Influence of Activated Platelet Fragments on *in vitro* Cultures of Peripheral Blood Mononuclear Cells

A DISSERTATION SUBMITTED

BY

Anupriya M G

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF PHILOSOPHY

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY

THIRUVANANTHAPURAM – 695 011

DECLARATION

I, **Anupriya M G**, hereby declare that I had personally carried out the work depicted in the dissertation entitled **"Influence of Activated Platelet Fragments on *in vitro* Cultures of Peripheral Blood Mononuclear Cells"** under the direct supervision of **Dr. Lissy K Krishnan, Scientist G, Thrombosis Research Unit,** Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. External help sought are acknowledged.

Anupriya M G

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES & TECHNOLOGY
THIRUVANANTHAPURAM – 695011, INDIA

(*An Institute of National Importance under Govt. of India with the status of University by an Act of Parliament in 1980*)

CERTIFICATE

This is to certify that the dissertation entitled **"Influence of Activated Platelet Fragments on *in vitro* Cultures of Peripheral Blood Mononuclear Cells"** submitted by **Anupriya M G** in partial fulfilment for the Degree of Master of Philosophy in Biomedical Technology to be awarded by this Institute. The entire work was done by her under my supervision and guidance at **Thrombosis Research Unit**, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Thiruvananthapuram-695012.

Thiruvananthapuram

10/08/2010

Dr. Lissy K Krishnan

The Dissertation

Entitled

Influence of Activated Platelet Fragments on *in vitro* Cultures of Peripheral Blood Mononuclear Cells

Submitted

By

Anupriya M G

For

Master of Philosophy

of

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY

THIRUVANANTHAPURAM – 695 011

Evaluated and approved

by

Signature

Name of Supervisor

Dr. LISSY K. KRISHNAN

Signature

Examiner's name and Designation

Dr. MIRA MOHANTY

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my guide, Dr. Lissy K Krishnan, Scientist G, Thrombosis Research Unit. I wouldn't have been able to execute my project successfully without her tremendous support and guidance.

I am extremely thankful to the Director, SCTIMST, Head, Biomedical Technology Wing and Deputy Registrar, SCTIMST for providing all the necessary facilities throughout the course.

I owe my thanks to the lab members of Thrombosis Research Unit. I am grateful to Mrs. Priyanka, Mrs. Juliet, Ms. Sabeela, Mr. Renjith S, Mrs. Mary Vasantha, Mrs.Ragaseema, Mrs. Tara, Mrs. Anumol, Mr. UnniKrishnan, Mr. Renjith P, Dr. Asha S Mathew, Dr. V Gayathri, Dr. Anugya Bhatt, and Mrs. Renu for their help and support throughout the project. I wish to extend my warmest thanks to the non-technical staff of TRU for helping me throughout the project.

I would like to thank Dr. Anil Kumar TV for providing the Confocal microscopy facility. I would like to extend my heartfelt thanks to the staff members of the Division of Academic Affairs for their immense support and help throughout the course.

I am very much grateful to Dr. Mira Mohanty and Dr. Kallyana Krishnan for their great help and support.

I would like to extend my heartfelt gratitude to my friends for standing by me in good and bad times and for being my strength.

I would like to thank everyone who has helped me to complete the course successfully.

Last but not the least I wish to express my deepest gratitude to my parents for their constant support.

Above all I gratefully acknowledge the Almighty.

Anupriya M G

ABBREVIATIONS

%	Percent
ACD	Acid Citrate Dextrose
ADP	Adenosine diphosphate
ASA	Acetylsalicylic acid
CAD	Coronary Artery Disease
CD	Cluster of Differentiation
COX	Cyclooxygenase
DC	Dendritic Cell
EC	Endothelial Cell
EPC	Endothelial Progenitor Cells
Epi	Epinephrine
FG	Fibrin composite
FSC	Forward Scattering
GP	Glycoprotein
HBSS	Hank's Balanced Salt Solution
HS	Human Serum
ICAM-1	Intercellular Adhesion Molecule-1
IL	Interleukin
IU	International Unit
LDL	Low Density Lipoprotein
LPS	Lipopolysaccharide
MAC	Macrophages
M-CSF	Monocyte-Colony Stimulating Factor
MIP-1	Macrophage Inflammatory Protein
ml	millilitre
MMP	Matrix Metalloproteinase
MO	Monocytes
MP	Microparticles
MPA	Monocyte-Platelet Aggregates
NBCS	New Born Calf Serum

nM	nanomolar
NO	Nitric Oxide
°C	Degree Celsius
PAF	Platelet Activating Factor
PAR	Protease Activated Receptor
PBMNC	Peripheral Blood Mononuclear Cells
PC	Platelet Concentrate
PCI	Percutaneous Coronary Intervention
PD	Platelet Debri
PDGF	Platelet-Derived Growth Factor
PF 4	Platelet Factor 4
PM	Proliferative monocytes
PMC	Platelet-Monocyte Complexes
PMP	Platelet Microparticle
PR	Platelet Releasate
PRP	Platelet Rich Plasma
SMC	Smooth Muscle Cell
SSC	Side Scattering
TCPS	Tissue Culture polystyrene
TF	Tissue Factor
TFPI	Tissue Factor Pathway Inhibitor
TGF ß	Tumor Growth Factor-ß
TLR	Toll-like Receptor
TM	Thrombomodulin
tPA	Tissue Plasminogen Activator
TXA_2	Thromboxane A_2
uPA	Urokinase-Type Plasminogen Activator
VCAM-1	Vascular Cell Adhesion Molecule
vWF	von Willebrand Factor
α-SMA	alpha-Smooth muscle Actin
µM	micromolar

Table of Contents

List of Figures

List of Tables

SYNOPSIS

Atherosclerosis is a disease of large and medium-sized muscular arteries and is characterized by endothelial dysfunction, vascular inflammation, and the build-up of lipids, cholesterol, calcium, and cellular debris within the intima of the vessel wall. A complex and incompletely understood interaction exists between the critical cellular elements of the atherosclerotic lesion. These cellular elements are endothelial cells, smooth muscle cells, platelets, and leukocytes. There is increased platelet activation in many cardiovascular diseases. The release of microvesicles ('platelet microparticles', PMPs) by activated platelets has been shown to be an integral part of the thrombotic process since they possess proatherogenic and procoagulant properties. At sites of vascular injury, the platelets bind to the endothelium of the blood vessel. Adhered platelets efficiently mediate monocyte rolling and arrest, even at high shear. Rolling is mediated by P-selectin on activated platelets and PSGL-1, constitutively expressed on monocytes.

The present study was conducted to investigate the possible relationship between released PMPs and the remaining platelet debris (PD) with circulating leukocytes. Specific emphasis was on platelet-monocyte interaction, their influence during *in vitro* culture of peripheral blood mononuclear cells which include $CD14^+$ proliferating monocytes and $CD34^+$ progenitor cells. There are literature reports on the possible interaction of monocytes with activated platelets which may lead to development of macrophages. Therefore, this study was designed based on the hypothesis that subcellular fractions isolated from activated platelets may influence peripheral blood mononuclear cells to get differentiated into macrophages. In order to test the hypothesis specific objectives were formed which include:

⇒ Activate washed platelets using physiologically relevant concentrations of epinephrine and thrombin.

⇒ Identification and quantification of platelet-leukocyte interaction and more specifically platelet-monocyte interaction using specific markers for each.

⇒ Isolate and label sub cellular fractions from activated platelets and study their interaction with monocytes and progenitors isolated from circulation.

⇒ Culture the isolated monocyte fraction in presence of platelet fractions and characterize the resultant cells by (i) morphology; (ii) immunochemical staining to identify the possible effect of platelet particles and debris on generation of macrophage, endothelial cells and smooth muscle cells in the culture

This dissertation is divided into 4 main chapters such as (i) Introduction; (ii) Materials and Methods (iii) Results and Discussion (4) Summary & Conclusions.

Chapter I include a brief introduction to the research topic and review of literature with citations. The reviewed literature proposes that concerted interaction of platelets with vascular cells and circulating monocytes or progenitor cells may manipulate a vessel injury to inflammatory response and atherogenesis. Platelets may support homing of circulating progenitors to the injured site and stimulate them to differentiate into endothelial cells & smooth muscle cells. A multitude of molecules released from activated platelets, membrane associated adhesive proteins and surface proteins on the debris of activated platelets etc are known to take part in the inflammatory response and/or vascular regeneration. Currently, many of the processes or mechanisms which lead to vascular disease or regeneration are less understood. There is data which suggest that circulating progenitors are influenced by platelets to result in the formation of macrophages in culture when $CD34^+$ cells and platelets are co-cultured *in vitro*. However, this area is relatively less understood and need further research to prove if the fragmented platelets play a definite role in vascular regeneration.

