The objective of this study was to detect Acute Myocardial Infarction (AMI) in blood samples by using biomarkers for monoclonal antibody Collagen Type IV related Perviromonas Gingivalis. Rupture of atherosclerotic plaque initially occurs through the degradation of Collagen Type IV and infection is one important risk factor in AMI. This research was experimental, in vitro with a descriptive cross sectional design. The subjects were (n=4) healthy people and (n=12) AMI patients with ethical clearance and was carried out in the Biomedical Laboratory, Brawijaya University. To show the expression of MMP-9 and to show the fragmentation of Collagen Type IV. Western Blot was employed. The monoclonal antibodies were obtained from mice which had sub coetaneous immunization, followed by isolating lymphocytes and then fusing them with myeloma cells to form hybridoma. To evaluate the MMP-9 product and fragmentation of Collagen Type IV, Dot Blot technique was employed. The result found the following the MMP-9 products: the band indicated 92kDa and the Collagen Type IV fragmentation band 60-80 kDa. The test for biomarker reaction to biomarker monoclonal antibody fragmentation of Collagen Type IV in blood samples 1 to 12 was positive for AMI. In conclusion, the biomarker for Collagen Type IV monoclonal antibody fragmentation were AMI positive.

Keywords: Monoclonal antibody collagen type IV, AMI, biomarkers.

BACKGROUND

Acute Myocardial Infarction (AMI) is a major medical problem and apart its rising prevalence, it is also the main clause of death (Braunwald 1997). In 2000, 16.7M people, or 30.3% of all deaths, died from AMI world wide with more than half coming from developing countries.

One factor in the rise of AMI is atherosclerosis the formation of which has many causes such as dislipidemia, hyperhomocystein, diabetes mellitus, hypertension, etc. However, infection factor is little known about the actual process of AMI.

According to Ross (1999) the process is an inflammatory response to microorganisms which invade the vascular endothel and induce an inflammatory response or indirectly a systemic effect.

In the blood vessel an inflammatory process, activated inflamed cells such as polimorfonuclear cell will trigger increases in pro enzyme production, such as Metallo
Protease Matrix (MMPS) and these pro enzymes can change into active enzymes which cause collagen lyses (Romanelli et al. 1999). When this process occurs on the surface of atherosclerotic plaque collagen fibers that protects plaque will experience lyses, thin and finally become susceptible to rupture.

Collagen type IV blood vessel is a major component of the vascular basal membranes that lie under the layers of endothel cells in veins. And collagen type IV is easily damaged by collagenase activity in the circulation. In addition, due to its location next to circulating blood and also because of its composition, which contains globular protein (non-collagenous domain, not fibrilar) susceptible to many collagenase substances (Lee and Libby, 1997). Collagen type IV degradation can occur directly from the protease produced by microorganisms or by serious inflammation and more severe degradation will come about from MMPs activity (Romanelli et al., 1999).

Ameriso et al (2001), Muliartha et al (2005) found there was a significant relationship between Perviromonas Gingivalis and periodontitis and cardiovascular atherosclerosis; other research has also discovered a connection between the two.

However, in the infection process, Perviromonas Gingivalis whole cells can travel through the circulatory system and help induce atherosclerosis by bacterial antigenic product.

According to Nassar et al (2001), bacterial antigenic lipopolisakarida bacterial in the circulation can direct and indirectly damage vascular endothelium by stimulating an immune response.

Whereas Ogawa (1994) discovered that lipopolisakarida bacterial and Perviromonas Gingivalis whole cells found in these microorganisms were virulent to the host. Whole cell antigenic protein circulating in the blood will induce an echo in which there is activity in cells involved in atheroma, due to the bacterial antigenic products, which in turn releases interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α) (O’Connor.S. 2001).

Perviromonas Gingivalis, and its other strains, is a bacterium whose whole cell is connected to pathogenesis as it is an antigen which can be recognized by both the nonspecific and specific immune systems. Based on the above factors, this study intends to prove the above hypothesis in vitro on whole cells Perviromonas Gingivalis which may induce an immune response when producing MMP enzymes and also to prove whether this enzyme can actively degrade collagen type IV and fragment its protein and the possibility that monoclonal antibodies can be formed to detect this fragmentation process in the blood of AMI sufferers, as it is well known that the main cause of oxygen and nutrient supply problems for the heart are thrombi.

