UNDERSTANDING PLATELET THROMBOGENICITY CASCADE OF THE BIODEGRADABLE CHITOSAN DERIVATIVES IN VON WILLEBRAND DISEASE *IN VITRO*

by

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2. activated factor 5……..…….……....(FVa)
3. activated FVIII……...…...…...(FVIIIa)
4. activated factor 9……...……...(FIXa)
5. activated FX…….……..(FXa)
6. activated factor 11……(FXIa)
7. activated factor 13……...……...(FXIIIa)
8. Activated partial thromboplastin time……..……..(APTT)
9. Adenosine diphosphate…….…..…….…(ADP)
10. Adenosine triphosphate…….…..……..…(ATP)
11. *a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13*……....*(*ADAMTS13)
12. Ammonium Persulfate……....……...…(APS)
13. Antibody……..……....…….....(Ab / Abs)
14. Antigen……...……..……...……..(Ag / Ags)
15. aqueous……..……...(aq)
16. βeta-tricalcium phosphate…….....……...(β-TCP)
17. β-mercaptoethanol……...(β-ME)
18. bovine serum albumin…….....…(BSA)
19. Calcium……...…..……..……..(Ca)

20. carboxymethylchitosan…….(CMC / CMCs)

21. chitosan-glycerol phosphate……...……...…….....(chitosan-GP)

22. Chitosan-Heparin……...…(Chitosan-Hep)

23. collagen and adenosine diphosphate…….….(CADP)

24. collagen and epinephrine…….(CEPI)

25. degree of deacetylation……...……(DDA)

26. deoxyribonucleic acid…….....(DNA)

27. desmopressin…….……...……...(DDAVP)

28. Enzyme-linked immunosorbent assay…….……...…….(ELISA)

29. ethylenediaminetetraacetic acid…….(EDTA)

30. Embryonic stem cells…….….(ESCs)

31. Extracellular matrix…….....…(ECM)

32. factor 1……..…….(F1/Fib)

33. factor 2…….(FII)

34. factor 3……...(FIII)

35. factor 4……...……...…….....…..(FIV)

36. factor 5……...……...…….…...…....(FV)

37. factor 7……..…(FVII)

38. factor 8…….……...(FVIII)

39. factor 9…….....…….....(FIX)

40. factor 10……...(FX)

41. factor 12……(FXII)

42. factor 13…….……..(FXIII)

43. Fibrinogen…….....……...…..….(Fib)

44. fluorescein isothiocyanate……..…….…….....(FITC)

45. Glycoprotein……..…….……(Gp)

46. Hematopoietic stem cells…….….(HSC / HSCs)

47. Horseradish Peroxidase-avidin……...(HRP-Av)

48. hours……..……(hr / hrs)

49. human skin allograft……...……..(HSA)

50. Immunoglobulin G…….…...…...…...……...(IgG)

51. Low Dose- Ristocetin-Induced Platelet Aggregation……...(LD-RIPA)

52. low molecular weight…….…...….(LMW)

53. Lymphocytes…….……...…….(Lym)

54. mean fluorescence intensity……..(MFI)

55. minutes……..……...(min / mins)

56. molecular weight……...……....(MW)

57. nanoparticles……..…….……...(NPs)

58. National Blood Centre / Pusat Darah Negara……...(PDN)

59. Nitrocellulose……..…….....(NC)

60. N, O-Carboxymethylchitosan……..(NO-CMC / NO-CMCs)

61. Oligo-chitosan…….....….(O-C / O-Cs)

62. phosphate buffer saline…….……..…..(PBS)

63. Platelet activating factors…….….(PAF)

64. Platelet derived growth factor-AB……...…….(PDGF-AB)

65. Platelet-poor plasma……...……...(PPP)

66. Platelet-rich plasma…….…....(PRP)

67. Polyacrylamide gel electrophoresis…….…….…….....(PAGE)

68. Prothrombin Time……...(PT)

69. P-selectin glycoprotein ligand-1…….…….....(PSGL-1)

70. Red blood cells……....(RBC / RBCs)

71. Repeated-measure analysis of variance…….……...…….…...……..(ANOVA)

72. Ristocetin-Induced Platelet Aggregation…….……(RIPA)

73. room temperature……...……....…….(RT)

74. scanning electron microscope……...…….……...…...(SEM)

75. skin regenerating template…….....…..(SRT)

76. sodium dodecyl sulfate…….....(SDS)

77. standard error of means…….……(S.E.M)

78. Standard and Industrial Research Institute of Malaysia…….....(SIRIM Berhad)

79. statistical Package for the social sciences…….…….....(SPSS)

80. Tetramethylethylenediamine…….……..……....(TEMED)

81. Thrombin Time……...……(TT)

82. Thromboxane…….……....……....…….…….…….(TX)

83. Thromboxane A_2…….…(TXA_2)

84. Tissue factor……..…..(TF)

85. TF pathway inhibitor……...…….....(TFPI)

86. tumor necrosis factor-alpha……..….(TNF-α)

87. Transforming growth factor-βeta 1……...…….…….…...(TGF-β1)

88. Universiti Sains Malaysia……....(USM)

89. von Willebrand disease…….....(vWD)

90. von Willebrand factor…….……..…….....(vWF)

91. von Willebrand factor: Collagen Binding Assay…….…….....(vWF: CB)

92. von Willebrand factor: Ristocetin Cofactor activity……...…...(vWF:RCof)

93. water-soluble chitin…….…….....…...(WSC)

94. western blot……...…....(WB)

95. White blood cells…….....(WBCs)

96. Wingless-Type MMTV Integration Site Family……..…(WNT)

LIST OF SYMBOLS

1. alpha……..…...(α)
2. Asterisk……...……..(*)
3. beta……...……..…(β)
4. celcius…….(°C)
5. correlation……...…….....(*r*)
6. daltons……..……..(Da)
7. deciliter…….…….……(dL)
8. International Unit…….……..(IU)
9. kilobases…….…...….(kb)
10. kilodaltons……..…….(kDa)
11. miliampere…….……...(mA)
12. milligrams……...…….…….(mg)
13. milliliter…….…...(mL)
14. millimeter…….…(mm)
15. microliter……...(µL)
16. micrometer……...….(µM)
17. liters……...……..(L)
18. magnification……...…....(x)
19. milliosmoles per kilogram……..(mOsm/kg)
20. nanogram……....(ng)

21. nanometer……..(nm)

22. Normality……(N)

23. ohm……...…...(Ω)

24. picogram……...….....(pg)

25. plus or minus……...…….(±)

26. Primary……..….(1°)

27. reciprocal centimeter / wavenumber…….…….….(cm^{-1})

28. Secondary……...(2°)

29. times gravity…….…….(× g)

30. voltage……...…….....(V)

