RESEARCH ARTICLE

Porphyromonas gingivalis Induced Fragmentation of Type IV Collagen Through Macrophage-Activated MMP-9

(In Vitro Study of Collagenolytic Mechanism in Pathogenesis of Atherosclerotic Plaque Rupture)

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Abstract

BACKGROUND: Periodontitis is caused mostly by Porphyromonas gingivalis (P.gingivalis) and it is related to acute coronary syndrome. P.gingivalis readily invades blood circulation and potentially induces collagenolytic activity of inflammatory cells that results in collagen vascular degradation leading to atherosclerotic plague rupture (APR). APR is responsible for the occurrence of fatal cardiovascular events such as acute myocardial infarction (AMI).

AIMS: To show that P.gingivalis potentially induces fragmentation of type IV vascular collagen due to macrophage-activated MMP-9.

MATERIALS AND METHODS: The ability of P.gingivalis to induce the type IV collagen fragmentation, shown by digesting type IV collagen with the supernatant of monocyte-derived macrophage activated by exposure to P.gingivalis suspension for 18 hours, 37oC, 5%CO2. The type IV collagen fragments were analyzed by SDS-PAGE and confirmed by Western-blotting. Antibody of type IV collagen produced and confirmed by dot-blotting prior to its being used as primary antibody of Western-blotting. The existence of MMP-9 was detected by Dot-blot and Western-blot technique, while the MMP-9 activity was assessed by SDS-PAGE and zymograms.

RESULTS: Our data showed that P.gingivalis induced macrophage to produce MMP-9 as one of collagenolytic components, and interaction with P.gingivalis proteases enhanced the proteolytic activity and resulted in degradation of type IV collagen with molecular weight of 88 kDa into two smaller fragments with molecular weight of 80 kDa and 60 kDa..

CONCLUSION: P.gingivalis induced macrophage to activate its MMP-9 that led to fragmentation of vascular type IV collagen in the pathogenesis of atherosclerotic plaque rupture.

KEYWORDS: P.gingivalis, macrophage, type IV collagen fragmentation, atherosclerotic plaque rupture, AMI.

Introduction

Periodontitis is an inflammatory disorder of the periodontium initiated by specific bacterial species and characterized by the destruction of the supporting connective tissue and, in severe cases, by exfoliation of the teeth. Porphyromonas gingivalis, a gram-negative anaerobic bacterium, has been identified as a major etiologic agent of chronic periodontitis (1). This bacterial species produces cysteine proteinases (gingipains) that can be cell bound or secreted (2, 3). Few studies have evaluated the contribution of P. gingivalis gingipains to tissue invasion and destruction processes. The degradation of extracellular matrix components such as fibronectin and collagens by gingipains has been reported (4). Moreover, gingipains indirectly contribute to tissue damage through the activation of latent host matrix

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metalloproteinases (MMPs) (5) and the inactivation of host proteinase inhibitors. MMP-1 and MMP-9 can be activated by Lys-gingipain, while MMP-3 can be activated by Arg-gingipain (4).

Recent epidemiological studies suggested a link between periodontal infections and the increasing risk of atherosclerosis and related cardiovascular and cerebrovascular events in human subjects (6, 7, 8, 9). In some studies, associations were observed between the degree of coronary atherosclerosis and alveolar bone loss or the number of missing teeth, suggesting that the degree of vascular activation correlates with periodontal disease severity.

P gingivalis and its key components have highly facilitated access to the systemic circulation. As this mode of delivery of the microorganism does not involve oral inoculation, the means by which P gingivalis ordinarily gains access to the tissues in human subjects, P gingivalis will modulate early atherogenesis in mice that spontaneously develop atherosclerosis. A recent report has suggested that large inocula of P.gingivalis delivered via weekly intravenous injections into the systemic circulation of heterozygous apolipoprotein E (apoE) -/- mice accelerated atherosclerosis (10).