The formation of thrombi is due to several factors, one of which is from the rupturing of atherosclerotic plaque and it is also now known that microorganism infections can damage endothel cells.

A part from being involved in atherosclerotic plaque rupture, microorganisms can also induce thrombi through endothel cell damage. According to Ogawa (1994) there are several possible infectious agents that can cause AMI, namely, invasive microorganisms that in veins will directly or indirectly give rise to an inflammatory response that increases the number of lymphocytes and macrophages as well as a rise in cytokine and factors for the growth of fibrous tissue.

In addition to secreting microorganism products like whole cells perviromonas gingivalis can also increase the binding of ester cholesterol by macrophages to form foam cells and also to induce smooth vein muscle contraction that pushes endothel towards the lumen, causing the formation of plaque and thus the narrowing and blocking of the blood flow.

In systemic infection, products lipopolisakarida from microorganisms can cause an echo. This echo can cause damage to endothel and thus rupture plaque, and when plaque ruptures from the rupture of collagen type IV it is carried by the blood stream giving rise to the blocking of veins in the heart resulting in AMI, and this can be detected by collagen type IV monoclonal antibody fragmentation. In addition, active inflammatory cells will increase the expression of surface tissue factor causing a rise in procoagulant. Furthermore, the plasminogen activator is blocked thus reducing trombomodulin endothel and proteoglican sulfate heparin, an increase in cytokine production by inflammatory marker activation which together with the stimulated procoagulant, will cause the formation of thrombosis and finally AMI.

At present diagnostic biomarkers AMI still develope. But conventional diagnostic among : CPK enzymes, myoglobin, troponine can detected when AMI was happen. Furthermore, its etiology factors are still not know. Additionally, diagnosis using invasive ultrasonography is very expensive. The purpose of this study is to prove that the biomarker monoclonal antibody fragmentation collagen type IV can detect the presence of antigen fragmentation collagen type IV in the serum of patients with AMI is visualized by the reaction with these biomarkers. This study also proves that the production of antibody biomarker of fragmentation type IV.

Therefore, it is our intention in this research to produce collagen type IV fragmented monoclonal antibodies as biomarkers that can detect ethiology of infection processes as well as AMI early and also detect such as perviromonas gingivalis antigen production.

**The Research Method**

This experimental laboratory research descriptive cross sectional, was carried out at the Central Biological
Collagen Type IV Degradation MMP

Enzyme Production and Activity Testing

Stimulating secretions and activating MMP enzymes was done by inserting Perviromonas Gingivalis into neutrophils that had been incubated for 1 hour at 30°C. Then this was cold centrifuged at 5000 rpm at 4°C for 10 minutes. The supernatant containing the MMP enzyme was collected, separated and mixed with a tris-glysin SDS buffer and left at room temperature for 10 minutes without heating. Then the sample was put into a well containing gel with 0.1% gelatin and gel-run with tris-glysin SDS running buffer and run at a constant 125V. At the start, current strength was 30-40 mA/gel, and 8-12 mA/gel at the finish after 90 minutes. The gel was renaturated through a zymogram renaturing buffer and agitated at room temperature for 30 minutes. The gel was moved to the developing Zymogram buffer at room temperature for 30 minutes and after that it was incubated for one night at 37°C. The gel was stained with glacial methanol. White bands in the blue gel indicated gelatinize activity. The results of the electrophoresis on the MMP enzymes from the Western Blot test used the anti-MMP-9 antibodies as the antibody primer. The MMP enzymes obtained were put on the collagen type IV labeled with biotin in a ratio of 1:2 and incubated for 18 hours at room temperature. RSB was added and it was then heated to 100°C for 5 minutes. A sample was placed in a well containing gel and then run with tri-glysin SDS running buffer at a constant 120 volts over 90 minutes, the current strength was 30mA. The bands from the collagen type IV fragmentation were observed using commassie brilliant blue R-250 stain which was removed by soaking the gel in a distaining solution, after this it was agitated in a shaker until it became clear. The material obtained by electrophoresis was given a Western Blot test using antibody fragments collagen type IV.

