CHAPTER I

INTRODUCTION

1.1 Statement of problems

The platelets play a crucial role in primary hemostasis and blood clotting at sites of vascular injury (thrombogenesis). The platelets in formation of the hemostatic plug imply that adhesion and aggregation are initial and mandatory function for contribution to the arrest of bleeding by sticking to the edges of a break in vessel wall to form a hemostatic plug (Davi and Patrono 2007). However, in similar way to nonhemorrhagic internal lesions, platelets were abnormally activated *in vivo*. Activation of platelet plays a key role in forming a thrombus within the lumen (Furie and Furie 2008) and cause numbers of thrombotic diseases such as peripheral vascular disease, deep vein thrombosis (Badimon and Vilahur 2007, Rodvien and Mielke 1976). This thrombosis complication is an important public health problem in Thailand because of continuously increasing number of patients and is the leading cause of death. Furthermore, numerous studies have documented that platelet activation occurs and plays a role in patients suffering from thalassemia (Eldor and Rachmilewitz 2002, Taher, *et al* 2008, Widlansky, *et al* 2003), tumor (Athale, *et al* 2008, Tilley, *et al* 2008) and diabetes mellitus (Carr 2001, Creager, *et al* 2003). Therefore, thrombosis caused by platelet activation is the major cause of death in these patients. There is therefore great interest in the measurement of *in vivo* platelet activation for identifying certain patients with thrombotic risk and decrease in number of dead (Badimon and Vilahur 2007).

From previous studies, researchers have been developed a sensitive and specific methods to detect the presence of *in vivo* activated platelets for the purpose of identifying a prethrombotic or thrombotic state. Currently, the most reliable markers of in *vivo* platelet activation are the measurement of plasma or urine substances released from activated platelets for example platelet factor 4, β-thromboglobulin (Kaplan and Owen 1981), ATP (Beigi, *et al* 1999) and metabolites of thromboxane A_2. However, measurement of these markers has been used in research setting but the technical problems related to sample collection and processing have limited for clinical use (Abrams, *et al* 1990).

In addition, flow cytometry has been used to detect activation-dependent markers on circulating activated platelets using specific monoclonal antibody (Shattil, *et al* 1987). Activated markers detected on the platelet surface include P-selectin, ligand-induced binding sites in glycoprotein IIb-IIIa, dense granule proteins (Bussel, *et al* 2000). Furthermore, various monoclonal antibodies to platelet activation markers have been reported (Klein, *et al* 2002). From this data led us to purpose the aim of our study is to produce and characterize the monoclonal antibodies against thrombin-activated platelets. The produced mAbs may offer numerous advantages include to replace the imported mAbs. Furthermore, the produced monoclonal antibodies to activated platelets can possibly be used in evaluating patients with thrombosis diseases and might offer advantages in the clinical management and prevention of patients at risk for thrombosis.

1.2 Literature reviews

1.2.1 Platelet structure

Blood platelets play a fundamental role in hemostasis and blood clotting at sites of vascular injury. Platelets are derived from the cytoplasm fragmentation of the megakaryocytes and released into peripheral blood lies between 140,000-400,000 per microliter. Platelets are the smallest corpuscular component of circulating blood and have a diameter of 2-4 μm (White 2004). In the absence of bleeding, platelets circulate in a basal resting state with minimal interaction with the other blood components and vessel wall. Platelets have a typical discoid shape with smooth surface. After activation by soluble agonists, platelets lead to a shape change with formation of pseudopods (spherical shape) to form a hemostatic plug and eventually contribute to wound healing (George 2000). The ultrastructure of platelets can be divided into four morphological regions, peripheral zone, structural zone, organelle zone and membranous systems (Figure 1.1) (Shapiro 2000).

1.2.1.1 Peripheral zone

The peripheral zone consists of the cytoplasmic membrane that is covered on its extracellular side by a thin layer composed of various glycoproteins, proteins and mucopolysaccharides (glycocalyx). The cytoplasmic membrane is made up of a typical polarized phospholipids layer containing the membrane proteins. Asymmetrical distribution of the phospholipids with phosphatidylcholine and phosphatidylethanolamine pointing outwards while phosphatidylinositol and phosphatidylspingomyelin pointing inwards is an important factor for platelet function (Marcus and Zucker-Franklin 1964). The phospholipids organization of the plasma membrane changes during activation of the platelets with formation of platelet

factor 3 as well as liberation of factors for signal transduction (secondary messengers) and arachidonic acid. In addition, the platelet plasma membrane expresses numerous integrated proteins that are receptors for soluble agonists such as ADP or thrombin and for adhesion proteins such as fibrinogen or von Willebrand factor (George 2000, Siess 1989).

1.2.1.2 Structural zone

The structural zone consists of microtubule located in the submucosa that represent tubulin threads and are surrounded by a network of other, widely differing structural proteins (Castle and Crawford 1980). The components of structural zone serve to maintain the typical discoid shape of resting platelets and actively participate in the shape changes of activated platelets. The structural proteins form the cytoskeleton which consists mainly of myosin and submembranous actin filaments (Pollard 1980). The actin is the most abundant protein in platelets accounting for 15% to 20% of the total protein. Actin exists in two functional states are G-actin (globular form) and F-actin (filament form). Filaments composed of actin and myosin are formed upon activation and these filaments have a connection to the cell organelles during the activation process (Hartwig, *et al* 1999).

1.2.1.3 Organelle zone

The zone of organelles is found in the cytoplasm and consists of mitochondria, glycogen stores and three different forms of storage granule (dense granules, alpha granules and lysosomes). The granules serve as storage sites for several proteins and other substances essential for platelet function (Loftus, *et al* 1984).

Dense granules contain a series of low-molecular-mass compounds such as ADP, ATP, Ca^{2+} and serotonin that promote platelet aggregation process.

Alpha granules are the most numerous in the platelet. This granule contains two major groups of proteins, the first group is hemostatic proteins such as fibrinogen, von Willebrand factor, P-selectin and the other group is non-hemostatic proteins such as epidermal growth factor. These proteins that influence widely differing biological functions like platelet adhesion, aggregation, coagulation, chemotaxis, proliferation and inflammation (Holt and Niewiarowski 1985).

Lysosomes contain hydrolytic enzyme such as α-arabinoside, β-galactosidase, collaginase and elastase.

1.2.1.4 Membranous systems

Membranous systems consist of the surface-connected open canalicular system (OCS) and the dense tubular system (DTS). The open canalicular system connect to the plasma membrane and is accessible from the extracellular space by pores (Behnke 1970). The dense tubular system is one of the main storage sites for calcium ion which play a major role in the regulation of platelet metabolism and activation. When the cytoplasmic free Ca^{2+} concentration exceeds a certain threshold the platelet undergoes a shape change with formation of pseudopods and degranulates (Cutler, *et al* 1978).

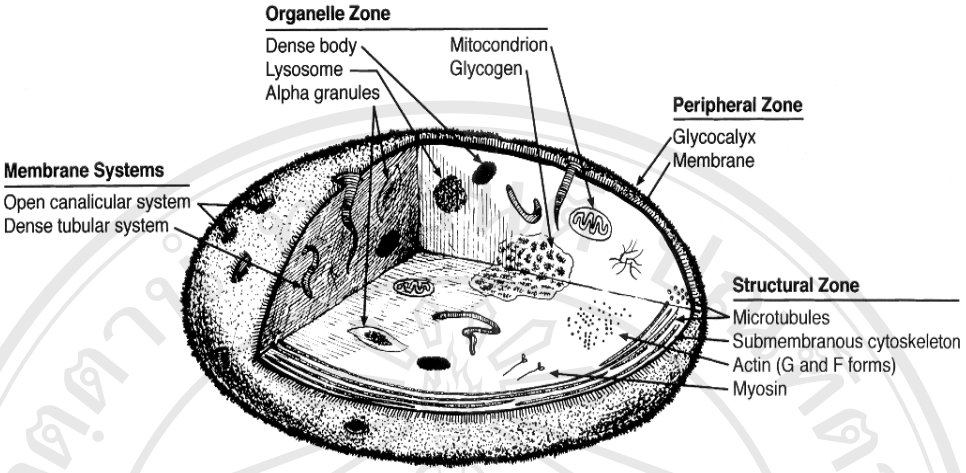


Figure 1.1 Platelet ultrastructure is composted of four morphological regions, peripheral zone, structural zone, organelle zone and membranous systems (McKenzie, 1996)**.**

1.2.2 Platelet function

Platelets provide for the initial arrest of bleeding and play a major role in primary hemostasis. When blood vessels have an injury, the platelet activity is responds to vascular damage and continues maintenance of vascular integrity by the rapid adherence of platelet to exposed endothelium. In addition, platelets spread, become to activated platelets and form aggregate to create a platelet plug initially arrest the bleeding. Activation of human blood platelets is associated with changes in platelet morphology, biochemistry and membrane composition. These events result in binding or increased surface expression of several endogenous platelet proteins and plasma proteins. In addition, the mechanism of platelet in primary hemostasis compose of major three parts; platelet adhesion, platelet activation and platelet aggregation.

