CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Human blood sample

Blood samples were obtained from healthy volunteer from Faculty of Associated Medical Sciences, Chiang Mai University. All of volunteer must not taking platelet antagonist drug for at least 2 weeks prior to the blood collection.

2.1.2 Mouse

Female BALB/c mouse at 6 weeks of age were purchased from the National Laboratory Animal Center, Mahidol University. The mice were maintained under air condition at 25^{o}C in individual cage.

2.1.3 Myeloma cells

X63-Ag8.653 myeloma cells were used for hybridoma cell production. The myeloma cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) with 10% fetal calf serum (FCS) contained 0.04 mg/ml gentamycin and 0.0025 mg/ml fungizone. The cell cultures were maintained under humidified atmosphere at 37^{o}C, 5% CO_2 incubator.

2.1.4 Chemicals

All chemicals and instruments used in this study were indicated in Appendix A

2.2 Methods

2.2.1 Production of monoclonal antibodies

2.2.1.1 Preparation of thrombin-activated platelets

Blood samples were collected in 3.2% sodium citrate as an anticoagulant at the ratio of 9:1. Then, blood samples were centrifuged at 250g, 22^{o}C for 10 minutes. Platelet rich plasma (PRP) were collected and washed with 0.5 % BSA-NaN_3-PBS for 3 times. Then, platelets were counted and adjusted to $1x10^9$ platelets/ml with sterile PBS. For platelet activation, thrombin was added at final concentration 0.1 U/ml and incubated at room temperature for 10 minutes. Thereafter, thrombin-activated platelets were washed at 1,500 g for 5 minutes. For mouse immunization, platelets were resuspended with 500 µl sterile PBS.

2.2.1.2 Mouse immunization

Female BALB/c mouse at 6 weeks of age was used. Before immunization, blood was collected by tail-bleeding as pre-immunized serum. Mouse was intraperitoneally immunized with 10^7-10^8 thrombin-activated platelets in 500 µl sterile PBS. The immunization was repeated every week for 3 weeks and mouse was immunized with $1x10^6$ thrombin-activated platelets at last time. Blood was collected one day before each immunization.

2.2.1.3 Determination of antibody response in the immunized mouse by indirect ELISA

2.2.1.3.1 Preparation of Platelet Rich Plasma (PRP)

Blood samples were collected in EDTA. For resting platelet samples, blood sample was added with 4% paraformaldehyde (PFA) while sample for activated platelets did not and were then incubated at room temperature for 30 minutes. After

incubation, blood samples were centrifuged at 250g, 22°C for 10 minutes. Platelet rich plasma (PRP) were collected and washed with 0.5 % BSA-NaN_3-PBS for 2 times. Then, platelets were counted and adjusted to $2.5x10^8$ platelets/ml with sterile PBS.

2.2.1.3.2 Determination of antibody response in the immunized mouse by indirect ELISA

Antibodies against thrombin-activated platelets in mouse serum were determined by indirect ELISA. The ELISA plate was coated with 10 µl of thrombin at final concentration 0.25U/ml for activated platelet and the well of resting platelet was coated with 10 µl sterile PBS. Then, the thrombin-coated wells were added with 40 µl of unfixed platelets and uncoated wells were added with fixed platelets. The mixture was incubated at 37°C for 30 minutes prior added 8 µl of 4% PFA, then incubated at room temperature for 5 minutes. After centrifugation plate at 250g for 15 minutes and removal supernatant, the ELISA plate was added 50 µl of sterile PBS and incubated at 4°C for overnight.

The coated wells were washed with washing buffer (0.05% Tween 20 in PBS) for 4 times. Then, 60 µl of 2% BSA-NaN_3-PBS was added into each well and incubated at 37°C for 1 hour. Fifty microlitters of diluted mouse serum were added into ELISA plate and incubated at 37°C for 1 hour. The plate was then washed for 4 times with washing buffer before added 50 µl of horseradish peroxidase (HRP) conjugated polyclonal rabbit anti-mouse Igs at dilution 1:2,000. After incubation for 1 hour, plate was washed 4 times with washing buffer. Then, 50 µl of tetramethylbenzidine (TMB) was added into each well and incubated at room

temperature in the dark for 5 minutes. The reaction was stopped by adding 50 µl of 1N HCl and the absorbance was measured at 450 nm using ELISA reader.