LIST OF MOLECULAR FORMULA

&

FUNCTIONAL GROUPS

1. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid…….…….....(HEPES)
2. Acetamide groups……..……..(-$NHCOCH_3$)
3. Amino group…….....(-NH_2)
4. Calcium carbonate…….($CaCO_3$)
5. Carbon……...…....(C)
6. Carbon-Hydrogen bond……..……(CH)
7. Carbon-Oxygen bond……...…….(C=O)
8. Hydrochloric acid……..(HCl)
9. Hydrogen……...……(H)
10. Hydrogen bond……..(-OH)
11. Hydroxymethyl group……..(CH_2OH)
12. Potassium chloride…….……(KCl)
13. Potassium hydroxide…….…….…..(KOH)
14. Potassium permanganate…….……...…..($KMnO_4$)
15. Nitrogen……...……..(N)
16. Oxalic acid……...($H_2C_2O_4$)
17. Sodium hydroxide……....……..(NaOH)

PEMAHAMAN MEKANISMA SEL PLATELET UNTUK MENGANALISA KEBERKESANAN KITOSAN TERHADAP PENYAKIT VON WILLEBRAND *IN VITRO*

ABSTRAK

Biobahan kitosan diperolehi daripada cengkerang hidupan laut mempunyai potensi yang besar bagi kegunaan klinikal kerana ia dapat bertindak balas dengan sel-sel platelet secara bebas untuk membantu proses pembekuan darah. Penyelidikan ini mengesahkan keupayaan biobahan kitosan untuk merangsangkan mekanisma platelet dari penderma darah normal dan pesakit von Willebrand disease (vWD) *in vitro*. Eksperimen ini meliputi kajian kebolehan degradasi; platelet: lekatan, pengaktifan, penggumpalan; analisis pembekuan darah dan analisa pengantara hemostatik: von Willebrand Factor (*vWF)*, Faktor 8 *(FVIII), Thromboxane* A_2 *(*TXA_2*), P2*Y_{12}*, glycoprotein IIbIIIa (GpIIbIIIa), Transforming Growth Factor- Beta 1 (TGF-β1),* dan *Platelet Derived Growth Factor-AB (PDGF-AB).* Kajian perbandingan telah dijalankan dengan menggunakan dua jenis kitosan yang terdiri daripada *7% N,O-Carboxymethylchitosan (NO-CMC)* (dengan 0.45 mL kolagen), *8% NO-CMC, Oligo-chitosan (O-C)* dan *O-C 53*. Kajian ini dijalankan dengan menggunakan teknik-teknik ujikaji seperti *enzyme-linked immunosorbent assay, westergren, coagulation analyzer, platelet aggregometery, western blotting, flow cytometry, scanning electron microscopy, light microscopy and automated hematology analyzer.* Seramai 14 orang pesakit von Willebrand *(vWD)* dan individu biasa telah direkrutkan dalam kajian ini. Hasil kajian ini menunjukkan bahawa kitosan jenis O-C mempunyai ciri-

ciri biodegradasi serta memiliki keliangan *(scaffold)* yang lebih baik. Liang *scaffold* ini membolehkan nutrien dan sel-sel menembus keluar dengan menggalakkan aktiviti platelet bagi mempercepatkan proses hemostasis dan proses penyembuhan luka. O-C memberi implikasi positif dengan menyebabkan platelet melekat, mengaktifkan, menggumpal serta membentuk rangkaian fibrin larut untuk mengukuhkan pembentukan platelet plug dengan merangsangkan platelet *mediators* yang dikaji. Berdasarkan hasil kajian yang diperolehi, kebanyakan pesakit di Malaysia dipengaruhi oleh penyakit vWD Jenis I. Memang tidak dapat dinafikan biobahan kitosan yang terdiri daripada kumpulan oligo mempunyai potensi yang mampu merangsangkan mekanisma platelet terhadap pesakit vWD. Kitosan O-C berpotensi memulakan tindakan platelet dan dikesan mempercepatkan proses pembekuan darah. O-C mampu menggalakan expresi reseptor vWF & FVIII *antigenicity* dan TXA_2 bagi tujuan proses penggumpalan platelet. Dalam pada masa yang sama, analysis GpIIbIIIa dan $P2Y_{12}$ juga menunjukkan yang kitosan kumpulan O-C boleh mengaktifkan activiti platelet dengan menyediakan permukaan membran yang baik untuk memudahkan generasi thrombin. Seterusnya, O-C juga boleh merangsangkan pembebasan faktor pertumbuhan, terutamanya TGF-β1 dan PDGF-AB. vWD adalah kelaziman gangguan pendarahan, dan kebanyakan pesakit memiliki penyakit vWD jenis I. Kitosan kumpulan Oligo berpotensi mampu mencetuskan platelet thrombogenicity cascades pada pesakit vWD. Kitosan berpotensi memulakan tindakan platelet dan dengan itu mempercepatkan proses hemostatik melalui 3 proses utama: lekatan platelet, pengaktifan dan pengumpulan. Kitosan O-C dan O-C 53 berfungsi lebih baik daripada jenis NO-CMC kitosan dalam mengaktifkan aktiviti platelet untuk membentuk hemostatik plug di kalangan penderma normal dan pesakit vWD *in vitro*.

UNDERSTANDING PLATELET THROMBOGENICITY CASCADE OF THE BIODEGRADABLE CHITOSAN DERIVATIVES IN VON WILLEBRAND DISEASE *IN VITRO*

ABSTRACT

Chitosan has become one of the most promising local hemostatic agents because it is of particular interest as it functions independently on platelets and normal clotting mechanisms. The present study was designed with the aim to test the ability of the mechanisms of blood platelets towards the action of biodegradable chitosan in normal donors and von Willebrand disease (vWD) patients *in vitro*. This work determined the underlying mechanism of chitosan-induced platelet thrombogenicity and comprises experimental tests such as degradation ability; platelet: adhesion, activation: aggregation; coagulation analysis and hemostatic mediators: von Willebrand Factor (vWF), Factor 8 (FVIII), Thromboxane A_2 (TXA_2), $P2Y_{12}$, glycoprotein IIbIIIa (GpIIbIIIa), Transforming Growth Factor- Beta 1 (TGF-β1) and Platelet Derived Growth Factor-AB (PDGF-AB). Comparative studies have been conducted to measure the hemostatic capacity of biodegradable 7% N,O-Carboxymethylchitosan (NO-CMC) (with 0.45 mL collagen), 8% NO-CMC, Oligo-chitosan (O-C) and O-C 53. Lyostypt, the topical hemostatic agent was used as a positive control. This study was conducted using enzyme-linked immunosorbent assay, westergren, coagulation analyzer, platelet aggregometery, western blotting, flow cytometry, scanning electron microscopy, light microscopy and automated hematology analyzer techniques. Fourteen vWD and normal donors were recruited in

this study with provided informed written consent. O-C type of chitosans are able to enzymatically degrade and possess better porosity to allow nutrients and cells to enter to accelerate hemostasis and wound healing process. O-Cs exert a combined effect on thrombogenesis by causing platelets to adhere, activate, aggregate and forms fibrin network to strengthen platelet plug formation by elevating the studied mediators. O-C was capable to induce the expression levels of vWF and FVIII antigenicity and TXA_2 receptor signals. This signaling pathway assists the platelet aggregation. Also, GpIIbIIIa and $P2Y_{12}$ analysis showed that O-C group of chitosan are capable of activating platelets by providing a good surface for blood hemostatic mediators and signals to facilitate thrombin generation. O-C-activated platelets lead to the release of growth factors, mainly TGF-β1 and PDGF-AB. Therefore, this exhibited that greater expression level of O-C group of chitosan assists in mediating wound healing process. vWD is the low prevalence hereditary bleeding disorder occurs in Malaysia, and most patients belong to vWD type I. Oligo group of chitosans are potentially capable to trigger platelet thrombogenicity cascades in vWD patients. Tested chitosan-stimulated-mediators potentially initiate the platelet actions and thus expedite the hemostatic processes via 3 major processes: platelet adhesion, activation and aggregation. This study demonstrated that the greater expression level of O-C assists in elevating platelet thrombogenicity cascades to achieve hemostasis. Biodegradable O-C and O-C 53 type of chitosan worked better than NO-CMC types of chitosan in activating platelet activities to form the hemostatic plug in normal donors and vWD patients *in vitro*.