Chapter II gives details of the equipments and reagents used, along with sufficient descriptions of the procedures employed for the study. Specific marker used for platelets was CD62 (P-selectin) whereas CD45 was used as a general leukocyte marker; CD14 was specifically used for monocytes and CD34 for circulating haematopoetic progenitors. Flow cytometric analysis was done to quantify the platelet/PMP/PD interactions with other circulating cells. Platelet activation was achieved using physiological concentrations of epinephrine and thrombin; sub cellular fragments were isolated using density gradient centrifugation and analyzed using SDS-PAGE. Peripheral blood mononuclear cells (PBMNC) isolated by density

gradient centrifugation were allowed to grow in culture in presence of platelet fragments. Morphological characterization was done using phase contrast microscopy. Markers used for identification of cells in culture were; CD14 for monocytes, CD68 for macrophages, CD31 for endothelial cells and smooth muscle actin (SMA) for smooth muscle cells. Fluorescence microscopy and confocal microscopy were employed for identifying positive cells for the above markers.

In Chapter III A & B, results are presented with appropriate illustrations/images and the findings are discussed in the light of current knowledge obtained from the literature. Major findings are: to activate the platelets, and up regulate the expression of CD62, 10 nM epinephrine was sufficient. When very low concentrations of thrombin were added after epinephrine addition, immediate synergistic up regulation of CD62 was found but it appeared to be reversible within 30 min to 60 min. When the concentration of thrombin is further increased (but still physiologically relevant) the changes on platelet membrane and interaction with leukocytes were stable and increased with time till 60 min. Significant quantity of PMP was released using the same epinephrine/thrombin combination and separated efficiently from platelet debri (PD) using density gradient centrifuge. Analysis of the PMP, PD and platelet releasate (PR) showed distinct bands in PMP which corresponded to collagen receptors and fibrinogen receptors. Both PMP and PD were found to interact with isolated $CD14^+$ monocytes and $CD34^+$ cells. An interesting observation was that all $CD34^+$ cells in the isolated PBMNC fraction were also $CD14^+$.

In cell culture experiments both PMP and PD were included with the culture substrate which consisted of a standard fibrin-gelatin composition, coated in polystyrene dishes as culture substrate. Results suggest that on bare polystyrene and on fibrin matrix cells get differentiated to $CD68^+$ macrophages. When the PD and PMP were included in the substrate, by day 9 in culture, endothelial cells ($CD31^+$) and smooth muscle cells (α-SMA^+) were more prominent. No $CD68^+$ cells were found in the cultures grown on PD-Fibrin or PMP-fibrin substrate. The cell attachment and spreading was faster on PMP-substrate whereas more cells were found to survive on PD-substrate in a concentration dependent manner.

The results obtained in this study suggested that PMP or PD is not likely to influence conversion of monocytes into macrophages, thus disproving the study hypothesis. It is concluded that PD and PMP are capable of stimulating the circulating progenitors into SMC and EC lineage even without the addition of exogenous growth factors into the cultures. The results indicate that $CD14^{+}/CD34^{-}$ progenitors may get differentiated into SMC and EC but further experiments are required to confirm the finding.

CHAPTER I

INTRODUCTION

I.1 BACKGROUND

Atherosclerosis is no longer considered as a disorder due to abnormalities in lipid metabolism. In fact, the inciting event of atherosclerosis is likely an inflammatory insult that occurs decades before the disease becomes clinically apparent. Once initiated, atherosclerosis progresses as a result of a well-studied series of changes in the constituent cellular make-up of the vessel wall. Specific cytokine-mediated events in this cycle are required for lesion growth. The clinical manifestations of atherosclerosis occur so late in this process that interventions such as percutaneous coronary interventions can deal with isolated areas of disease; however, they do not influence the underlying disease process.

Atherosclerotic lesions are composed of three major components. The first is the cellular component comprised predominately of smooth muscle cells and macrophages. The second component is the connective tissue matrix and extracellular lipid. The third component is intracellular lipid that accumulates within macrophages, thereby converting them into foam cells. Atherosclerotic lesions develop as a result of inflammatory stimuli, subsequent release of various cytokines, proliferation of smooth muscle cells, synthesis of connective tissue matrix, and accumulation of macrophages and lipid.

The atherosclerotic process is characterized, in its earliest stages, by perturbations in endothelial function. Atherosclerosis is likely initiated when endothelial cells over-express adhesion molecules in response to turbulent flow in the setting of an unfavourable serum lipid profile. Increased cellular adhesion and associated endothelial dysfunction then "sets the stage" for the recruitment of inflammatory cells, release of cytokines and recruitment of lipid into the atherosclerotic plaque.

It is now widely accepted that the earliest stages of the development of atherothrombosis are mediated, in large part, by the inflammatory cascade. Expression of adhesion sites increases recruitment of monocytes and T-cells to sites of endothelial injury; subsequent adhesion of leukocytes magnifies the inflammatory cascade by recruiting additional leukocytes, activating leukocytes in the media, and causing

recruitment and proliferation of smooth muscle cells. In response to signals generated within the early plaque, monocytes adhere to the endothelium and then migrate through the endothelium and basement membrane by elaborating enzymes, including locally activated matrix metalloproteinase (MMP) that degrade the connective tissue matrix. Recruited macrophages release additional cytokines and begin to migrate through the endothelial surface into media of the vessel. This process is further enhanced by the local release of monocyte-colony stimulating factor (M-CSF), which causes monocytic proliferation; local activation of monocytes leads to both cytokine-mediated progression of atherosclerosis, and oxidation of low-density lipoprotein (LDL).

Platelet activation can directly influence the clinical course of atherosclerosis; it is likely that acute myocardial infarction and unstable coronary syndromes are due to thrombin and von Willebrand factor-mediated platelet activation and aggregation. Additionally, platelet activation can play a role earlier in the pathogenesis of atherosclerosis. For example, PDGF and tumor growth factor (TGF)-ß are two very potent mitogenic cytokines elaborated by activated platelets that act at the site of the thrombus to promote atherosclerotic lesion development. Thrombogenesis is promoted by loss of endothelium, which may be caused by direct physical damage such as that occurs with angioplasty, hemodynamic stress, use of tobacco products, high blood cholesterol levels, or enzymes released from platelets and leukocytes. The shedding of endothelial cells exposes the sub endothelium to platelets and blood coagulation factors. Platelets that adhere to the sub endothelium undergo shape change, aggregate, and secrete their granular contents, thereby recruiting more platelets to form aggregates. In addition to collagen, a variety of other agonists, including thrombin, epinephrine, and thromboxane A_2 (TXA_2), also promote platelet aggregation. Whereas all of these agents stimulate the synthesis of TXA_2, collagen, thrombin, and TXA_2 also induce the release of adenosine diphosphate (ADP) from platelet granules, which amplifies the aggregation process. In addition to these pathways, thrombin-induced platelet aggregation occurs through a third mechanism that may involve the activation of platelet calpain.

The release of platelet microparticles (PMPs) from activated platelets has been shown to be an integral part of the thrombotic process. As they possess various platelet membrane proteins and bioactive lipids, PMPs are believed to mediate many biological processes. They are involved in all stages in the pathobiology of atherosclerosis. In addition to their role in thrombosis, PMPs may also have a pro-inflammatory effect, which promotes the development of atherosclerosis. Microparticles are more abundant and more thrombogenic in human atherosclerotic plaques than in plasma. Therefore, interaction of subcellular fragments of platelet with monocytes and circulating progenitors is an important area to be studied.

I.2 REVIEW OF LITERATURE

I.2.1 Coronary Artery Disease:

Atherosclerosis or coronary artery disease (CAD) is caused by an inflammatory process which involves various biochemical and cellular processes. The earliest process of atherosclerosis is perhaps the accumulation of lipids in the intima of arteries, which supposedly results from endothelial dysfunction because of insult-induced damage [Yan ZQ *et al*, 2007]. Possible causes of endothelial cell (EC) dysfunction leading to atherosclerosis include elevated and modified low density lipoproteins (LDL); hypertension, and diabetes mellitus; genetic alterations; free radicals caused by cigarette smoking, elevated plasma homocysteine concentrations etc. The injury induces the endothelium to have procoagulant instead of anticoagulant properties and to form vasoactive molecules, cytokines, and growth factors. The inflammatory response stimulates migration and proliferation of smooth muscle cells (SMC) that become intermixed with the area of inflammation to form an intermediate lesion. If these responses continue unabated, they can thicken the artery wall, which compensates by gradual dilation, so that up to a point, the lumen remains unaltered, a phenomenon termed "remodeling" [Glagov S *et al*, 1987]. LDL, which may be modified by oxidation, glycation (in diabetes), aggregation, association with proteoglycans, or incorporation into immune complexes, is a major cause of injury to the endothelium and underlying smooth muscle [Steinberg D, 1997]. In response to the stimulatory modified LDL, endothelial cells express adhesion molecules and vascular smooth muscle cells (SMCs) release chemokines and chemoattractants [Simionescu M, 2007], leading to recruitment of monocytes and T cells into the arterial wall at specific sites where modified LDL deposits are located [Jonasson L *et al*, 1985].