1.2.2.1 Platelet adhesion

The adhesion process is regulated by glycoprotein of the platelet membrane. Platelets possess a number of membrane-bound, adhesion receptors that regconize specific structure components of the extracellular matrix (ECM) in the subendothelial of vessels. The first contact between circulating blood platelets and the vessel wall lesion (called contact phase) is established by an interaction of glycoprotein Ib-V-IX on platelet membrane with von Willebrand factor (Andrews, *et al* 1997). After contact phase, the stabilization phase of the platelet adhesion proceeds for highly affinity *via* further membrane adhesion receptor GP Ia/IIa, GP Ic/IIa, GP Ic'/IIa bind with collagen, fibronectin and laminin, respectively (Figure 1.2) (Ruggeri 1994, Surin, *et al* 2008). These bindings between platelet receptors and subendothelial extracellular matrixs lead to the activation phase of the adherent platelets.

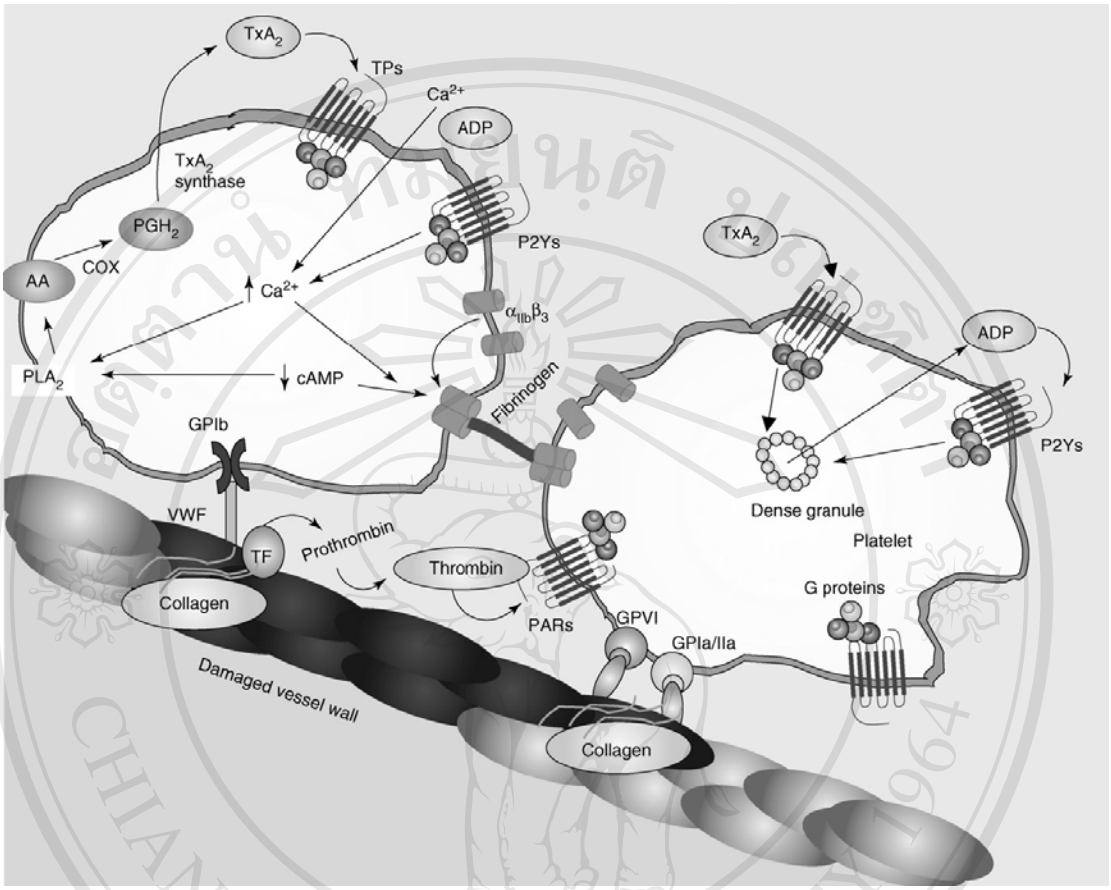


Figure 1.2 Platelet adhesion. Glycoprotein on platelet membrane adhere to the extracellular matrix in the subendothelial of vessels, become activated and trigger the recruitment of more platelets forming a platelet plug (Gresele, *et al* 2008).

1.2.2.2 Platelet activation

When platelets adhere to collagen fibers in the subendothelial, a series of morphology and function will change. This occurance is known as activation. Which involves in changing of platelet metabolic biochemistry, shape, surface receptors, and membrane phospholipids orientation (Table 1.1) (Siess 1989).

Platelet activation involves a series of morphologic and functional changes. Changes to platelet metabolic biochemistry result from substances generated by the platelet itself as well as cells in the injured tissue. Substances that induce platelet activation are called agonists (Engelmann, *et al* 1998). Each agonist attaches to a specific receptor on the platelet, causing a series of reactions inside of the platelet. The first of these biochemical changes occurs when vWF and collagen bind to glycoprotein Ib receptor on the surface of the platelet. This activates enzymes in the membrane that cleave specific membrane phospholipids resulting in products called "second messengers" (Stewart, *et al* 1990). These "second messengers" enter the platelet cytoplasm and transfer signals to interior parts of the cell, triggering a variety of biochemical changes.

One of the biochemical changes that occur within the platelet is an increase in internal calcium levels. When these levels reach a threshold, a change in the shape of the platelet occurs. The activated platelet is transformed from a disc-shaped cell to a sphere with spiny projections called pseudopods. This change in shape results in an increase in the surface area available for biochemical reactions and increased chance of contact with other platelets. As they change shape, platelets spread over the surface of the collagen to which they adhere, filling in the spaces between pseudopods and creating a "jigsaw puzzle effect" (de Gaetano, *et al* 2003).

Table 1.1 Properties of resting and activated blood platelet (Meinrad, 2001)

Property	Resting platelet	Activated platelet
Shape	Discoid	Spherical with pseudopods
Actin	F-form	G-form
Phospholipids	Inactive	TXA_2 synthesis (AA)
Phospholipase A_2	Inactive	Activate
Phospholipase C	Inactive	Activated cleaves PIP_2 to IP_3
Adenylate cyclase	Inactive	Active
Cytoplasmic Ca^{2+}	Low	High
GPIIb-IIIa	Inactive, no binding of soluble fibrinogen possible	Active, fibrinogen binding

1.2.2.2.1 Biochemical mechanism

Activation and adhesion are followed by the secretion of substances that drive the hemostatic process by stimulating other platelets to adhere, secrete, and aggregate. This mechanism begins by platelet activator (agonist) when activation and primary adhesion have been achieved, platelets discharge of granule contents (Tonon, *et al* 2002). Granule contents include ADP, adenosine triphosphate (ATP), serotonin, calcium, vWF, Factor V, and fibrinogen. The secreted substances further promote formation of the platelet plug by means of stimulating other platelets to adhere, aggregate and secrete. Each agonist binds at its specific receptor on the platelet surface and effects the formation of signal factors (secondary messenger) *via* signal transduction pathways (Siess 1989). Three enzyme complexs play a major part in the formation of secondary messenger are phospholipase C, phospholipase A_2 and adenylate cyclase (Figure 1.3).