Production Monoclonal Antibody Fragmentation Collagen Type IV Together With Electrophoresis

Fragmentation collagen type IV band seen in the NC membrane became the antigen injected for immunizing the mice. The electro elusion was done by cutting the bands transferred to the NC membrane and then placed in celofane (dilution membrane) already containing 1 ml running buffer. The dilution membrane was placed into the electro elusion chamber and run at 25 volts, 3OmA for 120 minutes. The electro elusion results were diluted for a night at 4°C at 6000 - 12000 rpm for 10 minutes. The supernatant was thrown away and the pellets were all dried by being placed in a refrigerator at 4°C. The taped pellets had tris-HC1 (pH 6.8) buffer added and stored as collagen type IV antigen fragment stock.

Isolating Lymphocyte cells from the Immunization

Antigen fragmentation collagen type IV from the electro elusion were injected into a 4-month-old male mouse of about 60 grams that had been kept in a cage and fed on immunization pellets. The immunization was done by injecting collagen type IV antigen fragments subcutaneously, 5 injections were administered at one weekly intervals. In the first week, collagen type IV antigen fragments were mixed with Complete Freund’s Adjuvant (CFA) in a ratio of 1:1 and the booster injections (booster 1 to 4) was enacted through repeat injections of collagen type IV antigen fragments mixed with Incomplete Freud’s Adjuvant (IFA).

Harvesting lymphocyte cells from the lien was done 1 week after the first injection and then every week thereafter for each of the next 4 injections and the lymphocyte cells were isolated. Then the myeloma cells were prepared, followed by fusion between the lymphocytes and the myelomas to form the hybridomas. After the hybridoma cells were formed, the best hybridoma clones were selected. The results from the clones with the highest titers were obtained by intraperitoneal insertion into the mouse to form mouse ascites, and these ascites were prototype monoclonal antibody fragments collagen type IV.

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
The evaluation of the AMI patients’ serum interaction and collagen type IV anti fragment antibodies was implemented by a Dot Blot method. To find the results of the interaction between the prototype monoclonal fragments collagen type IV, tests were run on numbers 1 - 12 AMI patients as well as 4 healthy persons. The design of this study was an descriptive cross sectional criteria for the sample of men less than 45 years and women are still menstruating. For inclusion criteria based on the results anamnese, diagnostic physical examination, examination elektrokardiography (ECG) and enzyme testing CPK, troponin and myoglobin. The test should show positive results. Also performed the function of kidneys and liver. To eliminate the examination of kidney fibrosis urea, blood creatinine. To eliminate the examination of liver fibrosis ALP, SGOT, SGPT that the results should be normal. With no fibrosis of the liver and kidneys.

To observe the perviromons gingivalis infection examined C-reactive protein and anti perviromons gingivalis blood tests. For exclusion criteria based on AMI patients who have these risk factors when more than one factor, then do exclusion. Risk factors include hypercholesterolemia, LDL, hypo HDL, diabetes mellitus, hypertension, smoking, hormonal contraceptive use and liver and kidney dysfunction. Patients with these risk factors not included in the study. To evaluate test on AMI patients, we use Dot Blot technique. The Dot Blot test is positive when dot blue appears.

**RESULTS AND EVALUATION**

**Research Results**

MMP Production and Testing Employing SDS-PAGE Gelatine Zymography. MMP enzyme production is thought to arise from one of the contents of neutrofil cells which are induced by Perperimonas gingivalis whole cells to produce MMPs enzymes. In order to uncover the presence and activity of MMPs, tests for MMP enzymes are done by employing SDS-PAGE gel gelatine on neutrofil supernatant infected by Perperimonas Gingivalis. The results of the MMP enzyme tests employing SDS-PAGE gel gelatine are shown in Figure 1.

Figure 1 indicates that in samples S1 to S3 (neutrofil supernatant from AMI patients) the band is a transparent brown with a molecular weight of 96 kDa and 72 kDa. The gel from 7.5% SDS-PAGE gelatine was then tested with Western Blot using anti MMP-9 antibodies as the antibody primer. Figure 2 shows the Western Blot test results where the anti MMP-9 antibody reaction indicate a molecular weight of 92-96 kDa.

MMP enzymes recognized by anti MMP-9 antibodies were thought to be MMP-9 enzymes. In samples S1 to S3, MMP-9 was obtained from AMI patients’ neutrofils invaded by Perviromonas Gingivalis whole cells, this indicates anti MMP-9 antibodies recognized had bands showing a molecular weight of 96 kDa and 72 kDa respectively. Whereas, in samples S3 and S4 there was MMP-9 from healthy persons’ neutrofils invaded by whole cell Perperimonas Gingivalis, showing that anti MMP-9 antibodies recognized molecules of 91.2 kDa in its band even thought indistinctly.

