REACTION BIOMARKER MONOCLONAL FRAGMENTATION OF COLLAGEN TYPE IV WITH BLOOD SERUM ACUTE MYOCARDIAL INFARCTION PATIENT RELATED INFECTION

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Abstrak

Penelitian ini bertujuan untuk mendeteksi penyakit jantung akut myocardial infark dengan menggunakan biomarker antibodi monoclonal fragmentasi kolagen tipe IV. Rupture plak aterosklerosis adalah terjadi karena degradasi kolagen tipe IV. *Perviromonas gingivalis* adalah salah satu faktor resiko yang terpenting untuk terjadinya akut myocard infark (AMI). Rancangan penelitian ini adalah ekperimental invitro dengan deksriptif cross sectional. Sampel penelitian adalah empat orang sehat (n=4) serta 12 penderita AMI (n=12). Untuk melihat ekpresi enzim MMP-9 dan fragmentasi kolagen tipe IV, kami menggunakan teknik western bloting. Untuk mengisolasi sel limfosit, kami mengimunisasi mencit dengan antigen fragmentasi kolagen tipe IV disertai dengan adjuvant. Selanjutnya dilakukan fusi antara sel limfosit dengan sel myeloma sehingga terbentuk hibridoma dan diuji dengan western bloting serta ELISA teknik. Kemudian dilakukan seleksi klon. Selanjutnya dilakukan insersi pada intra peritoneal sehingga terbentuk ascites. Hasil penelitian ini menunjukkan bahwa MMP-9 muncul pada 92 kDa dan fragmentasi kolagen tipe IV dengan band 60 – 80 kDa. Untuk menguji reaksi monoklonal antibodi fragmentasi kolagen tipe IV dilakukan uji 12 sampel penderita AMI. Hasilnya semua sampel AMI positif. Kesimpulan menunjukkan bahwa biomarker monoklonal antibodi fragmentasi kolagen tipe IV ternyata bereaksi positif pada penderita AMI.

Kata kunci : monoklonal antibodi fragmentasi kolagen tipe IV, AMi, biomarker

Abstract

The objective of this study was to detect Acute Myocardial Infarction (AMI) by biomarker monoclonal antibody fragmentasi collagen type IV. Rupture of atherosclerotic plaque is initially occured by degradation of collagen type IV. Infection *Perviromonas gingivalis* is one of important risk factors for AMI. The design of the research was experimental in vitro with descriptive analytic cross sectional design. The subject was (n=4) healthy and (n=12) AMI patients. To show the expression of MMP-9 and the fragmentation of collagen type IV we also used western blotting technique. Isolation lymphocyte

used mice sub cutan immunization. Continue by fusion with myeloma cell forming of hybridoma test by Western blooting and Elisa technique. Selection of clone to insertion intraperitonial to forming the ascites evaluated by Elisa technique. The result found the product of MMP-9 obtained the band showed 92 kDa and fragmentation collagen type IV band 60-80 kDa. To test the reaction of monoclonal antibody fragmentasi collagen type IV on the 1th-12nd blood sample AMI show the all of the blood sample AMI positive. The conclusion research show biomarker monoclonal antibody of fragmentasi collagen type IV showed AMI positive.

Key words: Monoclonal antibody collagen type IV, AMI, biomarker

Introduction

Heart disease of Acute Myocardial Infarction (AMI) at this time is still a major health problem in the world. In addition to the increased prevalence of this disease, it has also become the main cause of death.[1] It was recorded in 2000 that as many as 16.7 million people, or approximately 30.3% of total mortality in the world was caused by AMI disease and more than half were reported from developing countries.

The factor that has been associated with the AMI development is atherosclerosis. There are many factors that can be formed by atherosclerosis like dislipidemia, hyperhomocystein, diabetes mellitus, hypertension, etc. Nevertheless, the infection process has not been revealed yet. The process of atherogenesis is like a chronic inflammation response against microorganism.[2] The microorganism can invade vascular endothel and induce inflammation response directly or indirectly through systemic effect.

In the inflammation process, the activated cells will increase proenzyme production, including Matrix Metalloproteases or MMPs. This proenzyme could be changed into active enzyme that causes collagen lease.[3] If this process occurs on the surface of atherosclerosis plaque, the fibers of collagen which protect the plaque will get thinner and eventually rupture. Collagen type IV is known as a major component of basal vascular membrane which is located under the endothel of vein cells. Collagen type IV is easily damaged by collagenese activity in circulation, due to its near location to the blood circulation and its structure containing protein globules (non-collagenous domain, not fibrous) which are susceptible to various collagenese.[4,5,6] The degradation of collagen type IV can occur directly due to protease produced by microorganisms; or when there is a significant inflammation, there will be a greater degradation by MMPs activity. [3]

Some researches related whole cell *Perviromonas gingivalis* infection to periodentitis disease such as cardiovascular disease, especially when a seroepidemiology research done by Ameriso and Muliartha et. al found that there was a significant correlation between patient of seropositive Whole cell *Perviromonas gingivalis* with patient of atherosclerosis. [7.8] However, in the infection

process, Whole cell *Perviromonas gingivalis* did participate in the blood circulation mechanism namely systemic effect.