Phospholipase C cleaves phosphoinositol 4,5-biphosphonate (PIP_2) to form inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 induces the release of Ca^{2+} from the dense tubular system while DAG activates protein kinase C and sends signal protein to activate GP IIb-IIIa complex (Kumjian, *et al* 1991, Lerer, *et al* 1990).

Phospholipase A_2 is activated by release of cytoplasmic Ca^{2+} and catalyzes the arachidonic acid from membrane phospholipids. Arachidonic acid is a precursor of thromboxane A_2 for vasoconstriction function (Krzystanek, *et al* 2007).

Adenylate cyclase is stimulated by antagonists of platelet activation such as prostacyclin or theophylline and leads to the formation of cAMP with inhibitory activity (Lerer, *et al* 1990).

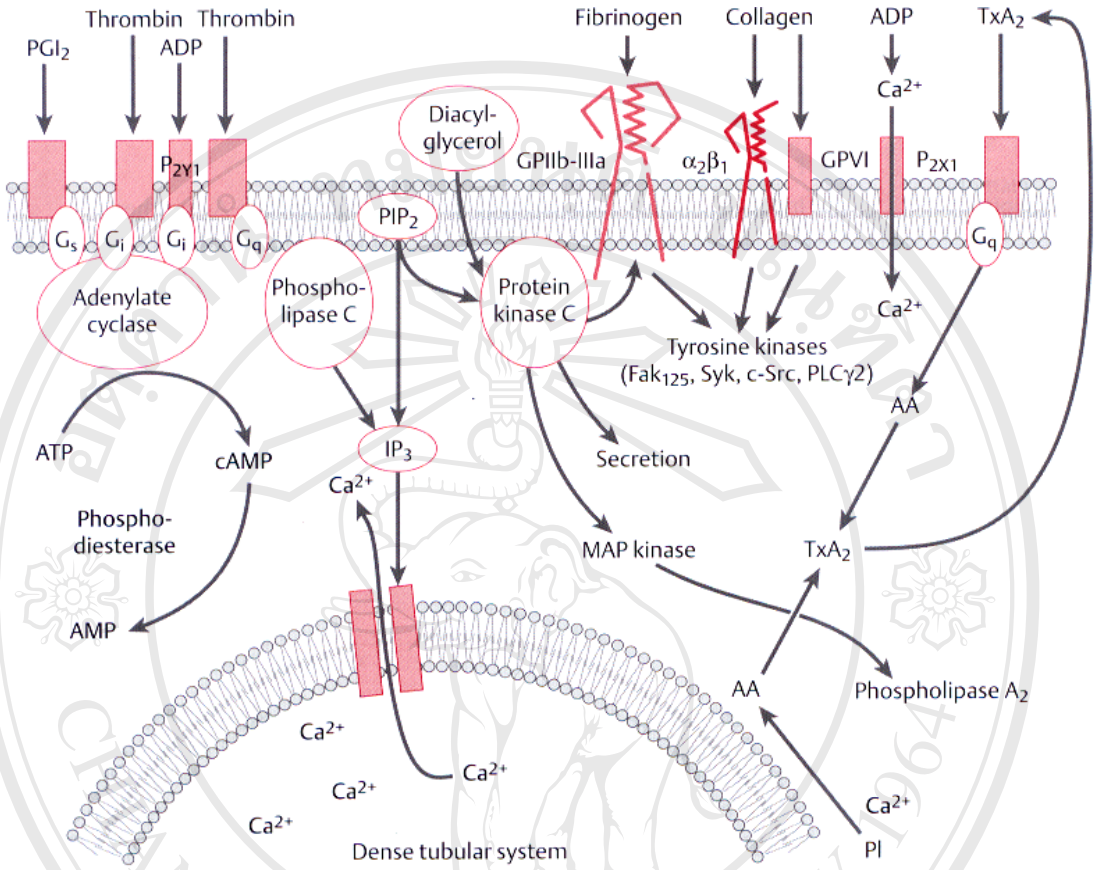


Figure 1.3 Mechanism for signal transduction in platelet activation (Brass, *et al* 1997)

1.2.2.2.2 Shape change

When the intracellular Ca^{2+} concentration exceeds a specific threshold, platelets undergo a shape change with formation of pseudopods (spheroid shape) (Yano, *et al* 1994). The shape change is accompanied by reorganization of the internal constituents of the platelet. As a result of contraction of the associated microfibrins, the peripheral band of microtubules undergoes central apposition and this has the effect of forcing the granules towards the plasma membrane, including that of the surface-connected canalicular system by facilitating secretion of their contents. The cytoplasmic microfibrins also appear to depolymerize and then reform within the pseudopods (Figure 1.4) (Zucker-Franklin 1969). Later, contraction of these microfibrins may account for the process of clot retraction, which helps consolidate the platelet plug.

(A) **(B)**

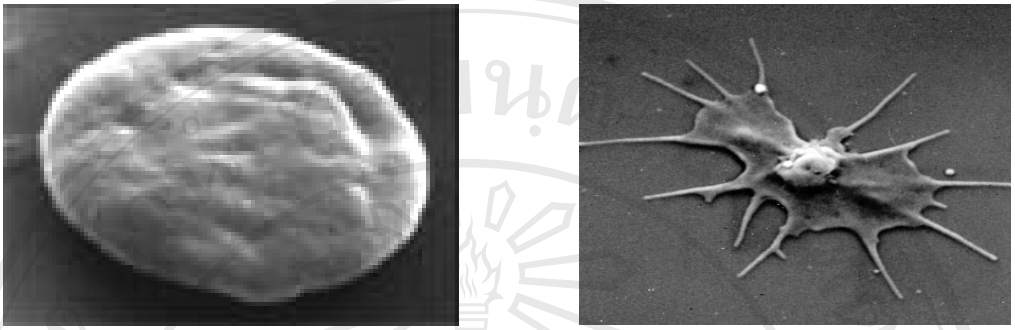


Figure 1.4 Platelet shape change. After activation, platelets undergo a shape change from discoid shape (A) to spheroid shape (B).

(http://www.acbd.monash.org/research/thrombosis-research-unit.html)

1.2.2.2.3 Platelet degranulation

Immediately following their adhesion and shape change, platelet commences a specific release reaction. This is sustained for several minutes and the intensity of which varies according to the stimulus. Weak inducers, such as low dose of ADP or adrenaline involve mainly the α-granule contents. A small proportion of which may even leaks out from unstimulated platelets in citrate blood. Higher concentrations of ADP or adrenaline but low concentrations of collagen induce secretion from both α-granules and dense granules. The release of ADP is then further amplifying the response. Strong stimuli such as thrombin or high concentrations of collagen also cause the release of lysosomal enzymes (Chronos, *et al* 1993). The release reaction occurs concomitantly with, and is dependent upon, phosphatidylinositol (PI) turnover and prostaglandin (PG) generation. Both of which also probably contribute to platelet aggregation (Polasek 2006).

1.2.2.2.4 Activation of surface receptor

During platelet activation, the glycoprotein IIb-IIIa is activated. The binding site for adhesive proteins appears shortly after activation with an agonist (Martin, *et al* 2007). It provides a site for fibrinogen and von Willebrand factor binding. Activation also causes a change in the membrane surface. This change enables fibrin-forming proteins (coagulation factors) to bind to the membrane.

1.2.2.3 Platelet aggregation

Following platelet activation, formation of the primary hemostatic plug continues with platelet aggregation. This is the phase in which activated platelets become attached to one another. After the release of agonists such as adenosine diphosphate (ADP), by platelets and the injured vessels, platelets undergo a change in shape and glycoprotein IIb-IIIa receptor sites become exposed for binding with fibrinogen (Chen, *et al* 2005). These changes enable the activated platelet to stick readily to those adhering to collagen. Fibrinogen thus forms direct bridges between adjacent platelets, leading to aggregation (Figure 1.5).

