I. INTRODUCTION

1.1 Statement and significance of the problem

Thalassemia is a congenital hemolytic disorder caused by a partial or complete deficiency of α- or β-globin chain synthesis due to their genes defects. These globin chains are the vital components of hemoglobin (Hb), which is oxygen/carbon dioxide carrying protein presenting in erythrocytes or red blood cells (RBC). Defect of genes encoding globin chain synthesis results in insufficiency of Hb levels. In case of an α-globin gene defect so called α-thalassemia, an excess in β-globin chain is found. On the other hand, in case of β-gene defect or β-thalassemia, an excess in α-globin chain is found. Homozygous state of β-globin gene defects shows severe anemia and some serious complications since the early childhood, identified as β-thalassemia major. The severity is controlled by chronic blood transfusion. However, this can cause severe iron overload resulting in progressive organ failures. Some forms of α-thalassemia are also associated with a similar clinical picture. Despite the difficulties associated with treatment, standard of care for thalassemic patients have improved in recent year, resulting in almost doubling of the average life expectancy. As a consequence, some more complications are now being recognized. In particular, profound hemostatic changes have been observed in patients with β-thalassemia major (β-TM) and β-thalassemia intermedia (β-TI) and also in patients with α-thalassemia (Hb H disease).

Cardiac failure is a cause of death in thalassemic patients, particularly those over 20 years of age. Left ventricular hypertrophy with and without dilatation due to anemia is often exacerbated by hypoxemia. Chronic pulmonary thromboembolism is an important cause of cor-pulmonale (Ferrer, 1975). In the patients with heart diseases,

the only manifestation of pulmonary thromboembolism is frequently worsening the cardiac function. In some thalassemic autopsy cases, pulmonary thromboembolic lesions were reported. Predisposing factors in these patients include perpetual hemolysis, recurrent infections, and thrombocytosis with increased young and active platelets and circulating platelet microaggregates, particularly in patients who undergone splenectomy (Winichagoon *et al.*, 1981; Tawarat *et al.*, 1987; Eldor *et al.*, 1989; Wong *et al.*, 1990; Isarangkura *et al.*, 1993).

The thromboembolic events in β-TM are usually associated with significant morbidity and mortality. These include recurrent and transient ischemic cerebral manifestations, stroke and a high frequency of thrombotic lesions in the pulmonary arteries. The prothrombotic anomalies in the majority of β-TI patients even in a very young age are found. This leads to the recognition of chronic hypercoagulable state in particular β-thalassemic patients.

Abnormal platelet functions have also been observed in patients with thalassemia. A significant decrease in platelet aggregation responses to various agonists especially in β-TM, Hb H disease and Hb E disease were noticed due to platelet exhaustion (Eldor, 1978; Eldor, 1978, Hussain *et al.*, 1979). These anomalies are possibly responsible for the observed mild hemorrhagic tendencies manifested by easy bruising and frequent epistaxis (Eldor, 1978; Eldor, 1978, Hussain *et al.*, 1979). Among these events recurrent and transient ischemic cerebral attacks and stroke as well as peripheral arterial and venous thrombosis are among the serious complication (Logothetis *et al.*, 1972; Sinniah *et al.*, 1977; Paolino *et al.*, 1983). The autopsy findings in thalassemic patients show a high frequency of thrombotic lesions in the pulmonary arteries and the development of atherosclerotic changes (Sonakul *et al.*, 1980).

The *in vitro* platelet aggregation anomalies could be associated with the mild hemorrhagic tendencies observed in thalassemia. They could also result from a hypercoagulable state and reflect an acquired functional defect due to "exhaustion"

of the platelets following their *in vivo* activation by substances released from lysed erythrocytes (O'Brien *et al.*, 1974). Evidence for this assumption is provided by the significant increase in the number of circulating platelet-aggregates in splenectomized thalassemic patients (Winichagoon *et al.*, 1981). This is also reassured by the finding of lower mean survival of platelets in adult patients with β-TM or β-TI both with and without splenectomy (Eldor *et al.*, 1989).

The roles of thromboxane A_2 (TXA_2) and prostacyclin (PGI_2) in the interaction of platelets with the vessel wall in thromboembolic and atherosclerotic disorders were expected (FitzGerald *et al.*, 1984; Oates *et al.*, 1988; Davi *et al.*, 1990). Since the excretion of urinary metabolites of TXA_2 and PGI_2 assayed by enzyme immunoassay showed the continuous platelet activation in the patients (Eldor *et al.*, 1991). Administration of ticlopidine, a platelet inhibitor which has no direct effect on prostaglandin metabolism, also resulted in a significant decrease in the urinary excretion of the metabolites in some thalassemic patients.

Excretion of 2,3,-dinor-6-keto-PGF_{1x} is an accurate index of biosynthesis of PGI_2 which reflects alterations in vascular synthesis of this ecosanoid (FitzGerald *et al.*, 1984; Oates *et al.*, 1988). Enhanced excretion of this metabolite was reported in severe atherosclerosis, unstable angina, peripheral vascular diseases and systemic sclerosis (FitzGerald *et al.*, 1984; Fitzgerald *et al.*, 1986; Oates *et al.*, 1988). A significant increase urinary excretion of this metabolite was found in all studied thalassemic patients (Eldor *et al.*, 1991). This finding could result from vascular intimal injury due to the hemolytic process or from oxygen radicals catalyzed by excess iron deposition in many organ and tissues (Shinar and Rachmilewitz, 1990). An alternative possibility is that increased platelet-vessel wall interaction leads to either physical contact or the release of platelet constituents (during activation), which are potent stimuli of vascular prostacyclin production (FitzGerald *et al.*, 1984; Oates *et al.*, 1988).

In thalassemia, *in vivo* platelet activation may be caused by the release of ADP or other thromboplastin-like materials from pathological hemolysed erythrocytes (Shinar and Rachmilewitz, 1990). The recent experiments showed that erythrocytes from non-transfused β–TI patient enhanced thrombin formation in a "prothrombinase" assay (using a chromogenic substrate). These results suggested that transbilayer movement of phospholipids in thalassemic erythrocytes with exposure of phosphatidylserine to the plasma may initiate intravascular coagulation and platelet activation.

The decreased levels of protein C, protein S and anti-thrombin III (AT-III) in β-thalassemia major were found in the majority of the patients (Shirahata *et al.*, 1992; Eldor *et al.*, 1993; Shebl *et al.*, 1999). About 60% had a decrease in one or more of these natural inhibitors of coagulation. These findings further support the existence of hypercoagulability in thalassemic patients.

Vaso-occlusive crisis does not occur in thalassemia, however there is accumulating evidence that the patients may develop a chronic hypercoaglable state. Some thalassemic patients were suffered from ischemic cerebral manifestations, including transient ischemic attacks and strokes (Sinniah *et al.*, 1977; Sonakul *et al.*, 1980; Paolino *et al.*, 1983). Some developed a major thrombotic event including pulmonary embolism, arterial and venous thrombosis. Furthermore, thrombosis in the pulmonary arterial tree may be more important. Pulmonary arterial thrombotic lesions and pulmonary atherosclerosis are very prevalent in patients with β-TM or Hb E disease (Sonakul *et al.*, 1980). Consequently, some of the thalassemic patients responded to treatment with aspirin and dipyridamole with a rise in their arterial oxygen content (Fucharoen *et al.*, 1981). Hypoxia may be involved in platelet activation since it causes damage to the vascular endothelium (Morrison *et al.*, 1977) resulting in the interaction of circulating platelets with the vessel wall.

The aims of this study are to measure platelets with their activation marker (CD63) expression, red blood cells with phosphatidylserine exposure on their

membrane and platelet activation *in vivo* including shape change and aggregated platelets and also platelet activation markers (β-thromboglobulin; β-TG and platelet factor 4; PF4) releasing. In addition, the platelet function or platelet aggregation tests using platelet rich plasma stimulated with various agonists *in vitro* are also planned to be studied. Finally the experiment to determine whether there is any factor on thalassemic erythrocyte membrane and / or in the plasma that is able to induce platelet activation *in vitro*. All of these are to be studied only in β-TM with multiple transfusions in comparison to normal healthy control subjects. After completion the more understanding on platelet activation and thromboembolic status in β-TM will be achieved. The prevention of such complications with prophylactic anti-platelet drugs such as low dose aspirin or others may be valuable.

1.2 Literature reviews

1.2.1 Platelet physiology

Platelets prevent blood loss or hemorrhage after injury of blood vessels. The complex hemostatic system comprises of interaction between blood vessels and various plasma proteins such as coagulation factors as well as the platelets themselves. The platelet membrane glycoproteins (GP) function as physiological receptors. They mediate the interactions between vascular subendothelium and the platelets so called adhesion. They also mediate platelet-platelet interaction so called aggregation. These interactions are the respond of platelets to thrombogenic stimuli.

1.2.2 Thrombopoiesis (platelet production)

Platelets are produced in bone marrow by megakaryocytes. The earliest platelet precursors are megakaryocytic burst-forming units (BFU-meg). Under the influence of various cytokines such as IL3, IL6 and growth factors such as GM-CSF, MK-CSF and thrombopoietin (Tpo), the BFU-meg develops into megakaryocytic colony-forming unit (CFU-meg). Thrombopoietin is produced from liver cells, in responses to low blood platelet levels. A single mature megakaryocyte is suggested to ultimately produce several thousand platelets. Normally, the megakaryocytes give rise to nearly 2×10^{11} platelets per day.

1.2.3 Megakaryocytic cells

1.2.3.1 Early development

BFU-meg, the most primitive progenitors, is committed to be megakaryocytic lineage under specific stimuli such as low total platelet and megakaryocytic cell mass. CFU-meg, the next cell type of megakaryocytic development, is a small, mononuclear marrow cell that expresses platelet-specific markers, but is not morphologically identifiable. These transitional cells represent approximately 5% of marrow megakaryocytic element. These transitional immature megakaryocytic cells can proliferate. However the more mature stages of megakaryocytic cells can not proliferate. They are actively undergoing endomitosis that refers as nuclear proliferation without cell dividing.

1.2.3.2 Megakaryoblast a round approximately 6-24 μm in diameter, has a loose chromatin structure, large nucleus and prominent nucleoli. Its cytoplasm contains platelet-specific granules and the characteristic of demarcation membrane system. This membrane system is invaginated from the surface of the megakaryocyte and undergoes extensive progression. At the end of the megakaryoblastic stage, it has extended through most of the cytoplasm of the cell. The megakaryoblast enlarges progressively by endomitosis. It is very likely that the endomitotic sequence characteristic of megakaryocytic maturation is a specialized function targeted to the production of intracellular membrane. Because of the active DNA synthesis, the megakaryoblasts have various ploidy stages starting from 8N, 16N, 32N to 64N with the majority of 8N to 16N.