Plaque inflammation plays an important role in the evolution of atherosclerotic lesions. Human atherosclerotic plaques consist of macrophages, activated T cells and, to a lesser extent, mast cells [Hansson GK *et al*, 1989]. Intra-plaque inflammation is a key factor in a process of plaque destabilization; plaque erosion, characterized by surface

denudation and exposure of the immediate subendothelial tissues, and plaque rupture, characterized by fissures often extending deep into the lipid core of the plaque, always co-localize with inflammation [van der Wal AC *et al*, 1998]. It thus appears that stable atherosclerotic lesions contain a scorching inflammation, which seems to be up-regulated once a clinically unstable condition appears.

I.2.2 Role of Monocytes in Atherosclerosis:

The recruitment of circulating monocytes into the arterial wall, followed by their differentiation into tissue macrophages is one of the earliest events in atherosclerotic plaque formation [Ross R, 1990; Swirski FK *et al*, 2007]. Human blood monocytes (MO) represent the immature precursor of the different types of macrophages (MAC), which are distributed ubiquitously in all tissues. They originate from bone marrow stem cells, circulate in the blood for up to 3 days, and finally migrate into the various tissues and body cavities where they mature into site-specific MAC under the influence of a special tissue microenvironment [Zucker-Franklin D *et al*, 1988]. Increasing evidence suggests that monocytes control smooth muscle cell (SMC) proliferation and migration, lipid metabolism, and inflammation within the vessel wall [Nozawa N *et al*, 2010]. Monocytes have thus been proposed to serve as markers, initiators, and promoters of arterial occlusive diseases [Yan ZQ & Hansson GK, 2007; Li AC & Glass CK, 2002].

Monocytes undergo phenotypic transformation, leading to their activation, even before they actively participate in the inflammatory process of atherosclerosis. CD14, which is monocyte endotoxin receptor-together with toll-like receptors (TLR)-bind lipopolysaccharides (LPS) evoking monocyte activation, and the interaction between leukocytes and endothelium results in an inflammatory cytokine cascade [Shantsila E& Lip GYH, 2009]. Activated monocytes may play a role in the pathogenesis of thrombosis via several pathways: they initiate the extrinsic pathway of coagulation via surface expression of tissue factor [Drake TA *et al*, 1989]. Moreover, activated monocytes rapidly show enhanced surface expression of the $ß_2$-integrin Mac-1. Mac-1 binds and converts factor X to Xa, leading to rapid fibrin formation. Mac-1 also binds to

intercellular adhesion molecule-1 (ICAM-1) on endothelial cells, facilitating tight adhesion as a prerequisite of monocyte trans-endothelial migration. In addition, activated monocytes release a variety of promoters of acute inflammatory response, such as metabolites of arachidonic acid, leukotrienes and interleukins. In patients with coronary heart disease, increased monocyte surface expression of both the adhesion molecule Mac-1 and tissue factor were found.

On being activated, monocytes modify their phenotype, thus enhancing their interaction with endothelial cells and ability to damage cardiac tissue. Increased monocyte expression of Mac-1 (CD11b/CD18) receptor, lymphocyte function associated antigen-1, and very late after activation antigen-4 promotes monocyte attachment to the endothelium. Monocyte associated levels of intercellular adhesion molecule (ICAM-1), vascular cell adhesion molecule (VCAM-1), and L-selectin are also elevated from the early stages of CAD, either with or without myocardial necrosis being present. Also, monocytes possess receptors to localize at sites of injured myocardium. The CAD-related upregulation of monocyte fibronectin receptor, VLA-5, may be involved in their migration to tissue fibronectin, the latter being an important component of cardiac extra cellular matrix [Trial J *et al*, 1999].

The infiltrated monocytes differentiate into macrophages, which uptake lipids and form lipid-laden foam cells in the unique microenvironment of the vascular intima, which is referred to as fatty streak lesions. Under the influence of various genetic and environmental risk factors, the early fatty streak lesion will progress into a complex lesion, typically characterized by a lipid-rich core covered by a fibrous cap, and a large number of activated inflammatory cells, particularly macrophages and T cells. Such atherosclerotic lesions will undergo a silent destructive process, if the inflammation cannot be controlled, eventually leading to plaque rupture and acute coronary syndromes [Farb A *et al*, 1996]. Monocytes are involved in the destabilization of atherosclerotic plaques by their production of MMPs. Macrophage/foam cells produce cytokines that

activate neighboring smooth muscle cells, resulting in extracellular matrix formation, fibrosis, and plaque instability.

Human monocytes are usually perceived as being a non proliferating cell type. However, recently it has been shown by different authors that a small percentage of human monocytes can enter the cell cycle in vitro in response to agents such as M-CSF and granulocyte cytokines and they are termed as proliferative monocytes (PM) [Clanchy FIL *et al,* 2006]. Based on the experimental data it has been proposed that they are found more predominantly in the CD14 positive population and they could enter sites of inflammation and contribute to the local macrophage proliferation, which has been observed clinically and in animal models of inflammation. Support for this concept comes from their finding that the more mature CD14low CD16positive subpopulation exhibited relatively reduced proliferative capacity. Assuming the PM is less mature than the bulk of the monocyte population, they are likely to have more potential to differentiate. In this context, there has been a deal of interest in the ability of $CD14^+$ human peripheral blood populations to be precursors of bone-resorbing osteoclasts [Shalhoub V *et al*, 2000], DC (reviewed in refs. [Banchereau J & Steinman RM, 1998; Banchereau J *et al*, 2000]) and even of nonhaemopoietic lineages, for example, smooth muscle cells, osteoblasts, and adipocytes [Zhao Y *et al*, 2003]. CFSE labeling enabled the sorting, culture, and morphologic characterization of the PM upon subsequent culture as distinctly spindle-shaped, delineating them by this criterion from the bulk of the $CD14^+$ monocytes. The delineation of the subsets lends itself to further work in elucidating the degree to which the subsets differ in other monocyte attributes such as response to lipopolysaccharide, motility, phagocytic ability, and differentiation potential.

I.2.3 Role of Smooth Muscle cells in Atherosclerosis:

Vascular smooth muscle cells (VSMCs) play an important role in angiogenesis, vessel maintenance, and the regulation of blood pressure. The appearance of SMCs in the intima is one of the early events in the pathogenesis of atherosclerosis [Ross R *et al,* 1977]. The phenotype of SMCs within atherosclerotic lesions differs from that of the

medial cells - that is, contractile and secretory SMCs. This difference is considered to be essential to the migration and proliferation of SMCs in the pathogenesis of atherosclerosis. These SMCs display a proinflammatory phenotype [Zeiffer U *et al,* 2004] and express genes sharing a similarity with proliferating stem cells. Current evidence indicates that bone marrow progenitor cells are a source of SMCs for transplant arteriopathy [Shimizu K *et al,* 2001], neointimal lesions of injured arteries [Han CI *et al,* 2001; Xu Y *et al,* 2004] and hypercholesterolemia induced atherosclerosis [Sata M *et al,* 2002]. In humans, circulating SMC progenitors have also been shown to exist [Simper D *et al,* 2002], and SMCs in transplant atherosclerosis are, at least in part, derived from progenitor cells [Caplice NM *et al,* 2003].

Tanaka *et al* reported that bone-marrow-cell involvement depends on the degree of arterial injury; for example, bone-marrow cells do not differentiate into mature SMCs within neointimal lesions of moderately injured arteries. Progenitor cells might migrate into the intima where they fuse with SMCs to form neo-SMCs, which might have a higher ability for proliferation. Researchers of SMCs have discovered that the normal ploidy of various fully differentiated SMCs in the vessel is tetraploid, which is related to induction of proliferation [Owens GK, 1989]. Fusion itself might also be a naturally occurring mechanism in the physiologic state, injury or atherogenesis. There are three possibilities for smooth muscle cells appearing in the intima. First, circulating SMC progenitors together with blood mononuclear cells attach to neo-endothelial cells and migrate into the intima when endothelial cells are replaced. Second, EPCs replacing dead endothelium might have an ability to differentiate into SMCs, as $CD34^{+}$ progenitors could differentiate into SMCs [Yeh ET *et al,* 2003]. Finally, adventitial and medial progenitor cells can be a direct source of SMCs within the early lesions [Torsney E *et al,* 2005]. The molecular mechanism of mobilization, homing and differentiation of putative smooth muscle progenitors remained to be clarified.