CHAPTER 1

INTRODUCTION

1.1 Blood

Blood is deemed so precious because it is the basic necessity for health since our body needs a steady provision of oxygen to reach billions of tissues and cells (Table 1.1)

Table 1.1 Blood physiology

Produced in	Bone marrow
Derived from	Hematopoietic stem cells (HSC / HSCs)
Physical characters	Denser, more viscous than water, sticky
Temperature	37 Celsius (°C)
pH	7.35-7.45
Consists of	20% extracellular fluid, 8% of total body mass
Made up of	Cellular elements (37-54 %) → Formed elements [red blood cells (RBC/ RBCs), white blood cells (WBCs) and platelets] Extracellular matrix (ECM) (46-63 %) → Plasma
Osmolality	275-295 milliosmoles per kilogram (mOsm/kg)
Blood volume	Male : 5-6 litres (L) Female : 4-5 L
Major functions	i) **Transports** – Oxygen from lungs to the cells of body, carbon dioxide from blood cells to the lungs, nutrients, waste products ii) **Regulates** – pH of all body fluids; Maintain homeostasis iii) **Protects** – From excessive loss of blood after an injury; against diseases

Source : (Tortora and Derrickson, 2005; Anatomy & Physiology, 2013).

1.1.1 Hematopoiesis process

All blood cells develop from hematocytoblasts. The hematocytoblasts is a type of stem cells produces blood cells. Hematopoiesis is the process where immature precursor cells develop into mature blood cells. The initial process generating new blood cells begins at the very early stage as embryo develops and this continues for the entire life span. Embryonic stem cells (ESCs) are characterized by their capability to self-renew indefinitely by not losing their pluripotencies. Billions of new blood cells produced in the body which derived from the HSCs. HSCs are categorized into long term, short term and multipotent progenitors depending on the degree of their self-replenishing abilities.

Hematopoiesis is the process that generates blood cells of all lineages. Signaling pathways such as Wingless-Type MMTV Integration Site Family (WNT) pathway help to regulate the stem cells in various types of organs like skin, nervous system and HSC. Stimulation of hematopoietic progenitors and stem cells with soluble WNT proteins or downstream activators of the WNT signaling leads to the expansion and regulation of hematopoietic system. Newly developed blood cells repeatedly originate from multipotent HSCs and became committed to the erythroid, megakaryocytic, granulocytic, monocytic and lymphocytic lineages. Blood cell formation results from a hierarchical progression of differentiation of multipotential HSCs (Robb, 2007; Hematopoiesis from multipotent stem cell, 2012). Matured blood cells comprise RBCs, WBCs: neutrophil, basophil, eosinophil, lymphocyte, monocyte and platelets (Figure 1.1; Table 1.2).

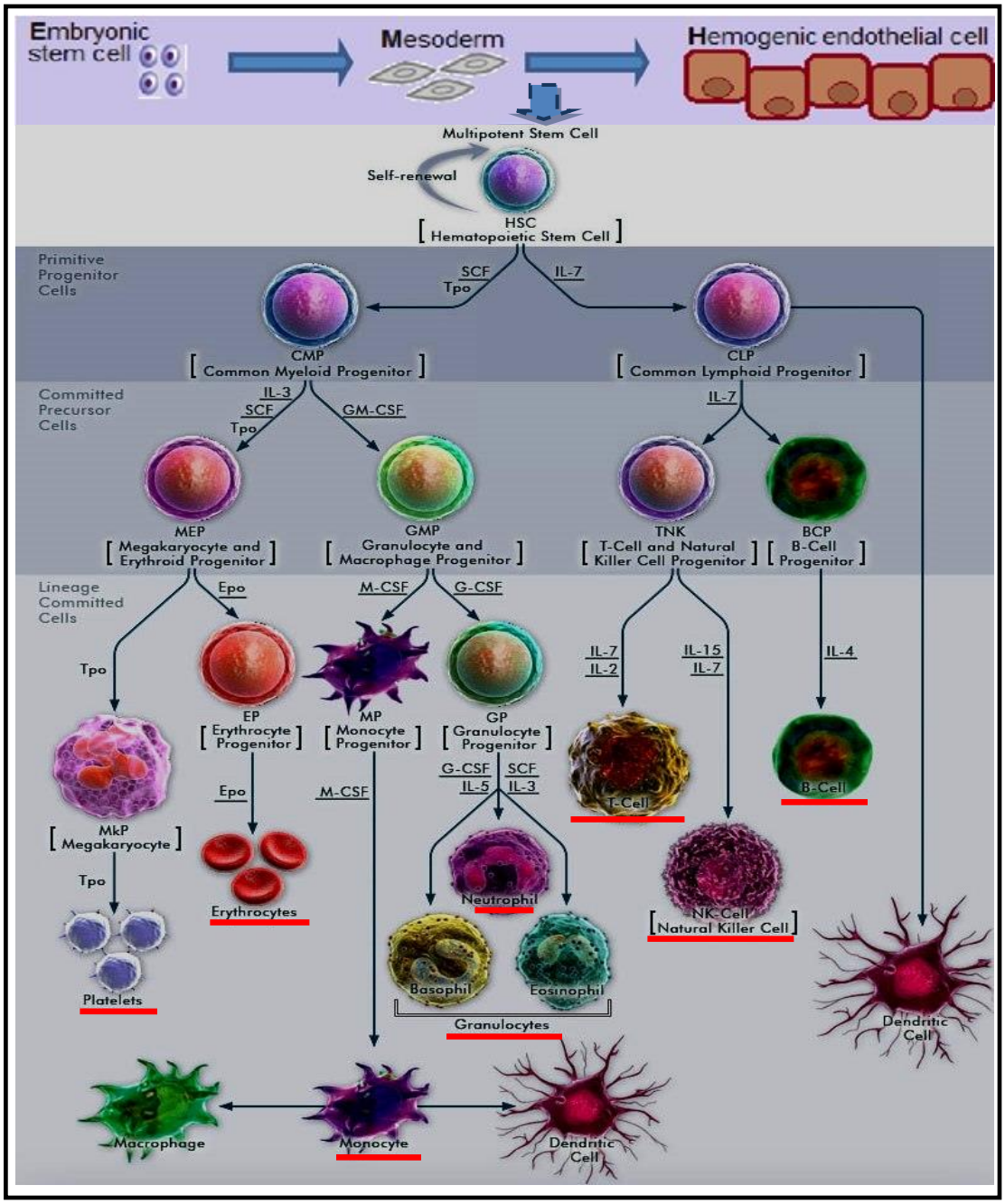


Figure 1.1 Regulation of hematopoiesis process. This figure shows the evolution of different types of cells that arise from HSC to mature blood cells (*underlined in red*: RBCs, WBCs and platelets). The image was adapted and modified from (Hematopoiesis from multipotent stem cell, 2012).