1.2.3.3 Promegakaryocyte or basophilic megakaryocyte a round approximately 14-30 μm in diameter, with lobulated nucleus, without nucleoli contains small amount of basophilic cytoplasm. After the completion of endomitosis and DNA synthesis, it enters a phase of cytoplasmic maturation. This phase is characterized by RNA production and protein synthesis. The RNA content gives rise to intense basophilia with Romanowsky staining. Electron microscopy shows prominent polyribosomes spread throughout the cytoplasm. The continuing development of the demarcation membrane system and azulophilic granules is found. These cells are mainly 16N, with some in the 8N and 32N and very few maturing as 4N and 64N.

1.2.3.4 Granular megakaryocyte a round or oval approximately 16-56 μm in diameter with polychromatic staining contains a lobulated nucleus. The cell volume continues to enlarge with ongoing cytoplasmic and membrane protein synthesis. As the cell mature the basophilic staining disappears. The nucleus becomes denser and moves to the center of the cell. The cytoplasm contains prominent specific granules. The demarcation membrane system becomes open and freely connects to the external environment.

1.2.3.5 Platelet release; The fully mature megakaryocyte begins to extend pseudopodia, which penetrate through the wall of adjacent marrow sinusoids. The pseudopodia are broken off by the force of the blood flow, giving rise to individual platelets or larger cytoplasmic fragments. The latter "proplatelet" are transported through the blood stream via the heart to the lung tissue. The final mechanical fragmentation is accomplished in the pulmonary microcirculation. Each megakaryocyte produces seven to eight proplatelets. The nucleus of the megakaryocyte remains in the marrow and is thought to degenerate and to be removed by the macrophages. Approximately 5 days are required for a megakaryoblast to development into platelets.

Normally, two-thirds of the total platelets that is released into the peripheral blood circulate in the blood stream at concentration of 150 to 400 x 10^9/L. The other one-third is sequestered into the spleen and is stored there until needing for equilibrium with those platelets in the blood. The average lifespan of platelets in the peripheral blood is approximately 7 to 10 days.

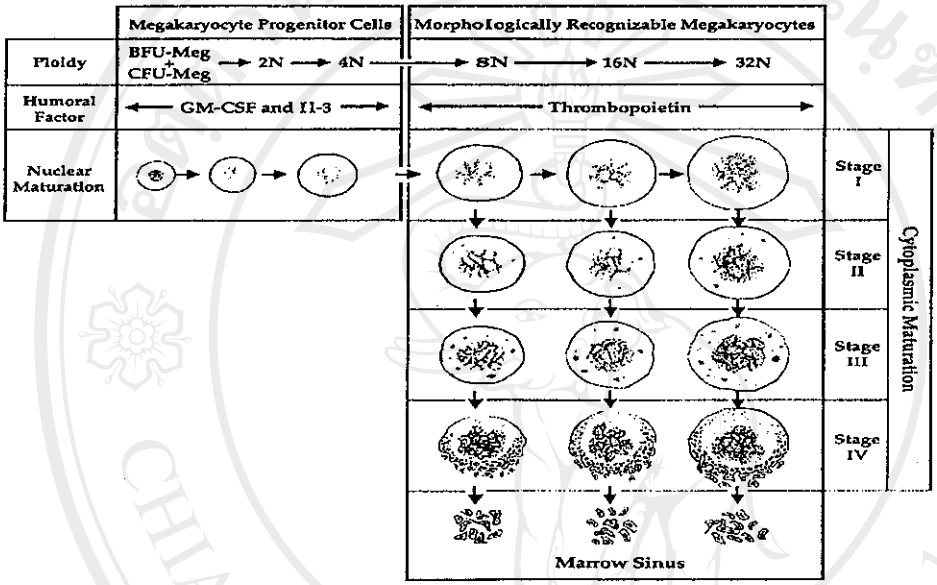

Figure 1 Development of megakaryocytic cells (Harmening, 1997)

1.2.4 Platelet structure

Platelets are small anucleated cells with diameters of 2 to 4 μm, and a cell volume of approximately 6-10 fL. The circulating resting platelets are discoid shape (biconvex) with smooth surface, unlike the exterior surfaces of erythrocytes and leukocytes. Platelets have several opening holes resemble as sponges. The openings are membranous channels, which extend deep into the interior of cytoplasm. After activation, platelets change from discoid shape to spherical shape with long extensions facilitating adhesion (starlet shape). The cytoplasmic actin and myosin mediate the change in morphology and the clot retraction. In addition to other cell

organelles, such as peroxisomes, mitochondria and lysosomes, platelets contain dense granules or delta granules (δ-granules) and alpha-granules (α-granules) which secrete various substances and proteins essential for the platelet function and coagulation reaction.

Platelet ultrastructure is composed of four zones:

Peripheral zone

Structural zone

Organelle zone

Membranous systems

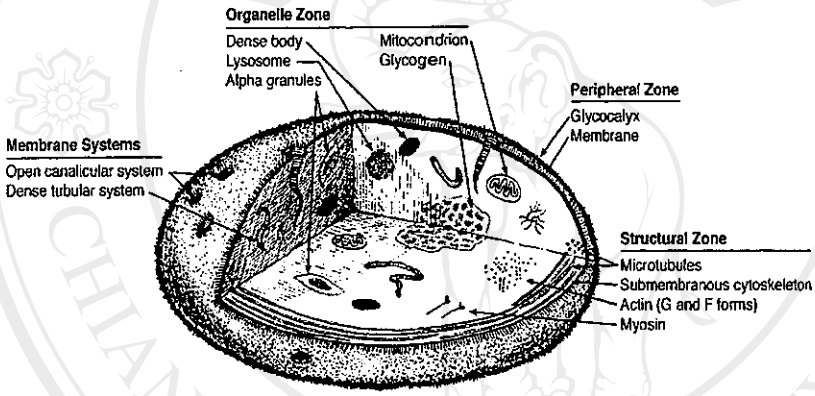

Figure 2 Ultrastructure of platelet (McKenzie, 1996)

1.2.4.1 Peripheral zone

The peripheral zone consists of a cytoplasmic membrane also has a typical trilaminar structure of bilayer of phospholipid embedded with integral proteins. The asymmetric arrangement of phospholipid is an important in the function of activated platelets. Phosphatidylcholine (PC) and phosphatidylinositol (PI) and sphingomyelin (SM) are concentrated on the outer half, while phosphatidylserine (PS), phosphatidylethanolamine (PE) predominate on the inner half of the bilayer. The membrane is covered on the exterior

by a fluffy surface coat so called the glycocalyx and on the interior by a thin submembranous region between the peripheral zone and the next layer.

The glycocalyx is also found on the surface membrane of the interior channel. Some of the surface proteins may be act as receptor for substances, which cause platelet activation so called agonists. The glycocalyx consists of several glycoproteins proteins and mucopolysaccharides. (Table 1)

Plasma membrane or cell membrane is a trilaminar unit composed of a bilayer of phospholipids in which cholesterol, glycolipids, and glycoproteins are embedded. The plasma membrane is thought to contain the sodium- and calcium-ATPase pumps that control the intracellular ionic environment of the platelet. Approximately 57% of platelet phospholipids are contained in the plasma membrane. The phospholipids are asymmetrically organized in the plasma membrane. The negatively charged phospholipids are almost exclusively present in the inner leaflet. The negatively charged phospholipids, especially phosphatidylserine, are able to accelerate several steps in the coagulation sequence. So they present in the inner leaflet of resting platelets, to prevent its contact to the plasma coagulation factors. This is thought to be a control mechanism for preventing inappropriate coagulation. During platelet activation induced by selected agonists, the aminophospholipids may become exposed on the outer platelet surface or platelet microparticles.

The phospholipid asymmetry in resting platelet may be maintained by an ATP-dependent aminophospholipid translocase that actively pumps phosphatidylserine and phosphatidylethanolamine from the outer back to the inner leaflet. Interactions of negatively charged phospholipids with cytoskeletal or other cytoplasmic elements may also contribute to the asymmetry.

The platelet plasma membrane is vital to the cell function for two reasons. The first, it contains a number of specific glycoprotein receptors. Though that platelet interacts with aggregating agents, inhibitors, coagulation factors (such as fibrinogen,

vWf and thrombin). Consequently, it also interacts with the vessel wall and *vice versa.* The best defined of these hemostatically important GPs are listed, along with their function. They belong to several different gene families. The best defined being the integrins (that include GPs Ia, Ic and the IIb-IIIa complex) and the leucine-rich GP family (that include GPs Ib, IX and V). Most share a common structure consisting of two subunits of α and β (e.g. GPIbα and GPIbβ) that being synthesized under separate genetic control. Both of which span the membrane and may thus effect signal transduction from the outer membrane to internal cytoskeletal components. Therefore influencing the secretory response is found. Other GPs, including those thought to mediate ADP-, adrenalin- and thrombin-induced aggregation, belong to a less well defined group so called the 7-transmembrane domain family.

Surface receptors, gpIIb and IIIa are complexed by signal transduction from agonists that activate the platelets. On the surface of resting platelets, GPIIb and IIIa not form any complex therefore cannot bind plasma fibrinogen. Upon activation of the platelet, these receptors undergo a complex with formation of "fibrinogen pouches" that initiate aggregation. In structural change of the activated platelets, the surface density of GPIIb and IIIa molecules are increased.

Change in the orientation of phospholipids in the vicinity of the plasma membrane allows the association of coagulation factors and the formation of a catalytic prothrombinase complex on the activated surface (platelet factor 3 with procoagulant activity). The increased formation of thrombin in the region of a platelet aggregation consolidates the hemostatic clot through fibrin cross-links.

