CHAPTER IV

DISCUSSION

Platelets play a central role in the physiology of primary hemostasis when the wall of a blood vessel is damaged, a hemostatic response is rapidly prevented excessive blood loss by the concerted action of blood platelets and the coagulation system (Davi and Patrono 2007). In various diseases and conditions, excessive platelet activation can lead to thrombosis. Activated platelets may adhere to each other and to the vessel wall leading to thrombotic occlusion. Platelets are transported to the site of the injury and aggregate to form a hemostatic plug and thromboembolic disease (Rodvien and Mielke 1976). Inappropriate platelet activation is common in the pathophysiology processes of arterial thrombosis and in a variety of clinical diseases including cardiovascular disease, diabetes mellitus, malignancies and thalassemia.

Previous studies have indicated the relationship between platelet activation and pathophysiology in patients with diabetes mellitus, thalassemia and malignancies and leading to thrombosis (Eldor and Rachmilewitz 2002, Huo and Ley 2004, Rickles and Edwards 1983, Taher, *et al* 2008). Hence, early detection of circulating activated platelets to assess risks for pre-thrombotic state is necessary and to evaluate the therapeutic strategies to prevent platelet activation. Since platelet activation and localization are fundamental events of injuries to blood vessels, there is a need for methods and reagents capable of detecting and discriminating between activated blood platelets and resting platelets. During hemostasis and thrombosis, resting blood

platelets are known to change morphologically in shape and biochemically in cell surface membrane composition during aggregation and secretion. Changes in the membrane glycoproteins on the platelet surface can be identified with specific monoclonal antibodies.

Several methods have been developed for measurement of platelet activation (Bates, *et al* 1991, Metzelaar, *et al* 1990, Shattil, *et al* 1987). To date, flow cytometry represents a valuable tool in the evaluation of platelet activation using monoclonal antibody against platelet activation marker (Tocchetti, *et al* 2001). Monoclonal antibodies have become important tools for many applications. They have been widely used in the medical research and development, diagnostic, trade and therapy (Breth, *et al* 2005, Chen, *et al* 2005, Matsumoto, *et al* 2007, McKenzie 2002, Nieuwenhuis, *et al* 1987, Xiang, *et al* 2008). In immunological test, specific antibodies are most important element. Numerous investigators have developed immunological tests for assaying *in vivo* activated platelets in patient with thrombotic risk, using specific monoclonal antibodies (Breth, *et al* 2005, Chen, *et al* 2005, Gobbi, *et al* 2003, Membre, *et al* 2008). Metzelaar, *et al* (Metzelaar, *et al* 1990) has been reported the detection of platelet activation in patients with thrombotic disease using monoclonal antibodies to activated platelet and analyzed by flow cytometry. Therefore, the detection of activated platelets might facilitate identifying certain patients with a thrombotic risk. The objective of this study is to produce and characterize the monoclonal antibodies to membrane molecules on thrombin-activated platelets. These produced monoclonal antibodies will be used in evaluating patients

with thrombotic risk and might offer advantages in the clinical management and prevention of patients at risk for thrombosis.

In this present study, mAbs specific to activated platelets were produced from BALB/c mouse immunized with thrombin-activated platelets by standard hybridoma technique. Since the specific changes on the surface of activated platelets that difference from resting platelets, such as GPIIb-IIIa (Ugarova*, et al* 1993) or P-selectin (Gawaz*, et al* 1999), thus, the identification of mAbs against activated platelets, resting platelets and thrombin-activated platelets were used as screening cells. Among various mAbs, three mAbs clone 138.7, 176.7 and 297.7 were interested and selected for further studies.