**Collagen type IV Degradation Stage**

**SDS-PAGE Results**

In the next stage, tests for collagen type IV degradation from MMP-9 were enacted using 10% SDS-PAGE on the
Figure 1. MMP test with SD S-PAGE gel showing SI results, marker samples S2 - S3 = 72 - 96 kDa.
M = Sigma protein detector marker using silver nitrate
S = Sample in well

Figure 2. Western Blot results from antigen reactivity - MMP-9 was able to identify the MMP enzyme,
M = Markers.
S1, S2 and S3 = 92-96 kDa.
S4 + S5 = Control.

Figure 3. SDS-PAGE results, 10% above collagen type IV induced for MMP-9 with brilliant blue 258R comassie stain.
Notes : M= sigma protein marker detector levels with brilliant blue R-256 comassie stain K = type IV collagen, Si - S2 = all collagen type IV cells (100 - 116 kDa), S4- S6 fragmented collagen type IV (66.2 - 97.6 kDa)

MMP-9 given to the collagen type IV are shown in Figure 3 and give a band size of 66.2 kDa to 97.6 kDa.

Western Blot Test Results
The anti type IV collagen antibody reactivation on the bands was enacted through MMP-9 put in collagen type IV is shown in figure 4b.
Figure 4B has the anti collagen type IV antibody shows the reactivations of to the sample bands of the collagen type IV with AMI patients’ neutrofil MMP-9, the bands show a molecular weight of 60 kDa – 80 kDa.
A Western Blot test had 2 bands which were positive for anti collagen type IV antibodies (figure 4b). Whereas, the anti collagen type IV antibody rectification to the pure collagen type IV 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 comassie brilliant blue stain could be detected by anti collagen type IV antibodies.

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

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

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

The selected clones, which were the highest antibody titers, were prepared for insertion intraperitoneally into the mice and aided by giving prestan to form ascites. The growth of ascites were tested with Western Blot and reconfirmed by Elisa. The results were shown in bands at 97.6 kDa by Western Blot, whereas the Elisa test found high antibody levels of 2.40 (Figures 10, 11, 12 and 13).

The selective clone that have highest antibody titers were injected via intraperitoneal route aided by giving prestant.

Results of the test run on the interaction between the anti sera 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 collagen type IV antigen fragments to the 1:5000 thinning (Figure 14) the Elisa test score of the highest titer was 2.32.

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).

DISCUSSION AND COMMENTS

DISCUSSION

The purpose of this study is to prove that the biomarker monoclonal antibody fragmentation collagen type IV can detect the presence of antigen fragmentation collagen type IV in the serum of patients with AMI in visualized by the reaction with these biomarkers.

This study also proves that the production of antibody biomarker of fragmentation type IV is in conformity with applicable standards for the production of monoclonal
**Figure 9.** Selected clones: Hybridoma cells selected from screened cells and tested with Western Blot and ELISA.
Clone 1 Clone 2 Clone 3

**Figure 10.** The growth of ascites and Western Blot testing shown at band 97.6 kDa.
Testing hybridoma clones, analyzing hybridoma supernatant specificity from selected clones on collagen type IV and fragmented collagen type IV with ELISA.

Notes:
- Columns 1, 2: Supernatant hybridoma
- Columns 3, 4: Supernatant hybridoma
- Columns 1, 3: Collagen type IV antigen
- Columns 2, 4: Collagen type IV antigen fragment
- Lines B, C, D, E, F, G, H: Sequence of antigen concentrations 200, 100, 50, 25, 12.5, 62.5, 3, 125 ng/ml
Figure 11. Elisa technique result of selected clones hybridomas.

Figure 12. Growth of ascites in mice
antibodies, which refers to an existing procedure created by Kohler dan Milstein (1977) and Butler (1987).