Epidemiological studies have shown that periodontitis may associate with the presence of atherosclerosis. DNA from periodontal pathogens has been detected in atherosclerotic lesions, regardless the fact viable oral bacteria may not be isolated from atherosclerotic plaques (11).

Lately, P.gingivalis is connected to acute coronary syndrome (ACS) because it can induce atherosclerotic plaque rupture (APR). APR is responsible in the occurrence of fatal cardiovascular events such as stroke, acute myocardial infarction (AMI) and sudden death. However, the evidence that confirms the role of P.gingivalis in ACS pathomechanism is yet to be further elucidated.

Inflammation is now suggested to play a key role in APR. A study by Susilawati (2008) has shown that P.gingivalis readily enters the blood circulation as demonstrated by the occurrence of its specific antibody (IgG) against P.gingivalis epitopes in the blood circulation. P.gingivalis induces acute inflammation due to the synthesis of collagenolytic components of neutrophil such as superoxide radicals and MMPs, which potentially increase collagenolytic activity on type IV collagen leading to APR. The inflammatory cell macrophage may contain proMMP-9, possibly activated by P.gingivalis, that plays a key role in the degradation of type IV collagen.

This study aimed to proof that P.gingivalis induces fragmentation of vascular type IV collagen due to macrophage-activated MMP-9. Result of this study may be used to produce monoclonal antibody that can be used for the diagnosis of early plaque rupture caused by infection of P.gingivalis.

Materials and Methods

Cell Culture. Monocytes were obtained from buffy coats of 50 ml of peripheral blood of healthy individuals. Fresh mononuclear cells for this procedure were obtained after Ficoll-Hypaque fractionation and 2 to 3 washes with RPMI medium containing 25 mM Hepes, 1,5 mL L-glutamine, 10 mg/mL gentamycine, 10% fetal bovine serum . Cells, were incubated for 2 h at 37°C (5% CO2 incubator). Then the floating cells were removed, dishes were rinsed twice with medium. Cells were inoculated at 25 x 103 cells per 1 cm well in thirty six wells, and half of the medium was replaced every 2 d and treated with 10-7 mg/ml phorbol 12-myristate 13-acetate (PMA). The cells were cultured for nine days for differentiation of monocyte-derived macrophage (Semizarov et al, 1998). The individual monocyte-derived cultures used in these experiments were obtained from donor after undergoing medical checkup which Hs-CRP is less than 2,5 mg/L. Ethical clearance and informed consent have been approved by the Instutional Ethical Committee for this investigation.

Bacterial culture. Porphyromonas gingivalis (obtained from ATCC 33277) was inoculated into BHI media and grown anaerobically for 48 h, enriched with hemin (10μg/mL) and vitamin K (1 μg/mL). P.gingivalis was described with gram staining, which take the safranin colour for this negative gram bacteria (Fig.1).

Bacterial infection. P.gingivalis was harvested from BHI media inoculation, the bacteria were harvested by centrifugation 600g for 10 min, washed twice in PBS, and resuspended in RPMI containing FBS 10 %. 106 bacteria were incubated into culture of 25 x 103 monocyte-derived macrophage per well for 18 h, 37°C and 5% CO2. The infected supernatant of macrophage that contained collagenolytic components, including MMPs were harvested by centrifugation 600g 4°C for 10 min. The supernatant was collected, and stored at -40°C refrigerator.

Detection of MMP-9 existence. Dot-blot technique was used to detect the existence of MMP-9 in the infected macrophage supernatant using antibody of MMP-9

(Sigma). Dark dot mean as positive result of this technique. Verification of the MMP-9 existence assessed by western-blot technique to find the MMP-9 molecular weight of 92 kDa.