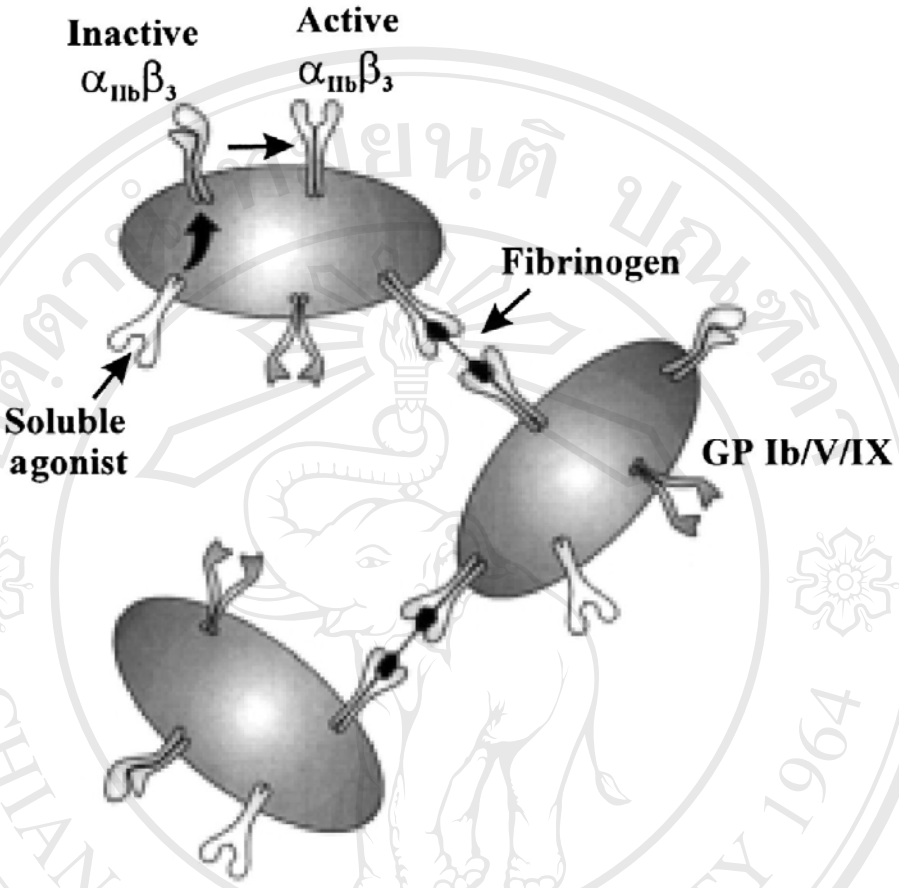


Figure 1.5 Platelet aggregation. After fibrinogen binding sites in the vicinity of GPIIb-IIIa receptor are exposed with subsequent platelet aggregation through formation of fibrinogen bridge.

(http://www.cfm.brown.edu/crunch/IMAG/projectsummary.html)

1.2.3 Thrombosis

Thrombosis is the formation of a clot or thrombus inside a blood vessel, obstructing the flow of blood. The formation of thrombus is usually caused by an injury to the vessel wall, either by trauma or infection, or by the slowing or stagnation of blood flow past the point of injury. In classical terms, thrombosis is caused by abnormalities in one or more of the following (Virchow's triad):

- The composition of the blood
- Quality of the vessel wall
- Nature of the blood flow

The two distinct forms of thrombosis are venous thrombosis and arterial thrombosis, each of which can be presented by several subtypes.

1.2.3.1 Venous thrombosis

Venous thrombosis is the formation of a thrombus (blood clot) within a vein. Deep vein thrombosis (DVT) is the formation of a blood clot within a deep vein. It most commonly affects leg veins, such as the femoral vein. Three factors are important in the formation of a blood clot within a deep vein, these are the rate of blood flow, the thickness of the blood and qualities of the vessel wall (van Stralen, *et al* 2008). Classical signs of DVT include swelling, pain and redness of the affected area.

1.2.3.2 Arterial thrombosis

Arterial thrombosis is the formation of a thrombus within an artery. In most cases, arterial thrombosis follows rupture of atheroma, and is therefore referred to as atherothrombosis (Badimon and Vilahur 2007).

1.2.4 Correlation of inappropriate platelet activation and clinical diseases

1.2.4.1 Thalassemia and hypercoagulability

Thalassemia is a hereditary anemic disease, which is characterized by insufficient synthesis of either α or β-globin (Weatherall, *et al* 1981). The severity of clinical course distinguishes this disease into 3 main subtypes: thalassemia major (TM), thalassemia intermedia (TI) and thalassemia minor (TMi). Patients with TI have, in general, a milder clinical phenotype than those with TM. The pathophysiology of TI is characterized by extravascular hemolysis, with the release into the peripheral circulation of damaged red blood cells and erythroid precursors because of a high degree of ineffective erythropoiesis. The life expectancy of β-thalassemia patients has markedly improved over the last few years, as a result of regular blood transfusions and compliance with tight iron chelation therapies. However, β-thalassemia patients still suffer from many complications of their chronic disease, and a series of serious previously undescribed complications is now being acknowledged. The presence of a high incidence of thromboembolic events, mainly in β-TI has led to the identification of a hypercoagulable state in thalassemic patients. Venous thromboembolic events such as deep venous thrombosis (DVT), pulmonary embolism (PE) and portal vein thrombosis have been observed. However, there are relatively few epidemiological data on the overall frequency of these complications. It has been reported that thromboembolic events occurred 4.38 times more frequently in TI than TM ($p<0.001$) when observed in 8,860 thalassemic patients(Taher, *et al* 2008). Moreover, thromboembolism was demonstrated with more venous events occurring in TI and more arterial events occurring in TM. Previous studies have reported the incidence of stroke in β-TM to range from 2% to 20%. In a study done to assess the rate of brain

damage in patients with benign hemoglobinopathies, 37.5% of patients with TI showed asymptomatic brain damage on MR imaging. In a series of β -TI patients, 29% of patients developed either DVT, pulmonary embolism, or portal vein thrombosis during a 10-year follow up (Cappellini, *et al* 2000). Borgna-Pignatti *et al*. (Borgna-Pignatti, *et al* 2004) observed that the risk of thromboembolism was 1.1% in 720 cases of TM. While Zurlo *et al.* reported that thromboembolism is the primary cause of death in 2.5% of patients with TE (Zurlo, *et al* 1989). Recently, data from autopsy findings in thalassemia patients have definitely established hypercoagulability as a pathologic state (Cappellini, *et al* 2005). Autopsy series in patients with β-TM and β-TI described the presence of DVT, pulmonary embolism and recurrent arterial occlusion, with thrombi in small and large pulmonary vessels. Autopsies of a large series patient with β-thalassemia/hemoglobin E disease revealed thrombotic lesions in the pulmonary arteries. This pulmonary arterial thromboembolism may have been due to circulating platelet aggregates. Similar findings of multiple microthrombi, which were composed mainly of platelets, were seen in the pulmonary arterioles and microcirculation in autopsies of two cases with splenectomized thalassemic disease. As a result of multiple recent clinical studies and laboratory data, thalassemia has been referred to as a ‘‘hypercoagulable state’’. On the basis of the available data, there is evidence of increased hypercoagulability in thalassemia. This coagulation activation is attributed to many factors. Defining the contribution of the hypercoagulable state to the pathophysiology of thalassemia requires further studies.

1.2.4.2 Platelet activation in thalassemic patients

The medical literature that contain evidence suggesting that patients with β-thalassemia have activated platelets (Atichartakarn, *et al* 2003, Setiabudy, *et al* 2008,

Sonakul, *et al* 1980). Moreover, flow cytometric studies have also confirmed the chronic platelet activation status (Del Principe, *et al* 1993). In β-thalassemia, there is evidence of increased platelet aggregation, an increased proportion of platelets expressing CD62P (P-selectin) and CD63, and a shortened platelet survival due to enhanced platelet consumption. The platelet life span (PLS) is shortened with mean PLS of 107 ± 36 h in splenectomized β-thalassemia cases (eight TM, two TI) vs. 248 ± 51 h in splenectomized controls. A shortened PLS in addition to increased platelet aggregates in thalassemic patients suggest platelet activation, which may be associated with active thrombotic disease or chronic hypercoagulable state. Further evidence of chronic platelet activation in patients with thalassemia was provided by the measurement of urinary metabolites of prostacyclin (PGI_2) and thromboxane A_2 (TXA_2). A significant increase (4–10-fold) in the urinary excretion of the stable hydrolysis products of TXA_2 and PGI_2 in β-thalassemia patients compared to controls has been reported (Eldor and Rachmilewitz 2002). No significant differences were found in the concentrations of these metabolites between TM and TI patients. These results showed that chronic platelet activation is present in β-TM and TI. The presence of morphologic platelet abnormalities as detected by scanning electron microscopy in splenectomized patients with β-thalassemia/hemoglobin E disease may also contribute to a high tendency of vascular complications, especially pulmonary thrombi.