The product of microorganism, which whole cell bacterial antigenic *Perviromonas gingivalis* in the blood circulation, can indirectly damage endothelium vascular by stimulating immune response.[9] Whereas, the whole cell bacterial antigenic *Perviromonas gingivalis* microorganism is still virulent to the host. Thus, the whole cell bacterial antigenic participating in the blood circulation will cause an echo. The activation of set-cells related to ateroma, which is cause by the product of bacteria, will produce cytokine. This cytokine will release interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α). Besides, the cytokine will stimulate hepalic synthesis from acute-phase reactants such as fibrinogen.[10]

Whole cell *Perviromonas gingivalis* is one of the bacterial periodentitis. Is known to be related to pathogenese and the whole cell bacterial antigenic *Perviromonas gingivalis* strain because it is an antigen which can be recognized by non-specific and specific immune system, and involves toll-like receptor (TLR4).

Based on the above statements, the researchers intended to prove the theory by conducting a research in vitro toward Whole cell *Perviromonas gingivalis* that may be able to induce immune response in producing MMP enzyme, and to investigate whether the enzyme can actively degrade the collagen type IV, and immunized to produce antibody monoclonal to detect fragmentation of collagen type IV on blood of AMI.

It has been noted that the major cause of the challenges for oxygen and nutrition supply to the heart is the existence of thrombus. The development of thrombus is caused by several factors like the rupture of atherosclerotic plaque and the factor of microorganism infection that can damage the endothelium cells.

Besides causing the rupture of atherosclerotic plaque, microorganism infection can also cause thrombus through the damaging of endothelium cells. Some of the potential infection agents may induce AMI. The direct microorganism invasion to the blood circulation will create inflammation response causing the increase of lymphocyte and macrophage, and the increase of cytokine production and tissue growth factors.

By systemic effect, the product produced by microorganism will indirectly create an echo, a condition in which there is an activity of cells producing cytokine as a response to the produced . This echo can damage the endothelium and caused thrombus induce AMI.

Up to now, biomarker diagnostic is still unsatisfactory. For example, enzyme Creatine Phospo Kinase (CPK), myoglobin, troponin A and B are only diagnosed after AMI process. The factor of etiology is also unknown. Also, the treatment using invasive ultrasonograph is very expensive. Therefore, our research is aimed at making biomarker antibody by antigen fragmentation collagen type IV to detect infection process and to detect AMI earlier, hope an antibody more effectively, more efficiently to detect AMI.

Research

This research was conducted in the central biomedical laboratory Malang. The design of the research was experimental in vitro with descriptive analytic cross sectional design. The subject was (n=4) healthy people and (n=12) AMI patients. The research was carried out in the vascular biomedical laboratory Malang. The research was approve by national commitee animal and human of ethical clearance. To examine the activity of MMP-9 enzyme, we used zymogene technique which was continued by electrophoresis, and strengthened by western blotting technique (Amer Sham Pharmacia Bio Tech).

This study comprised from two phases namely the phase of degrading collagen type IV using MMP enzyme which was produced by neutrophil due to the induction of Whole cell *Perviromonas gingivalis*, and the phase of producing and testing monoclonal antibody of anti fragments of collagen type IV.

Production and Activity Test of MMP Enzyme and Degradation of Collagen Type IV

Stimulation of secretion and activation of MMP enzyme was done by putting Whole cell *Perviromonas gingivalis* to the neutrophil which has been incubated for an hour under the temperature of 30 degree Celsius. The isolation of MMP-9 production was done using zymography electrophoresis technique. The result showed that the white band formed on blue gel was metalloproteinase enzyme. This test was continued by western blotting technique to show the molecular weight. The Western Blotting technique was shown through using the MMP-9 antibody as the primary antibody. (Sigma) MMP enzyme that was exposed on collagen type IV, that has been labeled biotin with 1 : 2 ratio was incubated for 18 hours at room temperature. Reducing Sample Buffer (RSB) was added and heated at the temperature of 100^{o} C for five minutes. The sample was put into the well which has been filled with gel, then it was run with tri-glysine SDS running buffer (sigma), then it was run on the condition of constant voltage (120V), and 30 mA current strength for 90 minutes. Bands resulted from the fragmentation of collagen type IV were observed by using blue R-250 blue comassie coloring. The removal of color was performed by soaking the gel in the destaining solution and shaking it using a shaker till the gel became clear. The result of the electrophoresis was treated by Western blotting using antibody anti fragmentasi collagen type IV.