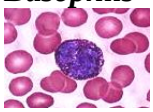
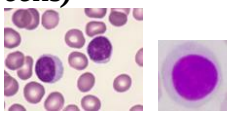
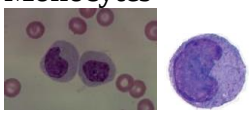
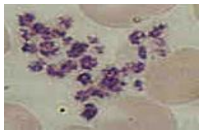
Source: http://www.ebioscience.com/resources/pathways/hematopoiesis-from-multipotent-stem-cells.htm.

1.1.2 Functions of blood

Table 1.2 Classification of 3 different types of blood cells according to their own functionality

Formed element	Number	Featured characteristics	Functions
RBCs 	$4.8x10^6$/ microliter (µL) in females $5.4x10^6$/µL in males	7-8 micrometer (µM)[t] ; Biconcave discs, no nucleus, lifespan : 120 days	Transport most of the oxygen and part of the carbon dioxide in the blood
Leukocytes / (WBCs)	$(5\text{-}10)x10^3$/µL	Live for few hours (hr / hrs) to few days	Battle against pathogens and foreign substances which invades the body
Granulocytes	**Of all WBCs:**		
Neutrophils 	60-70%	10-12µM[t], nucleated have 2-5 lobes are interconnected by thin strands of chromatin. Cytoplasm has very fine, pale lilac granules Lifespan: Minutes (min / mins) to days	Involved in phagocytosis process and destruction of bacterial with lysozyme by providing strong oxidants, such as superoxide anion, hydrogen peroxide and hydrochlorite anion
Eosinophils 	2-4%	10-12µM[t]. Nucleus has 2 lobes connected by a thick strand of chromatin; large; bright red-orange granules fill the cytoplasm. Lifespan: Mins to days	Involved in allergic reactions; phagocytized antigen (Ag / Ags)-antibody (Ab / Abs) complexes; destroy parasites infections

Table 1.2. Continued

Basophils 	0.5-1%	8-10µM[t] Nucleus has 2 lobes but difficult to notice the nucleus due to heavy dense dark purple granules Lifespan: Unknown	Release heparin, histamine and serotonin in allergic reactions that stimulate the inflammatory response
Agranulocytes ***Lymphocytes (Lym)*** ***(T cells, B cells and Natural killer cells)*** 	20-25%	Spheroid cells with a single and bigger size nucleus Small Lym:6-9µM[t] Large Lym:10-14µM[t]. Cytoplasm forms a rim around the nucleus, appears to be in sky blue; The larger the cell, the more the cytoplasm visible	Mediate immune responses including Ag-Ab reactions **B cells**: secrete Abs **T cells**: Attack invasive viruses, cancer cells and transplanted tissue cells **Natural killer cells**: Attack infectious microbes and tumor cells
Monocytes 	3-8%	Lifespan: Many years; 12-20µM[t], nucleus kidney shaped	Effective phagocytic cells and act as a garbage collecting cells in the immune system
Platelets 	1.4 x 10^5 – 4.4 x 10^5/µL	2-4 µM[t], Contain many vesicles and has no nucleus. Lifespan : 5-9 days	Aid in hemostasis process by releasing chemicals which promotes vascular spasm and blood clotting

All the information and figures were obtained from: (Tagliasacchi *et al*., 1997; Manning, 2004, Tortora and Derrickson, 2006; look for diagnosis, 2009; Connor E and Faraci J, 2009; Wellsphere, 2010; Medfriendly lymphocytes, 2012**;** Anatomy & Physiology, 2013; Circulatory system, 2013; Anatomy blood, n.d.); [t]Indicates *in diameter*.

1.2 Hemorrhage

Bleeding is the loss of blood from the circulatory system and technically known as hemorrhage. Fundamentally, bleeding can occur internally (blood escapes from blood vessels or organs) and externally (blood loss through natural opening such as mouth, nose, ear, urethra, vagina and anus; through a break in the skin) (Blake, 2014). Hemorrhage is the most common cause of death for severely injured patients when prompt action was not taken within a critical time period. Over the past 3 decades, improved methods have been widely introduced in the civilian settings. Battlefield wounds differ from other usual injuries in terms of epidemiology, mechanism of injury and pathophysiology of the body's response (Champion *et al*., 2003). Forty percent of traumatic mortality deaths and up to 90% of all civilian deaths took place in pre-hospital settings (Bellamy, 1984; Sauaia *et al*., 1995). Based on American College of Surgeon's Advanced Trauma Life Support, hemorrhage can be classified into 4 different categories depending on the level of severity and the classification is clearly depicted in Table 1.3 (Manning, 2004).

Table 1.3 Classification of hemorrhage levels varying from type 1, 2, 3 and 4 based on the volume of loss, sign & symptoms, volume resuscitation, behavioral changes and blood transfusion. Information adapted from Manning, (2004).

Type	Volume of loss	Sign & Symptoms	Volume resuscitation	Behavioral changes	Blood transfusion
1	>15%	No	No	No	No
2	15-30%	Often tachycardiac; Skin begin to appear pale	Required with crystalloids (saline solution or Lactated Ringer's Solution)	Moderate	No
3	30-40%	Blood pressure drops, heart rate increase and shock	Required with crystalloids	Moderate to severe	Yes
4	>40%	Body compensation is reached	Required aggressively	Severe	Yes

1.3 Platelets

When platelets decrease in number or become malfunction, the risk of hemorrhage is very high upon injuries. Platelets, which circulate within the blood, are the essential mediators that trigger the mechanical pathway of the coagulation cascade upon encountering any damage to the blood vessels. Platelets promote primary (1°) hemostasis via 3 major processes: activation, adhesion and aggregation. When the integrity of the vascular endothelium is interrupted, various macromolecular elements of the vascular subendothelium become exposed and readily accessible to platelets (Nakamura *et al*., 1999).