1.2.4.3 Malignancies and hypercoagulability

A hypercoagulable or prothrombotic state of malignancy occurs due to the ability of tumor cells to activate the coagulation system. It has been estimated that hypercoagulation accounts for a significant percentage of mortality and morbidity in

cancer patients (Rickles and Edwards 1983). In addition, treatments for cancer including surgery, hormonal therapy, cytotoxic chemotherapy, and also the placement of central venous catheters, contribute to the hypercoagulable state. Surgery, usually the first choice for benign solid tumors, can increase the risk of TE due to activation of the hemostatic system (Levine, *et al* 1988). The risk of postoperative thrombosis is raised approximately two-fold in cancer patients compared to noncancerous patients, and the risk of fatal PE postoperatively is even increased four-fold in cancer patients (Geerts, *et al* 2001, Kakkar, *et al* 1970). A preoperative laboratory evaluation of thrombosis markers as thrombin–antithrombin levels may be useful in identifying at risk patients for postoperative DVT (Falanga, *et al* 1993). The chemotherapy can increase the risk of thrombosis in cancer patients. Thrombotic complications have been shown in association with specific chemotherapeutic agents, including L-asparaginase, mitomycin C, cisplatin as well as high-dose chemotherapeutic regimens for bone marrow transplantation (Falanga 1998). Controlled studies have shown that conventional chemotherapy routinely used for treating breast cancer can also increase the risk of TE (Levine, *et al* 1988) and that prophylactic treatment with warfarin can reduce this risk (Levine, *et al* 1994). Moreover, hormone therapy with tamoxifen is an additional risk factor for thrombosis in breast cancer (Barbui, *et al* 1996, Falanga, *et al* 1999). Additionally, the use of hematopoietic growth factors such as granulocyte colony stimulating factor (G-CSF) or granulocyte–macrophage colony-stimulating factor (GM-CSF) may be implicated in hypercoagulation and clot formation in breast cancer. DVT and PE are the two most common thromboembolic complications in cancer. Cancer patients presents with VTE up to 15% (Green and Silverstein 1996, Luzzatto and Schafer 1990). Interestingly, VTE is not equally

common in all types of cancer. The highest incidence is found in mucin-producing adenocarcinomas, pancreas, gastrointestinal tract, lung cancer, and ovarian cancer. TE occurs less often in breast and renal cell carcinoma and rarely in patients with prostate cancer and melanoma (Caine, *et al* 2002).

1.2.4.4 Platelets activation in malignancies

Patients with advanced cancer have been shown to exhibit increased platelet activation, also indicated by increased platelet turnover and decreased platelet survival time. Mitogenic cell extracts, cell membrane fragments, and secreted chemicals from various animal and human cancers can directly aggregate platelets, as well as increase platelet turnover and decrease platelet survival time (Hara, *et al* 1980, Pearlstein, *et al* 1980). A link has also been proposed among the degree of cell surface sialylation of tumor cells, their ability to aggregate platelets, and the incidence of thrombosis in cancer patients (Scialla, *et al* 1979). Tumor cells and tumor vesicles shed from many tumor cells bind to platelets, raising the possibility of platelet aggregation by physical bridging (Raz and Lotan 1987). Other causes of the increased platelet activation in cancer include cancer-induced thrombin generation, adenosine diphosphate (ADP), and a cathepsin B–like cysteine proteinase production by tumor cells and raised levels of von Willebrand factor (Grignani, *et al* 1988, Nierodzik, *et al* 1995).

1.2.4.5 Diabetes mellitus and hypercoagulability

Diabetes mellitus affects approximately 100 million people worldwide (Boccara and Cohen 2004). Five to ten percent have type 1 (insulin-dependent) and 90% to 95% have type 2 (non–insulin-dependent) diabetes mellitus. Vascular diseases are the principal causes of death and disability in people with diabetes (Creager, *et al* 2003).

These complications are responsible for 50% of the deaths in patients with type 2 diabetes mellitus (DM), 27% of the deaths in patients with type 1 diabetes for 35 years or less, and 67% of the deaths in patients with type 1 diabetes for 40 years or more. In normal endothelial cells, biologically active substances are synthesized and released to maintain vascular homeostasis, ensuring adequate blood flow and nutrient delivery while preventing thrombosis and leukocyte diapedesis. Among the important molecules synthesized by the endothelial cell is nitric oxide (NO), which is constitutively produced by endothelial NO synthase (eNOS) through a 5-electron oxidation of the guanidine-nitrogen terminal of L-arginine (Moncada and Higgs 1993). The bioavailability of NO represents a key marker in vascular health. Nitric oxide causes vasodilation by activating guanylyl cyclase on subjacent vascular smooth muscle cells. In addition, NO protects the blood vessel from endogenous injury as atherosclerosis by mediating molecular signals that prevent platelet and leukocyte interaction with the vascular wall and inhibit vascular smooth muscle cell proliferation and migration (Radomski, *et al* 1987, Sarkar, *et al* 1996). Conversely, the loss of endothelium-derived NO permits increased activity of the proinflammatory transcription factor nuclear factor kappa B (NF-κB) resulting in expression of leukocyte adhesion molecules and production of chemokines and cytokines (Zeiher, *et al* 1995). These actions promote monocyte and vascular smooth muscle cell migration into the intima and formation of macrophage foam cells, characterizing the initial morphological changes of atherosclerosis and develop to thromboli causes of death in people with diabetes (Libby 2000, Nomura, *et al* 2000).

1.2.4.6 Platelets activation in diabetes mellitus

Multiple studies offer evidence of enhanced activation or increased platelet activity in patients with diabetes mellitus. It has been reported that platelets from patients with NIDDM maximally aggregate with low concentrations of ADP and arachidonic acid (Garcia Frade, *et al* 1987). A heightened response to ADP in several insulin-dependent diabetics is also demonstrated (Rosove, *et al* 1984). In a study of type 2 diabetics, Hughes *et al* found evidence of enhanced platelet *in vitro* hyperaggregation or increased circulating platelet aggregate (Hughes, *et al* 1983). In a study of platelet function in patients, Krishnaswami *et al* (Krishnaswami, *et al* 2002) found very high platelet contractile force. Activated platelets release multiple chemical substances and proteins from dense and alpha granules. Level of some of these release products serve as markers of *in vivo* platelet activation. Various studies have found high levels of thromboxane B_2 (Badimon and Vilahur 2007, Garcia Frade, *et al* 1987, Small, *et al* 1986), β-thromboglobulin (Hughes, *et al* 1983, Rosove, *et al* 1984, Small, *et al* 1986), platelet factor 4 (Borsey, *et al* 1984) in patients with diabetes. Moreover, expression of both glycoprotein Ib and IIb-IIIa is increased, augmenting both platelet–von Willebrand factor and platelet–fibrin interaction. The intracellular platelet glucose concentration mirrors the extracellular environment and is associated with increased superoxide anion formation and protein kinase C (PKC) activity and decreased platelet-derived nitric oxide. Hyperglycemia further changes platelet function by impairing calcium homeostasis and thereby alters aspects of platelet activation and aggregation, including platelet conformation and release of mediators.