Fragmented band of collagen type IV which was seen on the Nitro Cellulose membrane (sigma) was used as antigen to be injected in the immunization of mices. Electroelution was carried out by cutting the bands transferred on NC membrane (sigma), then inserted into the selofan (dialysis membrane) that has contained 1 ml running buffer. Dialysis membrane was inserted to electroelution chamber and run on 25 volt, 30 mA for 120 minutes. The result of the electroelution was dialysed and let overnight at the temperature of 4^{o} C. The result of the dialysis was put in effendorf which had been filled with an absolute ethanol (1 : 1), and incubated for a night at 4^{o} C. After incubated, the sample was sentrifugated at 4^{o} C in the 6000-12000 rpm for 10 minutes. Supernatant was removed and the pellet was dried by

tapping it in the refrigerator with the temperature of 4° C. The pellet that had been tapped was added with tris-HCl buffer (6.8 pH) and stored as a stock of antigen of fragmented collagen type IV.

Production and Test of Monoclonal Antibody of Fragmented Collagen Type IV

To isolate the antibody of fragmented Anti-collagen type IV, we did the preliminary steps as follows. First, the fragmented antigen of collagen type IV resulted from the electroelution was injected to male mices aged about 4 months (the body weight was around 600 g) which was kept in a cage and fed with pellet food. The immunization was done by injecting the antigen of fragmented collagen type IV. The injection was done five times with one week injection time interval. In the first week, the antigen fragment collagen type IV was mixed with Freund's Complete Adjuvant (CFA) using the ratio of 1:1, while booster injection (booster 1 to 4) was injected again by using the antigen of fragmented collagen type IV mixed with incomplete Freund's Adjuvant (sigma).

The Harvest period of the blood lymphocyte was done one week after the first week injection and every week after the injection of each booster. Isolated of the lymphocyte cell and fusion with the myeloma cell to forming the hibrydoma cell and next to selective a clone, and inserted the best clone to intraperitonial of the mice and forming of the ascites mice. The Blood serum ascites was then placed in a centrifuge tube at the temperature of 4° C for one night and centryfuged at 2000 rpm for 15 minutes. Anti-serum, which was supernatant was separated and added with glycerol solution and stored in serum bottles.

Antibody purification was done through the precipitation of 50% saturated ammonium sulphate. Solid ammonium sulphate was added to serum (SAS) in a ratio of 1:1, and was the processed in a vortex 3 times each for 3 minute at 10 minute intervals, followed by placing the samples in a low temperature for 1 - 2 hours. Then it was put in a centrifuge at 3000 rpm for 20 minutes, the pellets were retrieved and 50% SAS was ad at a ratio of 1:2 and again placed in a centrifuge at 3000 rpm for 20 minutes, the supernatant was disposed of and the pellets dissolved in 5ml 0.2m phosphate buffer of 7 pH, this was then placed in a vortex for 3 minutes, followed by dialysis with l000ml 0. 1M phosphate buffer with a pH of 7 at 4°C for one night. Antibody concentrations absorption levels were measured based on optic speed (OD) equal to 1.4 or equal to a protein concentration of 1 mg/ml.[11]

Dot Blot Specificity Test for Ascites Antibodies

Evaluations and antibody specificity tests were implemented by using a Dot Blot test in accordance with Priou at al and Suryadi et al's methods. The NC membrane was cut into 5 x 7 cm pieces, put in a Dot Blot test device and moistened with 50ul Tris Buffer Saline solution (TBS) per well and degassed. Then the NC membrane was incubated in a blocking solution of 37° C TBS skim for 1 hour. After the incubation, it was washed with 0.05% TBS-T 3x3 minutes and an antibody solution as an antibody primer at a ratio of 1:200 and was

Inserted into the NC membranes and incubated for one night at 4°C. After this incubation, they were washed with 0.05% TBS-T 3x3 minutes, and dissolved secondary antibodies (alkaline phosphatase conjugated antibodies 1200 in TBS) were added for 60 minutes at room temperature and shaken, and further washed with 0.05% TBS-T 3 x 3 minutes and then given a substrat for staining (enzymatic reaction) and a western blue substrat solution for 10-30 minutes in a dark room. Reactions are stopped by washing the samples with aquades whenever there is a change in colour in the drops from light to dark purple. A change in colour to purple in drops indicates a positive reaction. Dot Blot results were Interpreted with a 12 version Corel.[11]

The Tests for AMI Patients' Serum Interaction and Type IV Collagen Anti FragmentAntibodies

The evaluation of the AMI patients' serum interaction and type IV collagen anti fragment antibodies was implemented by a Dot Blot method. To find the results of the interaction between the type IV collagen prototype monoclonal fragments, tests were run on numbers 1 - 16 patients, consist 1 – 12 AMI patients and 4 healthy persons and test using Dot Blot technique. The Dot Blot test is positive when blue appears.