Assay for MMP-9 activity. Gelatine zymography was used to assess matrix metallogelatinase activity. Briefly, 7,5 % polyacrylamide gels containing 2% gelatine, a substrate for the enzyme of interest, were prepared. The supernatant of infected macrophage were electrophoresed. Samples were not boiled prior to electrophoresis. After migration at 120 V, constant A, for 90 min, the gels were rinse in a solution of 25 % Triton-X for 30 min to remove SDS. The gels were incubated in a solution of 50 mM Tris-Cl pH 7,6, 0,2 mM NaCl, 5 mM CaCl2, 0,2 (v/v) brij 35, at 37°C for over night. The gels were stained in commasie brilliant blue R-250, as described elsewhere

According to Lee and Libby (1997) (12), the presence of polypeptide with molecular weight of 10 kDa also indicating the existence of full activated MMP-9. This supporting analysis carried out by means the protein profile of infected macrophage supernatant using sodium dodecyl sulphate - 12% polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970).

Type IV collagen degradation. The supernatant of infected macrophage that suggested to contain collagenolytic components such as MMP-9 were reacted with 50 μg/mL type IV collagen (Sigma) (2:1 volume equivalent), incubated at 37°C waterbath for 18 h. Incubation at 70°C for 10 min was used to inactivate the enzyme activation prior to degraded collagen profiling.

Type IV collagen degradation profile. The type IV collagen degradation profile was analyzed by SDS-PAGE (12%) (13). Prior to electrophoresis, the type IV collagen (Sigma) were degraded by the supernatant of infected macrophage as described above, the separated proteins were stained with silver stain. The degradation profile would next confirmed by Western-blot using antibody of type IV collagen to find the degraded fragments.

Antibody of type IV collagen production. 100 μg/ml type IV collagen (Sigma) mixed with complete Freund's adjuvant (1:1) prior to immunize to mice. Boosters were given once a week for three weeks. Yield of polyclonal antibody of type IV collagen obtained from the blood serum. The serum contained polyclonal antibody of type IV collagen were precipitated from the albumin using ammonium oxalate prior to suspend in tris-Cl buffer solution of pH 8,8 and stored at -40°C refrigerator.

Assay for the yield of type IV collagen antibody. Dot-blot technique was used to assess the yield of type IV collagen antibody using the type IV collagen (Sigma) as the antigen.

Confirmation of the type IV collagen fragments degraded by the supernatant of infected macrophage. Western-blot technique semi dry was used to confirm the type IV collagen fragments, the antibody of type IV collagen that has been produced before used as the primary antibody.

Results

The monocyte-derived macrophage cells matured after 9 days culturing. On day 9th, the culture were incubated with the presence of P.gingivalis. How the bacteria adhere to macrophage after 30 minutes is shown in Fig.1.

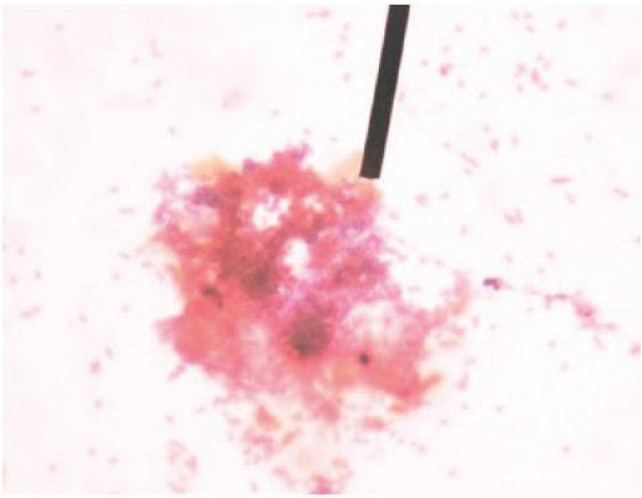

Figure 1. **Imaging of P.gingivalis adhesion to macrophage after 30 min incubation (400x magnification).**

The existence of MMP-9 in the infected macrophage supernatant. After 18 h at 37oC, 5%CO2 incubating the macrophage culture with P.gingivalis, the culture supernatant contained secretion of metalloproteinases and also proteinases of P.gingivalis was evaluated for the existence of MMP-9, the MMP-9 activity and subsequently the ability to fragment the type IV collagen. Figure.4 showed the existence of MMP-9 in the supernatant by the positive dot-blot result that depicted the dark colour of dot. However, the macrophage supernatant receiving no P.gingivalis also showed positive results, which mean MMP-9 already exist in the macrophage culture before infected with bacteria.