To produce the desired mAbs, the hybridomas producing mAb clone 138.7, 176.7 and 297.7 were subjected for large scale production in two ways, *in vivo* production by injection hybridoma-producing mAb into the abdominal cavity BALB/c mice and *in vitro* production by culture hybridoma cells in serum free media. The ascitic fluid or serum free media containing interested mAbs were subjected for purification using protein G sepharose column and AKTA purifier. The protein G sepharose affinity chromatography is the fastest methods for purifying antibodies, easy performance and scale-up (Andrew and Titus 2001). From purification results, SDS-PAGE showed two protein bands correlated with heavy chain and light chain of immunoglobulin were observed under reducing condition. While under non-reducing condition, only one major protein band was observed on the gel. The activity of purified mAb was also checked by indirect immunofluorescent staining and flow cytometry. The immunoreactivity of all purified mAbs, clone 138.7, 176.7 and 297.7,

to thrombin-activated platelets showed strongly positive. These results indicated that purified mAbs are suitable for further studies.

Cellular distribution of the molecules recognized by mAbs clone 138.7, 176.7 and 297.7 on various peripheral blood cells and hematopoietic cell lines was performed by indirect immunofluorescent staining and flow cytometry. The staining results showed that mAb clone 138.7 and 176.7 were positive with membrane molecules on activated platelets. While mAb clone 297.7 showed positively react with membrane molecules on both resting and activated platelets. From resting platelets staining, we found some platelet population was positive with mAbs clone 138.7 and 176.7. It means *in vitro* spontaneous platelet activation due to blood sample preparation was occurred (George 1981). However, this staining pattern also observed with CD62P mAb that used as positive control for activated platelet. Moreover, all of three mAbs were negative with other peripheral blood cells and hematopoietic cell lines tested. The results indicated that the molecule recognized by mAb clone 138.7 and 176.7 are molecules expressed only on activated platelets whereas 297.7 molecule is a molecule specific to platelets include resting and activated platelets. From this expression pattern, 138.7 and 176.7 molecules were proposed to be markers for activated platelets. Furthermore, 297.7 molecule, a platelet specific molecule, was also interested. The 297.7 mAb was selected for further study because its staining pattern similar to CD42b mAb. The mAb 297.7 was expected to be used instead of an imported CD42b mAb. Currently, many platelet membrane glycoproteins were discovered and studied (Mannel and Grau 1997, Moroi and Jung 2004). Based on their function and expression on platelets, CD42b (GPIbα),

the platelet marker, is a members of the leucine-rich repeat family and approximate 160 kDa (Ruggeri 1997) (http://www.sciencegateway.org/resources/prow/). Monoclonal specific CD42b (anti-CD42b) was used to separate platelets from other peripheral blood cells. Furthermore platelets also express some proteins or glycoproteins on their surface membrane after activation for example CD62P or P-selectin that is an integral membrane protein with 120 kDa and 140 kDa under non-reducing and reducing condition, respectively (Blann and Lip 1997) (http://www.sciencegateway.org/resources/prow/). The CD63 is a 40-60 kDa member of the tetraspanin superfamily present in dense granules and lysosomal membranes (http://www.sciencegateway.org/resources/prow/).(Becker 2001). P-selectin (CD62P) and CD63 were used as a principle of platelet activation (Gawaz, et al 1999, Hartwell, et al 1998).

The biochemical characterization of the molecules recognized by the produced mAbs clone 138.7, 176.7 and 297.7 was studied by SDS-PAGE and Western blotting. Under non-reducing condition, mAb clone 176.7 and 297.7 reacted to a protein band with the molecular weight of 120 kDa and 160 kDa respectively, while only mAb clone 138.7 did not react with any protein. Whereas, none of protein band was reacted with all mAbs under reducing condition (data not shown). In the present study, mAbs clone 176.7 and 297.7 bind to a conformational determinant that involve with disulfide bond. Whereas, mAb 138.7 recognize the native protein and react to the conformational determinant. Therefore, the immunoprecipitation technique will be used for further study (Young, *et al* 1994).