Results and Evaluation

The MMP production and test using Sodium Dodecyl Sulphat Poliacrilamide Gel Elektroforese (SDS-PAGE) Gelatin Zymography were conducted. The MMP enzyme production was suspected to be obtained from one of the neutrophil contents inducted to *Perviromonas gingivalis*. To find out the existence and activities of the MMP enzyme, the MMP enzyme test was conducted by using SDS-PAGE of gelatin gel toward the supernatant from neutrophil which was inducted to Whole cell *Perviromonas gingivalis*. The results of the MMP enzyme test using SDS PAGE gelatin gel can be seen in figure 1.

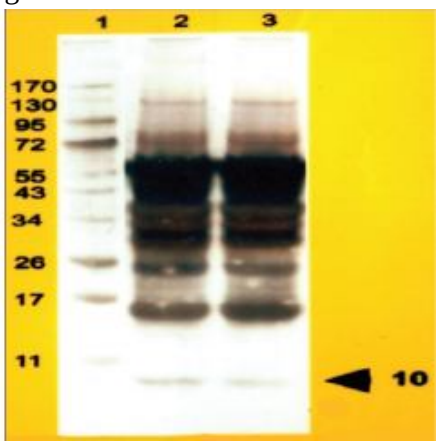

Figure 1 : MMP test with SD S-PAGE gel showing S1 results, marker samples
S2 - S3 = 72 - 96 kDa.

M = Sigma protein detector marker using silver nitrate
S = Sample in well
S_1 = sample on the well,
M = high marker protein detector.

The picture above shows in the wells of S2 to S3 there are white transparent bands with molecular weight of 96 kDa and 72 kDa. In the well S1 it was M Marker. The gel resulted from SDS-PAGE 7.5% gelatin was tested using Western blotting by using the antibody anti-MMP-9 as the primary antibody.

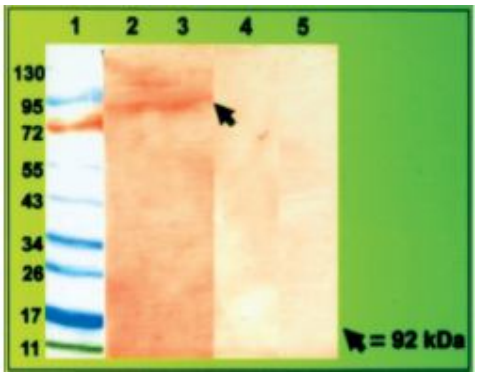

Figure 2 : Western Blot results from antigen reactivity - MMP-9 was able to identify the MMP enzyme. M = markers Si, S2 and S3 = 92-96 kDa., MMP-9 kDa: S4 + S5 = control.

Figure 2 shows the image on the Western blotting results in which anti-MMP-9 antibody reactivity can recognize MMP-9 enzyme. Show the MMP-9 on the band S2-S3, were 92 kDa. The MMP enzyme recognized by anti MMP-9 antibody was suspected to be the result of MMP-9 enzyme.

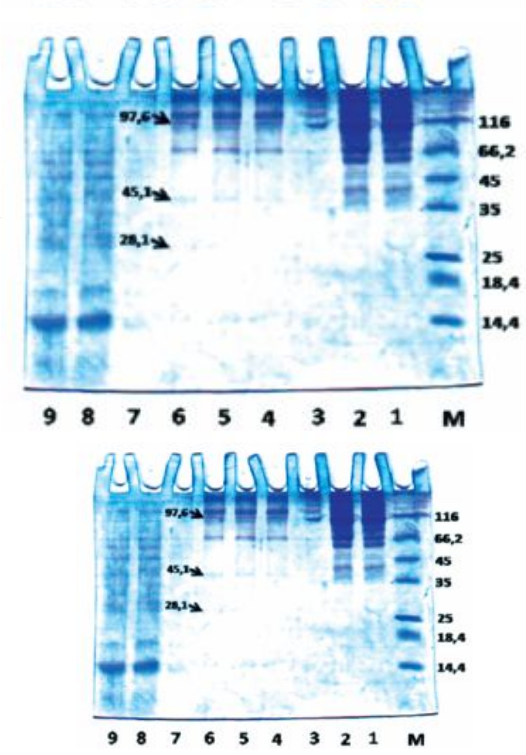

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Figure 3 : SDS-PAGE results, 10% above type IV collagen induced for MMP-9 with brilliant blue 258R commassie stain.