Table 1.4 Properties, structure, function and mechanism of platelets

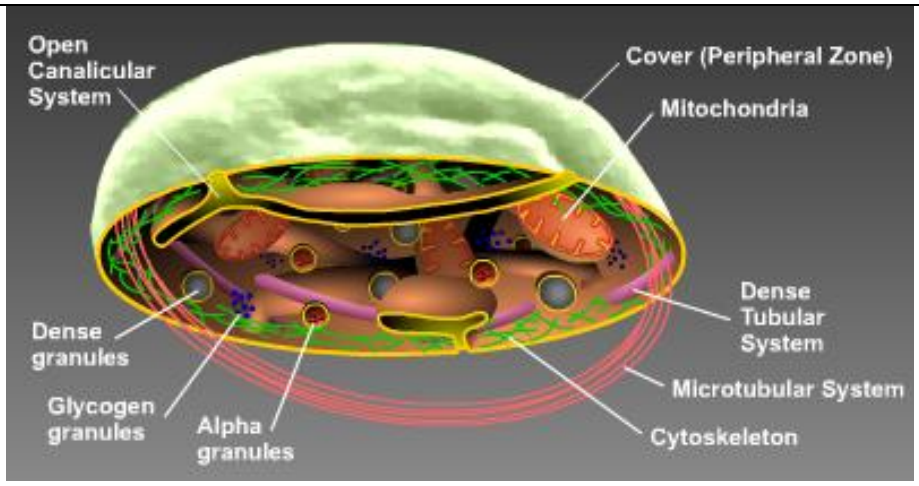
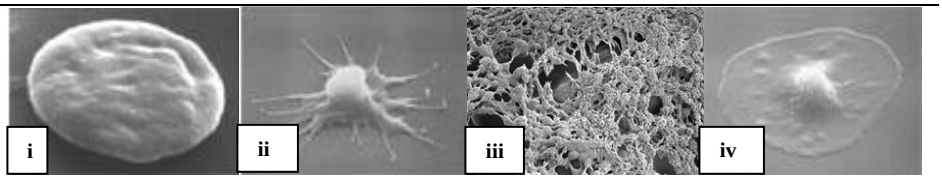
Action	**Descriptions**	
Platelets	Known as thrombocytes	
Produced from	Very large bone marrow cells called megakaryocytes	
Megakaryocytes	Develop into giant cells to release <1 X 10^3 platelets/ megakaryocytes	
Circulate	Only 7-10 days	
Structure	2.5µM in average normal diameter, biconvex discoid shape, sticky in nature	
Count	Normally (1.4 x 10^5) to (4.4 x 10^5) / µL	
Bleeding risk depending on the platelet counts	≥50,000 / µL 20,000 – 50,000 /µL <20,000 / µL <5000 / µL	▪ Minimal ▪ Minor bleeding after trauma ▪ Spontaneous bleeding ▪ Severe, possibly life threatening
Detailed structure:		
Open Canalicular system	Formed by invaginations of platelets. Provide a space for platelet products to enter	
Dense granules	Act as a storage pool. Consists of non-metabolic Adenosine triphosphate (ATP) and Adenosine diphosphate (ADP), serotonin and calcium (Ca). Platelets release their granules upon activations to interact with other platelet cells	

Table 1.4. Continued

Glycogen granules	Supply the energy source for platelet interactions
Alpha granules	Act as the metabolic or cytoplasmic pool. They mainly contains Fibrinogen (Fib), thrombospodin, factor 5 (FV), von Willebrand factor (vWF), beta-thromboglobuline and factor 4 (FIV). Upon activation, platelets release their granules to interact with other platelets
Cytoskeleton	The actin and myosin cytoskeleton organizes a network to sustain the platelet's discoid shape. Upon activation, membrane receptors interlink through this network to allow platelets to change shapes into pseudopodia forms and eventually release their granule contents
Microtubular system	Helps the actin membrane cytoskeleton maintain the discoid shape of platelets. Reorganize platelet shape changes, contract internally and granules content will release upon platelet activation
Dense tubular system	An internal smooth endoplasmic reticulum membrane, which helps to store Ca to activate platelets and aid in prostaglandin & thromboxane (TX) synthesis
Mitochondria	Serve as energy source because resting platelets generate their energy via oxidative phosphorylations
Cover	Contains typical phospholipid bilayer membranes and glycoproteins (Gp) and membrane phospholipids allow the coagulation proteins to interact
1° function	To stop hemorrhage following vascular injury

Table 1.4. Continued

Other functions	Fight microbial infections, trigger inflammation to promote tumor angiogenesis and metastasis process, secrete inflammatory mediators and aid in wound therapy
Mechanism	• Under normal circumstances, platelets do not adhere to the vessel wall. However, upon tissue injury, platelets adhere to the ECM by exchanging signals with many receptors and mediators to coordinate rolling of platelets to adhere at the sites of vascular injury. Firm platelet adhesion stimulates a signaling mechanism mediates via tyrosine kinases and G-protein coupled receptors, which supports platelet activation, resulting in granule release and increasing the number of platelets. • Platelet adherences and activations initiate platelet aggregation to provide a procoagulant surface engaged in the formation of fibrin-rich hemostatic plug at the injured area. Activated platelets stimulate endothelial cells to synthesize and secrete molecules that control and limit the formation of thrombus.
Stained smear	Appears as a dark purple spot on Geimsa-stained peripheral blood smear. Used to study the size, shape, qualitative number and clumping. Upon biomaterial adherences, platelets can be fixed in 2.5% glutaraldehyde for viewing under a scanning electron microscope (SEM)
Shape changes	 **(i)** Platelet in resting mode **(ii)** Activated platelets change into a pseudopodia shape (**iii**) Aggregated platelets (**iv**) Platelet spreading

All the information and figures were adapted from (Periayah *et al*., 2013; 2014; Platelet Research Laboratory, 2014).

1.4 Hemostasis

Hemostasis is a process to prevent hemorrhage by arresting and keeping the blood within the damaged vessel walls. Hemostasis is a complex process that is contingent on the complex interaction of platelets, plasma coagulation cascades, fibrinolytic proteins, blood vasculatures and cytokine mediators. Upon tissue injury, the hemostatic mechanism employs a plethora of vascular and extravascular receptors in accordance with the blood components, to seal off the impairments to the vasculature and closing it off from the encircling tissues. Normal hemostatic responses can be organized into 6 different important phases classified under 3 major categories of hemostasis (Kulkarni, 2004; Stassen *et al*, 2004; Stroncek and Reichert, 2008; Davidson, 2013; Moake, 2013) (Table 1.5).

Table 1.5 Mechanical pathway of 3 different types of hemostasis

Type of hemostasis	**Mechanism**
1° hemostasis	•Blood vessel contraction /vasoconstriction •Platelet plug formation upon platelet adhesion and aggregation
Secondary (2°) hemostasis	•Activation of the coagulation cascade •Deposition and stabilization of fibrin
Tertiary hemostasis	•Dissolution of fibrin clot •Dependent on plasminogen activation

1.4.1 Vasoconstriction

Vascular spasm occurs whenever there is an injury or damage to the blood vessels. This will trigger a vasoconstriction which could eventually stop the blood flow. This reaction can respond in up to 30 mins and is localized to the injured area. At this stage, exposed collagen fibers will release ATP and other inflammatory mediators to recruit macrophages. In addition, the ECM becomes highly thrombogenicity; promoting platelet adhesion and aggregation (Figure 1.2) (Kumar *et al.*, 2009; Hidalgo, 2011).