Platelet activation occurs and plays a role in several vascular disorders (Caine, *et al* 2002, Davi and Patrono 2007, Eldor and Rachmilewitz 2002, Green and Silverstein 1996, Panigrahi and Agarwal 2007). Therefore, the detection of activated platelets might facilitate identifying certain thrombotic disorders and evaluating therapeutic strategies

To facilitate understanding the physical and biochemical changes when platelets are stimulated to undergo a secretory response, they produce thromboxane A_2 and release the contents of their storage granules into the extracellular. Some of these released substances, such as platelet factor 4 and β-thromboglobulin, are platelet-specific and can be detected in plasma by sensitive radioimmunoassays. The measurement of these proteins in plasma and thromboxane A_2 metabolites in plasma and urine has provided evidence for platelet activation in several clinical disorders (Fitzgerald, *et al* 1987). These indirect methods have technical limitations for routine clinical use. For example, extreme care must be taken during blood sample collection and processing to prevent the *in vitro* release of platelet factor 4 and β-thromboglobulin. Therefore, several monoclonal antiplatelet antibodies were recently developed that permit the direct detection of activated platelets in whole blood by flow cytometry (Abrams and Shattil 1991, Abrams, *et al* 1990, Shattil, *et al* 1987). Use of antibodies provides information on several different aspects of the platelet-activation process: 1) conversion of glycoprotein (GP) GP IIb-IIIa into a competent adhesion receptor, a process required for normal platelet adhesion and aggregation: GP IIb-IIIa activation can be dissected into three sequential phases by using antibodies specific for the exposed adhesive ligand-binding site within GP IIb-IIIa, for the receptor-bound ligand, or for neoepitopes within GP IIb or IIIa exposed during

ligand binding; 2) exocytosis-related surface exposure of integral membrane proteins specific for either alpha, dense, or lysosomal platelet granules; 3) surface binding of proteins, such as thrombospondin, released during platelet secretion; 4) development of platelet procoagulant activity, manifested by the surface expression of binding sites for coagulation factors Va and VIIIa on both activated platelets and platelet-derived microparticles (Sims, *et al* 1989) 5) flow cytometry can be used to study platelet activation in whole blood and plasma (Shattil, *et al* 1987).

1.2.5. Platelet surface markers

1.2.5.1 Activated platelet surface markers

1.2.5.1.1 Glycoprotein IIb-IIIa

The functional state of the receptors on resting or activated platelets can be defined by specific monoclonal antibodies directed against the glycoprotein IIb-IIIa complex (Frelinger, *et al* 1991, Ginsberg, *et al* 1990). The surface density of GPIIb-IIIa can be evaluated using the monoclonal antibody to CD41 that binds the complex irrespective of whether it is in the resting or activated state. The high affinity activated receptor complex can be characterized by the binding of the monoclonal antibody PAC-1 (Shattil, *et al* 1987). This antibody recognizes the fibrinogen binding sites in the GPIIb-IIIa complex and binds exclusively to the activated GPIIb-IIIa receptor provided that they are not already occupied by fibrinogen (Calvete 1995). By means of further specific antibodies (directed against ligand-induced binding site; LIBS), ligand-occupied receptors can be identified. Anti-LIBS monoclonal antibodies such as LIBS-1 or PMI-1 can bind exclusively to the receptor when it binds fibrinogen and not to resting or activated receptors in the absence of fibrinogen. The

advantage of activation-specific anti-GPIIb-IIIa antibodies (LIBS-1, PAC-1) is that they can very sensitively recognize the activation state of the platelet even before the release reaction has occurred (Chen, *et al* 2005). Another possibility to characterize the functional state of GPIIb-IIIa involves the direct determination of the fibrinogen associated with the activated platelet membrane by means of antibodies directed against the ligand of fibrinogen. Similar to LIBS antibodies, anti-fibrinogen-RIBS antibodies have been developed (Chen, *et al* 2005). These antibodies specifically recognize, conformational changed fibrinogen receptor (induced binding site) (Ugarova, *et al* 1993). Furthermore, the accessibility of GPIIb-IIIa binding site can be examined by means of fluorescein-conjugated, RGD-containing ligands such as FITC-ecstatin; this is useful for monitoring GPIIb-IIIa antagonists (Michelson, *et al* 2000b).

1.2.5.1.2 Degranulation markers

The glycoproteins P-selectin (CD62P) and GP53 (CD63) are express exclusively on activated platelets provided that a degranulation of α granules (P-selectin) or lysosomes (GP53) has occurred. An increased binding of anti-CD62P or anti-CD63 indicate an irreversible degranulation of the platelets. P-selectin is constitutively present in α-granules of platelets and Weibel-Palade bodies of endothelial cells, and is only translocated to the cell surface after activation. Therefore, the P-Selectin is a marker of platelet activation (Gawaz, *et al* 1999, Hartwell, *et al* 1998).

1.2.5.1.3 Thrombospondin

Thrombospondin (TSP), a multifunctional glycoprotein, has many features in common with extracellular matrix constituents known to mediate platelet adhesion. It is secreted from platelet α-granules and binds to the activated platelet surface upon platelet stimulation. TSP also may play a role in platelet-substratum adhesion and has

hemagglutinating (lectin-like) activity and forms a specific complex with fibrinogen for stabilizes platelet aggregates (Kowalska and Tuszynski 1993, Leung 1984).

1.2.5.2 Resting platelet surface markers

1.2.5.2.1 Glycoprotein Ib-V-IX

In contrast to GPIIb-IIIa, the surface density of glycoprotein Ib-V-IX is reduced by receptor internalization after thrombin activation of platelets. The degree of activation of platelet can also be characterized by flow cytometric determination of the surface expression reduction of GPIb-V-IX (Michelson 2000, Michelson, *et al* 2000a).

1.2.5.2.2 Glycoprotein IV (CD36)

CD36 is an membrane glycoprotein expressed platelets and this molecule has been shown to serve as the receptor for two extracellular matrix proteins, collagen and thrombospondin. The role of CD36 as a cell surface receptor has been extended to a signal transduction molecule in platelets, being associated in platelets to protein tyrosine kinases (Watson, *et al* 2000).

1.2.5.2.3 Glycoprotein VI (p62)

Glycoprotein VI is a platelet membrane protein that was identified as a physiological collagen receptor. GPVI is present as a complex with the Fc receptor (FcR) γ-chain, probably composed of two GPVI molecules and one FcR γ-chain dimer. GPVI must form such a dimeric complex to exhibit high affinity binding to collagen. The GPVI-induced activation mechanism is initiated by tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) of the FcR γ-chain, and then this signal is transduced to many related proteins, mainly by tyrosine phosphorylation. GPVI is widely recognized as a requisite factor for the

formation of platelet aggregates on a collagen surface under blood flow (Moroi and Jung 2004).

1.2.6 Monoclonal antibody

The antibodies are produced and secreted by B lymphocytes. Many clones of B lymphocytes can produce different antibodies that respond to different epitopes of an antigen, so called polyclonal antibodies. In 1975, the hybridoma technique was developed by Kohler and Milstein (Kohler and Milstein 1975). This technique developed for producing monoclonal antibody as a single specific antibody and produced the immortalizing individual antibody-secreting cells from an immunized animal by hybridomas.

1.2.6.1 Hybridoma technique

Monoclonal antibodies (mAbs) are antibodies produced by a single clone of hybridoma cells. These cells are derived from the fusion of B-cell (antibody producing cell) and myeloma cell by hybridoma technique. In 1975, Kohler and Milstein devised a method of growing large numbers of antibody producing cells from fusing B-cells with immortal myeloma cancer cells (Figure 1.6). The immortalized antibody-producing cell lines are called hybridomas and the antibodies they produced are termed monoclonal antibodies. Myeloma cell lines that used for fusion are hypoxanthine phosphoribosyltransferase or HGPRT deficiency, an enzyme of a salvage pathway for purine biosynthesis, while the myeloma cells can use the *de novo* pathway to survive (Figure 1.7) (Goding 1986). Splenocytes of the immunized mouse are fused with myeloma cells by using polyethylene glycol. After cell fusion, the fused cells can be selected in a medium containing hypoxanthine, thymidine, and an

antifolate drug, aminopterin (HAT medium). Aminopterine is a folate antagonist that blocks the *de novo* pathway. Therefore, unfused myeloma cells and fused cells that have negative HGPRT will die (Yelton and Scharff 1980). Hybridoma cells can survive in the selective medium because the salvage pathway of splenic B lymphocyte partner can be function. Wells containing the desired antibody produced by growing-hybridomas can be identified by a number of immunoassays, such as immunofluorescence assay or enzyme-linked immunosorbent assay (ELISA). The limiting dilution is a technique using isolate single hybridoma cells or single clone and the production from single clone is a monoclonal antibody. The hybridoma clones that produce a desire antibody can be expanded or induced ascitic fluids in a mouse to produce large scale of monoclonal antibodies.