M = sigma protein marker detector levels with brilliant blue R-256 commissie stain

K = type IV collagen,

S1-S2 = all type IV collagen cells (100 - 116 kDa), S4- S6 fragmented (66.2 - 97.6 kDa)

Western Blotting result the reactivity of antibody of anti-collagen type IV toward the band treatment MMP-9 that was inducted to collagen type IV can be seen in figure 4B.

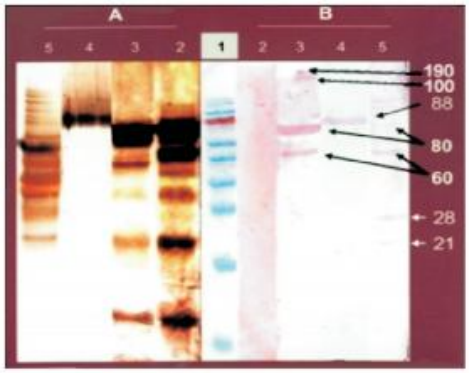


Figure 4B : Western Blot results from type IV collagen induced for MMP-9.

Sigma high protein indicator with brilliant blue R-258 commissie stain,

S1 = markers,

S2 = pure type IV collagen at 100-196 kDa,

S3-S4=fragmented type IV collagen at 60-80 kDa

Figure 4B shows the reactivity anti-collagen type IV on the band samples of collagen type IV inducted to MMP-9 from the neutrophil of AMI patients. The band show a molecular weight of

60 kDa – 80 kDa.

A Western Blot test had 2 bands which were positive for anti type IV collagen antibodies (figure 4b). Whereas, the anti type IV collagen antibody rectification to the pure type IV collagen samples (to be control on well 2) indicated that all bands responded, moreover, some bands, previously not detected in the 10% SDS-PAGE, when given comussie brilliant blue stain could be detected by anti type IV collagen antibodies.

The interaction test for type IV collagen anti fragment antibodies was enacted by using Dot Blot to find out the interaction between type IV collagen antigen fragments produced by the mice and the type IV collagen antigen fragments induced by the Peperimonas Gingivalis whole cells.

Tests on the Immunization Results and the Type IV Collagen Monoclonal Antibody Fragmentation Production.

The mice immunization using type IV collagen fragments with added complete and incomplete freund adjuvant produced lymphocyte cells (Figures 5 and 6).

Figure 5 : Preparations for the immunization.

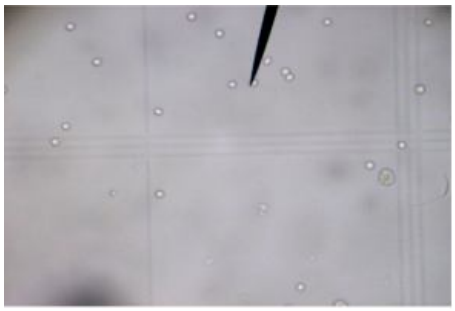


Figure 6 : Isolating lymphocyte cells

This was followed by lymphocyte cell isolation as well as myeloma cell isolation and growth. Then the fusion of lymphocyte and myeloma cells to form hybridoma. After this, hybridoma clone selection was initiated by employing an Elisa test (Figures 7, 8 and 9).

Continue by Western Blooting (Figures 10).

The result were shown in band at 97.6 kDa by Western Blooting, whereas the Elisa test found high antibody levels of 2.584 (Figures 10, 11)

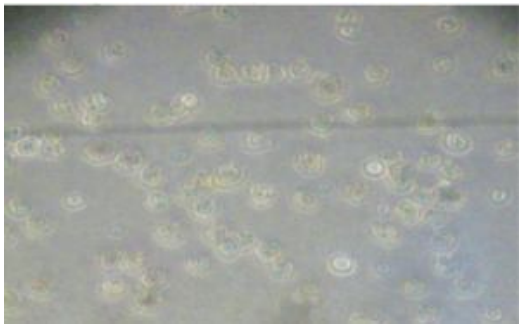


Figure 7 : Identifying myeloma cells, the results of the lymphocyte cell fusion with myeloma cells forming hybridoma cells

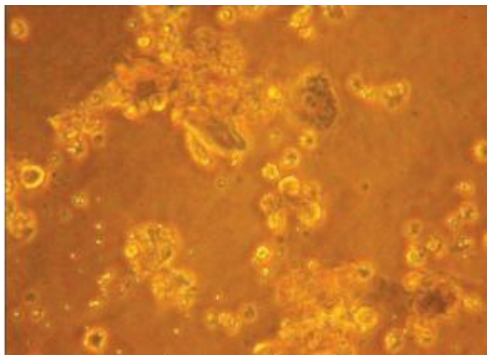
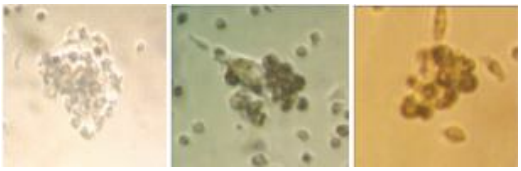