I.2.4 Endothelial Dysfunction and Progression of CAD:

The vascular network of fully grown adults is a dynamic organ with an estimated surface area of >1000m^2. The structural and functional integrity of this network is maintained by continuous renewal of the endothelial cell layer, with a low basal replication rate of 0·1% per day [Hunting CB *et al*, 2005]. The endothelium, a thin monolayer of cells covering the inside of both arteries and veins, has emerged as one of the pivotal regulators of hemostasis through its ability to express anticoagulant and vasodilatory molecules in health and, in disease conditions, to release vasoconstrictors and to express procoagulant and cell adhesion molecules and cytokines [Gresele P *et al*, 2010]. A non-thrombogenic endothelial surface is maintained through a number of mechanisms, including the production of thrombomodulin (TM), an activator of anticoagulant protein C, the expression of heparan and dermatan sulphate, which accelerate the thrombin-inhibitory activity of antithrombin III and of heparin cofactor II, the constitutive expression of tissue factor pathway inhibitor (TFPI), an inhibitor of tissue factor, and the local production of tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), the main effectors of physiologic fibrinolysis. The synthesis of prostacyclin (PGI_2) and of nitric oxide (NO) is a crucial event for the antithrombotic activities of endothelium [Gresele P, 2010]. NO is a powerful vasodilatory agent, but is also a strong antiplatelet substance, an inhibitor of leukocyte adhesion and activation and a suppressor of smooth muscle cell proliferation. PGI_2 is a vasodilator, an inhibitor of platelet activation and a suppressor of leukocyte adhesion and activation [Loscalzo J *et al*, 2008]. Upon activation, endothelial cells respond with an increased surface expression of cell adhesion molecules (such as P- or E-selectin, ICAM-1 or VCAM-1) that promote the adhesion and activation of leukocytes, an event that initiates and amplifies inflammation and contributes to thrombosis. Activated leukocytes, in particular monocytes, express tissue factor (TF), a strong trigger of blood clotting [Gresele P *et al*, 2010]. Endothelial dysfunction is associated with an increased oxidative stress and with inflammatory changes that play a role in the development of

atherosclerosis in the early stages, while later they increase the vulnerability of fully developed plaques facilitating their rupture [Verma S *et al*, 2002].

I.2.5 Vascular Regeneration:

The maintenance of the endothelial monolayer may prevent thrombotic complications and atherosclerotic lesion development [Urbich C & Dimmeler S, 2004]. The regeneration of injured endothelium has been attributed to the migration and proliferation of neighbouring endothelial cells. A rapid regeneration of the endothelial monolayer may prevent vascular disease development by endothelial synthesis of antiproliferative mediators such as nitric oxide. Whereas the regeneration of the endothelium by EPCs protects lesion formation, bone marrow–derived stem/progenitor cells may also contribute to plaque angiogenesis, thereby potentially facilitating plaque instability [Hu Y *et al,* 2003].

The finding that bone marrow–derived cells can home to sites of ischemia and express endothelial marker proteins has challenged the use of isolated hematopoietic stem cells or EPCs for therapeutic vasculogenesis. Infusion of various distinct cell types either isolated from the bone marrow or by ex vivo cultivation was shown to augment capillary density and neovascularization of ischemic tissue. In animal models of myocardial infarction, the injection of *ex vivo* expanded EPCs or stem and progenitor cells significantly improved blood flow and cardiac function and reduced left ventricular scarring [Kawamoto A *et al,* 2001; Kocher AA *et al*, 2001]. The initial step of homing of progenitor cells to ischemic tissue involves adhesion of progenitor cells to endothelial cells activated by cytokines and ischemia and the transmigration of the progenitor cells through the endothelial cell monolayer [Vajkoczy P *et al,* 2003]. Integrins are known to mediate the adhesion of various cells including hematopoietic stem cells and leukocytes to extracellular matrix proteins and to endothelial cells [Springer TA, 1994; Carlos TM *et al,* 1994; Muller WA, 2002]. The homing of progenitor cells to different tissues is dependent on distinct adhesion molecules [Scott LM *et al,* 2003].

I.2.6 Role of platelets in CAD:

Atherosclerosis is a chronic inflammatory disease. However, the contribution of platelets to the process of atherosclerosis was unclear until this millennium. Currently, there is conclusive evidence produced by many authors for the involvement of platelets in inflammatory process of vascular disease development. A growing body of evidence indicates that platelets play a main part in inflammation. Activated platelets interact with various cell types at the vascular wall. During these cellular interactions, which involve direct receptor interactions as well as autocrine and paracrine pathways, platelets and their respective cellular counterpart activate each other in a mutual and vicious circle-like fashion. These processes lead to multiple inflammatory processes, including atherosclerosis, restenosis, thrombosis, and coagulation. Platelet activation is a common feature in inflammatory diseases and occurs in cardiovascular pathologies, such as unstable angina or acute myocardial infarction. In addition, platelets can actively initiate the development of severe cardiovascular complications, such as unstable angina, acute myocardial infarction, or stent thrombosis, and influence the outcome of cardiovascular interventions, such as percutaneous interventions or bypass surgery. Consequently, effective platelet inhibition reduces major adverse cardiovascular events in acute cardiovascular syndromes and cardiovascular interventions.

Platelets contain various compartments, such as 3 different granules (α-granules, lysosomes, dense core granules), and a complex membranous system that allows them to store and rapidly release a variety of factors, such as adhesion proteins (eg, fibrinogen, fibronectin, von Willebrand factor [vWF], thrombospondin, itronectin, P-selectin, and GP IIb/IIIa), growth factors (eg, PDGF, transforming growth factor [TGF]-α, EGF, bFGF), chemokines (RANTES, platelet factor 4 [PF4; CXC chemokine ligand 4, CXCL4], stromal cell-derived factor-1 [CXCL12, SDF-1], epithelial neutrophil-activating protein 78 [ENA-78; CXCL5]), cytokine-like factors (eg, IL1-β, CD40L, β-thromboglobulin),and coagulation factors (eg, factors V, XI, plasminogen activator inhibitor [PAI]-1, plasminogen, protein S). These proteins act in a concerted and fine-regulated manner,

influencing widely differing biologic functions, such as cell adhesion, cell aggregation, chemotaxis, cell survival and proliferation, coagulation, and proteolysis, all of which accelerate inflammatory processes and cell recruitment. Under inflammatory conditions, platelets can physically interact with other circulating cells such as leukocytes or progenitor cells by coaggregation within the blood stream or when adherent to the vascular wall. Once recruited to the vascular wall, platelets can attract circulating leukocytes or endothelial progenitor cells (EPCs) through mediators such as platelet activating factor (PAF), macrophage inflammatory protein (MIP-1), RANTES, or SDF-1.

I.2.7 Platelet-Endothelium Interaction:

Platelets usually do not interact with the intact vascular endothelium. Whereas the endothelium normally controls platelet reactivity through inhibitory and modulating mechanisms involving COX-2, PGI2, or prostanoid synthetic systems, inflamed endothelial cells develop properties that render them adhesive for platelets. During the adhesion process, platelets become activated and release an arsenal of potent inflammatory and mitogenic substances into the local microenvironment, thereby altering chemotactic, adhesive, and proteolytic properties of endothelial cells. For example, GP IIb/IIIa receptor engagement during platelet adhesion signals upregulation of CD62P and CD40L on platelets, resulting in CD40Ldependent endothelial activation. Platelets that adhere to the vessel wall at sites of endothelial-cell activation contribute to the development of chronic atherosclerotic lesions, and when these lesions rupture, they trigger the acute onset of arterial thrombosis [Dav G & Patrono, C *et al*, 2007].

The initial tethering of platelets at sites of vascular injury is mediated by glycoprotein Ib/V/IX, a structurally unique receptor complex expressed in megakaryocytes and platelets. Von Willebrand factor is the major ligand for one component of this complex, glycoprotein Ib, and the absence of the factor causes defects in primary hemostasis and coagulation [Mannucci, PM, 2004]. After the initial adhesion of platelets to the extracellular matrix, the repair process requires a rapid response to autocrine and paracrine mediators, including adenosine diphosphate (ADP), thrombin,

epinephrine, and thromboxane A2. These mediators amplify and sustain the initial platelet and they recruit circulating platelets from the flowing blood to form a growing hemostatic plug [Patrono, C *et al*, 2007]. Activated platelets can influence the progression of plaque formation by releasing adhesive ligands, such as P-selectin, that become expressed on the platelet membrane and mediate platelet–endothelium interactions [Ruggeri ZM, 2002]. Signaling by P-selectin stimulates monocytes and macrophages to produce chemoattractants or growth factors. Engagement by P-selectin of the P-selectin glycoprotein ligand 1 on the monocyte surface initiates the formation of platelet–monocyte aggregates and outside-in signaling that induces the transcription of COX-2 [Dixon DA *et al,* 2006]. Prolonged adhesion-dependent signaling promotes the expression of interleukin-1β.