2.2.1.4 Hybridoma technique

The BALB/c mouse that response for immunization was sacrificed and spleen was removed. Then, splenocytes were carefully isolated by homogenization. The obtained splenocytes were counted with Turk's solution. Myeloma cells were collected from cultured flasks and counted with 0.2% trypan blue. Then, the splenocytes were fused with mouse myeloma cells using 50% polyethelene glycol (PEG) by standard hybridoma techniques with some modification. Briefly, splenocytes were mixed with myeloma cells at the ratio 2:1 (2 splenocytes: 1 myeloma), then, were centrifuged at 400 g, at room temperature for 10 minutes. After removing the supernatant completely, cell pellets were mixed by hand and warmed at 37^{o}C for 5 minutes. The fusion procedure was started by dropping 1.5 ml of 50% PEG into the cell mixture within 1 minute followed by gentle stirring for another 1 minute. Then, 1 ml of pre-warmed IMDM medium was continuously added into the tube within 1 minute. Thereafter, 3 ml of pre-warmed IMDM was then added into the tube for over 1 minute and 16 ml of pre-warmed IMDM was added into the tube for 2 minutes. After centrifugation at 400 g for 10 minutes, the cell pellets were placed in a 37^{o}C waterbath for 5 minutes followed by carefully removing supernatant. Then, the fused cells were resuspended in 100 ml HAT medium and transferred to 96-well culture plates at 10 plates/fusion. The hybridoma cells were incubated at 37^{o}C in 5% CO_2 incubator for 5 days. After five days of culture, 150 µl of HT supplement medium was added into each well. The plates were then incubated at 37^{o}C in 5% CO_2

incubator to expand the cells and monitor the hybridoma clone by inverted light microscope.

2.2.1.5 Screening for hybridomas producing monoclonal antibodies to activated platelets by indirect immunofluorescent staining and flow cytometry

Antibodies reactivity against thrombin-activated platelets in culture supernatant from well containing hybridoma cells were determined by indirect immunofluorescent staining and flow cytometry. The preparation of thrombin-activated platelets was described in 2.2.1.1. Then, activated platelets were counted and adjusted to $1x10^7$ platelets/ml with 0.5 % BSA-NaN_3-PBS. The plaletets were blocked Fc receptor by incubating platelets with 20% human AB serum and incubated at room temperature for 30 minutes. Fifty microlitters of culture supernatant were then added into 50 µl of the blocked activated platelets and incubated at room temperature for 30 minutes. After the incubation, the samples were washed twice with 0.5% BSA-NaN_3-PBS. Then, 25 µl of FITC-conjugated $F(ab')_2$ fragment of sheep anti-mouse immunoglobulins antibody was added and incubated at room temperature for 30 minutes in dark place. Finally, the samples were washed 3 times with 0.5% BSA-NaN_3-PBS and fixed with 1% paraformaldehyde. The stained cells were analyzed by a flow cytometer.

2.2.1.6 Limiting dilution

The hybridoma cells from the positive wells were counted and adjusted to 4, 2, and 1 cell per 150 µl with IMDM medium containing 10% FCS and 10% BM condimed. Then, 150 µl of cell suspension were added into 96-well culture plates. The culture plates were incubated at 37°C in 5% CO_2 incubator for 2 weeks. Culture supernatant was collected from each well that containing single clone and determined

antibody reactivity by indirect immunofluorescent staining and flow cytometry as previously described.

2.2.2 Isotyping of monoclonal antibodies

An Isostrip Mouse Monoclonal Antibody Isotyping Kit was used for determining the isotype of the produced mAbs. One hundred-fifty microlitters of culture supernatant was added into development tube and incubated at room temperature for 30 seconds. After incubation, isotyping strip was dipped into development tube and incubated at room temperature. The result was showed on strip about 5-10 minutes.