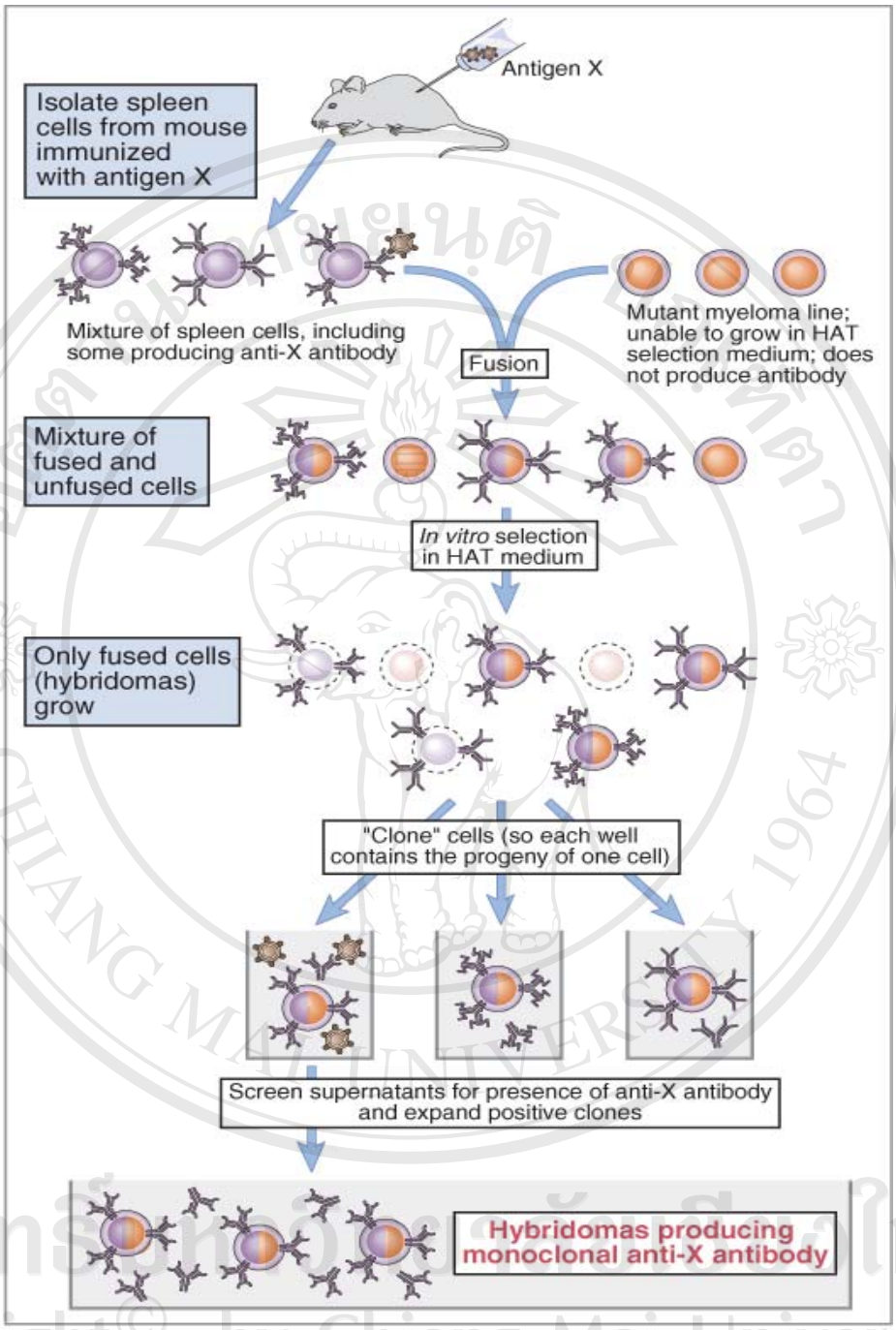


Figure 1.6 Monoclonal antibody production (Abbas and Lichtman 2005)

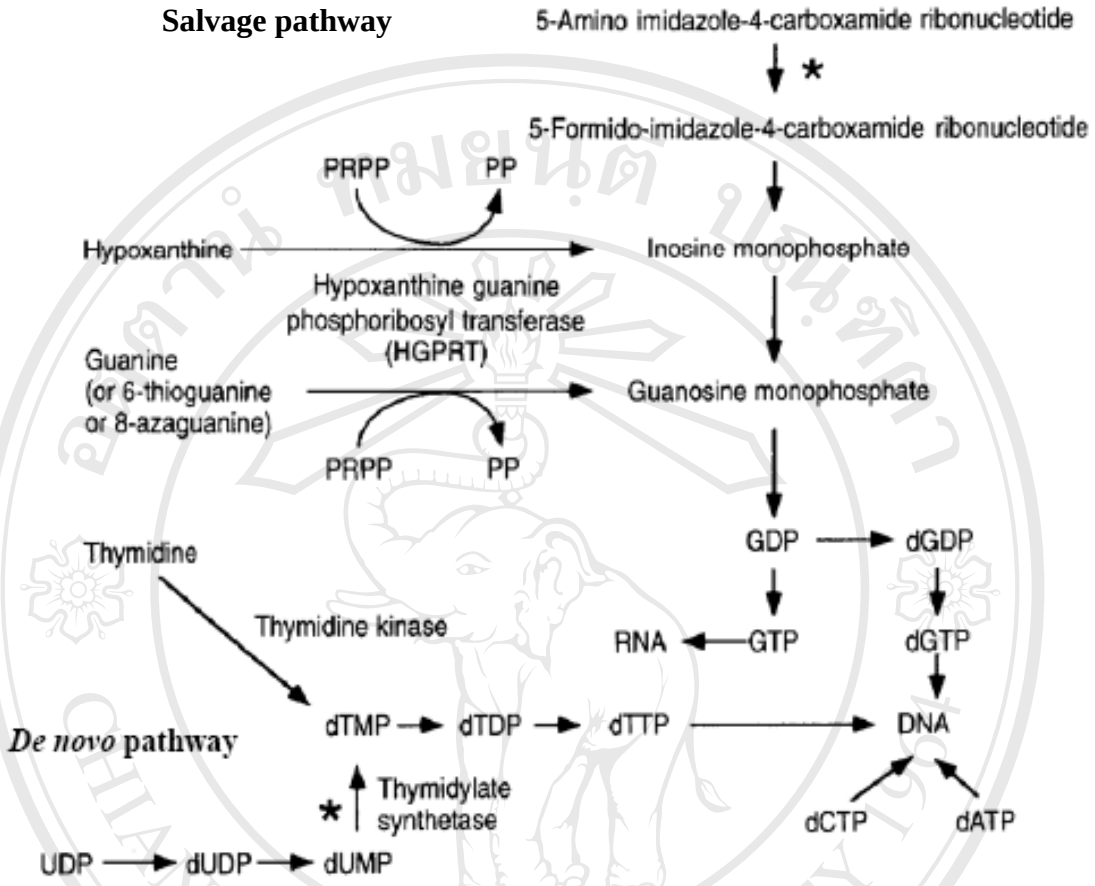


Figure 1.7 Metabolic pathway of nucleotide synthesis when the *de novo* pathway are blocked with folic acid analogue (*), such as aminopterin, cell must depends on the salvage pathway (Goding 1986).