I.2.8 Platelet -Leukocytes Interaction:

An increased association of activated platelets with leukocytes contributes to the pathophysiology of unstable angina, myocardial infarction, cardiopulmonary bypass, thrombosis, and sepsis. Leukocyte recruitment requires multistep adhesive and signalling events, including selectin-mediated attachment and rolling, leukocyte activation, integrin-mediated firm adhesion, and diapedesis, which result in the infiltration of inflammatory cells into the blood vessel wall. Activated platelets promote leukocyte arrest on the vascular endothelium, which is believed to be a key process in the development of atherosclerosis. Platelets physically interact with both leukocytes and with the vascular wall. This interaction can occur in variable sequences: first, platelets can coaggregate with leukocytes and thereby support leukocyte recruitment to the endothelium by activating leukocyte adhesion receptors, or by directly serving as bridging cells. For example, platelet- monocyte coaggregates can attach to the vascular endothelium by both platelet-endothelium or by monocyte-endothelium contacts. Second, when adhered to the endothelium, platelets can chemoattract leukocytes and then provide a sticky surface for their adhesion to the vascular wall. During these interactions involving platelets, leukocytes, and the endothelium, all cell types involved become activated in a cascade-

like manner. These cellular interactions are part of a fine-regulated and orchestrated activation cascade involving autocrine and paracrine pathways, as well as direct adhesion receptor interactions.

On adhesion or activation, platelets rapidly translocate P-selectin from α-granules to the plasma membrane. This allows leukocytes to tether to platelets via PSGL-1/P-selectin interaction. Subsequently, monocytes or polymorphonuclear cells firmly adhere to platelets in a Mac-1– dependent (CD11b/CD18) manner. On platelets, various counter receptors of Mac-1 have been identified: GP Ib junctional adhesion molecule-C (JAM-C, JAM-3), CD40L, ICAM-2 as well as bridging proteins, such as fibrinogen (bound to GP IIb/IIIa) or high molecular weight kininogen (bound to GP Ib). Nevertheless, the exact contribution of each receptor system awaits clarification.

During this adhesive process, receptor engagement of PSGL-1 and Mac-1, together with platelet-derived inflammatory compounds, induces complex activation cascades in monocytes. These activation processes involve the intracellular activation pathways, including NFkB activation, and promote monocyte or neutrophil adhesion (up regulation and activation of Mac-1 and VLA-4), thrombosis (monocyte secretion of tissue factor), monocytic chemokine and cytokine release (interleukin [IL]-1β, IL-8, MCP-1, tumor necrosis factor [TNF]-α) or the oxidative burst of neutrophils. In addition, engagement of PSGL-1 by P-selectin also drives translationally regulated expression of proteins, such as the urokinase receptor (uPAR), a critical surface protease receptor and regulator of integrin-mediated leukocyte adhesion *in vivo*. Additional adhesion receptor pairs appear to be involved and to signal inflammation.

Platelet-monocyte interactions have an important role in the procoagulant state typically seen in CAD [Shantsila E & Lip GYH, 2009]. Activated and de granulated platelets very rapidly form circulating aggregates with monocytes, the so-called monocyte-platelet aggregates (MPA). Microparticles (MP) are small (~0.1-1μm) membrane vesicles released from a variety of cells upon activation or death and are released from activated platelets as well [Garcia AB *et al,* 2005]. Microparticles contain

membrane, cytoplasmic and nuclear constituent characteristic of their precursor cells and differ in size and composition from other subcellular structures such as apoptotic bodies and exosomes [Distler, JHW, *et al,* 2005]. Platelet-derived MP increase the adhesive interactions between endothelial cells and both monocytes and monocytoid cells. MP-induced adhesiveness occurs via upregulation of monocyte and CD11a and CD11b and endothelial cell intracellular adhesion molecule–1 (ICAM-1) [Barry OP *et al,* 1998]. Furthermore, platelet MP increase cell chemotaxis and induce upregulation of CD14.

Circulating MPAs have been found to be a more sensitive marker of *in vivo* platelet activation than platelet surface P-selectin. Interactions with platelets stimulate monocyte expression of Mac-1, NFkB activation, and increased production of IL-1ß, IL-6, IL-8, TF; indeed, MCP-1 changes were suggested to be an important factor of microvascular reflow abnormalities after reperfusion. Enhanced generation of MPA is associated with high risk of future cardiovascular events.

I.2.9 Platelet-Endothelial Progenitor Cell Interactions:

Currently, one of the most challenging topics in atherosclerosis research is the investigation of the contribution of circulating endothelial progenitor cells. Although stem cell biology in general is not well-understood currently, and the exact role of endothelial progenitor cells (EPCs) for atherosclerosis in particular, there is a consensus that circulating EPCs derive from bone marrow, typically surface express CD34 or CD133 and have the capability to differentiate to endothelial cells and, therefore, to repair vascular damage. A variety of factors have the potential to mobilize EPCs from bone marrow, including SDF-1. A variety of physical or clinical conditions appear to influence the number and function of circulating EPCs, including exercise, statin use, age, smoking, diabetes, chronic heart failure, and acute coronary syndromes. Although EPCs can repair vascular damage by differentiation to an endothelial cell phenotype, they also may contribute to atheroprogression or restenosis, because they can also differentiate to smooth muscle cells or foam cells.

Platelets are the first cell type that attaches to the exposed subendothelium or altered endothelium and platelets can direct circulating EPCs to the site of arterial thrombi. Platelets were found to store SDF-1 in their alpha granules and to secrete this chemokine into the microenvironment on activation, which supports the recruitment of EPCs to surface of arterial thrombi *in vivo*. *In vivo*, antibodies against P-selectin and GP IIb inhibited the recruitment of $CD34^+$ bone marrow–derived progenitor cells to intra-arterial thrombi. Similar to platelet-leukocyte coaggregates (see above), platelets form coaggregates with circulating $CD34^+$ progenitor cells. Antibodies against P-selectin and GP IIb inhibited the recruitment of $CD34^+$ bone marrow–derived progenitor cells to intra-arterial thrombi. Similar to platelet-leukocyte coaggregates, platelets form coaggregates with circulating $CD34^+$ progenitor cells.

Platelets do not only recruit and bind EPCs to the altered vascular wall, but also support the differentiation process. On one hand, platelets can induce EPC differentiation to cells with an endothelial phenotype and a typical surface receptor pattern. On the other hand, coincubation of $CD34^+$ progenitor cells with platelets for 5 to 10 days induced morphological changes in $CD34^+$ cells toward macrophages and foam cells. A key mechanism in this differentiation process is the phagocytosis of platelets within the first 24 hours. Surface-bound LDL on platelets appears to play a relevant role in this process. Up to 30% of the original cells showed a 3-fold increase in size (diameter approximately 25μm), round morphology, and high granularity.

I.2.10 Activation of Platelets and CAD Progression:

Both superficial and deep intimal injury disrupts the intact endothelium, which normally prevents the adherence of platelets by the production of the antiplatelet agents, nitric oxide and prostacyclin. When disruption of the endothelium exposes collagen, adherence of platelets to the subendothelium takes place both directly and via von Willebrand factor and subsequently lead to platelet activation. Platelet adhesion is mediated by binding of platelet receptors to a number of arterial wall receptors, including

subendothelial collagen (whose corresponding platelet receptor is Gp Ia/IIa), von Willebrand factor (Gp Ib/IX and Gp IIb/III), and fibrinogen (Gp IIb/IIIa).

Binding of platelets to these structural proteins in concert with the action of soluble receptor-mediated stimulants, such as thrombin, adenosine diphosphate (ADP), and thromboxane A2 (TxA2), induces platelet activation. This process involves the mobilization of calcium from intracellular stores, the activation of several intracellular kinases, and the release of arachidonic acid from membrane phospholipids, resulting in the generation of TxA2. Platelet activation produced *in vivo* is enhanced by circulating catecholamines [Lauri D *et al*, 1985].

While thrombin and collagen are considered to be the strong physiological agonist, epinephrine (Epi) per se can also activate platelets [Hjemdahl P *et al,* 1994]. However, direct platelet activation by Epi is usually seen at supra physiological concentrations (micro molar range) and experimentally only under certain conditions. Lower, more physiological concentrations of Epi can sensitize platelets to other agonists, such as ADP *in vitro*, and Epi infusions causing high physiological levels of Epi in plasma also seem to activate platelets *in vivo*. Such activation of platelets by epi can result in platelet volume increase, enhanced serum thromboxane B formation, stimulation of platelet release, and enhanced platelet aggregation *in vivo*. Seemingly paradoxically, platelet activation *in vivo* may be accompanied by reduced platelet sensitivity to agonist stimulation *in vitro*. Thus, the picture emerges that Epi is a poor platelet activator per se but nevertheless has the ability to enhance the effects of other agonists *in vitro* or to act in concert with other phenomena *in vivo* to cause platelet aggregation.

The accumulation of thrombin at sites of vascular injury provides one of the major mechanisms of recruiting platelets into a hemostatic plug. Thrombin works by activation of the G protein-coupled protease activated receptors PAR1 and PAR4 on human platelets to initiate signaling cascades leading to increases in [Ca]i, secretion of autocrine activators, trafficking of adhesion molecules to the plasma membrane, and shape change, which all promote platelet aggregation. The thrombin receptors work in a

progressive manner, with PAR1 activated at low thrombin concentrations, and PAR4 recruited at higher thrombin concentrations [Brass LF, 2003].