Figure 8 : Hybridoma cells on day 10



CLONE 1 CLONE 2 CLONE 3

Figure 9 : Selected clones : Hybridoma cells selected from screened cells and tested with Western Blot and ELISA

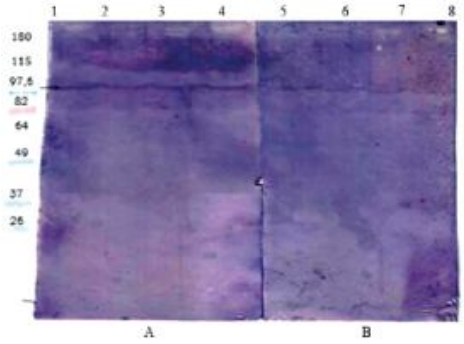


Figure 10 : Selected clones Western Blot analysis results.

Western Blooting analysis results for the interaction between antigen collagen type IV fragments indication that S3, S4, S7 and S8 molecule weights were 97.6 kDa.

1 2 = Biomedik
3 4 = Pusvetma
1 3 = Collagen
2 4 = Fragmentasi Collagen
Absorbance Report
Single Wavelength
Mes = # 1

Antibodi primer 1 : 1
Blank Mean 0.036
Std. Dev. 0.000

	1	2	3
A	0.000	- 0.001	0.008
B	1.315	1.648	2.156
C	0.989	2,062	2.302
D	2.400	1,897	1.911
E	1.862	1.975	1.808
F	1.646	1.924	1.919
G	2.159	2.112	2.042
H	1.623	2.580	2.008
	1	2	3
A	0.000	- 0.034	- 0.035
B	1.787	- 0.029	- 0.033
C	1.987	- 0.028	- 0.032
D	1.784	- 0.028	- 0.033
E	2.059	- 0.028	- 0.032
F	1.967	- 0.030	- 0.033
G	1.710	- 0.031	- 0.034
H	2.584	- 0.029	- 0.034

Figure 11 : Testing hybridoma clones, analyzing hybridoma supernatant specificity from selected clones on type IV collagen and fragmented type IV collagen with ELISA.

Collumns 1,2 : Supernatant hybridoma
Collumnus 3,4 : supernatant hybridoma
Collumnus 1,3 : type IV collagen antigen
Collumnus 2,4 : type IV collagen antigen fragment

Lines B, C, D, E, F, G, H: sequence of antigen concentrations 200, 100, 50, 25, 12.5, 6,25, 3,125 ng/ml

The selected clones, which were the highest antibody titers, were prepared for insertion intraperitonally into the mice and aided by giving prestan to form ascites.

The growth of ascites mice (figure 12) evaluated by Elisa Technique and continue by delution test of antibody (see figure 12, 13 and table 1).

Figure 12 : Growth of mice ascites

Absorbance Report
Single Wavelength
Mes = # 1
Blank Mean 0.053
Std. Dev. 0.000

	Fragmen Collagen I	Collagen Total	Kontrol Sekunder
A	0.000	0.003	0.011
B	2.658	0.131	0.116
C	1.671	0.104	0.117
D	1.014	0.141	0.116
E	0.614	0.146	0.109
F	0.365	0.152	0.110
G	0.243	0.109	0.695
H	0,223	0.099	0.110

Figure 13 : Testing type IV collagen monoclonal prototype antibody fragmentation, ascites laboratory ELISA test results. Notes:

Coloumn 1: Antigen fragments 1 from type IV collagen infection.

Coloumn 2 : Type IV collagen antigens
Antigen concentrations by Elisa technique

A. 0 ng E. 12,5 ng
B. 200 ng F. 25 ng
C. 60 ng G. 6,25 ng
D. 50 ng H. 3,125 ng

Results of the test run on the interaction between the anti serums induced by the type IV antigen fragmentation in the mice are in Figure 14. The total number blues indicates a positive reaction between the mice anti serum and the type IV collagen antigen fragments to the 1:5000 thinning and the Elisa test score high this was 2.67 (figure 13, 14 and table 1).

Table 1
Dilution Antibody Anti Fragmentasi Collagen Type IV

Table 1
Dilution Antibody Anti Fragmentasi Collagen Type IV

Concentrate Antigen 1/100	Delution antibody anti fragmentation collagen type IV					
	1	1/10	1/100	1/500	1/1000	1/5000
Control	0	0	0	0	0	0
Booster 1	136.04	82.04	120.59	144.01	157.13	179.65
Booster 2	189.03	194.27	209.08	211.56	213.7	213.2
Booster 3	122.21	51.1	87.63	120.8	142.12	174.74
Booster 4	64.04	81.22	80.65	101.8	138.43	162.18

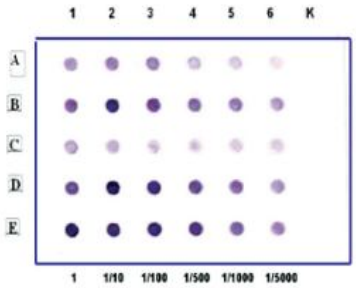


Figure 14 : Results from the interaction between the mice serum antigen and the collagen type IV antigen fragments from the Dot Blot test indicating positive purplish blue interaction results from the dilution to 1/5000, as well as corel analysis version 12

In addition, tests results from AMI patients 1 - 12, together with 4 samples for control from healthy patients; were positive for the AMI patients but negative for the control (Figure 15).