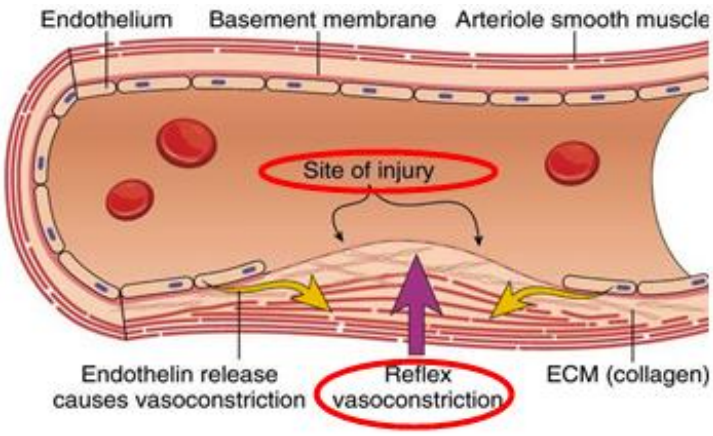


Figure 1.2 Vasoconstriction phase. 1° hemostasis is characterized by vasoconstriction, which is the initial phase for stopping the blood flow. The figure was extracted from the source: Kumar, V., Abbas A.K. & Aster, J.C. (2009). Robbins and Cotran Pathologic Basis of Disease. 9th ed.: Saunders Elsevier.

1.4.2 Platelet plug formation

Following vasoconstriction, exposed collagen from the damaged surface will encourage platelets to adhere, activate and aggregate to form a platelet plug and sealing off the injured area.

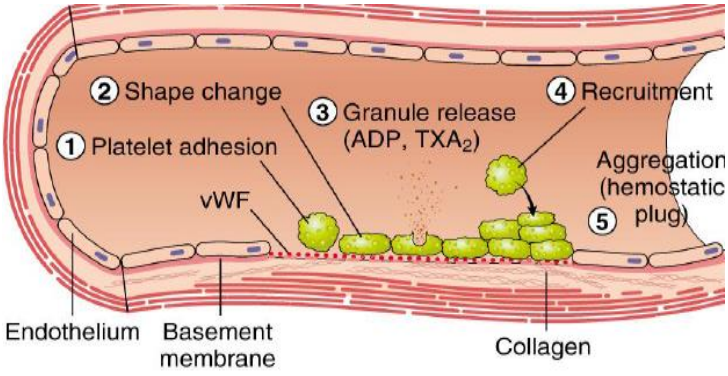


Figure 1.3 Platelet plug formation. Injuries on the endothelial cells highly exposes to thrombogenic, subendothelial ECM to ease platelet adherences and activation. Platelet activation triggers platelet shape changes by releasing secretory granules. Released secretary granules will recruit additional platelets to form platelet plug which is referred to as 1° hemostasis. The figure was extracted from the source: Kumar, V., Abbas A.K. & Aster, J.C. (2009). Robbins and Cotran Pathologic Basis of Disease. 9th ed.: Saunders Elsevier.

1.4.2.1 Platelet adhesion

Platelet adhesion mechanism is generally supported by the particular interactions between the membrane receptors and the absorbed plasma proteins. The platelet membrane receptors are enriched with Gp receptors embedded in the phospholipid bilayer including tyrosine kinase receptors, integrins, leucine rich receptors, protein G coupled transmembrane receptors, selectins and immunoglobulin domain receptors. These are the important proteins involved to facilitate hemostatic function by mediating the interactions between cell-platelet and platelet-substrates (Marguerie *et al*., 1979; Andrew *et al*., 2003; Corum, 2011). The initial event that occurs upon hemostasis is the rolling and adherences of the platelets to the exposed subendothelium. Platelet adhesion is mediated by vWF which binds to Gp Ib-IX in the platelet membrane. vWF is a blood Gp that serves as an adhesive protein, which could bind to other proteins, especially Factor 8 (FVIII) at the wound sites (Packham and Mustard, 1984; Sadler, 1998; Kumar *et al.*, 2009; Ruggeri, 2009; Rumbaut and Thiagarajan, 2010).

1.4.2.2 Platelet activation

A variety of stimuli can activate platelets. Platelet cells can also be activated upon biomaterial surface stimulation. Adhered platelets undergo degranulation and release cytoplasmic granules that contain serotonin, platelet activating factors (PAF) and ADP.

ADP is an important physiological agonist stored in the dense bodies of platelets that play an essential function in normal hemostasis and thrombosis. Platelet are activated to change shapes into a pseudopodal form upon the adhesion to the injured area which will activate the collagen receptors on their surface membrane named, GpIIbIIIa, to undergo release reactions. The GpIIbIIIa complex, organized through Ca-dependent association of GpIIb and GpIIIa that is a necessary step in platelet aggregation and endothelial adherence (Calvete, 1995; Shattil, 1999). At the same time, platelets tend to synthesize and discharge thromboxane A_2 (TXA_2), aiding in vasoconstriction and platelet aggregation. In addition, GpIIbIIIa integrins and P-selectin move from the α-granule membrane to the platelet membrane to support platelet aggregation. Additionally, these are the receptors that could act as the catalytic surface and facilitate the hemostasis process. (Figure 1.4) (Niiya *et al*., 1987; Comfurius *et al*., 1996; Gupta, 2013).

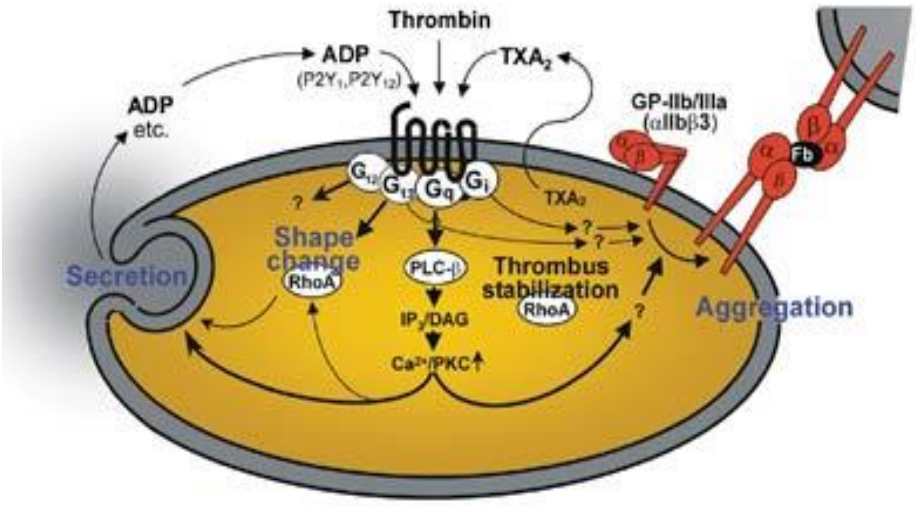


Figure 1.4 Platelet activation process. The schematic diagram portrays the internal organelles with prominent crucial storage contents that are involved in platelet activations and aid in platelet aggregation. This figure was adapted from the source: Moers, A., Wettschureck, N. & Offermanns, S. (2004).

1.4.2.3 Platelet aggregation

Platelet aggregation begins once platelets become activated, triggering the GpIIbIIIa receptors (50-100/platelets), which attach to vWF or Fib. Each activated platelet extends pseudopods, clumping and becoming aggregated. These activations are further heightened by the generation of thrombin via the hemostasis mechanism. Platelet aggregation promotes 1° platelet plug. The ADP receptor interconnects with a family of

ADP receptors ($P2Y_1$ and $P2Y_{12}$), which could be detected on platelets as helping with aggregation. $P2Y_1$ receptors assist in stimulating the initial platelet shape changes and platelet aggregation. At the same time, $P2Y_{12}$ is an important mediator for blood clotting. It increases significantly, responding to ADP to complete the aggregation process. Eventually, the formed platelet plug ought to be stabilized by the formation of fibrin (Figure 1.5) (Coller *et al*., 1991; Dorsam and Kunapuli, 2004; Yip *et al*., 2005; Offermanns, 2006; Kumar *et al*., 2009).