At different phases of blood coagulation, thrombin is generated in an extremely wide range of concentrations, varying from picomolar and nanomolar amounts to the maximal level of 0·8–1·4 μmol/l activity of these thrombin concentrations correspond to 0·0001, 0·1 and 86–151 National Institutes of Health (NIH) units/ml, respectively, for thrombin with specific activity of 3000 NIH units/mg. Hence, during blood coagulation, platelets can be exposed to very low and very high thrombin concentrations, which may have different impact on platelet activation and apoptosis. Up-regulation of P-selectin (CD62P) is reported in human whole blood with 64 nmol/l thrombin. Primarily known as an inducer of blood coagulation and platelet activation, thrombin also triggers platelet apoptosis. Thrombin concentrations of 0·5–1 nmol/l activated almost all platelets, but only a small fraction underwent apoptosis, suggesting that at these relatively low thrombin concentrations, platelets may perform haemostasis but not be involved in programmed cell death. At high thrombin concentrations of 10–100 nmol/l, generated during blood coagulation, 30–40% of platelets became apoptotic, indicating that hypercoagulable states may be associated with increased numbers of apoptotic platelets. [Leytin V *et al,* 2007].

Upon activation, platelets release numerous platelet microparticles (PMP) from the plasma membrane and express activation markers. The interactions of activated platelets with leukocytes are believed to play an important role in ischemic reperfusion injury and other thrombotic conditions. CD62P expressed on activated platelets mediates adhesion of platelets to leukocytes, chiefly neutrophils, but little is known of the interaction of PMP with neutrophils.

Upon activation, platelets adhere to monocytes and neutrophils (PMN) through specific ligands, and this adhesion was shown to be mediated mainly through the granular membrane protein P-selectin (CD62P; formerly GMP-140 or PADGEM) expressed on the platelet surface after activation, and Lewis x (CD15) or sialyl Lewis x molecules on

the neutrophil or monocyte surface [Jy W *et al*, 1995]. Monocytes and neutrophils may also bind non-activated platelets, although with lower affinity and independently of expression of P-selectin. The majority of lymphocytes do not bind platelets. Platelet glycoprotein (GP) IIb/IIIa and fibrinogen appear to play only minor roles in platelet-leukocyte interaction.

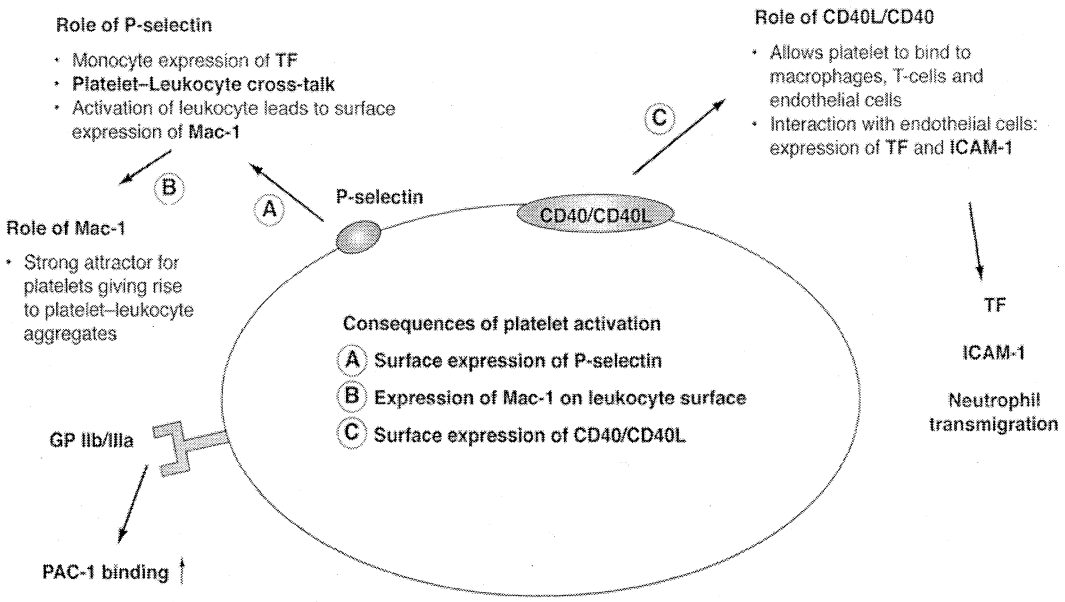

Fig.I.1 Cascade of events contributes to inflammation and thrombosis following platelet activation. (A) Following activation, degranulated platelets express P-selectin, enabling 'cross-talk' between platelets and leukocytes and resulting in leukocyte surface expression of integrins such as Mac-1. P-selectin expression on platelets also results in monocyte expression of tissue factor, and causes monocytes to release cytokines. (B) When Mac-1 is expressed on leukocytes, it is a strong attractor for platelets, giving rise to platelet–leukocyte aggregates. (C) Platelet activation also results in surface expression of CD40 and CD40 ligand (CD40L). CD40/CD40L interactions occur between platelets and the other main cell types involved in the pathophysiology of atherosclerosis (macrophages, T-cells and endothelial cells). TF, tissue factor; ICAM-1, intercellular adhesion molecule-1; Mac-1, leukocyte integrin (CD11b/CD18). PAC-1, antibody specific for activated form of the GP IIb/IIIa receptor. Bold font indicates key inflammatory markers/players.

I.2.11 Use of Antiplatelet agents:

Platelets play a major role in the clinical manifestations of ischemic heart disease and it is understood that activated platelets exert activated fibrinogen receptors (GP IIb/IIIa). Therefore, patients who are prone to CAD and who have hyper-reactive platelets with exaggerated adhesion, aggregation and thrombin generation are generally treated with anti-platelet drugs. The cheapest and most widely used agent is aspirin. Ticlopidine and clopidogrel are newer, and are recommended for patients with aspirin allergy or intolerance, and when clinical events arise despite ASA therapy. Clopidogrel is recommended over ticlopidine because it is associated with less serious side effects and provides superior benefit to aspirin in patients with vascular disease, such as stroke, myocardial infarction or peripheral arterial disease. This benefit of clopidogrel is enhanced in patients at higher risk, such as those with hyperlipidemia, diabetes, prior coronary bypass surgery and disease in multiple vascular beds. Aspirin and clopidogrel have a synergistic effect to provide better cardiovascular protection.

Since platelets play a key role in vascular inflammation, through release of their own pro-inflammatory mediators and interactions with other relevant cell types (endothelial cells, leukocytes, and smooth muscle cells), antiplatelet drugs may prevent many different cascades in atheroma progression. An increasing body of literature shows that inflammatory biomarkers can be used to predict atherothrombotic risk and that antiplatelet therapy may reduce the levels of these markers. Acetylsalicylic acid (ASA) has been attributed with reducing levels of the transcription factor nuclear factor κB (NF-κB), C-reactive protein, and soluble CD40 ligand, although the evidence relating to the latter two markers is conflicting. There is also substantial evidence that therapy with clopidogrel, a specific antagonist of the platelet P2Y12 ADP-receptor, also leads to reductions in serum levels of CD40 ligand, C-reactive protein, P-selectin, and platelet—leukocyte aggregate formation. Beneficial effects of clopidogrel on inflammatory markers have been demonstrated across the spectrum of atherothrombotic disease (acute coronary syndrome patients, patients undergoing percutaneous coronary intervention

(PCI), acute ischemic stroke patients, and those with peripheral arterial disease. Oral glycoprotein (GP) IIb/IIIa receptor antagonists, at doses that achieve moderate levels of receptor blockade, may paradoxically be associated with platelet-mediated pro-inflammatory effects. A similar phenomenon has been observed with intravenous GP IIb/IIIa antagonists *in vitro*, but most often at low doses, and data from clinical studies suggest that these agents may actually attenuate release of inflammatory mediators when administered at doses producing more complete receptor blockade.

I.2.12 Invasive Procedures to reduce CAD outcome:

I.2.12.1 Angioplasty:

Percutaneous transluminal coronary angioplasty (PTCA) or balloon angioplasty was introduced by Dr Andreas Gruentzig in1977 as a technique for the treatment of proximal, non-calcified, concentric lesions involving a single coronary Artery [Waller BF. 1989]With improvements in angioplasty equipment and techniques, the use of PTCA was expanded to more complex lesions and in patients with multivessel disease. In the mid-1980s and 1990s, the focus of investigation shifted towards the preferred method of revascularization — CABG vs PTCA. PTCA is catheter based and less invasive. But the major drawback is the occurrence of restenosis. Restenosis is defined as the stenosis of more than 50% of the luminal diameter.