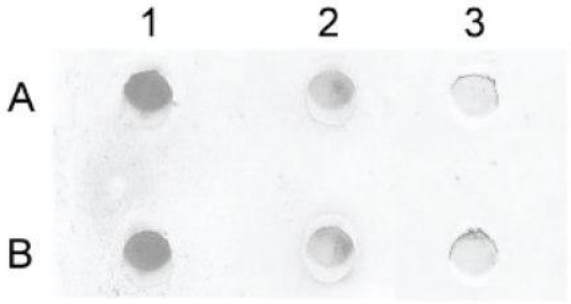

Figure 2. **Detection of MMP-9 existence of monocyte-derived macrophage supernatant induced by P.gingivalis.** Shown by dot-blot technique using antibody of MMP-9 (Sigma). The infected macrophage supernatant showed positive dot-blot result (A2, B2). Indicating the existence of MMP-9. A1 & B1 = MMP-9 (Sigma) as the antigen (positive control). A3 & B3 = Supernatant of macrophage receiving no bacteria (negative control).

Figure.3 shows the MMP-9 molecular weight of 92 kDa by Western-blot technique, which verifies the MMP-9 existence in the infected macrophage supernatant.

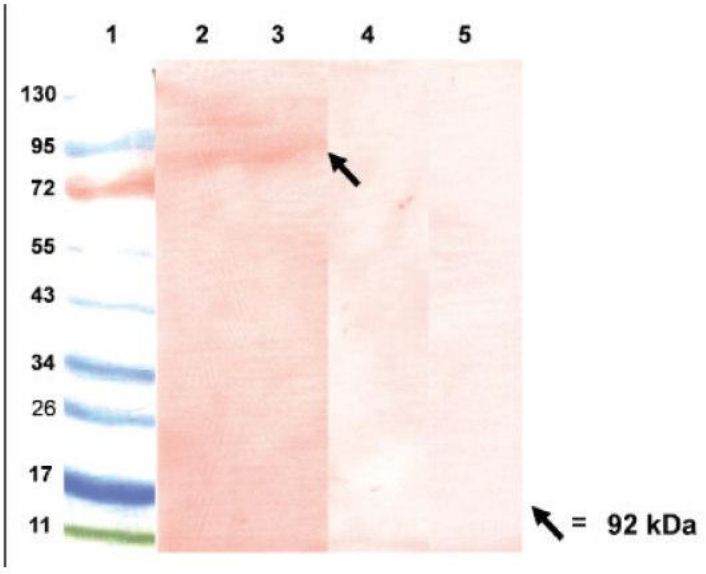

Figure 3. **From Western-blot result indicated the MMP-9 molecular weight of 92 kDa of the macrophage supernatant induced by P.gingivalis.** Shown by western-blotting of MMP-9 which depicted the molecular weight of 92 kDa (Line 2 and 3). The medium of macrophage receiving no bacteria (Line 4) and the negative control (Line 5) receiving no primary antibody depicted no protein bands. Lane 1 = prestained protein ladder. Molecular weight (MW) are indicated in thousand of Daltons.

The activity of monocyte-derived macrophage. The culture supernatant which contained proteases of P.gingivalis, also proved to contain MMP-9 or named metallogelatinase that was secreted by monocyte-derived macrophage. The MMP-9 was evaluated by gelatin zymography in order to assess gelatinase activity (Figure.4). In this technique, gelatinolytic activity was shown by the appearance of gelatinolytic on zones 72 kDa, 37 kDa and 32 kDa for the supernatant or macrophage medium before receiving P.gingivalis. While the gelatinolytic after infected with P.gingivalis was on zones above 76 kDa, 64-72 kDa and 54 kDa. The interaction between macrophage secreted enzyme and P.gingivalis proteases after infection made the alteration of gelatinolytic zones.

