells with plasmin or plasminogen. *S. mutans* M51 cells were incubated with plasmin (Pl), plasminogen (Plg), or plasminogen followed by urokinase (uPA). Cell-associated plasmin activity was determined using S2251. Data represent the mean optical density (405 nm) values from at least three triplicate samples and are representative of at least three experiments.

S. mutans cells in the presence of plasminogen could activate plasminogen and generate a protease activity that could cleave S2251. This level of proteolytic activity was similar to that generated when *S. mutans* cells were incubated with plasminogen and urokinase.

Inhibition of plasminogen activation

To determine the manner by which activation of plasminogen to plasmin occurs, two different classes of inhibitors were used. The first class includes lysine and

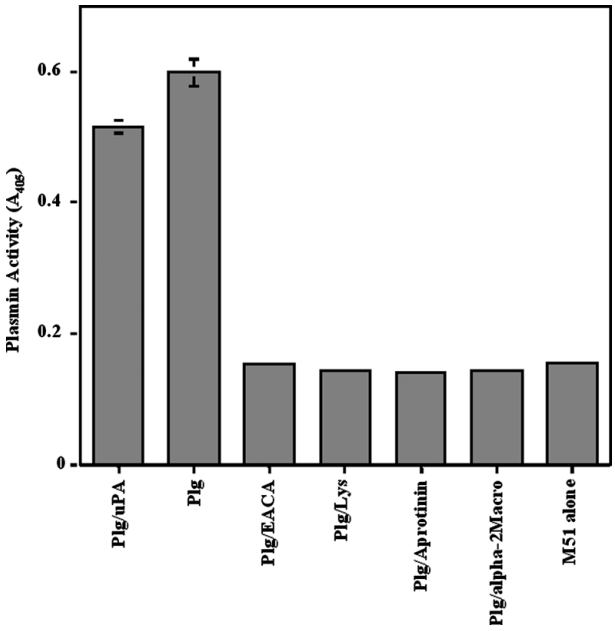

Fig. 3. Inhibition of plasminogen activation. *S. mutans* M51 cells were incubated with plasminogen in the presence of ε-ACA, lysine (lys), aprotinin, and α_2-macroglobulin. All inhibitors were added to a final concentration of 100 μg/ml. Cell-associated plasmin activity was determined using S2251. Data represent the mean optical density (405 nm) values from at least three triplicate samples and at least three experiments.

ε-ACA, while the second class includes aprotinin and α_2-macroglobulin. The first class of inhibitors actually competes with plasminogen receptors for the kringle domains of plasminogen, which are involved in the binding interactions of plasminogen [2]. The latter class, however, are general serine protease inhibitors for proteases that degrade in a trypsin-like manner [6]. Fig. 3 demonstrates that ε-ACA, lysine, aprotinin, and α_2-macroglobulin are all able to inhibit plasminogen activation by *S. mutans* cells to a similar degree.

Acquisition of bound plasmin activity by incubation of S. mutans in the presence of plasma or serum

The results presented in Fig. 4 indicate that *S. mutans* cells have the capacity to acquire substantial cell-associated plasmin activity when incubated in the presence of 20% human plasma or serum. Bacteria incubated in BHI alone failed to hydrolyze the plasmin-specific substrate, S2251. The level of activation of plasminogen by *S. mutans* from plasma or serum was similar.

Degradation of fibronectin by plasminogen-coated S. mutans cells

To determine if *S. mutans* cells that had been incubated with plasminogen could generate a plasmin activity that could degrade the extracellular matrix molecule,

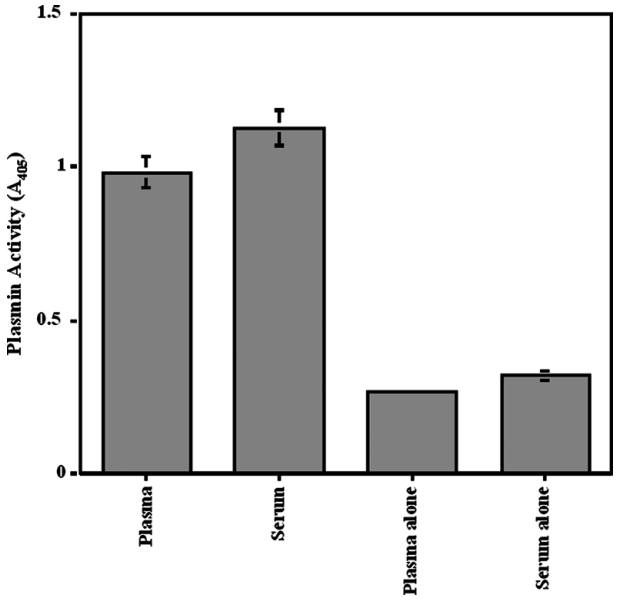

Fig. 4. Surface-associated enzymatic activity acquired by *S. mutans* following incubation in the presence of either human plasma or serum. Washed *S. mutans* M51 cells were incubated in PBS containing 20% human plasma or serum. Cells were pelleted and washed three times with PBS. Cell-associated enzymatic activity was determined using S2251. Data represent the mean optical density (405 nm) values from triplicate samples and are representative of three experiments.

fibronectin, *S. mutans* cells were coated with plasminogen and then incubated with fibronectin. SDS–PAGE and Western blot analyses of fibronectin that had been incubated with plasmingen-coated bacteria demonstrated that the plasmin activity produced on the surface of *S. mutans* cells could proteolytically degrade fibronectin into smaller polypeptides. Furthermore, the degradation of fibronectin was specific for plasmin since it could be inhibited by lysine, ε-ACA, and aprotinin. These results are shown in Fig. 5.