Table 1 Some important platelet membrane glycoproteins (GP)

GP	CD	MW (kDa)	Gene family	Complex	Ligand for the complex	Platelet function	Other cells expressing GP
IIb (α)	41	125	Integrin	IIb/IIIa	Fibrinogen, vWf,	Aggregation,	Megakaryocyte
IIb (β)		23	Integrin		fibronectin,	adhesion at	Megakaryocyte
			Integrin		vitronectin	high shear	
IIIa	61	95				rates	Megakaryocyte
Ib (α)	42b	141	Leucine-rich	Ib/IX/V	vWf, thrombin	Adhesion	Megakaryocyte, endothelial cells
Ib (β)	42c	22	Leucine-rich			Adhesion	
IX	42a	20	Leucine-rich			Adhesion	
V	42d	80	Leucine-rich			Function uncertain	
Ia	49b	155	Integrin	Ia/IIa	collagen	Adhesion, aggregation	Activated T lymphocytes,
IIa	29	138	Integrin				Endothelial cells, other cell types
IV=IIIb	36	97	-		Thrombospondin, collagen	adhesion	Monocytes, endothelium, erythroblast

Collagen and a series of soluble agonists (e.g., ADP, thrombin, thromboxane A_2) bind to specific receptors on the platelet membrane and lead to activation of the platelets. These activating receptors are linked to G-proteins that amplify the activation step. In parallel, platelets are also inhibited by antagonists by the way of specific receptors. Human platelets are known to express heterotrimeric G-proteins of all four major subfamilies. Activated platelets release different classes of G_α subunits (G_s, G_i, G_q, and G_{12}) and concomitantly the $\beta\gamma$ complexes. Receptors that directly inhibit platelets (such as receptors for adenosine: β-adrenergic agents, prostacyclin and prostaglandin E_1) stimulate adenylate cyclase via Gs-proteins. While platelet activating receptors are thought to couple to more than one G-protein, i.e., G_q, G_{12}, G_{13}, G_{i2}, G_{i3} (in the case of thromboxane A_2), and G_{i2} (in the case of ADP). (Brass *et al.*, 1997; Offermanns, 2000)

ADP receptors: Platelet activation and aggregation by ADP plays a key role in the development and pathogenesis of arterial thrombosis (Gachet *et al.*, 1997).Therefore, the mechanisms of platelet activation by ADP are of considerable pharmacological and medical interest (Cattaneo and Gachet, 1999). Platelets are presently the only cells known to express ADP-specific purino-receptors. Several purino-receptors have been characterized within the last few years including a P_{2X1} ionotropic receptor and P_{2Y1} receptor linked to G-proteins, which stimulate phospholipase C/Ca^{2+}-mobilization from intracellular stores and /or inhibit adenylase (Kunapuli and Daniel, 1998). Evidence for the presence of a third distinct platelet ADP-receptor identified as P_{2Y1} which mediates ADP-induced calcium mobilization (Cattaneo and Gachet, 1999). Recently, this receptor has been cloned with the help of a genetic defect in a patient with bleeding disorder and is designated as P_{2Y12} (Hollopeter *et al.*, 2001). A further receptor, aggregin, has been described but its function is still mostly unknown. In addition, binding sites for adenine nucleotides on the glycoprotein IIb-IIIa complex

have been described. The P_{2X1} receptor mediates ADP-induced Ca^{2+} influx and the shape of platelets. This activation pathway does not seem to be influenced by substances of the thienopyridine class. The other purinergic receptor P_{2Y1} possesses seven transmembranous domains and is coupled to inhibit G-proteins. P_{2Y1} mediates the ADP-induced release of intracellular Ca^{2+}, the inhibition of adenylate cyclase, the activation of the GPIIb-IIIa receptor, and the subsequent platelet aggregation. All of these mechanisms are affected by thienopyridines (ticlopidine, clopidogrel) (Kunapuli and Daniel, 1998; Puri and Colman, 1998).

Thrombin receptors: Thrombin is a serine protease that is converted from an inactive from (prothrombin) in the plasma by the activation during the coagulation cascade. The protease activity of thrombin leads to the formation of fibrin by cleavage of fibrinogen. Thrombin can also activate the signal transduction of numerous types of cells including platelets by stimulation of G-protein-linked receptors (Coughlin, 2000).

Human α-thrombin consists of a pair of A- and B-chain joined to each other by disulfide bridges. α-Thrombin cleaves the G-protein-linked receptor downstream of the amino acid arginine and lysine. The anion binding region (anion binding exosite) represents the binding region for fibrinogen within the thrombin molecule and the cloned thrombin receptor. γ-Thrombin, formed by cleavage of tyrosine from α-thrombin, has no procoagulant activity but can still activate platelets (Coughlin, 1999; Coughlin, 2000).

Thrombocytes have two types of principal binding sites for thrombin: a high affinity binding site in the region of glycoprotein Ib and a moderate affinity site that is formed by G-protein-coupled receptor with 7 transmembrane domains. Thrombin interacts with the latter receptor through two binding sites: the active center binds to the extracellular domains of the thrombin receptor in the vicinity of the cleavage site LDPR-S (Leu=L, Asp=D, Pro=P, Arg=R, Ser=S) while addition of thrombin to the hirudin-like domain of the receptor takes place at the anion binding exosites. After

binding thrombin the extracellular domain of the receptor is cleaved and the receptor activated by a tethered ligand. In addition, a proteolytic free polypeptide SFLLRN-x (Ser=S, Phe=F, Leu=L, Arg=R, Asn=N) is formed. Synthetic peptides with the amino acid sequence SFLLRN (thrombin-receptor activating peptide, TRAP) directly activate the platelet-thrombin receptor (Jamieson, 1997).

The high affinity binding site is in the region of GPIb, which in turn is a component of the vWf receptor GPIb-V-IX. The thrombin binding sequence in GPIb resembles the thrombin binding regions in hirudin and in the thrombin receptor. The significance of the thrombin-GPIb interaction for platelet function has not been completely clarified.

Collagen receptors: During adhesion platelets come into contact via specific receptors with collagen fiber presented in the subendothelium. Besides GPVI (p62) and GPIV (CD36), integrin $\alpha_2\beta_1$ plays a major role in the adhesion of platelets to collagen. The adhesion of resting platelets to collagen leads to activation with subsequent secretion and aggregation (Watson, 1999; Watson *et al.*, 2000).

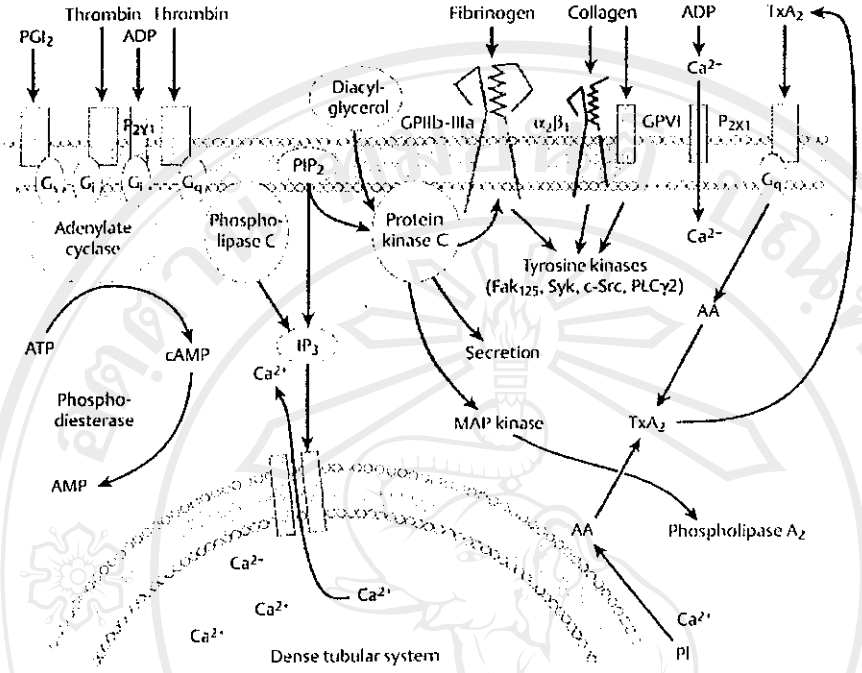

Figure 3 Mechanism for signal transduction in platelet activation (Meinrad, 2001)

1.2.4.2 Structural zone

The structural zone consists of microtubules and a network of proteins. They are arranged in the form of an inner ring beneath the surface of the platelet and are distinct from the canalicular (or open canalicular) and dense tubular system of the membrane system. The functions of the structural zone are to support the plasma membrane, maintain the resting discoid shape of the platelet and to provide a mean of change in the shape when the platelet is activated.

The microtubule consists of the protein tubulin. They are a bundle of 8 to 24 tubules that are located beneath the submembranous region. The microtubules completely surround the circumference of the platelet. They are important in maintaining the platelets discoid shape.

The protein network (or contractile proteins) largely consists of myosin and submembranous actin filaments that are anchored to the surface of the platelet by the transmembrane glycoprotein actin. The actin is the most abundant protein in platelets accounting for 15% to 20% of the total protein. Its two forms are G or globular and F or filamentous forms. The F form consists of several polymerized G molecules. The resting platelet has the F form and the G form approximately equal (50%). The actin binding protein is attached to the cytoplasmic side of the glycoprotein Ib/IX complex and anchors actin to the membrane.

1.2.4.3 Organelle zone

The organelle zone is beneath the microtubule layer. It consists of mitochondria, glycogen particles and at last three kinds of granules dispersed within the cytoplasm. They are dense-granules, alpha-granules and lysosomal-granules. The granules serve as storage sites for several proteins and other substances essential for platelet function.

Dense-granules have characterized by high electron density, with 20 to 30 nm in diameter. It contains substances and mediators of platelet function (platelet aggregation) and hemostasis which are non-proteins such as adenosine-diphosphate (ADP), adenosine-triphosphate (ATP) and other nucleotides as well as phosphate compounds, calcium ions (Ca^{2+}) and high concentrations of serotonin. Trapping of serotonin may occur as a result of the lower pH (~6.1) maintained in dense-granules due to the action of an H^+ pumping ATPase on the dense body membrane.

Alpha-granules are the most numerous in the platelet. It contains two major groups of proteins. The first group is hemostatic proteins such as fibrinogen, factor V (FV), von Willebrand factor (vWf), plasminogen activator inhibitor (PAI-1), α_2-antiplamin, and plasminogen. The FV and vWf are synthesized in the megakaryocytes during the platelet development. However other proteins such as fibrinogen, PAI-1 are not synthesized in the megakaryocytes. They are absorbed from the plasma and packaged

in the α-granules. The last group is non-hemostatic proteins. They are proteins with a variety of functions. Some found exclusively in platelets and are not found in other cells.

Lysosomal granules contain acid hydrolase typical of these organelles. Among the enzyme thought to originate from platelet lysosomes are β-glucuronidase, cathepsin, aryl-sulphatase, β-hexosaminidase, β-galactosidase, endoglucosidase (heparinase), β-glycerophosphatase, elastase, and collagenase (Holmsen, 1994). When platelets undergo secretion, lysosomal contents are slowly and incompletely released. When compared to the content of α-granules and δ-granules (Holmsen *et al.*, 1982; Verhoeven *et al.*, 1984). Moreover, stronger inducers of activation are required to induce release of lysosomal contents. These enzymes may contribute to the intracellular effects of phagocytosis and may create an uncertain amount of damage extracellularly at the site of platelet release. Proteins present in lysosomal membrane (e.g. CD63) have been identified. Their appearance on the plasma membrane serves as a marker of the platelet release reaction (Nieuwenhuis *et al.*, 1987; Abrams and Shattil, 1991). The elastase and collagenase activities may contribute to vascular damage at the sites of platelet thrombus formation. The heparinase may be able to cleave heparin like molecules from the surface of endothelial cells.

Table 2 Some important contents of platelet δ-granules and α-granules

Granule	Function
Dense-granules (δ-granules)	
ADP	Aggregation, vasoconstriction
ATP	Energy source
5-HT	Vasoconstriction, aggregation
Calcium ion (Ca^{++})	Function uncertain
Pyrophosphate	Function uncertain
Alpha-granules (α-granules)	
Platelet factor 4 (PF4)	Heparinoid neutralization
β-thromboglobulin (β-TG)	Chemotaxis
Trombospondin (Tsp)	Aggregation
Platelet derive growth factor (PDGF)	Mitogenesis, vessel repair
von Willebrand factor (vWf)	Adhesion
Fibrinogen	Aggregation, coagulation
Factor V	Prothrombinase activity
Fibronectin	Fibroblast and platelet adhesion
Plasminogen activation inhibitor-1 (PAI-1)	Inhibition of fibrinolysis
α_2-antiplasmin	Inhibition of fibrinolysis

Mitochondria are found in the platelets but not in the mature red blood cells. Platelet contains approximately seven mitochondria of relatively small size. They are involved in oxidative energy metabolism. They also contain enzymes for oxidative metabolism and provide a major source of energy through the generation of ATP. Platelet also contains occasional ribosomal particles and small amount of RNA.