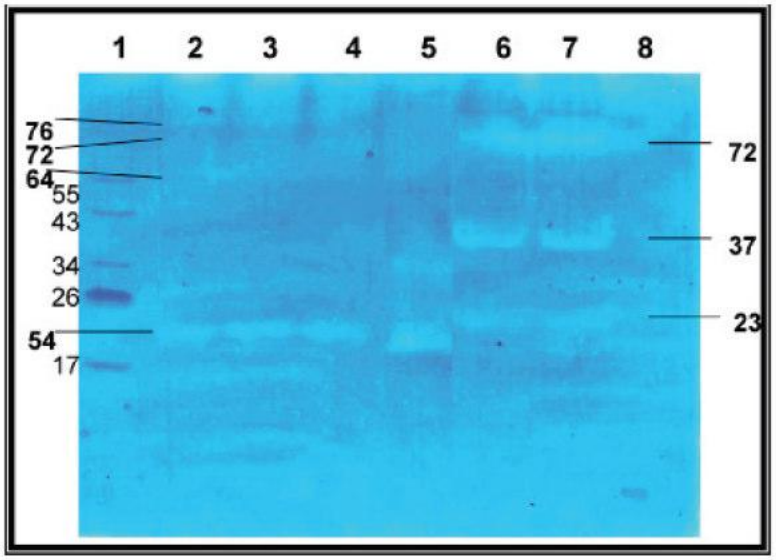

Figure 4. **The gelatinase activity of metallogelatinase contained in supernatant of monocyte-derived macrophage induced by P.gingivalis.** Assay for the gelatinase activity shown by zymogram. Lanes 2,3,4 = gelatinase activity of metallogelatinase contained in supernatant of monocyte-derived macrophage receiving P.gingivalis. Lane 5 = The pellet of macrophage supernatant receiving P.gingivalis Lanes 6,7 = Medium of monocyte-derived macrophage before receiving P.gingivalis. Lane 8. Pellet of macrophage before receiving P.gingivalis. Molecular weight (MW) are indicated in thousand of Daltons.

As supporting data analysis, we show the SDS-PAGE profile of infected macrophage supernatant, the presence of polypeptide with molecular weight of 10 kDa (Figure 5) also indicating the existence of full activated MMP-9, according to Lee and Libby (1997) (12).

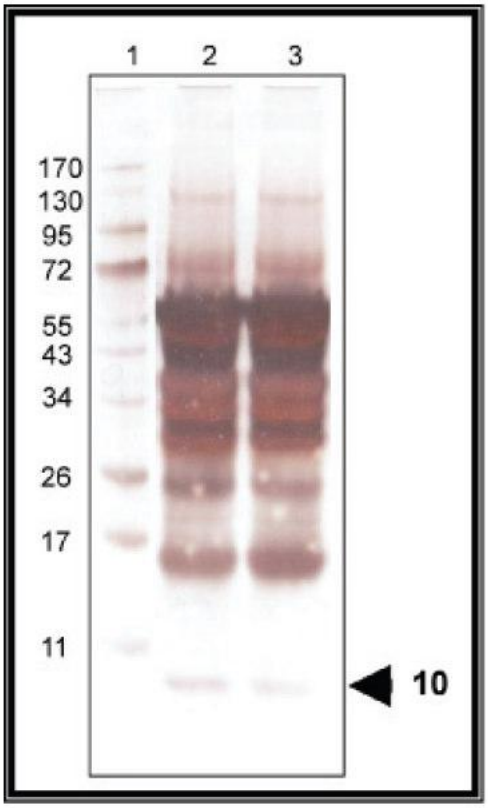

Figure 5. **Profile of 10 kDa polypeptide. Indicating presence of full activated MMP-9** Shown by Silver-stained SDS-PAGE of monocyte-derived macrophage supernatant induced by Pophyromonas gingivalis (lane 2,3). Lane 1 = Prestained protein ladder. MW indicated in thousand of Daltons.

