**ABSTRACT**

**Background:** *Streptococcus mutans*, the main bacteria of dental caries, can easily invade circulatory system and was reported to have the ability to survive and colonize on atherosclerotic plaque, therefore was suspected to contribute in pathomechanism of atherothrombotic disease. It was assumed that *S. mutans* play a role in atherothrombotic disease due to its potency to produce proteases which were directly or indirectly via (Matrix metalloproteinases, MMPs) causing subendothelial vascular collagen fragmentation leading to platelet aggregation. **Purpose:** This study purposed to demonstrate *in vitro* that *S. mutans* could (directly or indirectly) cut subendothelial vascular collagen (type IV collagen) to become fragments which subsequently stimulated platelet aggregation. **Method:** Collagen fragmentation was analyzed by means of Sodium Dodecyl Sulphatopolyacrylamid Gel Electrophoresis (SDS-PAGE) and Soluble Biotinylated Assay (SBA), while platelet aggregation was analyzed microscopically and spectrophotometrically. **Result:** Result showed that *S. mutans* (directly and indirectly), could fragmentate type IV subendothelial vascular collagen leading to platelets aggregation. **Conclusion:** The potency of *S. mutans* on subendothelial collagen degradation and platelet aggregation may suggest the molecular mechanism of *S. mutans* involvement in pathogenesis of atherothrombotic disease.

**Key words:** *Streptococcus mutans*, protease, MMPs, collagen, platelet.

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**INTRODUCTION**

Studies related to *S. mutans* (the primary bacterial cause of dental caries) are mostly reviewing its role in dental caries. This is due to the ability of *S. mutans* to metabolize glucose to produce acids that cause tooth demineralization. Meanwhile, based on genome analysis, *S. mutans* can also produce proteases.1 Unfortunately research that concerned with its proteolytic potency of *S. mutans* is very limited.

*Streptococcus mutans*, from deep carious lesion can easily invade into systemic blood circulation6 and was reported to have ability to survive and colonize on endothelium7 and coronary atherosclerotic plaque,4 therefore, it was suspected to contribute in pathomechanism of atherothrombotic plaque. It was reported that 40% *S. mutans* enter blood stream during tooth brushing and 88% after periodontal surgery.2 Our previous study found that antibody anti-*S. mutans* exist in blood serum of acute myocardial infarction patients.5

Atherothrombotic disease is a complex vascular disease in which cholesterol deposition, inflammation, and thrombus formation play a major role.5 It causes
coronary heart disease when the disease involving coronary arteria and causes stroke when involving the arteria that supply blood to the brain, i.e internal carotid arteria. The potency of Streptococcus mutans role in pathogenesis of atherothrombotic disease has not been studied yet.

New paradigm that emerged recently shown that atherothrombotic disease is caused by inflammation response against the invasion of bacteria into the circulatory blood vessel wall. Bacterial invasion induce activation of phagocytes to produce proteinases mainly matrix metalloproteinase (MMPs), that causes the degradation of vascular collagen matrix. Collagen vascular damage readily induce platelet aggregation followed by activation of the coagulation cascade resulting in thrombus formation.7-9

It was reported that some bacteriae could induce production of MMPs zymogen (pro-MMPs) in phagocytes such as monocyte.10 Meanwhile there were also reported that proteases of bacteria could activate pro-MMPs to become active MMPs.11,12 Those enzymes play a key role in the degradation of extracelluar matrix, including collagen. Streptococcus mutans capable to produce proteinases, therefore it was assumed that Streptococcus mutans proteases can activate monocyte pro-MMPs to become active MMPs, that can break down collagen into fragments. In addition to its potency to activate MMPs, Streptococcus mutans proteases was thought to be able to degrade collagen directly.

The aim of this study was analyzing the role of Streptococcus mutans in pathogenesis of atherothrombotic disease, in vitro, by proving that Streptococcus mutans directly or indirectly (by its interaction with monocyte) break down vascular type IV collagen to become fragments, and those fragments stimulated platelet aggregation.

**MATERIALS AND METHODS**

Peripheral venous whole blood from healthy donor. Wild type Streptococcus mutans was obtained from Microbiology Laboratory Faculty Dentistry University of Airlangga. TYC medium was purchased from Topley House UK, Type IV collagen (Sigma), BHIB medium (Sigma), Biotin-N-Hydroxysucci-nimide Esters (Sigma), N-Hydroxysuccinimide Esters (Sigma), N-Hydroxysuccinimide esters (Sigma), Rabbit anti-human MMP-9 (Sigma); Anti-rabbit IgG-biotin (MP Biomedicals, Inc), Nitrocellulose membrane (NC, KPL), SA-HRP (streptavidin avidin-Horse Reddish peroxidase, Dako), TMB (Tetra methylbenzidine, KPL), high-range protein molecular weight marker (Bio-Rad).