2.2.3 Large scale production of monoclonal antibodies

2.2.3.1 *In vivo* production (ascitic fluid)

The BALB/c mice were injected by intraperitoneal route with 500 µl pristane oil solution. One week after injection, mice were intraperitoneally injected with $1x10^7$ hybridoma cells in 500 µl sterile PBS. The ascitic fluid were collected after 1-2 weeks and centrifuged at 14,000 g, 4°C for 10 minutes. The supernatant was collected and stored at -20°C. The activity of monoclonal antibodies in ascitic fluids were checked using indirect immunofluorescent staining and analyzed by a flow cytometer as described in 2.2.1.5.

2.2.3.2 *In vitro* production (cell-culture techniques)

MAb-producing hybridoma cells were cultured in IMDM with 10% fetal calf serum (FCS). To avoid contamination with bovine immunoglobulin, hybrid cells were adapted and cultured in serum free media (SFM). Briefly, culture medium was changed to 50% IMDM-SFM and 75% IMDM-SFM, respectively. After cells were

grown in 75% SFM, hybrid cells were washed with sterile PBS for 3 times. Thereafter, cells were adjusted to $1x10^7$ cells and cultured in 10 ml of 100% SFM. Hybridoma cells were continuously maintained until cells death about 70% in culture flask. Monoclonal antibodies were then harvested from the medium and centrifuged at 400 g,4^{o}C for 5 minutes.

2.2.4 Purification of monoclonal antibodies by affinity chromatography

The mAb of the IgG isotype was purified from the ascitic fluid or serum free media using protein G sepharose column (Amersham). Briefly, ascitic fluid was diluted in binding buffer (20 mM sodium phosphate buffer pH 7.0) at the ratio of 1:2 and clarified by centrifugation at 14,000 g, 4°C for 10 minutes. Then clarified ascitic fluid or undiluted serum free media containing mAb were applied into protein G sepharose column and purified using AKTA Purifier. The antibodies were eluted from the column using eluting buffer (0.1M Glycine-HCl, pH 2.7) and immediately adjusted the pH of eluate by neutralizing buffer (1M Tris-HCl, pH 9.0). The purified mAb fractions were collected and measured at OD 280 nm for the presence of proteins. Thereafter, purified mAb fractions were pooled and dialyzed overnight against PBS at 4^{o}C and protein concentration was determined by measuring absorbance at 280 nm. Then, the purified mAbs were aliquot and stored at -20°C prior to use.

The purified mAbs were further checked activity by indirect immunofluorescent staining and flow cytometry as was described in 2.2.1.5.

2.2.5 Determination of the purified monoclonal antibodies using SDS-PAGE

SDS-PAGE was used to determine the purity of purified mAbs. Purified mAbs were adjusted to 5 μg/ml and mixed with reducing or non-reducing sample buffer. Then, samples were loaded onto 10% SDS-polyacrylamide gel and electrophoresed at 120 V for 2 hours. The heavy chain and light chain of immunoglobulin were migrated according to their molecular weight. Protein bands were visualized by staining with coomassie brillant blue.

2.2.6 Cellular distribution of the molecules recognized by monoclonal antibodies to thrombin-activated platelets

To study the cellular distribution of molecules recognized by mAbs of interest, various hematopoietic cell lines (Daudi, supT1, Jurkat, Molt4, HL60, K562, THP1 and U937) and peripheral blood cells include resting platelets, lymphocytes, monocytes, granulocytes, erythrocytes were stained with produced monoclonal antibodies and determined by flow cytometry .

2.2.6.1 Hematopoietic cells and cell lines preparation

2.2.6.1.1 Resting Platelet

Blood samples were collected using 3.2% sodium citrate as an anticoagulant and immediately fixed with 4% paraformaldehyde at room temperature for 30 minutes. The blood samples were centrifuged at 250 g, 22^{o}C for 10 minutes. Platelet rich plasma (PRP) were collected and washed with 0.5% BSA-NaN_3-PBS for 3 times. Then, platelets were counted and adjusted to $1x10^9$ platelets/ml with 0.5% BSA-NaN_3-PBS. For Fc receptor blocking, platelets were incubated with 20% human AB serum at room temperature for 30 minutes.