Peroxisomes are very small organelles present in platelets. They contain catalase, which protects the platelet from oxidative damage in connection with periodically intense metabolic activities. They are thought to contribute to lipid metabolism. They may participate in the synthesis of platelet activating factor (PAF). They contain acyl-CoA:dihydroxyacetonephoshate acyltransferase, which catalyzes the first step in the synthesis of phospholipids. Deficiencies of this enzymatic activity in platelet have been identified in the cerebro-renal Zellweger syndrome.

1.2.4.4 Membranous systems

The membranous systems consist of two major networks. The first one is platelet canalicular system (open canalicular system) and the second is dense tubular system.

The open canalicular system (OCS) is the surface-connected open canalicular system that begins as indentation of the plasma membrane and course throughout the interior of the platelet. (Behnke, 1970) It serves several functions such as providing a mechanism for entry of external elements into the interior of the platelets. It also provides a potential route for the release of granule contents to the outside. This function needs the granule fusion with the plasma membrane itself (White, 1974). This function is especially important. The platelet granules appear to move to the center after platelet activation rather than to the periphery (Stenberg *et al.*, 1984). Controversy remains, however regarding to the relative frequency of which secretion occurs via the OCS versus direct fusion with the plasma membrane (Ginsberg *et al.*, 1980).

The OCS also represents an extensive internalization of membrane. It is created by numerous invaginations of the platelet surface and interspersed among these structures. Both filopodia and platelet spreading after adhesion result in a dramatic increase in surface plasma membrane compared to the plasma membrane of resting platelets. It is not possible for new membrane to be synthesis during the shot time-course of these phenomena. Thus the membrane of the OCS most likely contributes to the increase in plasma membrane under these conditions. The membrane of δ-granules, α-granules and a lesser extent, lysosomes may also contribute. This only if the stimuli are sufficient to induce the fusion of these organelles with the plasma membrane (release reaction). Finally, the membrane of the OCS may sever as a storage site for plasma membrane glycoproteins.

The dense tubular system (DTS) is a closed channel network of residual endoplasmic reticulum characterized by the presence of peroxidase activity (Breton-Gorius and Guichard, 1972; White, 1972). The channels of the DTS are less extensive than those of the OCS. It tends to cluster in the region closing to the OCS. The DTS has been liked to the sarcoplamic reticulum of muscle. Since its can sequester ionized calcium and release when platelets are activated (Robblee *et al.*, 1973; Menashi *et al.*, 1982). Calreticulin, a calcium-binding protein presents in the DTS, probably help to sequester calcium ions (Michalak *et al.*, 1998). Release of calcium ions from the DTS involves the binding of inositol 1,4,5 trisphosphate (IP_3), a messenger molecule formed during signal transduction, to receptor on the DTS membrane (Kaser-Glanzmann *et al.*, 1978). Cyclic AMP (cAMP) inhibits calcium ion release from the DTS, either by enhancing the calcium pumping mechanism or by inhibiting release induced by IP_3 (Tertyshnikova and Fein, 1998).

The DTS membrane is also probably a major site of prostaglandin and the thromboxane synthesis. In fact, the peroxidase activity that can identify the DTS is an enzymatic component of prostaglandin synthesis (Gerrard *et al.*, 1976; Picot *et al.*, 1994).

In addition the major inner structures of the platelet are the cytoskeletal, the microtubules and a system of contractile proteins. The cytoskeletal provides a framework to anchor the platelet membrane and allow signal transduction to take place. Moreover the cytoskeletal is a framework of the contractile proteins of the platelet that can initiate shape change and protrusion of pseudopodia at the onset of spreading.

1.2.5 Platelet function

The platelets play a major role in the prevention of blood loss when blood vessels are damaged. Platelets normally move freely through the lumen of blood vessel as a component of the circulatory system. Maintenance of normal vascular integrity involves nourishment of the endothelium by some platelet constituents or the actual incorporation of platelet into the vessel wall. This process requires less than 10% of the platelets in the circulating blood (Mary, 1999). For the hemostasis to occur, the platelets must not only be present in normal quantities but also must function properly. The primary role of a platelet is to plug damaged vascular endothelium by adhering to the injury site.

When damaging of endothelium presents, a series of event occur, including platelets adhere to the injured vessel wall, shape change, aggregation and secretion. Each structure and function change is accompanied by a series of biochemical reaction that occur during the process of platelet activation. The platelet membrane is the focus of interactions between extracellular and intracellular environments. Agonists that lead to platelet activation are many. These include ADP, lipid (thromboxane A_2, platelet-activating factor), a structural protein (collagen), and a proteolytic enzyme (thrombin) (Mary, 1999). The platelet activity is responded to vascular damage and continued maintenance of vascular integrity by the rapid adherence of platelet to exposed endothelium. In addition, platelet spreads, become activated, and forms larger aggregates to create a 'platelet plug' initially arrests the bleeding.

Mechanism of platelet plug formation

Platelet adhesion

Platelet shape change

Platelet release reaction

Platelet aggregation

1.2.5.1 Platelet adhesion

Platelets in an injured endothelial site subsequently contact with and adhere to, subendothelial components, in particular collagen and microfibrins. The two reactions seem to require different cofactors. Adhesion to collagen involves interaction between the platelet GPIa-IIa complex and two sites (an Asp-Gly-Glu-Ala sequence and lysyl side-chains) on the α-chain of collagen. The minimal structural unit is microfibrin unit on collagen. The initial process requires little amount of cofactors and magnesium ions. Linkage of the GPIa-IIa receptor to laminin on the inside of the platelet initiates microtubule contraction and potentiates the release reaction. Collagen also binds vWf that serve to anchor the platelet to the collagen. The GPIa-IIa complex is found on other cells too (e.g. endothelium cells, leukocytes and fibroblasts). It promotes their adhesion to the vascular collagen.

Platelet adhesion and their receptors, by many proaggregatory substances induce a series of metabolic processes can provoke the shape change, the release reaction, and aggregation of the platelets. The first event is the activation of polyphosphoinositide phosphodiesterase (phospholipase C) and phospholipase A_2. Consequently, several other enzymes that stimulate prostaglandin metabolism (Figure 4) and phosphoinositide turnover are involved (Ronald A *et al.*, 1999).

1.2.5.2 Platelet shape change

Within seconds of their adhesion to vessel wall components, or to non-physiological surface such as glass, platelets undergo a change in shape, becoming more spherical and putting out long, spiny pseudopods. The latter form the initial points of contact with, and enhance interaction between, adjacent platelets. 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. Later, contraction of these microfibrins may account for the process of clot retraction, which helps consolidate the platelet plug (Ronald A *et al.*, 1999).

1.2.5.3 Platelet release reaction

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 δ-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. 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 (Ronald A *et al.*, 1999).

1.2.5.4 Platelet aggregation

A wide variety of soluble substances, including ADP, TXA_2, adrenaline, 5-HT, vasopressin and platelet activating factor (1.0.alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine) induce platelet aggregation. These agonists may influent aggregation *in vivo*. Their actions are in synergistic fashion under physiological condition. Only ADP and TXA_2 are likely to reach the concentration required to induce aggregation locally and transiently at the point of blood vessel damage.

They appear to operate *via* a common pathway, wherein binding to specific but poorly-defined receptor that are thought to belong to the 7-transmembrane domain family. This is followed by a series of signal transduction processes. The GPIIb-IIIa complex is activated (permitting fibrinogen binding and platelet aggregation), PI turnover and PG metabolism are stimulated both directly and indirectly by inhibiting adenylate cyclase, causing a fall in cAMP (which normally suppresses aggregation by enhancing Ca^{2+} uptake by the dense tubular system).

The binding of fibrinogen to the platelet GPIIb-IIIa complex involves at least three peptides, two on the α-chain (both of which contain the sequence Arg-Gly-Asp) and the third forming the last 12 amino acids at the carboxy-terminal of the γ-chain of fibrinogen. Being a dimer, fibrinogen thus forms direct bridges between adjacent platelets, leading to aggregation. Other adhesive proteins, such as vWf, fibronectin and thrombospondin, could presumably operate in a similar fashion, either through the GPIIb-IIIa complex or, in the case of thrombospondin, *via* their own specific binding sites. Platelet aggregation, however it is caused, leads to activation and release reaction that induced a self-sustaining cycle of events, which result in the formation of a platelet plug at the site of injury.

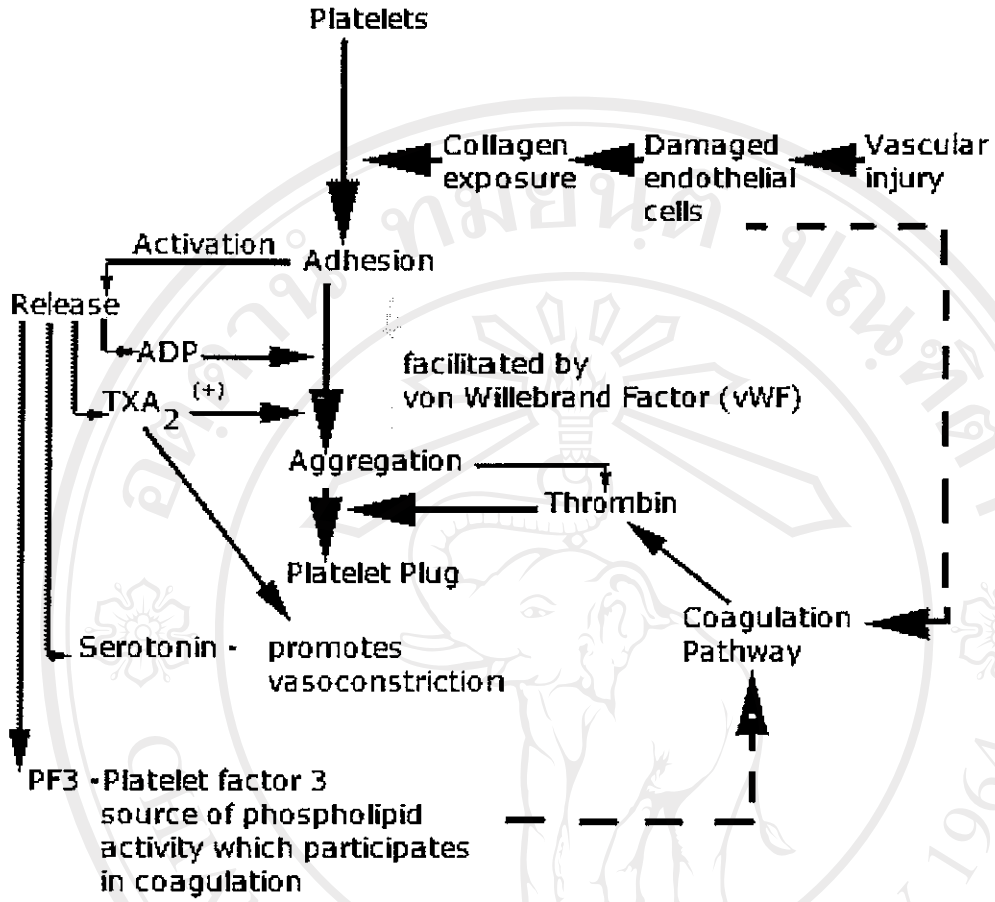

Figure 4 Mechanism of platelet plug formation

(http://www.medicine.mcgill.ca/physio/209A/Blood/blood6a.htm)

1.2.6 Flow cytometry

1.2.6.1 Flow cytometry is a technique for measurement of cellular properties both extracellular and intracellular. The cells must be free and suspended in fluid that is pumped to flow through a laser beam. The direct measured properties are size and granularlity, while the indirect measured properties are surface and intracellular markers. The direct properties can be measured without any staining but indirect properties must be fluorescent or immunofluorescent staining. This technique has an advantage over fluorescent microscopy in a higher number of cells analyzed and subjective. Therefore it is now very popular in hematological measurement including platelet studies.