Collagenolytic activity of macrophage-activated MMP-9 induced by P.gingivalis on the type IV collagen. The SDS-PAGE profile (Figure 9.A) showed the type IV collagen fragmentation pattern due to degrading activity of proteolytic enzyme, especially MMP-9 which contained in the supernatant of macrophage induced by P.gingivalis (in line 2A and 3A) compared to profile of the type IV collagen (line 4A) and profile of the type IV collagen fragmentation due to MMP-9 (Sigma) activity (line 5A). To accurate the pattern of type IV collagen fragmentation and eliminate the other bands that wasn't the fragments, confirmation using type IV collagen was obviously necessary.

We made the antibody of type IV collagen prior to western-blotting. The antibody of type IV collagen produced by collagen immunized collagen mice. The antibody verification using dot-blot technique showed the positive result (Figure 6), assuming that the antibody responded by mice was due to the type IV collagen.

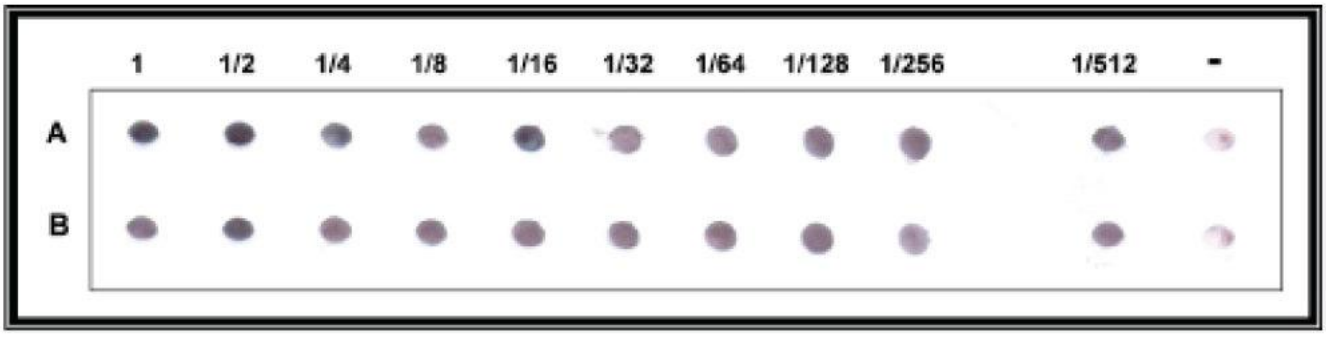

Figure 6.**Verification of the antibody type IV collagen production.**
Shown by positive dot-blot result of the antibody using the type IV collagen (Sigma) as the antigen. 1-1/512 of this figure was the lowered concentration of the antibody. All antibody concentration gave positive result which depicted as dark dotting. Whereas the negative control using bovine serum albumin (BSA) as the antigen gave a lighter dot.

The Western-blot data confirmed the fragmentation of type IV collagen due to degrading activity of proteolytic enzyme, especially MMP-9 which contained in the supernatant of macrophage induced by P.gingivalis (Figure 7.B). The data showed that the supernatant had the ability to fragment the type IV collagen with molecular weight of 88 kDa (line 4B) to be at least two smaller fragments which molecular weights are 80 kDa and 60 kDa (line 3B). Fragmentation of type IV collagen due to MMP-9 (Sigma) activity (line 5B) resulted in about four fragments with molecular weight of 80 kDa, 60 kDa, 28 kDa and 21 kDa. Two fragments were degraded by the collagenolytic components of activated macrophage which are same with the two of four fragments degraded by MMP-9 (Sigma) indicated the component that may responsible for the type IV collagen degradation in the supernatant was indeed MMP-9.