Although coronary angioplasty immediately reduces angina, in about 8% of patients restenosis occurs requiring repeated angioplasty or bypass surgery in about 30%. Recently, several clinical trials have shown that the implantation of coronary stents or treatment with blockers of platelet glycoprotein IIb/IIIa receptors reduces the occurrence of acute complications and restenosis in patients undergoing coronary angioplasty. These new therapies have spread rapidly and have changed the practice of interventional cardiology remarkably since 1994. New coronary devices have expanded the clinical and anatomical indications for revascularization initially limited by balloon catheter angioplasty.

I.2.12.2 Coronary stenting & Restenosis:

First introduced in 1980's the coronary stent has been used to reduce the rate of arterial restenosis. Coronary-stent placement is a new technique in which a balloon-expandable, stainless-steel, slotted tube is implanted at the site of a coronary stenosis which provides a scaffold within the coronary arteries to treat acute vessel dissection and reduce the risk of restenosis. The implantation of coronary stents has become a major form of revascularization therapy for coronary artery disease [Goy JJ *et al*, 1998; Topol EJ *et al*, 1998; Windecker S *et al*, 2000]. Coronary stenting has been shown to improve initial success, reduce angiographic restenosis, and reduce the need for repeat revascularization compared with conventional balloon angioplasty (PTCA). Abrupt or threatened vessel closure after coronary angioplasty is associated with increased risk of myocardial infarction, emergency coronary artery bypass graft surgery and in-hospital death. When dissection or prolapse of dilated plaque into the lumen is unresponsive to additional or prolonged balloon catheter inflation, coronary stenting offers a nonsurgical mechanical means to rapidly restore stable vessel geometry and adequate coronary blood flow. In selected patients with acute myocardial infarction, primary stenting can be applied safely and effectively, resulting in a lower incidence of recurrent infarction and a significant reduction in the need for subsequent target-vessel revascularization compared with balloon angioplasty.

I.2.12.3 Restenosis:

Restenosis is characterized by the inflammatory response, smooth muscle cell proliferation and production of extra cellular matrix particularly collagen, with resultant stenosis and remodeling [Gershlick AH & Baron J, 1998]. Following blood vessel injury, the injured endothelium exposes the inner layer, internal elastic lamina to the blood flow. Platelets get adhered and activated at the injured vessel, SMC migrate through the internal elastic lamina and proliferate in the newly formed intimal layer. Inflammation and thrombus formation following vessel injury causes activation of various cellular components of blood which may lead to the release of various cytokines and growth

factors like interleukins, transforming growth factor; platelet derived growth factor etc. to the blood stream. Among them, IL-1, PDGF and TGF-β1 promote the proliferation of SMC [Jawien A *et al*, 1992; Otsuka G *et al*, 2006].

The collagen matrix constitutes a major portion of the vascular extra cellular matrix and imparts blood vessels with tensile strength and, even more important, modulates smooth muscle cell (SMC) responses via specific receptors and signaling pathways. Better understanding of the interactions of SMCs with the collagen matrix, how these interactions are involved in sensing the local environment and the receptors that mediate these processes promises to provide novel therapeutic targets and treatment strategies for the prevention of arterial occlusive diseases such as atherosclerosis and restenosis.

I.2.13 Summary:

The reviewed literature suggests that concerted interaction of platelets with vascular cells and circulating monocytes or progenitor cells may manipulate a vessel injury to inflammatory response and atherogenesis. Therefore, anti-platelet drugs are considered to be very important to prevent the progression of coronary artery disease and restenosis or if the regeneration of the injured vessel will take place. Platelets seem to play significant role in vascular regeneration after injury to endothelium also by directing the migration and proliferation of vascular cells and also by stimulating the circulating progenitors to home at the injured site and differentiated into endothelial cells & smooth muscle cells. A multitude of molecules released from activated platelets, membrane associated adhesive proteins and surface proteins on the debri of activated platelets etc are known to take part in the inflammatory response and/or vascular regeneration. Currently, many of the processes or mechanisms which lead to vascular disease or regeneration are less understood. There are sufficient data which suggest that circulating progenitors are influenced by platelets to result in the formation of macrophages in culture when monocytes and platelets or $CD34^+$ cells and platelets are co-cultured *in vitro*.

Thus it becomes very fascinating to know that platelets play multiple roles; in haemostasis, thrombosis, vascular regeneration and inflammation leading to cardiovascular diseases and restenosis. It is understood that these processes are triggered by activation of platelets. It is also understood very low levels of thrombin for initiating the platelet activation and subsequent changes on the platelet membrane exposes different receptors necessary for initiation and progression of various physiological and pathological events. Synergistic action of epinephrine also results in the accelerated expressions of various receptors on platelet membrane. Due to such dynamic and regulated functions of activated platelets it is often difficult to predict the conditions at which platelet activation might lead to inflammatory response and how vascular regeneration is up regulated.

I.2.14 Gap area:

It is now understood that the $CD14^+$ monocytes in circulation are proliferative and they are less mature than other subsets and based on the availability of stimulus cytokines like M-CSF, they can get differentiated into different cell types in culture. Co-culture of monocytes with platelets has been shown to induce differentiation of CD34 positive cells isolated from peripheral blood get differentiated into macrophages. However, it is not confirmed if the CD14 positive monocytes are included in the isolated CD34 positive cells. Because the co culture of $CD34^+$ cells were done with platelets, if the activated platelets or its sub fractions would influence the monocyte differentiation is less understood.

I.2.15 Hypothesis:

Mild activation of platelets with physiological agonists is sufficient for membrane shedding and formation of active sites on platelets for interaction with leukocytes and specifically the monocytes. Fate of circulating monocyte may be different depending on whether interaction of monocyte is between released platelet microparticles or platelet debri. It is possible that immobilized platelet debri may attract CD14 positive monocytes

to the injured site and they may get differentiated into macrophages and promote inflammatory response and atheroma progression. On the other hand platelet debri can act as a homing site for monocytes and get signals for their differentiation into endothelial cells and/or smooth muscle cells.

I.2.16 Objectives:

⇒ Activate washed platelets using physiologically achievable concentration of epinephrine and sub threshold concentrations of thrombin

⇒ Using activation specific platelet markers and leukocyte markers, identify:

- Platelet-leukocyte interaction
- Platelet-monocyte interaction

⇒ Isolate and label sub cellular fractions from activated platelets and study:

- binding of platelet membrane particles to monocytes
- binding of platelet debri to monocytes
- binding of platelet membrane to CD34 cells
- binding of platelet debri to CD34 cells

⇒ Isolate the circulating monocyte fraction from buffycoat using density gradient centrifugation

⇒ Culture the isolated monocyte fraction in presence of platelet fractions and characterize the cells by

- Morphology
- Cytochemical staining

⇒ Analyze the data and identify the possible effects of various fractions of platelets on generation of macrophage, endothelial cells and smooth muscle cells in the culture.

CHAPTER II

MATERIALS AND METHODS

II.1 Study of platelet-leukocyte interaction

II.1.1 Preparation of Washed Platelets:

Blood was collected from human volunteers after informed consent and mixed with the anticoagulant, acid citrate dextrose (ACD) at a ratio of 8.5:1.5. The blood sample was centrifuged at 116 g for 20 minutes using a table-top centrifuge (*Heraeus Labofuge 300, UK)*. Platelet rich plasma (PRP) was aspirated along with the white blood cells using a Pasteur pipette into a fresh tube. One part ACD was added to nine parts PRP (by volume) and mixed gently. The cells were pelleted by spinning at 726 g for 10 minutes and the supernatant was discarded. The pellet was resuspended in one part ACD and nine parts Tyrode's buffer. The platelet suspension was centrifuged at 726 g for 10 minutes and the supernatant was discarded. The pellet obtained was resuspended in one part ACD, nine parts Tyrode's buffer and $CaCl_2$ was added to get a final concentration of 5 mM.

II.1.2 Agonist-induced activation of platelets:

Washed platelets in Ca-Tyrode's buffer were incubated for 30 min before they were activated using agonists. Stock solution of (-)-epinephrine (*Sigma chemicals, USA*) was added into platelet-leukocyte suspension to get a final concentration of 10nM. The cells were incubated for 10 minutes at 37°C in incubator shaker (*Kuhner Shaker, Switzerland)*. A 100 µl sample was collected for labelling with selected fluorochrome conjugated antibodies to identify platelets/leukocytes/monocytes and analyzed using flow cytometer (*Beckman Coulter Epics XL, USA*).

To the 10 nM (-)-epinephrine-activated cell suspension, various volumes of stock solution of thrombin was added to achieve 0.01 IU, 0.02 IU, 0.04 IU, 0.1 IU, 0.2 IU and 0.4 IU concentrations and a sample (100 µl) was collected immediately for labelling with selected markers and analysis. The remaining cell suspensions were incubated at 37°C, with agitation at a speed of 75 rpm using incubator shaker (*Kuhner Shaker, Switzerland)*. Samples (100 µl) were collected at different time intervals (0 min, 10min, 30 min and 1 h) for labelling and analysis.