**Streptococcus mutans** were suspended in BHIB medium and incubated 2 x 24 hours at 37° C. Using spectroscopic methods, the concentration of Streptococcus mutans was adjusted to 10^9 per ml. Protease of Streptococcus mutans was prepared from culture medium after being incubated for 2x24 h, rt. The medium was centrifuged 3500 rpm, 10 min, rt. Supernatant contain proteases was then filtered using microfilter 0,2 μm (Sartorius).

Monocyte was isolated by means of ficoll hypaque centrifugation. Six ml heparinized whole blood was diluted in HBSS (1:3), then layered on ficoll (1:3) and centrifuged 30 min, 1400 rpm, at room temperature (rt). The buffy coat layer contain mononuclear cells are aspirated, put in falcon tube, washed 3 times and resuspended with 2 cc HBSS. Suspension of mononuclear cells were then layered on gelatin (0.5%) and incubated 1 h, 37° C, rt. Non adherent cells were removed and rinsed 3 times. The adherent cell (monocytes) were gently scaped and suspended in 3 cc HBSS, then washed 2 times. Pellet cells were then resuspended in 300 μl HBSS and ready for further analysis.

MMPs was prepared from monocyte medium after being incubated with Streptococcus mutans. A total of 250 μl monocytes suspension were exposed to 250 μl suspension of Streptococcus mutans and incubated for 18 h, 37° C. After centrifuged at 3500 rpm, 10 min, rt, supernatant contain active MMPs was aspirated and filtered (Sartorius 2μm). To confirm the presence of MMPs products, we tested MMP-9 secretion by monocytes with immunochemistry assay.

Collagen type IV (5 mg) was dissolved in 2.5 ml of acetic acid 25%. For biotin labelling, 500 μl collagen suspension was added to 500 μl d-biotin and stirred 4° C, 24 h. Dialysis was then performed to remove excess biotin in sterile H2O, 24h, 4° C, followed by sterile PBS, 24 hours, 4° C.

Collagen fragmentation was analyzed by means of SDS-PAGE dan SBA (modified from methods by Romanelli et al, 1999).13 For direct fragmentation we reacted biotinylated collagen with Streptococcus mutans proteases, while for indirect fragmentation with monocytes MMPs. Briefly 100 μl suspension containing Streptococcus mutans protease or MMPs was reacted with 100 μl biotinylated type IV collagen for 18 h, 37° C. This process caused collagen degradation into fragments. This reaction was stopped by addition of 100 μl RSB and heating 100°C for 5 min. Collagen fragments were then separated by SDS-PAGE (12.5%) and transferred to NC membrane. After blocking non-specific binding with Skim milk blotto, the membrane was washed and incubated with
SA-HRP for 2 h. After being washed 2 times, membranes were incubated with TMB substrate, stop reaction with aquades. Collagen fragments are visualized as a colored band on the membrane blot. The character (molecular weight) of collagen fragment were identified by comparing it with standard molecular weight proteins (Poncheau Red staining).

Platelet aggregation were analyzed using microscopic and spectrophotometric assay. Firstly, collagen fragments was prepared by SDS-PAGE, the identified fragments were isolated from gel using electroelusion methods. Eluent consist of collagen fragments were ready for further assay. For microscopic assay, 50 ul collagen fragments suspension were coated on cover slide in humid chamber, then, 500 ul of platelet suspension was layered on it and incubated for 18 h, 37° C. After removing the medium, cells was rinsed, fixed and stained with Giemsa. Platelet aggregation was observed under light microscope 400x magnification. Spectrophotometric assay was done to compare the optical density (334.4 nm) of platelet suspension before and after being reacted with suspension of collagen fragments.

RESULTS

*Streptococcus mutans* produced proteases which can directly degrade type IV collagen into specific fragments. To perform fragmentation, biotinylated collagen was reacted with medium culture of *S. mutans* that was assumed to contain proteases, this was presented by SDS-PAGE (Figure 1A). To visualize collagen fragments only, SBA (biotinylated collagen) or ligand blotting method was used (Figure 1B).

This study also demonstrated that *S. mutans* could induce monocyte to produce MMPs, one of them was MMP-9 (Figure 2). Furthermore, using SDS-PAGE method and SBA, it was proved that these MMPs can degrade type IV collagen to become fragments, they were fragments with molecular weight of 70; 62 and 7 kDa (Figure 3).

Figure 1. Fragmentation of type IV collagen by *S. mutans* proteases.