2.2.6.1.2 Peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) were separated by ficoll-hypaque density gradient centrifugation. Briefly, EDTA blood samples were diluted with PBS at the ratio of 1:2. Then, 7 ml of diluted blood was overlayered onto 3 ml of ficoll-hypaque solution and centrifuged at 400 g for 30 minutes. The PBMCs were collected and washed with 1%BSA-NaN_3-PBS for 3 times. Then cells were counted and adjusted to 1×10^7 cells/ml in 1%BSA-NaN_3-PBS. The PBMCs were blocked Fc receptor by incubating with 10% human AB serum and incubated at room temperature for 30 minutes.

2.2.6.1.3 Granulocytes

To separate granulocytes, EDTA blood was diluted with PBS at the ratio of 1:2. Then, 20 ml of diluted blood was mixed with 20 ml of 6% dextran in PBS. The mixtures were incubated in a slant position at 37°C for 30 minutes. The granulocytes-enriched supernatant were collected and centrifuged at 400 g for 5 minutes. The contaminated erythrocytes were lysed by adding 10 ml of hypotonic ammonium chloride solution and allowed to stand for 5 minutes. The obtained leukocytes were washed with PBS for 3 times and adjusted to 1×10^7 cells/ml in 1% BSA-NaN_3-PBS. Then, cells were blocked Fc receptor by incubating with 10% human AB serum and incubated at room temperature for 30 minutes.

2.2.6.1.4 Erythrocytes

To prepare red blood cells for staining, EDTA blood was washed with PBS for 3 times. Then, red blood cells were adjusted to 0.3% red blood cells with 1%BSA-NaN_3-PBS.

2.2.6.1.5 Hematopoietic cell lines

All hematopoietic cell lines were cultured in RPMI-1640 containing 10% fetal calf serum (FCS), gentamycin 40 mg/ml and fungizone 5 mg/ml (10% FCS-RPMI) at 37°C in 5% CO_2 incubator. For staining, cells were washed with 1% BSA-NaN_3-PBS for 3 times and resuspended at 1×10^7 cells/ml with 1% BSA-PBS-NaN_3. Thereafter, cells were blocked Fc receptor with 10% human AB serum and incubated on ice for 30 minutes.

2.2.6.2 Indirect immunofluorescent staining

Produced monoclonal antibodies of interest were stained with hematopoietic cell lines and peripheral blood cells using indirect immunofluorescent staining and detected by flow cytometer. Fifty microlitters of culture supernatant containing interested mAb was added into 50 µl of the tested cells and incubated on ice for 30 minutes. After the incubation, the cells were washed twice with 1% BSA-NaN_3-PBS. Then, 25 µl of FITC-conjugated $F(ab')_2$ fragment of sheep anti-mouse immunoglobulins antibody was added and incubated on ice for 30 minutes in dark place. After washing, stained cells were fixed with 1% paraformaldehyde and analyzed by a flow cytometer.

2.2.7 Biochemical characterization of the molecules recognized by mAbs of interest by Western blotting

2.2.7.1 Preparation of platelet lysate

The preparation of thrombin-activated platelets as was described in 2.2.1.1. Then activated platelets were adjusted to final concentration of 2×10^8 platelets in 1 ml of 1% NP-40 lysis buffer (Tris lysis buffer; 50 mM Tris-HCl pH 8.2, 100 mM NaCl, 2

mM EDTA, 0.02% NaN_3, containing 1% NP-40 as detergent and protease inhibitor (1mM phenylmthyl-sulphonylfluoride (PMSF), 5 mM iodoacetamide, 10 μg/ ml aprotinin). The mixture was incubated on ice for 30 minutes and gentlely mixed. Finally, activated platelet lysates were collected by centrifugation at 12,000 g, 4°C for 30 minutes.