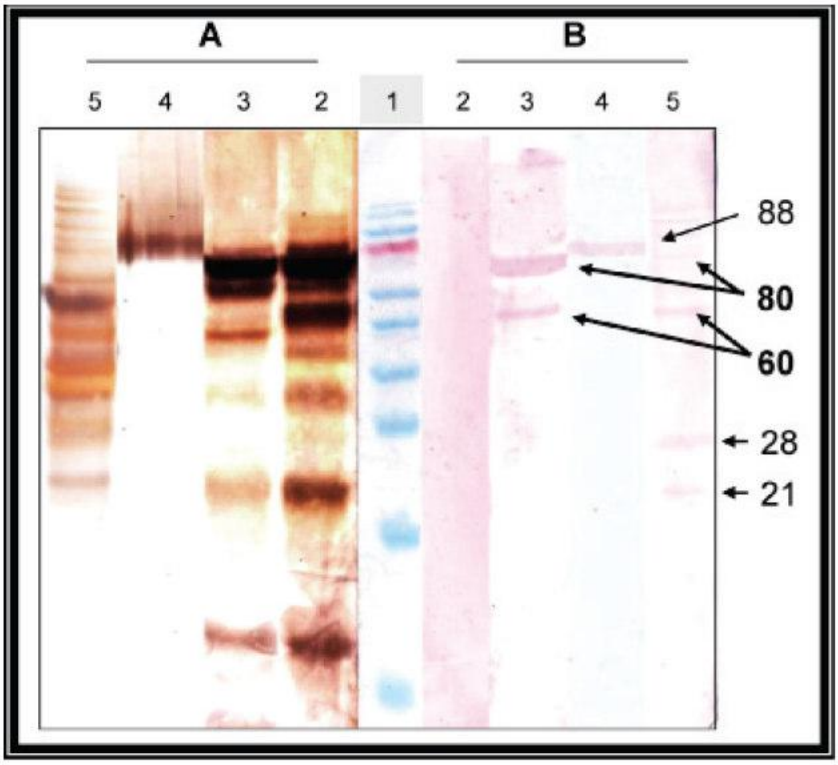

Figure 7. **Fragmentation of type IV collagen caused by MMP-9 contained in supernatant of macrophage induced by P.gingivalis**. Shown by Silver-stained SDS-PAGE (A), confirmed by western-blot technique using antibody of type IV collagen (B). Lane 1 = Prestained protein ladder. Lane 2A & 2B = supernatant of infected macrophage. Lane 3A & 3B = type IV collagen reacted with supernatant of infected macrophage. Lane 4A & 4B = The type IV collagen (Sigma). Lane 5A & 5B = type IV collagen reacted with MMP-9 (Sigma). Molecular weight (MW) are indicated in thousand of Daltons. Shown that the type IV collagen of 88 kDa (4B) fragmented to be 80 kDa and 60 kDa (3B). Molecular weight (MW) are indicated in thousand of Daltons.

Discussion

The majority of acute clinical manifestations of atherosclerosis are due to physical rupture of advanced atherosclerotic plaques. It is now believed that physical rupture of advanced plaques, not lesion stenoses, are responsible for the majority of acute clinical manifestations of atherosclerosis such as myocardial infarction and stroke (14,15,16). Studies of human atherosclerotic plaques have revealed that lesions that tend to rupture are rich in activated macrophages and have a thin fibrous cap, implicating the macrophage as a key regulator of atherosclerotic plaque stability (17). Given that the ECM components collagen and elastin are responsible for the structural integrity of the lesion's fibrous cap, the balance between the synthesis and degradation of these matrix components appears critical for plaque stability. Macrophages are thought to play an important role in this balance due to their ability to produce enzymes capable of degrading the ECM. Members of the MMP and cathepsin families of enzymes appear to have the requisite collagenolytic and elastinolytic activity to destabilize advanced plaques, with several MMPs and cathepsins expressed by macrophages at sites of plaque rupture (18).