1.2.6.2 Flow cytometry for platelet studies: A major development in the functional studies of thrombocytes using flow cytometry is growing 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. Flow cytometry involves the analysis of individual cells that are detected by a laser beaming on a continuous flow. The scattered light and the intensity of the fluorescence are recorded by specific photodiodes. In this way, the size (forward scatter), granularity (side scatter), and surface expression of antigens following binding of a fluorochrome-conjugated antibody (immunofluorescence) can be determined. Using different fluorochromes with differ light frequency spectra is possible to concomitantly analyze different functional change to the platelet membrane.

1.2.6.3 Platelet surface markers

Glycoprotein IIb-IIIa on resting, activated or ligand-occupied circulating platelets can be defined by specific monoclonal antibodies to glycoprotein IIb-IIIa complex. 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. 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. 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 were 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. 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. These antibodies specifically recognize, conformationally 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.

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.*, 2000).

Table 3 Monoclonal antibodies for flow cytometric determination of immunological platelet activation

Surface antigen	Monoclonal antibody	Purpose
GPIIb-IIIa	4F10, 2G12	To determine the surface expression of GPIIb-IIIa complex
	Anti-CD61	Surface expression of the β_3 chain
	Anti-CD41	Surface expression of the α_{IIb} chain
	PAC-1	To detect the activated fibrinogen receptor
	LIBS-1, LIBS-2, LIBS-6, PMI-1	To detect ligand-bound GPIIb-IIIa
Fibrinogen	RIBS	To detect bound fibrinogen on GPIIb-IIIa
GPIb-V-IX	Anti-CD42	Internalization after thrombin activation
P-selectin	Anti-CD62P	Degranulation of α granules
GP-53	Anti-CD63	Degranulation of lysosomes

1.2.7 Thalassemia

1.2.7.1 Basic knowledge of thalassemia

Thalassemia is a hereditary anemic disease, which is characterized by insufficient synthesis of either, α or β-globin (Weatherall *et al.*, 1981). It is the most common genetic disorders among the people living in Southeast Asia. Where α-thalassemia, β-thalassemia, Hb E and Hb constant spring (CS) is prevalent. The gene frequencies of α-thalassemia reach 30-40% in Northern Thailand and Laos (Fucharoen and Winichagoon, 1992). While β-thalassemia gene frequencies vary between 1 and 9%. In addition, Hb E is the hallmark of Southeast Asia contributing to a frequency of 50-60% at the junction of Thailand, Loas and Cambodia. The gene frequencies of Hb CS vary between 1 and 8% in the same area. These abnormal genes in different combinations lead to over 60 thalassemia syndromes (Fucharoen and Winichagoon, 1992). The two major α-thalassemia diseases are Hb Bart's hydrops fetalis or homozygous α-thalassemia 1 and Hb H disease which could occur from the interaction between α-thalassemia 1 and α-thalassemia 2 or between α-thalassemia 1 and Hb CS. Interaction between β-thalassemia and β-thalassemia/Hb E which are majority among β-thalassemia syndromes in the region (Nienhuis *et al.*, 1982; Fukumaki, 1985; Fucharoen and Winichagoon, 1992).

There is no α-globin chain production in Hb Bart's hydrops fetalis resulting in the most serious form of thalassemia disease. The fetus dies in utero or soon after birth because Hb Bart's does not release O_2 to the tissues. The affected features are hydropic with severe growth retardation and abnormal development in utero of vital organs such as brain and lung contribute to the severe morbidity that makes the condition incompatible with life (Weatherall *et al.*, 1981; Fucharoen and Winichagoon, 1987; Tanphaichitr *et al.*, 1987).

Homozygous β-thalassemia causes a severe disease knows as Cooley's anemia or thalassemia major. The clinical manifestation of this disease develops in the first year of life. The patients have growth retardation, thalassemic faces and hepatosplenomegaly. Regular blood transfusions are needed to reduce the degree of anemia. β-Thalassemia/Hb E contains a wide range of clinical severity. The hemoglobin levels ranges between 3 to 13 g/dL. The patients with very low hemoglobin levels may be as severe as homozygous β-thalassemia. Extramedullary hemopoiesis leads to hepatosplenomegaly. Iron overload, infections, leg ulceration, pulmonary thromboembolism, and hypoxemia are among the common complications seen in the thalassemic children (Keens *et al.*, 1980; Sonakul *et al.*, 1980; Fucharoen and Winichagoon, 1987; Sonakul *et al.*, 1987; Songkhla *et al.*, 1987; Youngchaiyud *et al.*, 1987; van Teunenbroek *et al.*, 1989; Sumiyoshi *et al.*, 1992).

1.2.7.2 Molecular defects of α-thalassemia

The α-globin gene cluster is located on the short arm of chromosome 16 and contains seven genes arranged in the order of ζ-ψ-ψα2-ψα1-α2-α1-θ1 (Weatherall *et al.*, 1981; Fukumaki, 1985). The two α-globin genes α1 and α2 have high degree of structural similarity and produce identical products of α-chain. DNA analysis revealed that α-thalassemia due to deletion of one or both α-globin genes is the most common. Deletion that causes α-thalassemia 1 in Thailand usually loses about 20 kb of DNA including both α1 and α2 genes in α-globin gene cluster (Yoshida *et al.*, 1990; Kattamis *et al.*, 1996). The 5' start of the deletion may exist with the third exon of the ζψ-gene and the 3' end of the deletion terminates close to the hypervariable region located at the 3' end of the α-gene complex. Deletion of the entire α-globin gene complex ($--^{THAI}$) is rare. In α-thalassemia 2, there is only one gene (-α) that is left functioning. Two types of deletions in α-thalassemia 2 have been found. One involves deletion of the

α 2-globin gene with 4.2 kb of DNA and only the α 1-globin genes is left functioning ($-\alpha^{4.2}$). The other loses 3.7 kb of DNA between the linked α1 and α2-globin genes and produces hybrid α-globin gene ($-\alpha^{3.7}$) (Winichagoon *et al.*, 1992). The latter has more distribution and is the most common form of α-thalassemia 2 in Thailand (Hundrieser *et al.*, 1990; Fucharoen *et al.*, 1991; Fucharoen and Winichagoon, 1992). Study of 406 cord blood samples by Southern blot technique revealed that the incidence of α-thalassemia 1 in Bangkok is 3.5% and for α-thalassemia 2 is 16.25%. Only 5% of this α-thalassemia has the 4.2 kb deletion type (Winichagoon *et al.*, 1992; Kattamis *et al.*, 1996).

Since the two α-globin genes (α1 and α2) are embedded within the highly homologous regions, unequal homologous recombination within these regions can lead to α-gene rearrangements and result in a single α-globin (α-thalassemia 2) or triplicate α-globin genes (Winichagoon *et al.*, 1992).

There are four α-globin genes in normal individuals; and four varieties of α-thalassemia can be defined depending on the number of α-globin gene deletions in the diploid genome. In population where α-thalassemia is common, the homozygous state for α-thalassemia (-α/-α) can produce a hematologic phenotype identical to that of the heterozygous state for α^0-thalasssemia (--/αα). This consisted of mild anemia with reduced MCV and MCH values. Hb H disease usually results from the compound heterozygous state for α^0-thalassemia and either deletion or nondeletion α^+-thalassemia. It occurs most frequently in Southeast Asia ($--^{SEA}/-\alpha^{3.7}$) and the Mediterranean region (usually $-^{MED}/-\alpha^{3.7}$). Hb H disease may also result from the homozygous state for nondeletion mutants affecting the α2 gene ($\alpha\alpha^{T\ Saudi}/\alpha\alpha^{T\ Saudi}$). The Hb Bart's hydrops fetalis syndrome usually result from the homozygous state for α^0-thalassemia, the most common types are $--^{SEA}/--^{SEA}$ or $--^{MED}/--^{MED}$. There have been a few reports of infants with this syndrome who synthesized very low levels of

α-chain at birth. Gene-mapping studies suggested that the interaction of α^0-thalassemia with nondeletion mutations is the cases ($\alpha\alpha^{T\ Saudi}$).

Hb CS is a variant with elongated α2-globin genes (TAA→ CAA). This was result in the longer message, which is translated to the next terminated signal and produces a globin with additional of 31 amino acids instead of a usual 141 amino acids. Although α^{CS}-mRNA can be detected in bone marrow of the patients, it is absent from their peripheral blood suggesting its instability (Winichagoon *et al.*, 1992; Zhang *et al.*, 2002). Because of the remarkable reduction in α^{CS}-chain productions, Hb CS behaves similar to the α-thalassemia 2 genes and leads to Hb H disease when interacting with α-thalassemia 1 ($--/\alpha\alpha^{CS}$). However, the loss in expression of the α2-globin locus by non-deletion mutation appears to be more severe than the loss of α-globin gene in the 3.7 kb deletion type of α-thalassemia 2 ($-\alpha^{3.7}$). Hb H levels and red blood cells containing inclusion bodies are significantly higher in Hb CS (α-thalassemia 1/Hb CS, $--/\alpha^{CS}\alpha$) than in the deletion Hb H (α-thalassemia 1/ α-thalassemia 2, --/-α) (Winichagoon *et al.*, 1992). Quantitation of α-mRNA also demonstrated a more significant loss of α-globin mRNA synthesis from the $\alpha^{CS}\alpha$ chromosome than from the $-\alpha^{3.7}$ chromosome (Winichagoon *et al.*, 1992). Furthermore, homozygosity for Hb CS ($\alpha^{CS}/\ \alpha^{CS}$) is symptomatic with mild anemia, jaundice and splenomegaly whereas, homozygous α-thalassemia 2 with the $-\alpha^{3.7}$ deletion type ($-\alpha^{3.7}/-\alpha^{3.7}$) is asymptomatic (Winichagoon *et al.*, 1992).