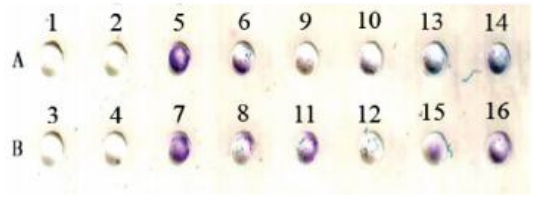


Figure 15 : Dot Blot analysis results for the interaction between biomarker antibody fragments collagen type IV with blood serum AMI patient's show 12 AMI patients positif.

Discussion

It is known that *Perviromonas gingivalis* has whole cell protein that is antigenic and an antigen that is recognized by the specific immune system, whereas, the lipid A group is an antigen that can be recognized by the non specific immune system. [12]

As has already been explained, microorganism whole cells can involve the toll-like receptor system (TLR4) which in turn can activate NF-KB and express genes that encode protein that are important in various non specific immune response components that include inflammatory cytokines (TNF-α, IL-2 and IL-12), leukocyte-endhotelial adhesion molecules (such as E and P-selectin) and proteins involved in the microbe killing mechanism (like iNOS.[12]

According to Abbas, increased cytokine production will activate phagocytes and TNF, produced by the cytokine, and will not only direct. neutrophils and monocytes to areas of infection but also activate them to destroy antigens.

This study has proved that the introduction of Perperimonas Gingivalis whole cells can induce neutrophils to produce MMP enzymes, and this was done by testing the MMP enzymes produced by neutroflis with zymography (Figure 1), neutrophil samples from AMI patients were given *Perviromonas gingivalis* whole cells. (S1 to S3), and indicate the presence of a transparent chocolate band formed at the 96 kDa and 72 kDa molecular weights (Figure 2).

MMP-9 activity between 72 kDa and 96 kDa molecular weights indicate that the neutrophil isolated from the AMI patients were neutrophil containing pro MMP9, so, at the time of the introduction of Perperimonas Gingivalis whole cells this pro IVIMP-9 became active immediately. This may have a connection with the fact that AMI patients had an inflammatory response throughout the development of their atherosclerosis, and according to Visse and Nagasse[13] inflammatory cytokine and its growth factor produced throughout the atherosclerosis formation process can be expressed by MMP' s and that MMP- expression can be triggered by any suitable inducer. [14] And according to Kalela (2000), MMP-9 has frequently been associated with arterial inflammation. Also, increased MMP-9 levels in serum have been reported in patients with myocardial infarct and unstable angina. [15]

MMP-9's can be activated by 3 mechanisms; namely, stepwise; intracellular and cell surface activation. Even though all type of MMP's have their familial protease, with the same structure and function, each type has its own separate initiating activation mechanism. [16] Initially division occurs in the propertides and then these move to the intra molecules through various intermediaries until the re is fragmentation. [13] This chemical activity can also he observed in vivo experiments where NO activates pro MMP-9 through out cerebral ischemia by reacting with thiol groups from cystein. [7] As long as there is inflammation, pro MMP-9 can be activated by reactive oxygen

species or ROSS such as hydrogen peroxide and radical hydrosol and both chemical and oxidative activation are considered important in inflammatory diseases. [17]

According to Worthly et al (2001), although several enzymes are involved in ECM destruction, MMP- I and MMP3 for example, only MMP-9 with the rupture of atherosclerotic plaque. They go on to state that MMP-9 can degrade matrix components whereas the other protolytic enzymes are unable to do so. [18]

It was with this in mind that this research was enacted to test the ability of MMP-9, obtained from neutrophils into which Perperimonas Gingivalis whole cells were introduced to it, to degrade type IV collagen. Based on the results of the SDS-PAGE, i.e. 97,6 kDa, (Figure 3) followed by the immunoblotting test (Figure 4b), which found a fragment profile from the degrading of type IV collagen using MMP-9 from AMI patient neutrophils with a weight of between 60 and 80 kDa. The confirmation of this degrading was based on the lowest band, type IV collagen has a molecular weight of 65 kDa, so it was assumed that the molecular weight of the product formed was less than 65 kDa and that it was this band that showed the results the degrading of the type IV collagen. Further confirmation of this was obtained by Suzanne E.G, et al (2002) in a study on the cleavage of intact type 1 collagen by MMP- 1. [19]