A. SDS-PAGE profile, line 1 protein marker, line 2 & 3 untreated collagen, line 3 & 4 mixture of collagen fragments and proteases-containing culture medium of *S. mutans*.

B. SBA profile, line 1 protein marker, line 2, 3 and 4 type IV collagen fragments, comprises six fragments: 114, 70; 50; 40; 23 and 7 kDa.

Figure 2. Immunostaining of MMP-9. A. Untreated monocyte did not express MMP-9; B. Monocytes induced by *S. mutans* produced MMP-9 (brown secrete)

Figure 3. Fragmentation of type IV collagen by *S. mutans*-induced monocytes MMPs.

A. SDS-PAGE profile, line 1 protein marker, line 2 & 3 untreated collagen, line 3 & 4 mixture of collagen fragments and MMPs.

B. SBA profile, line 1 protein marker, line 2, 3 and 4 type IV collagen fragments, comprises three fragments: 70; 62 and 7 kDa.
Formation of collagen fragments related to the action of *S. mutans* were tested for its ability to induce platelet aggregation. Presentation of platelet aggregation test (interaction between platelets with fragments of collagen IV) were visualized in the form of microscopic picture and histograms of spectrophotometric data. Based on the microscopic image, it can be determined whether the aggregation of platelets occurred around the collagen fragments (Figure 4A, 4B). While spectrophotometric data presented the comparison of the optical density of platelet suspensions before and after being exposed to collagen fragments. Low optical density indicated that platelet aggregation occurs, and vice versa, a high optical density showed little platelet aggregation (Figure 4C).

**DISCUSSION**

*S. mutans* is well-known as an asidogenic bacterium, because they have enzymes for metabolizing carbohydrates that the final product are acids that causes a decrease in pH in the environment surrounding the growth of *S. mutans*. This acids production suggests that *S. mutans* probably can lead to damage host proteins through a decrease of pH. However, in this study the hypothesis that collagen can be damaged by acidic pH was not proven, because after culturing *S. mutans* for 2 x 24 h, did not cause significantly changes in the pH to becomes acidic (data not shown). As an explanation for this is, the production of acid by the glycolytic enzymes of *S. mutans* may occur in cytoplasm, and it takes time to dispose acidic products into the extracellular space.

Therefore, the result of this study suggested that collagen degradation occurs not by acids but because of the activity of proteolysis enzymes.

Based on genomic analysis, *S. mutans* has a gene that encodes the expression of several types of protease, i.e., serine protease HtrA, HtpX, Zn-dependent protease, two types of collagenase-like protease, serine protease RgpF and membrane proteases. These *S. mutans* proteases, however, has not been characterized and identified its specificity. It is therefore important to do more research on the *S. mutans* proteases.

In context of collagen degradation, in addition to direct collagenolytic properties, *S. mutans* was suggested to be able to induce monocyte to produce MMPs, while other notions indicated that various bacterial proteases may activate proMMPs. Therefore it is reasonably suspected protease *S. mutans* can also enable to activate MMPs that cause collagen degradation. The results of this study proved, *S. mutans* induced the expression of MMP-9 monocytes (evidenced by imunocytochemistry). Also shown that *S. mutans* proteases can activate monocytes MMPs that cause degradation of collagen type IV. This study specifically analyzed the involvement of MMP-9, because it has been known that substrate specification of MMP-9 is type IV collagen.

Type IV collagen is the principal constituent of subendothelial basement membrane, so it is a collagen of vascular wall which located at the most luminal surface. When there is invasion or attachment of foreign bacteria on vascular wall, there will be a vascular inflammatory response, in these circumstances, collagen type IV will be exposed firstly to the destructive effects of vascular inflammation. Therefore alleged that
fragmentation of collagen type IV is the key step for platelet aggregation and thrombus formation.

Formation of collagen fragments related to the action of *S. mutans* was proved to be able to induce platelet aggregation. This results study supported the notion by Constantinides (1994), who stated that vascular collagen degradation is the most powerful stimulus for platelet aggregation and thrombus formation.\(^\text{15}\)

Potency of *S. mutans* to induce vascular collagen degradation and platelet aggregation, concomitantly with the ability of *S. mutans* to spread into blood circulation, to invade endothelium and survive in coronary atherosclerotic plaque, provide better understanding regarding the mechanism role of *S. mutans* in pathogenesis of atherothrombotic diseases.

It can be concluded that *Streptococcus mutans* produce proteases which directly or indirectly via activation of MMPs monocyte can be able to degrade type IV vascular collagen, and its fragments induce platelet aggregation. This mechanism may suggest the role of *S. mutans* cause atherothrombotic disease.

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