Hb CS variants have an almost normal mean cell volume (MCV) and the anemia is more severe when compares with other α-thalassemic variants. The underlying cause of this MCV "normalizing" affects of Hb CS and the severe anemia is not fully explained. Hb CS containing RBCs are distinctly overhydrated relative to deletional α-thalassemic variants and the dearangement of volume regulation and cell hydration early occur in erythroid maturation and are fully expressed at the reticulocyte stage.

The membrane rigidity and membrane mechanical stability of Hb CS containing RBCs is increased when compares with Hb H and α-thalassemia 1 trait RBCs. The cause underlying these cellular alterations was sought by analyzing membrane from Hb CS and deletion α-thalassemic variants and it was found that in addition to oxidized β-globin chains, oxidized α^{CS}-globin chain are also associated with in the membrane and their skeletons in Hb CS containing RBCs. It has been proposed that the membrane pathology of Hb CS is cased by combination of the deleterious effects induced by membrane–bound oxidized α-CS and β-globin chain.

1.2.7.3 Molecular defect of β-thalassemia

The β-thalassemia (β-thal) is characterized by decrease or absent β-globin chain synthesis due to various abnormalities of the β-globin gene. The production of an excess of α-globin chain is occurred. Those α-globin chains are incapable of forming a viable hemoglobin tetramer, which precipitated in red blood cell precursors. In contrast to α-thalassemia, gene deletion does not appear to be a common underlying abnormality in β-thalassemia. The molecular defects in β-thalassemia are very heterogeneous. Different types of point mutations are found to be major basic abnormalities of β-thalassemia.

The β-globin gene cluster is located on the short arm of the chromosome 11 in a 50 kb region that contains ε, $^{G}\gamma$, $^{A}\gamma$, ψβ, δ and β-globin genes. β-Thalassemia is a very heterogeneous disorder because of various defects in the β-globin genes. Single base substitutions or insertions in the nucleotide sequences are mainly responsible for the molecular defects of β-thalassemia. These mutations affect transcription factor interaction, in the process of transcription, RNA processing, and RNA translation. Mutations that affect the promoter region of the genes or processing of mRNA and reduce the level of functional mRNA usually cause mild β-thalassemia (β^{+}-thalassemia). While the mutations affecting the abolition of mRNA production or producing the

non-functional mRNA cause severe β-thalassemia (β^0-thalassemia) (Weatherall *et al.*, 1981; Nienhuis *et al.*, 1982; Fukumaki, 1985; Lynch *et al.*, 1987; Fukumaki *et al.*, 1992; Laosombat *et al.*, 1992; Winichagoon *et al.*, 1992).

More than twenty mutations have been detected in Thai subjects by using allele specific oligonucleotide (ASO) probes or DNA sequencing techniques (Antonarakis *et al.*, 1982; Yenchitsomanus *et al.*, 1987; Petmitr *et al.*, 1989; Thein *et al.*, 1990; Fucharoen *et al.*, 1991; Fukumaki *et al.*, 1992; Laosombat *et al.*, 1992). Among these, four are common, which were firstly, a 4-bp deletion leading to frameshift at codon 41/42 (-CTTT); secondly, a non-sense mutation at codon 17 (A→T); thirdly, a C→T mutation at position 654 at the IVS-2; fourthly, A→G transition at position –28 of the TATA box (Petmitr *et al.*, 1989; Thein *et al.*, 1990; Fukumaki *et al.*, 1992).

Three mutations have been found in Thai patients C→A mutation in codon 35, a C→G mutation at position –86 in the promoter region which cases a mild β-thalassemia and an insertion of adenine (A) in codon 95 which result in a shift of the reading frame with termination at the new codon at 101. The molecular basis of β-thalassemia is still unknown in 3.7% of the patients with β-thalassemia/Hb E and in 10.5% of patients with homozygous β-thalassemia (Lynch *et al.*, 1987).

Hb E a β-globin variant ($\beta 26^{glu \rightarrow lys}$) commonly found in Thailand behaves like a mild β-thalassemia. The codon change, GAG → AAG, at position 26 (Antonarakis *et al.*, 1982; Orkin *et al.*, 1982) activates an alternative splice site at codon 25. Thus, in addition to normally spliced mRNA the utilization of this cryptic splicing site results in a deletion of a portion of first exon. This mechanism results in reduced β^E-mRNA and hence reduces synthesis of β^E-globin chains accounting for the β-thalassemia phenotype of Hb E (Traeger *et al.*, 1982).

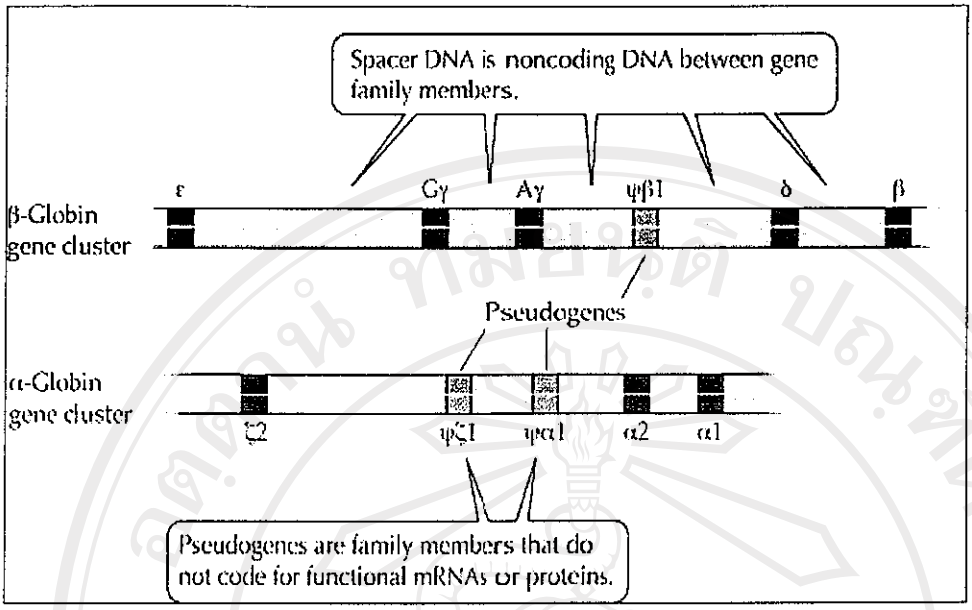

Figure 5 Structure of β-globin and α-globin genes

(http://www.blc.arizona.edu/marty/181/181Lectures/S02Lecture17.html)

1.2.7.4 Clinical features of β-thalassemia

β-Thalassemia

The most clinical severe forms of β-thalassemia are thalassemia major. A milder clinical picture characterized by a later onset and either on transfusion requirements or a fewer transfusion required, is designated as β-thalassemia intermedia. β-Thalassemia minor is the term used to describe the heterozygous carrier state for β-thalassemia.

β-Thalassemia minor

The heterozygous state for β-thalassemia is not usually associated with any clinical disability. The abnormality is discovered only on performing a blood examination. It is most commonly discovered during periods of stress, such as pregnancy or during severe infections, when a moderate degree of anemia may be found. Some patients with

thalassemia minor have increased iron stores, but this may often be due to injudicious iron therapy stated because of misdiagnosed microcytic anemia.

β-Thalassemia intermedia

The clinical phenotype of patients designated to have thalassemia intermedia is more severe than usual symptomatic thalassemia trait but milder than transfusion-dependent thalassemia major (Camaschella and Cappellini, 1995; Rund *et al.*, 1997; Ho *et al.*, 1998). The syndrome encompasses disorders with a wide spectrum of disability. In the severe stage, patients present with anemia later than usual in transfusion-dependent forms of homozygous β-thalassemia and are just able to maintain a hemoglobin level of about 6 g/dL without transfusion. However, their growth and marked skeletal deformities, arthritis, bone pain, progressive splenomegaly, growth retardation and chronic ulceration above the ankles are found. At the other end of the spectrum, there are patients who remain completely asymptomatic until adult life and are transfusion-independent, with hemoglobin levels as high as 10 to 12 g/dL. All varieties of intermediate severity are observed and some patients become disabled simply due to the effects of hypersplenism. Intensive studies of the molecular pathology of this condition have provided some guidelines about genotype-phenotype relationships that are useful for genetic counseling.

β-Thalassemia major

The clinical course is characterized of severe anemia with frequent complications. These children are particularly prone to infection, which is a common cause of death. Because of increased folate utilization by the hypertrophic marrow, folic acid deficiency frequently occurs. Spontaneous fractures commonly occur as a result of the expansion of the marrow cavities with thinning of the long bones and skull. Maxillary deformities often lead to dental problems. The formation of massive

deposits of extramedullary hematopoietic tissue may cause neurologic complications. With the gross splenomegaly that was presented secondary to active sequestration of abnormal blood cells. Then thrombocytopenia and leukopenia frequently develop leading to a feature of bleeding tendency and prone to infection respectively. Epistaxis is particularly common. These hemostatic problems are associated with poor liver function in some cases. Chronic leg ulceration may occur, although this is more common in thalassemia intermedia.

Children who have grown and developed normally throughout the first 10 years of life as a result of regular blood transfusion usually begin to develop the symptoms of iron overload as they enter puberty. In particular if they have not received adequate iron chelation. The first indication of iron overload is usually the absence of the pubertal growth spurt and a failure of the menarche. Over the succeeding years, a variety of endocrine disturbances may develop particularly diabetes mellitus; hypogonadotropic and growth hormone deficiencies; hypothyroidism and adrenal insufficiency also occur but are less common. Toward the end of the second decade, cardiac complications arise, and death usually occurs in the second or third decade as a result of cardiac siderosis. This may cause an acute cardiac death with arrhythmia, or intractable cardiac failure. Both of these complications may be precipitated by intercurrent infections.

1.2.7.5 Thalassemia and oxidative stress

In thalassemia the oxidative damage *via* free radical formation, lipid peroxidation (Chiu *et al.*, 1989; Chiu and Lubin, 1989) and iron toxicity (Hebbel, 1990) has been elucidated. The mechanisms facilitating oxidative damage are multi factorial and result from the presence of excess unpaired globin chains, high intracellular content of non-hemoglobin iron, and low concentration of normal Hb in thalassemic red blood cells (RBC).

As direct result of genetic defect, thalassemic RBC contain an excess amount of Hb subunit which is α-globin chains accumulate in β-thalassemic RBC and β-globin chain of α-thalassemic RBC (Nathan and Gunn, 1966). Following oxidation, these unstable Hb are known to generate *in vitro* free oxygen radicals. These free radical forms were methemoglobin of both reversible and irreversible hemichromes. Finally, they disintegrate to heme and globin moieties, loading the RBC membrane with denatured globin chain, heme and iron (Saltman, 1989).

Free heme is capable of interacting with either lipid bilayer or with cytoskeletal membrane proteins such as spectrin, actin, and protein 4.1 (Sears and Luthra, 1983). Hemin degradation is accompanied with iron release, which plays a significant role in RBC oxidation.