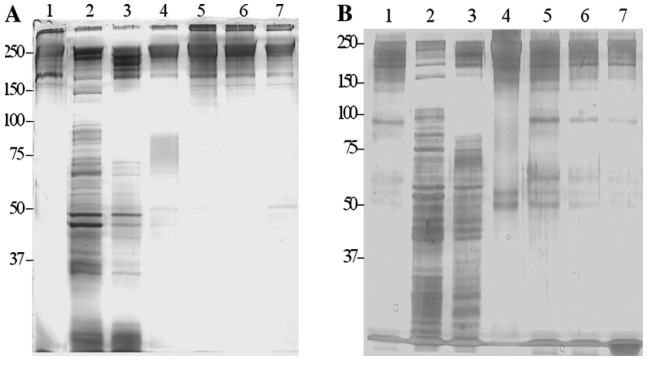

Fig. 5. Degradation of fibronectin by plasminogen-coated *S. mutans* cells. Lane 1, fibronectin; lane 2, urokinase activated plasminogen-coated bacteria and fibronectin; lane 3, plasminogen-coated bacteria and fibronectin; lane 4, untreated bacteria and fibronectin; lane 5, plasminogen-coated bacteria and fibronectin in the presence of ε-ACA; lane 6, plasminogen-coated bacteria and fibronectin in the presence of lysine; and lane 7, plasminogen-coated bacteria and fibronectin in the presence of aprotinin. Silver-stained SDS–polyacrylamide gel (A); blot probed with anti-fibronectin sera (B).

Discussion

In this study, we demonstrate that the oral streptococcus, *S. mutans*, can bind plasminogen and that the bound plasminogen can then be converted into the serine protease, plasmin. This finding to our knowledge is the first to demonstrate the activation of plasminogen by an oral streptococcus. The results of this study further indicate that *S. mutans* can bind and activate plasminogen that is present in serum or plasma to plasmin and can then acquire the ability to degrade extracellular matrix molecules, such as fibronectin. Activation of plasminogen by *S. mutans* could potentially aid in the formation and spread of vegetations on damaged endothelial tissue by degradation of exposed components of extracellular matrices and basement membranes. This degradation of matrix components could also foster the spread of vegetations to other damaged heart tissue in a scenario that involves fragments of vegetations generated by the proteolytic activity of plasmin attaching to other sites of damaged endothelial tissue and resulting in a new cycle of vegetative growth and damage.

We have demonstrated that plasminogen activation by *S. mutans* can be inhibited by lysine, ε-ACA (a lysine analog), aprotinin, and α_2-macroglobulin. The results of inhibition with lysine and ε-ACA suggest that the activation of plasminogen occurs by a binding mechanism that involves interaction with the kringle domains within the plasminogen molecule. These findings are consistent with a number of other studies on plasminogen activation by bacteria [10].

When *S. mutans* cells were incubated with plasminogen alone, plasmin activity was generated at a level similar to that of a positive control, which was *S. mutans* cells incubated with plasminogen and the exogenous plasminogen activator, urokinase. This result suggests that *S. mutans* produces its own cell surface localized plasminogen activator. The production of endogenous plasminogen activators has been reported for a number of bacteria, including group A and C streptococci, *Staphylococcus aureus*, and *Yersinia pestis* [10]. Further studies on the plasminogen activator of *S. mutans* are needed to elucidate its specificity and its genetic relatedness to known plasminogen activators.

Stinson et al. [19] were the first investigators to demonstrate that oral streptococci, including *S. mutans*, have the capacity to invade cells in HUVEC monolayers. The invasion of HUVEC by *S. mutans* could only be accomplished if the *S. mutans* cells were incubated with human plasma prior to exposure to the HUVEC. It is reasonable to speculate that the requirement for incubation of cells with plasma for cell invasion to occur involves a critical need for *S. mutans* cells to bind and activate plasminogen from plasma before the cells can adhere to and subsequently enter the HUVEC. This scenario would suggest that the plasminogen activation

system may be a virulence determinant of *S. mutans* that may facilitate destruction of the extracellular matrix and eventually invasion of endothelial cells. In support of this hypothesis, it has been reported that the binding of *Borrelia burgdorferi* to plasminogen causes an increased ability of the spirochete to penetrate endothelial cell monolayers [20]. However, further studies are needed to substantiate this hypothesis. Overall, the results of these studies demonstrate for the first time that *S. mutans* can bind plasminogen and facilitate processing of cell surface associated plasminogen to plasmin by an endogenous plasminogen activator. Future studies will involve the characterization of plasminogen-binding proteins and plasminogen activators of *S. mutans* and elucidation of the role of plasminogen activation in the pathogenesis of systemic infections caused by *S. mutans*.

Acknowledgments

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