It has been hypothesized that macrophages play a key role in inducing plaque rupture. This investigation reveal the role of macrophage in inducing plaque rupture by secreting proteolytic enzyme that destroy the extracellular matrix type IV vascular collagen that provides physical strength to the fibrous cap, due to inducement of periodontal bacteria P.gingivalis.

All data showed that collagenolytic components secreted from activated macrophage culture due to inducement of P.gingivalis interacted each other resulting

type IV collagen fragmentation. Macrophage-activated MMP-9 suggested as the main enzyme taking part on this collagenolytic activity. MMP-9 produced as inactive zymogen (proMMP-9). Certain mechanism is necessary to activate it. Chlamydia pneumoniae (19) reported to be able activating it. This study demonstrated how zymogen MMP-9 activated by proteases from P.gingivalis.

The macrophage-activated MMP-9 as one of matrix metallogelatinases was shown to have gelatinase activity from the zymogram, suggestive of the ability of P.gingivalis to activate or increase the activity of macrophage-activated MMP-9. This suggestion is supported by the presence of polypeptide with molecular weight of 10 kDa shown in the profile of infected macrophage supernatant (Fig.7), indicating the presence of full activited MMP-9 (12). This 10 kD protein describes peptide fragment of N-terminus bound of MMP-9 predomain. MMP-9 investigated in this study was found related to fragmentation of type IV vascular collagen. However, other MMPs also play key role in collagenolytic activity (20).

Bacterial proteases activate MMPs through proteolytic mechanism by releasing one or more polypeptide bonds in NH2-terminal, resulting in the exposure on caltalytic site of MMPs that leads to enzyme activation. P.gingivalis proteases (thiol proteinase) is reported to have the ablility to activate zymogen MMPs through proteolytic activity, among which are MMP-1, MMP-3 and MMP-9 (5). MMP-1 and MMP-9 can be activated by Lys-gingipain, while MMP-3 can be activated by Arg-gingipain. Pure gingipain also reported to activate whole MMPs significantly (4).

The SDS-PAGE profile of type IV collagen fragmentation which was confirmed by Western-blotting showed that P.gingivalis may potentially induced macrophage to activate its MMP-9 and fragmented the type IV collagen into two smaller fragments with the molecular weight of 80 kDa and 60 kDa.

The type of fragments degraded related to bacteria inducement seemed to be different among several bacteria (20). This assumption may relate to specific component secreted by the bacteria and also the difference of collagenolytic mechanism among those bacteria.

The type IV collagen was the main ECM that gave structural integrity of plaque fibrous cap. Some fragments of the type IV collagen plays a role in controlling adhesion, proliferation or cell apoptosis. Many enzyme (MMPs, elastase, cathepsin) shown to have ability in degrading the type IV collagen and resulting fragments that have unique character (22). Interestingly, Pfister et al, 2006 (22) reported that peptide fragments from collagen fragmentation identified as neoantigens. More studies about fragments that associate with P.gingivalis are necessary to confirm if those fragments are neoantigens or not.

Collagen degradation is a complex process that occurs in many stages. Physiologically, the main components involved in collagen degradation are MMPs. Type IV collagen is specifically degraded by metallogelatinase (MMP-2 and MMP-9). Degradation is continued by specific proteinases and the collagen fragments are phagocytozed by cells and processed by lysosomal enzymes (23).

The role of P.gingivalis in inducing fragmentation of type IV collagen trough macrophage-activated MMP-9 in the pathogenesis of plaque rupture occurin in at least three stages (21), namely: (1) P.gingivalis invades the systemic circulation and reaches the fibrous cap of atherosclerotic plaque, (2) P.gingivalis induces local inflammation responses of macrophage on the atherosclerotic plaque, and (3) enhances macrophage proteolytic activity through MMP-9 activation that increases the collagenolytic activity leading to type IV collagen fragmentation and resulting in acute plaque disruption.

Conclusion

Porphyromonas gingivalis indirectly induces plaque rupture of acute myocardial infarction through activating MMP-9 of monocyte-derived macrophage that leads to fragmentation of type IV vascular collagen.

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