2.2.7.2 SDS-PAGE and Western immunoblotting

Platelets lysate was prepared in non-reducing sample buffer and boiled for 5 minutes. Then, platelets lysate was performed on 4% stacking gel over 7.5% separating gel. The proteins were subsequently transferred to a polyvinylidene fluoride membrane (PVDF) by semi-dry electrophoretic blotting system at 40 mA for 2 hours. The membrane was blocked in 5% skim milk-PBS at room temperature for 2 hour. After washing, membrane was incubated with interested mAbs at 4°C overnight. The membrane was washed with 0.1% PBS-Tween 20 for 2 times and PBS 3 times. Then membrane was incubated with horseradish peroxidase (HRP) conjugated anti-mouse immunoglobulins in PBS containing 1% skim milk (1:5,000) at room temperature for 1 hour. After washing, the protein bands were visualized by the enhanced chemiluminescence detection system. Briefly, peroxide-luminol/enhancer solution was added to the membrane and allowed to incubate for 5 minutes at room temperature. Then, the membrane was then wrapped in plastic wrap and exposed to Kodak MXB film. Finally, the films were developed with Kodak GBX solution.

2.2.8 Functional study of monoclonal antibodies against thrombin-activated platelets

2.2.8.1 Effect of monoclonal antibodies on platelet adhesion by indirect ELISA

2.2.8.1.1 Biotinylation of thrombin-activated platelets

The preparation of thrombin-activated platelets as described in 2.2.1.1. Platelets were adjusted to final concentration of 2 x 10^8 platelets in 1 ml of 5 mM Sulfo-NHS-LC-biotin in PBS and incubated at room temperature for 1 hour. The biotinylation reaction was stopped by washing with PBS 2 times and biotinylated platelets were adjusted to 1x10^7 platelets/ml

2.2.8.1.2 Effect of monoclonal antibodies on platelet adhesion by indirect ELISA

To investigate the monoclonal antibodies of interest on platelet adhesion, indirect ELISA was performed. The ELISA plate was coated with 50 µl of 2 mg/ml fibrinogen or 100 µg/ml collagen at 4^{o}C for overnight. The coated wells were washed with 0.85% sodium chloride for 2 times. Then, 60 µl of 2% BSA-NaN_3-PBS was added into each well and incubated at 37^{o}C for 1 hour. Twenty-five microlitters of biotinylated platelets (1x10^7 platelets/ml) were incubated with 25 µl of various concentrations antibodies at room temperature for 30 minutes prior added into ELISA plate. The mixture was incubated at 37^{o}C for 1 hour. The plate was washed non-adherent platelets for 4 times with washing buffer (0.05% Tween 20 in PBS) before added 50 µl of horseradish peroxidase (HRP) conjugated streptavidin at dilution 1:20,000. After incubation for 1 hour, plate was washed 4 times with washing buffer. Then, 50 µl of tetramethylbenzidine (TMB) was added into each well and incubated at room temperature in the dark place for 5 minutes. The reaction was stopped by adding 50 µl of 1N HCl and the absorbance was measured at 450 nm using ELISA

reader. The percentage of platelet adhesion was calculated with the formula (Eriksson and Whiss 2005)

$$\text{Percentage of platelet adhesion} = \frac{(\text{sample - conjugate control})}{(\text{positive control - conjugate control})} \times 100$$

2.2.8.2 Effect of monoclonal antibodies on platelet aggregation by platelet aggregation assay

Blood samples were collected using 3.2% sodium citrate as an anticoagulant at the ratio of 9:1. Blood samples were centrifuged at 250 g, 22°C for 10 minutes. Then, platelet rich plasma (PRP) was collected into a plastic tube and adjusted to $300x10^9$ platelets/L with platelet poor plasma (PPP).

Platelet aggregation was measured using a Platelet aggrecorder II PA 3320 (Figure 2.1). Briefly, 360 µl of platelet suspension was incubated with 90 µl of various concentrations of mAb at 37°C for 5 minutes prior added 50 µl of 20 µg/ml collagen or 200 µM ADP. Platelet aggregation was then recorded into 10 minutes after the addition of an agonist.

2.2.8.3 Statistical analysis

Statistical analyses were performed using SPSS software (version 11.0). All the results are expressed as means ± SD. The significance of differences between the means of two group was determined using a Wilcoxon signed-rank test. The differences were considered significant when *P* value was less than 0.05.

Figure 2.1 Platelet aggregometer model Aggrecorder II PA 3320