Whereas the fragment characteristics of the type IV collagen degradation using MMP-9 from the neutrophils of healthy patients had a molecular weight of 60 kDa with a very low distribution, which may have dependent on the activity of MMP-9 produced. In addition, it is possible that as the neotrofils obtained from the healthy persons had not been infected with prior inflammatory, and that possibly the pro MMP-9 in the neutrophils of the AMI patients had already been formed previously, as the introduction of whole cell. *Perviromonas gingivalis* will quickly produce active MMP-9. It is also possible that in the MMP-9' s obtained from the healthy group, there was some form of partial activity which was able to degrade type IV collagen even if it only had a molecular weight of 60 kDa.

In the inflammatory process, activated inflamed cells will increase the production of pro enzymes, among them Matrix active enzymes and can thus cause collagen degradation. [3] When this process occurs on the surface of atherosclerotic plaque, the collagen fibres that protect the plaque will degrade, become thinner and finally rupture.

MMP-9 or gelatinase B is one of a group of gelatinase that have a molecular weight of 92 kDa, is known to be a gelatine-binding-protein synthesized by leucocyte cells,. MMP-9 expression can be induced by any suitable trigger. It is also known that monocytes, neutrophils, dendric cells, lymphocytes, endothelial cells, eplitel cells and osteoblasts can all produce gelatinase B. [14]

Type IV collagen degradation occurs directly through the protease formed by microorganisms or when there is significant inflammation, degradation comes about which is increased by MMP's activity. [3] It is known that both MMP-2 and MMP-9 have the same 3 areas of type II fibronectin in the catalytic area, and it is hypothesized that at the time MMP-9 becomes active these same 3 areas, all from

type II fibronectin, well interact with type IV collagen, apart from this, the C-terminal-hemopesin-like domain, an integral part of MMP-9, is used to split the triple helix form of collagen. [9]

In this study, production antibody by immunization on mice and also doing isolation of lymphocyte cell (Figure 5,6).

Lymphocyte cells, produced normally in the mice, gave a response to most of the antigens (Ag). In preparation for the fusion, myeloma cells were grown (Figure 7).

Then the fusion to form hybridoma cells (Figure 8), this resulted in healthy and productive hybridoma cells that produced antibodies, after this, the best hybridoma clones were selected (Figure 9). The antibody selected clone was test by western blot which produce a band of 97.6 kDa (Figure 10), this was confirmed by an Elisa test which gave a relatively high figure of 2,584 (Figures 11, 12 and 13), as well as high titers of clone to be prepared for insertion intraperitonially into the mice to form ascites and growth the ascites with prestan. The results were type IV collagen monoclonal antibody fragmentation in the form of monoclonal prototype antibodies. Following this, the type IV collagen prototype antibody fragmentation was tested by Elisa Technique high titer antibody and continue by delution test. From the type IV collagen antibody fragment collagen with type IV collagen antigen fragments, indicated a reaction of 1/5000 thinning (Figure 14 and Table 1). This shows that the type IV collagen anti fragment antibodies had a positive interaction so that the antibody produced was truly an antibody obtained from the induction of type IV collagen fragment antigen.

According to Ortega (2002), Ab is, amongst others, determined by a number a factors, namely, the species of animal, dosage and form of Ag, the use of adjuvant, procedure and the number of injections administered and the time period between injections.[20]

The Dot Blot test on the 12 serum samples from the AMI patients with type IV collagen anti fragment antibodies indicated that there was a positive interactive, confirmed by dot which had blue spots. Dots for all AMI patients, the sign of a positive reaction, whereas the samples from the healthy patients were negative.

Therefore, we concluded that interaction the monoclonal antibody marker for type IV collagen antibody anti collagen could reveal the reaction in the serum of the 12 AMI patients. Other enzymes such as CPK, troponin A and B and myoglobin cannot detect AMI, thus type IV collagen antibody markers can detect AMI early on Yet, until the present no other enzymes have been able to detect AMI in its early stages, thus, our biomarker, known as type IV collagen fragmentation monoclonal antibody novel biomarker, can uncover the cause of AMI form *Perviromonas gingivalis*

Microorganism infections (Figure 15), for example.

Competing interests

We don't have competing interests

Authors' contributions

1. Have made substantial contribution to conception and design or aquisition of data, analysis and interpretation.
2. Have been involved in drafting the

manuscript or revising it critically for information in intelectual context.

3. Give final approval of verside to be publisher. Each author should have participate sufficiently in the work to take public responsibility for appropriate portion of the concept. Aquisition offending collection data or general supervision of the research group.

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