Step-by-step procedure of making a monoclonal antibody briefly, as follows:

The initial step is the production of matrix metalloproteinase-9 (MMP-9), collagen type IV fragmentation process, the process of immunization, process production of monoclonal antibody fragmentation collagen type IV, isolation of lymphocytes, lymphocyte cell fusion with myeloma cells to form hybridomas, clon selection, insertion clon to intraperitonial produce the fragmentation of monoclonal antibody collagen type IV as well as with the test (Butler et al, 1987). Monoclonal antibodies are produced should be able to react accurately to the antigen. For that antigen must be imunogensitas (Boldicke et al., 1991). The results showed protein antigenic fragmentation collagen type IV antigen is a result of fragmentation by enzyme matrix metalloproteinase-9 (MMP-9) to forming fragmentation collagen type IV with a molecular weight of 92-96 Kd (figure 1), which polymorfonuclear cell (PMN) from patients with AMI induction by perviromonas gingivialis cells. In the process of immunization carried out in vitro as did the Boldicke et al, (1991), which is injected as much as 60 g of collagen type IV fragmentation in sub cutan then to be repeated again at 1 week intervals, accompanied by complete Freund adjuvant with a ratio of 1: 1. Done so on back booster with a ratio of 1 : 4, along with incomplete Freund adjuvant. This procedure is in accordance with the procedures that we did in our study. To production of monoclonal antibodies based on results of production hybridoma is based on Mac Farlane Burnet hypothesis that B lymphocytes produce one type of antigen with monospesifisitas. In the research we’ve done in accordance with the technique of Butler et al (1987). The initial stage is to isolate the lymphocytes, then do fusion with myeloma cells to form hybridomas. In the procedure of Butler do poliethylene glycol (PEG). This material will isolate the fusion product and continue by grow hybridoma which hybridoma eliminates and which one continue to grow and which ones die.

The idea of fusion technique is motivated by Kohler dan Milsten (1977) to produce a monoclonal antibody. Selection of clones was done by using elisa by selecting the highest titer of antibody (hybridomas) are produced. To produce antibodies with high quality injection intraperitonial performed on mice. Further laboratory testing is done in the monoclonal antibody fragmentation collagen type IV produced by the technique delution (dilution) followed by a dot blot technique. The result of dilution up to 1/5000 (figure 14 table 1).

Collagen type IV monoclonal fragmentation of the resulting research is in the form of a prototype that has undergone testing in the laboratory and the final test in patients with AMI. To do this biomarker testing in patients with AMI, we conducted a selection of AMI patients will be
included in this study. The design of this study was an observational descriptive cross sectional for criteria inclusion sample of men less than 45 years and women are still menstruating. The inclusion and exclusion criteria both already mention in previews explanation. We cannot use size sample, because our research in for of prototype biomarker. And design of the research is descriptive cross sectional. We only use minimal sample of AMI without statistic analyze.

The results showed that 12 patients with AMI who examined all showed positive results against biomarker monoclonal antibody fragmentation collagent of type IV according to the CPK enzyme test in patients with AMI.
COMMENTS

The research has many comments. The first comment is monoclonal antibody biomarkers fragmentation collagen type IV is not specific to the heart. Also increased for kidney disease and hepatic fibrosis. In anticipation of the organs of kidney fibrosis, liver and others, we've been doing the selection through the inclusion criteria. Patient samples that are only associated with myocardial infarction acute disease. Where the organ have fibrotic disease, we do exclusion. This study has the advantage that the etiology of the AMI can be detected in the presence of perviromonas gingivalis infection.

The other comment is this research use very small population sample patient. The research was conducted with research design qualitative experimental, descriptive cross sectional. To watch for the reaction of monoclonal antibodies generated against the fragmentation of collagen type IV antigen by dot blot test compared with controls. To study use to many sample, we will conduct a phase II study with more complete preparation of the monoclonal antibody test collagen type IV fragmentation in the existing research center in Indonesia on a regional area. So that it takes a lot of patient acute myocardial infarction (AMI) for testing of this diagnostic tool.

The last comment is about regarding specificity, sensitivity, and predictive value these varieties have not been implemented. As already mention in the previous explanation that the study is only qualitative descriptive cross sectional testing of patients with AMI without specificity, sensitivity analysis, and predictive value. Phase II study we will do the testing and the specificity, sensitivity and predictive value with enough samples.

CONCLUSION

The conclusion from this study show that the monoclonal antibody fragmentation collagen type IV give a reaction positive blood AMI patients’ with Dot Blot technique.

Competing Interest

We don't have competing interest.

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