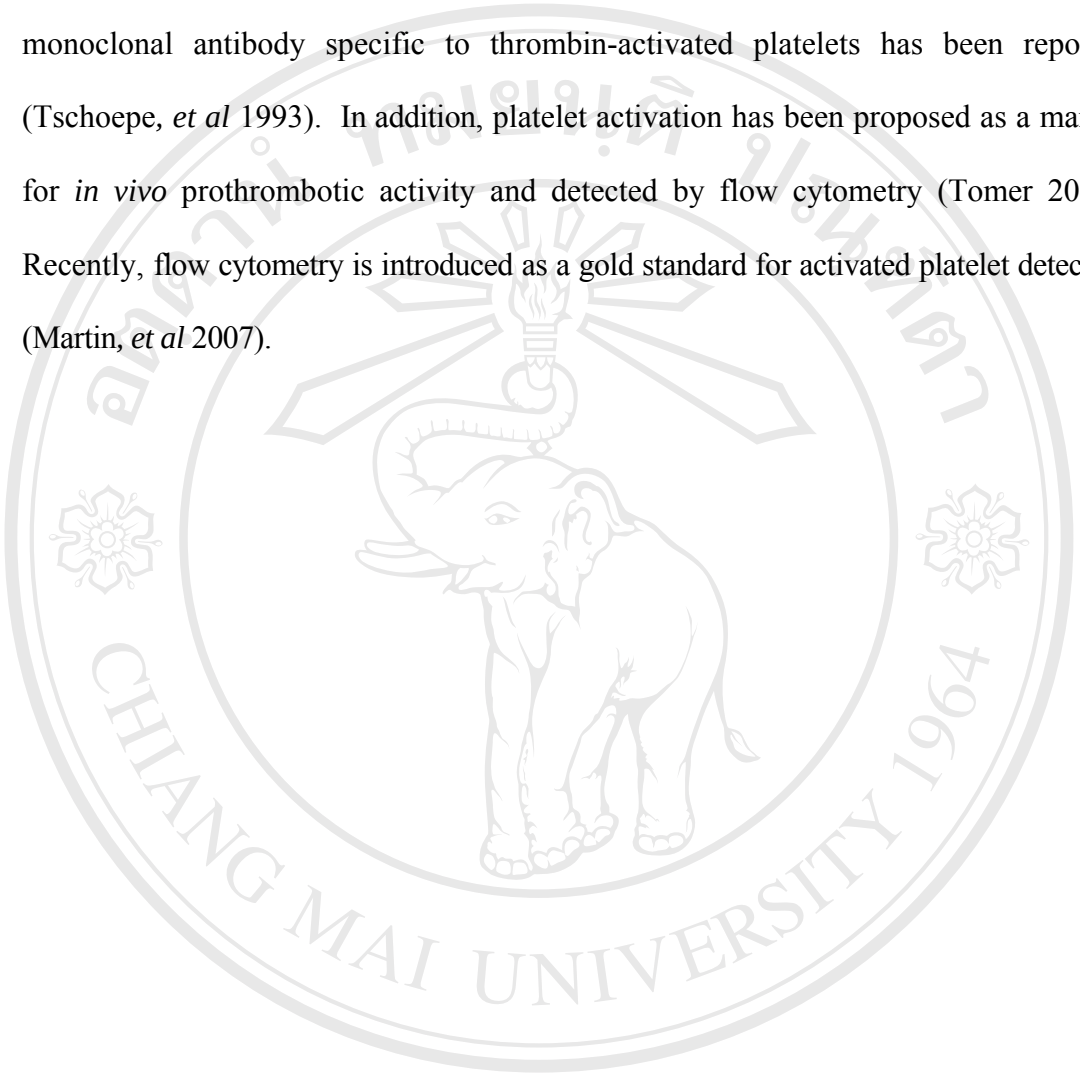
1.2.7 Flow cytometry

Flow cytometry uses the principles of light scattering, light excitation, and emission of fluorochrome molecules to generate specific multi-parameter data from particles and cells in the size range of 0.5 to 40 µm diameter. Cells are hydro-dynamically focused in a sheath of PBS before intercepting an optimally focused light source (Figure 1.8). Lasers are most often used as a light source in flow cytometry (Fouchet, *et al* 1993). A number of detectors are aimed at the point where the stream passes through the light beam; one in line with the light beam called Forward Scatter or FSC and several perpendicular to it called Side Scatter or SSC. Each suspended particles passing through the beam scatters the light in some way and fluorescent chemicals found in the particle or attached to the particle may be excited into emitting light at a higher wavelength than the light source. This combination of scattered and fluorescent light is picked up by the detectors. FSC correlates with the cell volume and SSC depends on the inner complexity of the particle such as shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness (Camplejohn 1994).

1.2.7.1 Flow cytometry in platelet study

A major development in the functional studies of thrombocytes using flow cytometry is developing rapidly (Shattil, *et al* 1987). Flow cytometry in combination with fluorochrome-labeled monoclonal antibodies or substances makes it possible to characterize specific changes on the surface of activated platelets. During platelet activation several changes occur at the platelet surface. Currently, flow cytometry is the most sensitive technique to detect increased surface exposure of activation antigens on the platelet surface. Whole blood flow cytometric techniques can

minimize the artifactual platelet activation often seen with other techniques such as platelet aggregation and measurement of β-thromboglobulin in plasma. Moreover, a monoclonal antibody specific to thrombin-activated platelets has been reported (Tschoepe, *et al* 1993). In addition, platelet activation has been proposed as a marker for *in vivo* prothrombotic activity and detected by flow cytometry (Tomer 2004). Recently, flow cytometry is introduced as a gold standard for activated platelet detection (Martin, *et al* 2007).



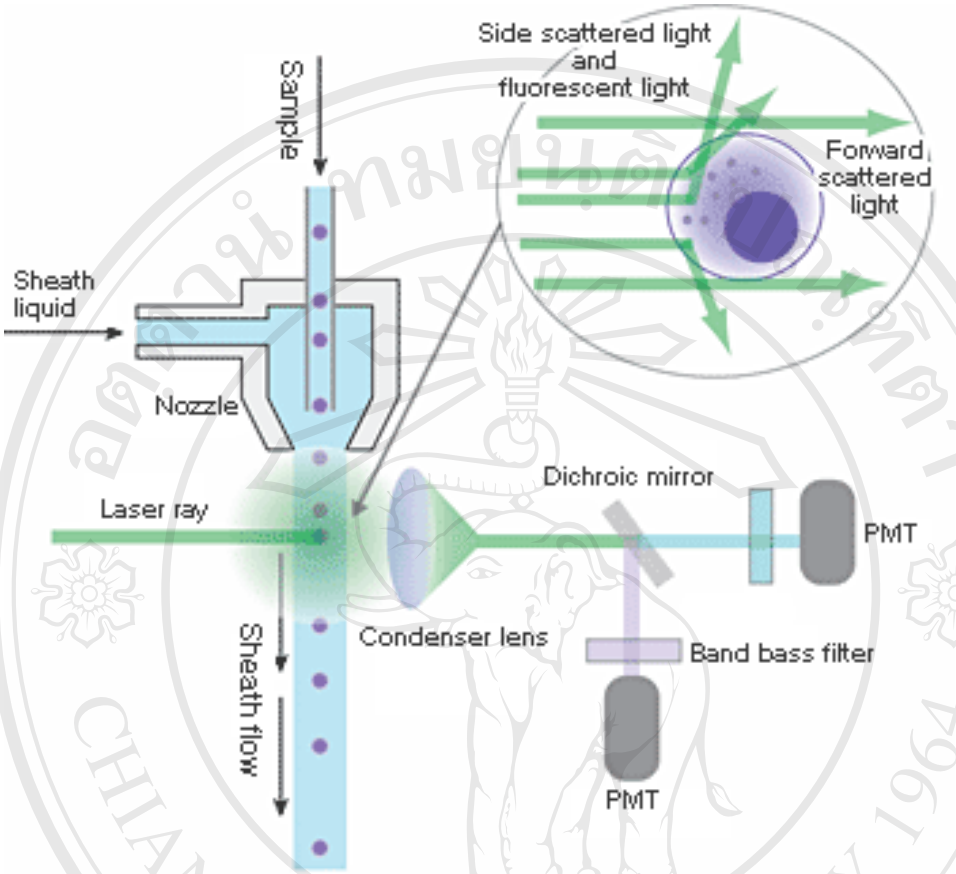


Figure 1.8 Flow cytometer uses the principle of hydrodynamic focusing for presenting cells to a laser.

(http://www.reprocell.com/en/service/researcher_01.html)

1.3 Objectives

1. To produce monoclonal antibodies against surface molecules on thrombin-activated platelets
2. To study the expression of molecules recognized by produced monoclonal antibodies
3. To characterize the biochemical property of molecule recognized by produced monoclonal antibodies