The results from the cellular distribution and biochemical characterization of the produced mAbs indicated that mAb clone 297.7 positively reacted to membrane molecules on resting and activated platelets. Moreover, this mAb reacted to a protein band with the molecular weight 160 kDa under non-reducing condition. This finding shown to be similar to anti-CD42b (Michelson, *et al* 2000a). Whereas, mAb clones 138.7 and 176.7 showed positive reactivity with activated platelets only. Furthermore, mAb clone 176.7 reacted to a protein band with the molecular weight 120 kDa as same as P-selectin (CD62P) molecule. However, the identification of recognized molecules by our generated mAb clone 297.7 and 176.7 should be confirmed by CD42b and CD62P recombinant proteins, respectively (Kijanka, *et al* 2009).

The mechanism of hemostasis and thrombosis require a close interplay between platelets, endothelium, coagulation factors and the structure of the vessel wall. Adhesion processes that are regulated by numerous specific adhesion receptors play a major role in this mechanism. Platelet adhesion is mediated by several key adhesion receptors essentially unique to platelets glycoprotein (GP) Ib-V-IX, GPIa-IIa, GPIc-IIa and GPVI attach to several adhesive proteins in the matrix of connective tissue such as von Willebrand factor, fibronectin, and collagen (Bussel, *et al* 2000, Laduca, *et al* 1987, Yip, *et al* 2005). Subsequent to aggregation, the fibrinogen receptor, GPIIb-IIIa on the activated-platelet surface become exposed to stabilization of the thrombus with polymerized fibrin (Hsu-Lin, *et al* 1984). Glycoproteins on platelets play a major role in the platelet function, adhesion and aggregation, then the proteins that specific binding to produced mAbs on adhesion and aggregation process were

investigated. Therefore, platelet adhesion and platelet aggregation assay were used to study the effect of generated mAbs to platelet function.

To study the effect of mAbs clone 138.7, 176.7 and 297.7 on platelet adhesion, indirect ELISA (Eriksson and Whiss 2005) was carried out. The 96-well plate was coated with collagen or fibrinogen prior, added the mixture of biotin-labeled activated platelets and various concentrations of mAbs. The reaction was then detected by horseradish peroxidase-conjugated streptavidin and measured using ELISA reader. The result showed that mAbs did not effect on platelet adhesion. Based on platelet adhesion process, surface platelet glycoproteins include GPIV, GPVI and GPIa-IIa play a major role in adhesion of platelets to collagen (Watson 1999), while GPIIb-IIIa is the fibrinogen receptor (Du and Ginsberg 1997). Therefore, our produced mAb clone 138.7, 176.7 and 297.7 did not specifically bind to GPIV, GPVI, GPIa-IIa and GPIIb-IIIa on activated platelets.

The effect of produced mAbs on platelet aggregation was performed based on turbidimetry (Born, *et al* 1978). After the addition of platelet agonists to platelet rich plasma, platelet aggregation was monitored. Adenosine diphosphate (ADP) and collagen are platelet agonists were used in the study. ADP is a weak agonist that binds to specific platelet membrane receptors to suppress membrane-associated adenylate cyclase activity and induce calcium mobilization to the platelet cytosol (Heemskerk, *et al* 2001). ADP at low concentration between 1-10 μM, induces biphasic aggregation, platelet undergo primary aggregation and followed by diaggregation (Samal and Loiko 2000). At higher concentration, the ADP reagent induces simultaneous irreversible shape change, secretion and formation of

aggregates, resulting in a monophasic curve (Fritsma 2007). For our experiment, we used 200 μM ADP, at high concentration, thus the result in aggregation showed monophasic curve. While, collagen induce aggregation depends on intact membrane receptors, membrane G-protein integrity and normal cyclooxygenase pathway function. Collagen, strong agonist, induces no primary wave of aggregation (Laduca, *et al* 1987). From platelet aggregation assay, we found the inhibitory effect of the produced mAbs on platelet aggregation. The results showed different aggregation curve from difference blood sample due to the polymorphisms on human platelets (Michelson, *et al* 2000b, Quinn and Topol 2001).