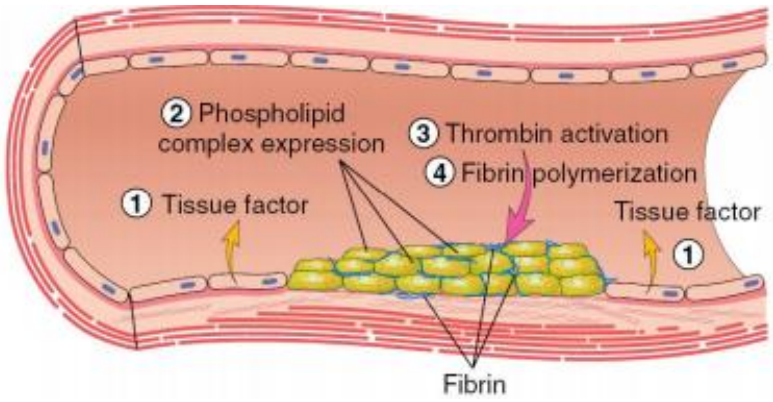


Figure 1.5 Platelet aggregation phase. Tissue factor (TF) also known as factor 3 (FIII) and thromboplastin, is a membrane-bound procoagulant. TF acts with factor 7 (FVII) as the major *in vivo* initiator of the coagulation cascade to generate thrombin. Thrombin adheres with circulating Fib and convert into insoluble fibrin by forming fibrin network. This fibrin network strengthens the initial platelet plug. This image was extracted from the source: Kumar, V., Abbas A.K. & Aster, J.C. (2009). Robbins and Cotran Pathologic Basis of Disease. 9th ed.: Saunders Elsevier.

1.4.3 The Coagulation Mechanism

Approximately fifty significant substances affect the blood coagulation mechanisms. The blood coagulation cascade of 2° hemostasis mainly consist of 2 main pathways. The pathways are the intrinsic (contact activation pathway) and extrinsic (TF pathway) pathways. The blood clotting process can be classified into 3 important steady steps as follows; (i) involvement of a complex cascade, triggering the chemical reactions that are mediated by the coagulation factors that respond to form fibrin strands for consolidating the platelet plugs; (ii) the conversion of prothrombin (PT) into thrombin which is catalyzed by the PT activator; and (iii) conversion of Fib into fibrin, which eventually enmeshes the plasma, platelets and blood cells to build a firmer clot (Figure 1.6) (Lefkowitz, 2006; Pallister and Watson, 2010; Hall and Guyton, 2011).

1.4.3.1 Extrinsic pathway

The newer blood coagulation cascade model was well elaborated by Jerry B. Lefkowitz. Thrombin was portrayed as the center of the coagulation universe. All the coagulation factors involved in the hemostasis process feed into the regulation and control of thrombin generation, which then forms clots at the sites of vascular injury. Thrombin is a proteolytic enzyme derived from PT, which aids in blood clotting by catalyzing the conversion of Fib to fibrin. The modified intrinsic coagulation cascade, which is displayed in Figure 1.6, is different from the older one and lacks the significance of factor 12 (FXII) and prekallikren. Apparently, these proteins are not considered to play a crucial role in the coagulation process *in vivo*.

There are 2 major processes that could initiate the blood clotting mechanism. They are extrinsic and intrinsic pathways. Firstly, TF binds to FVII or activated FVIII (FVIIIa) in 1:1 ratio complex. A limited proteolysis process extends to TF / FVIIIa complex which activates factor 10 (FX) or factor 9 (FIX), further activating FX / FIX and activating serine proteases via the cleaving an activation peptide. Proteolysis is the hydrolysis process that involves the breakdown of proteins into smaller polypeptides. Once the extrinsic pathway is triggered, the activation of FX / FIX in the TF/ FVIIa complex is instantly inhibited by TF pathway inhibitor (TFPI), which is generated from endothelial cells. Freshly activated factor 9 (FIXa) subsequently adheres to its cofactor, factor VIIIa, upon the phospholipid surface to stimulate the tenase complex which results in the activation of FX to activated FX (FXa).

Finally, the common pathway for thrombin activation is initiated via the activation of FXa. The activated FXa merges with the cofactor, activated factor 5 (FVa), and Ca on the phospholipid surfaces to construct prothrombinase complex. This complex eventually helps to convert PT to thrombin by cleaving the PT, which is the activation peptide. Thrombin activation will be generated to a very major extent by the extrinsic pathway, which is adequate and crucial to initiate the coagulation cascade which subsequently triggers and expands thrombin generation via the intrinsic pathway (Green, 2006; Leftowitz, 2006; Pallister and Watson, 2010).

1.4.3.2 Intrinsic pathway

The activation of factor 11 (FXI) to activated FXI (FXIa) and more thrombin generated via FIXa and FVIIIa leads to the activation of FX, which is involved in the intrinsic pathway. FV and FVIII, which are partially proteolyzed or activated are known to be involved in and facilitated hemostasis process. Subsequently, the activation of FV and FVIII by thrombin triggers more mechanical action of the coagulation pathway by enhancing the bioactivity of tenase and prothrombinase complexes.

As described in Table 1.6, factor I (FI / Fib) plays a crucial role in forming a fibrin clot to seal the injured area with fibrin meshes. Fib typically consists of 3 globular domains, which is the central E domain attached or flanked by 2 exactly alike identical D domains. At this stage, thrombin sticks to fibrinopeptides A and B, which are derived from the A alpha (α) and B beta (β) chains, to build a fibrin monomer. These monomers gather into protofibrils in a half-distributed manner, which is stabilized by the noncovalent interactions among fibrin molecules. Eventually, the photofibrils are obliquely organized into dense fibrin networks to form a temporary fibrin clot that is not covalently crosslinked.

Nevertheless, to form a stable blood clot, thrombin needs to activate factor 13 (FXIII) to the transglutaminase enzyme activated factor 13 (FXIIIa). Factor XIIIa will stimulate the glutamic acid and lysine side chains, producing a stable clot. Factor XIIIa is the fibrin stabilizing factor of the blood coagulation system that crosslinks with fibrin. Furthermore, factor XIIIa also plays a significant role towards tissue repair and the

angiogenesis process (Chandler, 2005; Green, 2006; Leftowitz, 2006; Pallister and Watson, 2010).