Trace metals, e.g. copper and iron have been implicated as causative agents in excessive generation of endogenous free oxygen radicals, which are capable of causing oxidative stress. In thalassemic RBC, non-heme iron is increased. Free iron or aggregates of ferritin and deposits of hemosiderrin are catalyst of lipids and protein peroxidation *via* Fenton reaction as the reaction (Hebbel, 1990) below:

$$Fe^{2+} + H_2O_2 \longrightarrow OH^0 + OH^- + Fe^{3+}$$

Data have been accumulated suggesting that increased lipid peroxidation takes place in thalassemic RBC. The organization of membrane phospholipids in thalassemic RBC is depicted with most of phosphatidylenthanolamine (PE) and phosphatidylserine (PS) on the outer leaflet (Wolfe *et al.*, 1982). Not only, the distribution of membrane lipids was abnormal, but a lower percentage of PE could be detected together with a decrease in the percentage of polyunsaturated fatty acids (PUFAs), such as arachidonic acid (Polliack *et al.*, 1974). The flip-flop of PS to the outer surface has also been found (Rachmilewitz *et al.*, 1985). Both PE and the PUFAs are known to be susceptible to peroxidation

(Jacob and Lux, 1968). Another indicator is the high malonyldialdehyde (MDA) contents of thalassemic RBC detected following exogenous oxidant stress. MDA is the result of PUFA oxidation. MDA is formed from the breakdown of PUFAs and serves as a convenient index for the determining the extent of peroxidation reaction. It reacts with thiobarbituric acid to give a red species with an absorbance at 535 nm (Stocks *et al.*, 1972).

Lipid peroxidation is known to cause the polymerization of membrane components, thus decrease cell deformability. Increase membrane rigidity is detected in RBC from patient with both α- and β-thalassemia. This could be explained by excess globin chains attached to membrane components and MDA which caused the cross linking of membrane proteins (Pfafferott *et al.*, 1982). Lipid peroxidation is known to play an important role in RBC removal by reticuloendothelial system because of the abnormality in membrane integrity.

Membrane proteins of thalassemic RBC are damaged by oxidative stress *via* increased cross linking of membrane proteins and decreased in membrane thiols (Kahane *et al.*, 1978). Electrophoretic analysis reviewed RBC membrane cytoskeletal damages. Cytoskeletal network is considered to be important in regulating different RBC function. Major cytoskeletal proteins are sensitive to oxidants because of topographical proximity of the sulfhydryl groups to RBC membrane PUFAs. The type of abnormalities in RBC cytoskeletal membrane protein is different between α and β-thalassemia due to the distinct properties of excess globin chains. In α-thalassemia, the precipitated β-subunits (Hb H inculsion bodies) are larger and bind more easily to their natural binding site on the membrane the cytoplasmic domain of protein band 3 and may mechanically distort the spectrin binding sites.

In α-thalassemia, the morphological appearance of Hb H inclusion bodies has been attributed to the unequal synthesis of globin chains. They are unstable and trend to precipitate or convert to hemichromes, upon oxidation. The pathophysiological role of

hemichrome in RBC membrane is to promote clustering of band 3 and other membrane proteins. The hemichromes aggregates contain globin chains, membrane proteins (e.g. band 3 ankyrin, protein 4.1, 4.9, actin and glycoprotein A or B).

It was demonstrated that membrane sialoglycoproteins are the site to protect thalassemic RBC from galactosyl exposure, which would enhance the removal of RBC from the circulation *via* the immune reaction. Antiprotein 3 is also responsible for removal of RBC *via* hemoglobin denaturation, hemichrome formation and the consequent clustering of protein 3. Phagocytosis is increasingly encountered in pathological RBC or RBC that undergoes experimental oxidation. Thalassemic RBC is phagocytosed more readily than control RBC by macrophages (Knyszynski *et al.*, 1979).

Hemoglobin denaturation to hemichrome following oxidation, increased membrane-bound hemichromes and the integral-membrane protein clustering may generate new antigenic sites that enable specific binding to auto-antibodies, thus leading to ultimate removal of those RBC from the circulation by reticuloendothelial system. The effects on α-thalassemia 4.1 protein and α-thalassemia ankyrin still need to be warranted since the oxidants are proved to be present in the thalassemia serum *via* the RBC hemolysis, causing insufficient phagocytosis and the inflammatory process.

1.2.7.6 Thromboembolic manifestations in β-thalassemia

Thalassemia and coagulation defects

The increasing knowledge about the potential hypercoagulable state in some forms of thalassemia has been more understandable (Rachmilewitz *et al.*, 1985; Schrier, 1994; Weatherall, 1998). There are some evidences that patients, particularly after splenectomy and with high platelet count, may develop progressive pulmonary arterial disease due to platelet aggregation in the pulmonary circulation.

Low levels of the coagulation inhibitors, protein C and protein S, have been observed in patients with β-thalassemia from a variety of ethnic backgrounds

(Shirahata *et al.*, 1992; Visudhiphan *et al.*, 1994; Eldor *et al.*, 1999; Cappellini *et al.*, 2000). In Israeli patients mostly of Kurdish Jewish, Yemenite Jewish, or Arabic origin, protein C (both antigen and activity) and free protein S were significantly decreased on both adults and children with thalassemia (Eldor *et al.*, 1999).

Low levels of heparin cofactor II (HCII), known to be associated with increased thrombotic risk, have been found in thalassemic patients (O'Driscoll *et al.*, 1995). Frequent blood transfusions resulted in a slow normalization of HCII levels, suggesting that the low HCII levels could be related to increased RBC turnover that had been suppressed by hypertransfusion (O'Driscoll *et al.*, 1995).

Deep venous thrombosis and pulmonary embolism

Deep venous thrombosis (DVT), pulmonary embolism, and recurrent arterial occlusion have been described in patients with β-thalassemia major (β-TM) and β-thalassemia intermedia (β-TI) from many countries. In most case, thrombosis was spontaneous and no known risk factor, although some patient with thrombocytosis after splenectomy developed venous thrombosis. Following these sporadic reports, several multi-centers studies were carried out to determine the incidence of thromboembolism in patients with β-TM and β-TI (Tso *et al.*, 1982; Paolino *et al.*, 1983; van Teunenbroek *et al.*, 1989; Michaeli *et al.*, 1992; Gillis *et al.*, 1999; Cappellini *et al.*, 2000). In one Italian multi-center study, 32 of 735 patients (4.35%) experienced thromboembolic events. The incidence was 3.95% among 685 β-TM patients and 9.61% among 52 patients with β-TI (Borgna Pignatti *et al.*, 1998). The same group reported a lower incidence (1.1%) of thromboembolic complications among 1,146 patients with β-TM in the follow up of 37 years (Borgna Pignatti *et al.*, 1998). Another study showed a 5.3% overall incidence of thrombotic complications among 495 patients with thalassemia whose median age was 28 years (Borgna-Pignatti *et al.*, 1998; Moratelli *et al.*, 1998). The prevalence of thromboembolic events was 3.3%

among 421 patient with β-TM and 16.2% among 74 patients with β-TI, although 15.3% of these patients had predisposing congenital or acquired factors contributing to the hypercoagulability (Moratelli *et al.*, 1998). Recently, a high incidence of venous thrombotic events (VTE) was observed in a group of 83 patients with β-TI who were followed for 10 years (Cappellini *et al.*, 2000). Twenty-four patients (29%) developed either pulmonary embolism, DVT, or portal vein thrombosis, and recurrent VTE occurred in 9 of these cases. All patients except one had undergone splenectomy.

Autopsy findings in patients with thalassemia have clearly demonstrated hypercoagulability as a pathologic feature (Sonakul *et al.*, 1980; Sumiyoshi *et al.*, 1992). Autopsies on 17 splenectomized and 2 non-splenectomized patients of 43 with β-thalassemia/Hb E disease showed atherosclerotic change and obstructive lesions consisting of organized, recanalized thrombi in the pulmonary arteries and microvasculature. No evidence of thromboembolism was found elsewhere, although routine dissection of the veins in the leg was not performed (Sonakul *et al.*, 1980). Similar findings of multiple microthrombi in the pulmonary arterioles, composed mainly of platelets, were found in autopsies performed on 2 thalassemic patients (Sumiyoshi *et al.*, 1992). Asymptomatic pulmonary vascular disease that could result from silent, recurrent thromboembolic event has been found in many patients with β-TM and β-TI. This was suggested by echocardiographic studies in 35 β-TM patients who had no clinical signs or symptoms of thromboembolic disease. Many of the patients showed pulmonary hypertension and right heart failure, which were more prevalent than left heart failure (Grisaru *et al.*, 1990). In addition, reduced lung volumes and flow rates, hypoxemia and pulmonary hypertension were found in these patients (Grisaru *et al.*, 1990). In another study of 15 thalassemic patients, the mean total lung capacity, mean residual volume, and mean forced vital capacity were significantly reduced (Luyt *et al.*, 1993). In addition, hypoxemia was present in 6 of 13 patients tested (Luyt *et al.*, 1993). These findings suggest that the early right

ventricular dysfunction, which precedes left heart failure in many patients with β-TM and β-TI that may be due to pulmonary hypertension and cardiomyopathy resulting from excessive iron deposition (Grant *et al.*, 1986; Koren *et al.*, 1987). In support of this idea, a Doppler echocardiography study in which pulmonary artery pressure was measured in 33 patients with β-TM (age 2-24 years) showed that 28 patients had evidence of pulmonary hypertension (Du *et al.*, 1997). Pulmonary artery hypertension was also detected by M-mode and Doppler echocardiography in 15 of 16 children, aged 5 to 14 years, with homozygous β-thalassemia and β-thalassemia/Hb E disease (Chotivittayatarakorn *et al.*, 1993). Right ventricular dysfunction was detected earlier than left ventricular dysfunction in these children, suggesting that the right heart failure and pulmonary hypertension seen in thalassemia could result from microembolization in the lungs. Indeed, autopsy findings revealed a high frequency of thrombotic lesions in the pulmonary arteries and the development of cor pulmonale consistent with a long-standing pulmonary vascular thromboemboli (Sonakul *et al.*, 1980; Sumiyoshi *et al.*, 1992). The venous and arterial thrombotic events have not received much attention and were not mentioned in comprehensive reviews on thalassemia (Olivieri, 1999).

Cerebral thrombosis

Thromboembolic complications associated with thalassemia, many describing cerebral thrombotic evens. As early as 1972, Logothetis et al, reviewing 138 case of β-thalassemia major (β-TM) in Greece, described a "stroke syndrome" in 2 patient and neurologic deficits compatible with transient ischemic attacks in about 20% of the case. An Italian multi-center study of cerebral thrombosis was also found in patients with β-thalassemia/Hb E disease and in α-thalassemia. All of these reports were from patients who were not given regular transfusions and were not associated with an individual blood transfusion. Other reports have described case of hypertension, convulsion, and cerebral hemorrhage in thalassemia patients following blood

transfusion (Wasi *et al.*, 1978; Constantopoulos and Matsaniotis, 1980; Sonakul and Fucharoen, 1992; Gurgey *et al.*, 1994). Asymptomatic brain damage has also been reported; results from magnetic resonance imaging (MRI) on 41 patients with β-TI revealed asymptomatic brain damage including ischemic lesion as a frequent occurrence affecting 37% of patients (Sonakul and Fucharoen, 1992; Manfre *et al.*, 1999). Damage was inversely correlated with hemoglobin levels in patients with β-TI and directly correlated with age.