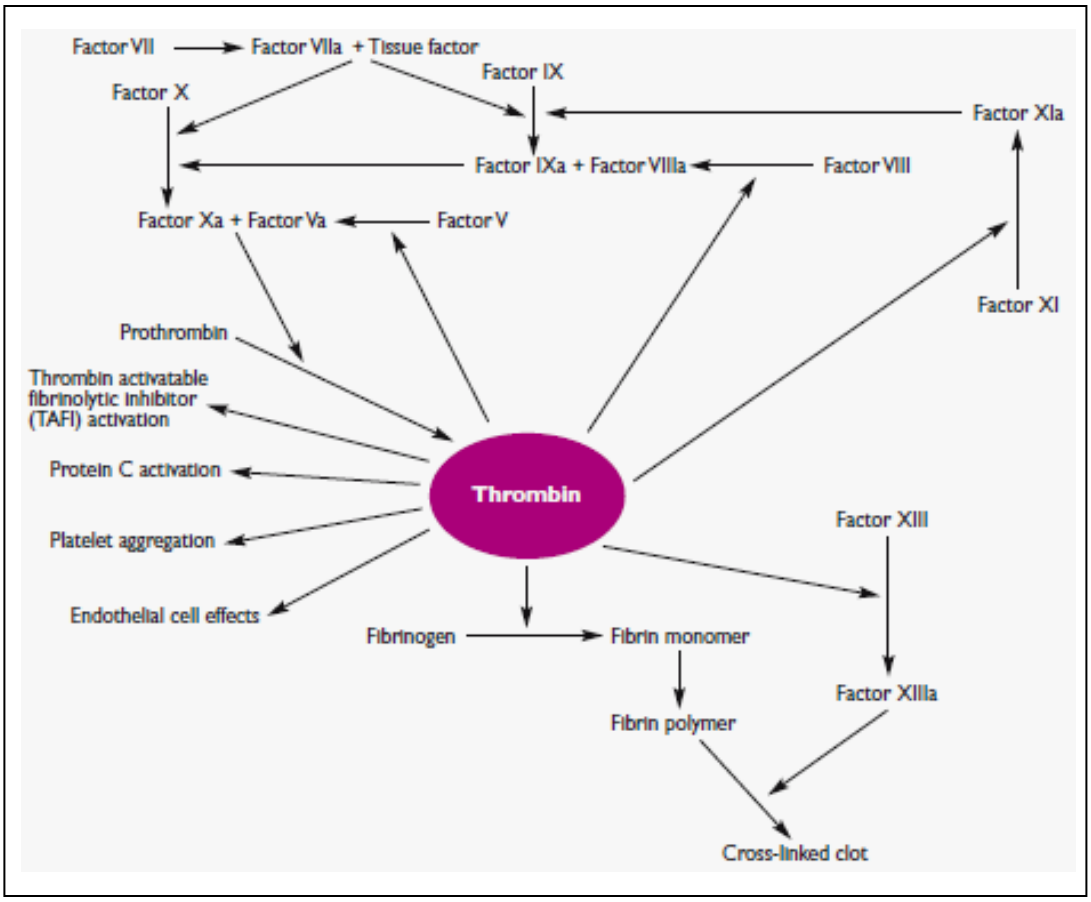


Figure 1.6 Coagulation mechanism. Thrombin plays a vital role in generating cross-linked fibrin by cleaving Fib to fibrin and activating a few other coagulation factors. Thrombin also modulates other important cellular activities via protease-activated receptors. Simultaneously, it will directly increase the platelet agglutination and the production of TXA_2 to express adhesion molecules. This diagram was adapted from the source: Lefkowitz, J.B. (2006). Chapter 1. In hemostasis physiology. Coagulation pathway and physiology. JB Lippincott Co, Philadelphia, 3-12.

1.4.3.3 Coagulation factors

Table 1.6 Coagulation factors aids in blood coagulation cascade

Factor	Name	Source	Pathway	Description	Function
I	**Fib**	Liver	Common	Plasma Gp; Molecular Weight (MW)= 340 kilodaltons (kDa)	Adhesive protein which aids in fibrin clot formation
2 (II)	**PT**	Liver	Common	Vitamin K-dependent serine protease; MW= 72 kDa	Presence in the activated form and the main enzyme of coagulation
III	**TF**	Damaged cells and platelets	Extrinsic and Intrinsic	Known as thromboplastin; MW= 37 kDa	Lipoprotein initiator of the extrinsic pathway
IV	**Ca ions**	Bone and gut	Entire process	Required for coagulation factors to bind to phospholipid	Metal cation that is important in coagulation mechanisms
V	**Proaccererin / Labile factor**	Liver and platelets	Intrinsic and extrinsic	MW = 330 kDa	Cofactor for the activation of PT to thrombin (prothrombinase complex)
VII	**Proconvertin (stable factor)**	Liver	Extrinsic	MW = 50 kDa; vitamin K-dependent serine protease	With TF, it initiates the extrinsic pathway (FIX & FX)
VIII	**Antihemophilic factor A (cofactor)**	Platelets and endothelium	Intrinsic	MW = 330 kDa	Cofactor for the intrinsic activation of FX (which it forms tenase complex)

Table 1.6. Continued

IX	**Christmas factor / Antihemophilic factor B (plasma thromboplastin component)**	Liver	Intrinsic	MW = 50 kDa; vitamin K-dependent serine protease	The activated form is an enzyme for the intrinsic activation of FX (forms a tenase complex with FVIII)
X	**Stuart-Prower factor (enzyme)**	Liver	Intrinsic and extrinsic	MW = 58.9 kDa; vitamin K-dependent serine protease	The activated form is the final enzyme for the common pathway activation of PT (forms prothrombinase complex with FV)
XI	**Plasma thromboplastin antecedent**	Liver	Intrinsic	MW = 160 kDa; serine protease	Activates the intrinsic activator of FIX
12 (XII)	**Hageman factor**	Liver	Intrinsic; (activates plasmin)	MW = 80 kDa; serine protease	Initiates the activated partial thromboplastin time (APTT)-based intrinsic pathway; Activates FXI, FVII and prekallikrein
XIII	**Fibrin stabilizing factor**	Liver	Retards fibrinolysis	MW = 320 kDa; Crosslinks fibrin	Transamidase which cross-links fibrin clot

Informations were obtained and modified from (Green, 2006; Leftowitz, 2006; Pallister and Watson, 2010; Sonawani *et al*., 2010)

1.4.4 Tertiary hemostasis

Once the fibrin clot has been formed, the activated platelets will be well organized and take position to contract their intracellular actin or myosin cytoskeleton. The intracellular actin network will directly connect to the integrin GpIIbIIIa and Fib receptor internally. Subsequently, the external component of GpIIbIIIa will adhere to the fibrin network of the blood clot, making the clot compact and decreasing the clot volume slowly, which is called clot retraction. A plasminogen activator is a serine protease that converts plasminogen to plasmin to promote fibrinolysis by cutting and degrading the fibrin networks. Plasmin slashes off the fibrin meshes formed around the wounded area, resulting in the formation of other circulating fragments that are cleared by other proteases or by the kidney and liver. The clot resolution mechanism aid in clearing the injured and obstructed vessels, regenerating blood flow that is directed to the normal blood flow pathway. GpIIbIIIa disrupts the fibrin binding capacity with platelets and complete the clot resolution process (Hoffbrand, 2002; Leftowitz, 2006; Pallister and Watson, 2010).

1.4.5 Wound healing

Wound healing is an innate revitalizing response in tissue injuries, and the interaction of the cellular mechanical pathway events results in resurfacing, reconstitution and refurbishment of cells on injured surface area. The healing process can be explained in 3