Hypercoagulable state in thalassemia

The mechanism of the hypercoagulable state in thalassemia has not been fully elucidated. However, evidence from studies of other types of hemolytic anemia, such as sickle cell disease (SCD) and paroxysmal nocturnal hemoglobinuria (PNH), in which thrombosis is also a major clinical entity, may be helpful in understanding the etiology of the phenomena (Middelkoop *et al.*, 1988; Tait and Gibson, 1994; Barker and Wandersee, 1999).

It is believed that in thalassemic RBC, some negatively charged aminophospholipids such as PS are moved to the outer leaflet and thus provide a surface on which coagulation can be activated. Other nonspecific changes in the coagulation pathway and its antagonists have also been observed in patients with different form of thalassemia. A comparison of normal RBC with RBC from patients with β-TM or β-TI suggests that thalassemic RBCs may provide a source of negatively charge phospholipids, which can increase thrombin generation, as measured by thrombinase assay (Helley *et al.*, 1996; Kuypers *et al.*, 1996; Kuypers *et al.*, 1996). The procoagulant effect of thalassemia RBC appears to be due to an increased expression of anionic phospholipids such as PE and PS on the red cell surface. This was demonstrated by experiments that showed by increase annexin V binding on thalassemic RBC (Helley *et al.*, 1996).

Aminophospholipids such as PS or PE are normally confined to the inner leaflet of the plasma membrane; while the outer leaflet contains mainly neutral phospholipids such PC and PI (Bevers *et al.*, 1989; Bevers *et al.*, 1996; Bevers *et al.*, 1999). This asymmetry in membrane aminophospholipid composition is achieved by the activity of enzymes such as the ATP-dependent aminophospholipid translocase (floppase) that preferentially transport PS and PE from the outer to back the inner leaflet (Williamson and Schlegel, 1994; Williamson and Schlegel, 2002). Active translocase counteracts spontaneous flipping of PS to the outer leaflet by relocating it to the inner leaflet. In the early stage of apoptosis, activation of the initiator caspase complex result in inversion of the direction of the translocase activity (Obringer *et al.*, 1997) or in activation of translocation proteins that transport aminophospholipids in a reverse manner (flippase) (Zwaal *et al.*, 1993). The final consequence of either event is the accumulation of PS in the outer membrane leaflet (PS flipping).

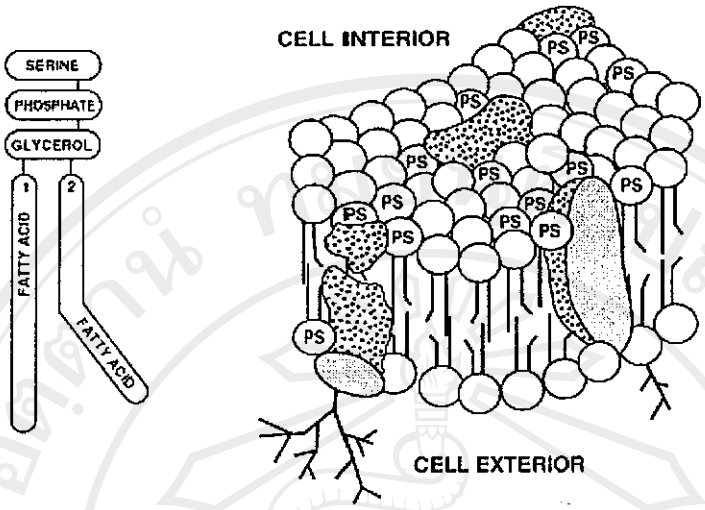

Figure 6 Structure of normal plasma membrane

(http://www.thorne.com/altmedrev/fulltext/phosserine1-2.html)

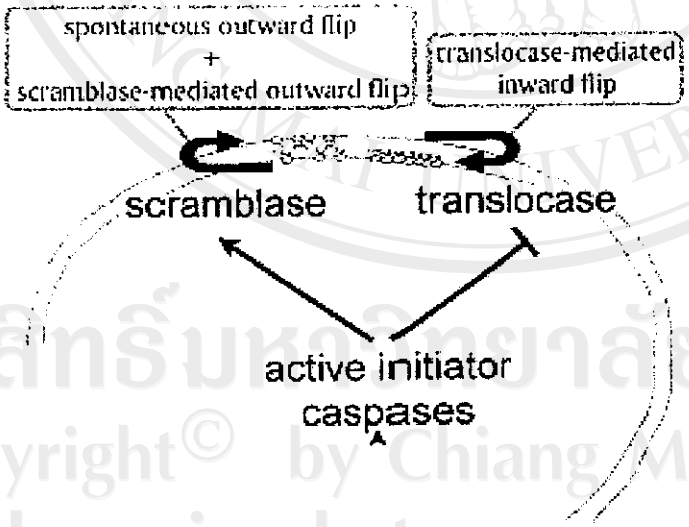

Figure 7 Mechanism phosphatidylserine (PS) distributions (Huppertz *et al.*, 1999)

1.2.7.7 Platelet activation

Further evidence for the existence of chronic platelet activation in thalassemia was provided by the measurement of urinary metabolites of thromboxane A_2 (TXA_2) and prostacyclin such as prostaglandin I_2 (PGI_2). A study of 9 splenectomized patients with β-TM who were regularly transfused, 5 non-splenectomized patients with β-TI who received occasional blood transfusion, and 20 healthy individuals (Eldor *et al.*, 1991; Eldor *et al.*, 1999) found a significant 4 to 10-fold increase in the urinary excretion of 2,3-dinor-TXB_2, 11-dehydro-TXB_2, and 23-dinor-6-keto-proataglandin (PG) $F_{1\alpha}$ in patients with β-TM and β-TI compared to healthy control. The concentration of metabolites in patients with β-TM and β-TI was not significantly different, and 6 patients who received aspirin (20mg/day) for 7 day showed a significant decrease in their urinary concentrations of 2,3-dinor-TXB_2 and 11-dehydro-TXB_2 derived from platelets. In contrast, levels of urinary 2,3-dinor-6-keto-$PGF_{1\alpha}$ reflecting vascular production, and TXB_2 and 6-keto-$PGF_{1\alpha}$ originating from the kidney were not significantly changed (Eldor *et al.*, 1991). The results of this study were consistent with enhanced production of TXA_2 due to chronic endogenous platelet activation and reflect the increased concentrations of urinary thromboxane metabolites found in other disease associated with *in vivo* platelet activation including unstable coronary disease, severe atherosclerosis, and type II diabetes mellitus (FitzGerald *et al.*, 1984; Fitzgerald *et al.*, 1986; Davi *et al.*, 1990). In another more recent study, (Eldor *et al.*, 1991; Eldor *et al.*, 1993; Eldor *et al.*, 1999) urinary prostaglandin metabolites were determined in group of 62 β-TM patients comprising 26 children (age 2-18) and 36 adults. All the thalassemic children and the adult had highly elevated levels of the urinary prostaglandin metabolites, 11-dehydro-TXB_2 and 2,3-dinor-6-keto-$PGF_{1\alpha}$. None of the thalassemic children had experienced clinical signs or symptoms suggestive of venous or arterial thrombosis, indicating that platelet activation in

thalassemic patients persists from early in childhood when thrombotic events are extremely rare.

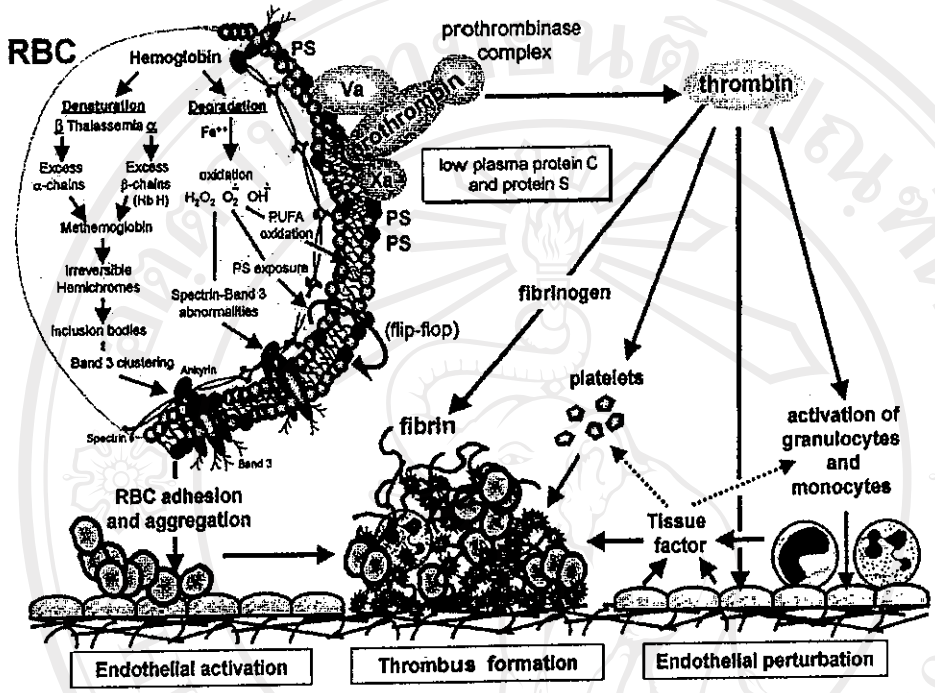

Figure 8 Hypercoagulable state in thalassemia (Eldor and Rachmilewitz, 2002)

In conclusion, studying the platelet activation and red blood cell membrane PS exposure along with platelet function as hypo-, normo- or hyper-aggregabilities is considerably importance especially in multiple transfused β-thalassemia major both with and without splenectomy. This kind of study has not been performed on the patients of Maharaj Nakorn Chiang Mai Hospital. The findings may be able to contribute to the improvement of standard of care on such patients. For example, the aspirin prophylaxis may be valuable for the prevention of thrombosis in such patients.

1.3 Objectives

1. To determine the platelet function as hyper-, normo-, or hypo-aggregabilities
2. To determine the ratio of red cells that expose membrane phosphatidylserine *in vivo*
3. To determine the platelet activation *in vivo* using indicators as follows
   - Shape change of platelet morphology
   - β-thromboglobulin (β-TG) and platelet factor 4 (PF4) release in the plasma
   - Platelet activation antigen (CD63) expression by flow cytometry
4. To determine platelet activation in the co-culture between normal platelets and thalassemic red cells or plasma
5. To determine the correlation among these parameters

All of these are in comparison of patients with β-thalassemia major and normal healthy subjects.