II.1.3 Labelling of cells with specific markers:

Resting platelets and those stimulated as described above were taken and diluted (1:25) using ACD-PBS. To 100 µl of cell suspension, 2 µL of fluorescent labelled antibodies [CD62-PE CD45-FITC (*Beckman Coulter, USA*), CD14 FITC (*Millipore, California*)] were added. The samples were kept at dark for 1h and were made up to 1 mL using 650 µL phosphate buffered saline (PBS) and 250 µL, 1% paraformaldehyde and kept under dark till the analysis was done.

II.1.4 Analysis of cell suspension using flow cytometer:

Analysis was done using a single laser (488 nm) 3-color flow cytometer (Epics XL Beckman coulter, USA). Unlabelled resting platelets were used for adjusting the gate position based on FSC and SSC. Protocol was developed for analysis of $CD62^+$ platelets, $CD45^+$ leukocytes and dual labelled $CD62^+/CD45^+$ cells in one set of experiments. The position of gate was adjusted in control to include almost all leukocytes and <0.1% positive in all three quadrants (Q1 [$CD62^+$], Q2 [$CD62^+/CD45^+$] and Q4 [$CD45^+$] signals). For the next set, identification of monocytes was done using monoclonal antibody against CD14 instead of CD45 in the former. As the target population was monocyte, gate position was adjusted to get almost all monocytes within the gate. Study was repeated using samples isolated from at least 4 donors. Samples were acquired and analyzed for 60 s for analysis of each samples. Percentage of positive cells in each quadrant was noted for comparison between different experimental conditions used and for determining statistical significance.

II.2 Preparation of Platelet fragments

II.2.1 Isolation of Platelets & Activation:

One unit of platelet concentrate (PC) isolated during component preparation was used for each batch of isolation of platelet membrane particles (PMP), platelet debri (PD) and platelet releasate (PR). A total cell count of PC was taken using hematology analyzer (*Sysmex K4500, Japan)* to estimate the recovery of all three fractions from a known number of platelets. Platelets were washed as described in

II.1.1 and the final pellet was resuspended in 5 mL of ACD-Tyrode's buffer and $CaCl_2$ was added to get a final concentration of 5 mM. Platelet number was analyzed using hematology analyzer (*Sysmex K4500, Japan).* Platelets were activated using the method used in *II.1.2.* Concentration of epinephrine was 10nM and the suspension was divided into two parts and to one, 0.04 IU thrombin and to the other, 0.4 IU thrombin were added and left at 37°C to activate platelets. Platelet fragments were prepared from 3 separate donors.

II.2.2 Separation of platelet fragments:

The activated platelet suspensions were layered over 27% sucrose containing 0.02% sodium azide and were centrifuged at 63000 *g* for 3 h at 4°C using the ultracentrifuge (*Beckman Coulter Optima™ L-90K Ultracentrifuge, USA).* The releasate (supernatant) and PMP (the white layer at the interface) were collected. The sucrose solution was aspirated and discarded. The cell debri was resuspended in 1 mL Tyrode's buffer. The releasate was filtered and stored at -40°C in small aliquots. To the PMP and cell debri, Hank's Balanced Salt Solution (HBSS) containing antibiotics (*GIBCO, USA*) was added and centrifuged at 100000 g for 1 h at 4°C using the ultracentrifuge *(Beckman Coulter Optima™ L-90K Ultracentrifuge, USA).* The pellets were resuspended in HBSS containing 1X antibiotics, aliquoted and stored at -40°C.

II.2.3 Estimation of Protein:

The concentrations of protein present in the samples prepared as per the procedures described in *II.2.1* and *II.2.2* were determined using Lowry's method [Lowry OH *et al*, 1951]. The samples were diluted using 1X PBS. 100 µL of the diluted samples were taken in 2 mL eppendorf tubes. 1 mL Reagent C was added to the samples and incubated for 10 minutes at room temperature. Then 0.1 mL Reagent D was added to the samples and kept at room temperature for 30 minutes. Absorbance at 600 nm was taken using UV-Visible Spectrophotometer (*Hewlett Packard Diode array 8453, Germany*). (The preparation of Reagent C and Reagent D were described in the APPENDIX).

Data from three different preparations were compiled, estimated the yield and normalized for 10^{10} platelets and compared the yield in all isolations. Comparison was also done to identify if the thrombin concentration used for platelet activation had any effect on the yield of PMP, PD and PR.

II.2.4 Gradient Gel Electrophoresis:

Analysis of the PMP, PD and PR obtained after the centrifugations were analyzed using Lammeli's method (non-reduced) on 6% to 12% gradient gel prepared using gradient gel mixer (*Bio-Rad, USA*). The samples were prepared in 1X gel loading buffer (*Sigma Chemicals, USA*). The proteins were separated using SDS-polyacrylamide gel electrophoresis. The gel loaded with higher concentration of protein was stained with Coomassie Brilliant Blue R 250 (*Sigma Chemicals, USA*); the one loaded with lower concentration was developed using silver staining [Morrissey JH, 1981]. Differences in protein molecular weight profile between PMP and PD were analyzed.

II.3 Study of platelet fragment-monocyte interaction

II.3.1 Labelling of PMP and PD with specific marker:

Isolated (*II.2.2*) platelet fragments (300 µg PMP or 300 µg PD) were mixed with 5 µl CD62-PE antibody and were incubated overnight at 4°C. The excess antibody was washed off by centrifugation at 100000 g for 1 h at 4°C. The platelet fractions were resuspended in 100 µL Ca^{2+}/Mg^{2+}-free HBSS and stored at -20°C.

II.3.2 Isolation of peripheral blood mononuclear cells (PBMNC):

Discarded buffy coat which is a by-product from component separation was obtained from the SCTIMST blood bank and transferred to two 50 mL tubes (*Nunc, Sweden*) and diluted using ACD-HBSS (1:10). PBMNC isolation and culture were done as described earlier (Asahara, *et al*, 1997 and Sreerekha & Krishnan, 2006). The samples were centrifuged at 1216 g for 10 minutes (*Contifuge Stratos, Heraeus, UK*). The thick white layer (buffy coat) in the interface was collected which is a mixture of leukocytes, platelets contaminated with lots of RBCs. Buffy coat was diluted to make

15 ml cell suspension using ACD-HBSS and was layered over two 15 ml tubes containing 7.5 ml Histopaque-1077 (*Sigma-Aldrich, Germany*) in each and the density gradient prepared were centrifuged at 400 *g* for 30 minutes. The thick white layer (PBMNCs) in the interface was collected and diluted using ACD-HBSS. The cell suspension was centrifuged at 150 *g* for 10 minutes at 4°C. The supernatant containing mainly platelets was discarded and the pellet was resuspended in ACD-HBSS for studying the monocyte interaction with platelet fragments.

II.3.3 Analysis of PMP/PD binding to monocytes:

Two 100 µL monocyte suspensions ($1x10^5$ cells) were taken and to one, 10 µL CD62 labelled PMP (30µg) and to the other CD62 labelled PD was added and the cells were incubated for 1 h at 37°C. 100 µL of monocyte suspension was used as the control. To label the monocytes/circulating progenitors, FITC-conjugated-CD14 (*Millipore, California*) and APC-conjugated CD34 (*Santa Cruz Biotec, USA)* were added. The cells with platelet fragments were incubated for 1 h. The cells were fixed using 1% paraformaldehyde and made up to 1 mL using HBSS. The data were recorded using fluorescent activated cell sorter (*FACS-Aria, BD Biosciences, USA*).

II.3.4 Instrument set up and Data collection:

Flow cytometric analysis was carried out on a FACS Aria flow cytometer (BD sciences, USA). In the case of dual stained samples, colour compensation was done for PE and FITC using single stained cells. Twenty thousand events were acquired for each assay. To include cells that may have changed granularity after culture, SSC was extended from the normal monocyte cluster in all analysis and gate position was fixed for all assays in both experiments. By the processing technique adopted here, RBCs and platelets were removed during the monocyte isolation process to rule out the chances of interference during analysis.

II.4 Cell Culture

II.4.1 Preparation of culture substrates:

Sub cellular fractions of platelets (PMP/PD/PR) were immobilized on tissue culture polystyrene using fibrin as the matrix. Cryoprecipitate isolated from human plasma was made up in sterile distilled water to adjust concentration to 2 mg/ml fibrinogen, ~ 0.2 mg/ml fibronectin and it contains plasminogen and FXIII (quantity not estimated). For making fibrin composite matrix [denoted as FG in this study], the cryo precipitated fibrinogen concentrate (1 ml) was added with gelatin (0.2%). Other components included with FG were PMP (specified concentrations) [to make FG-PMP] and PD (specified) to make [FG-PD] or both PMP & PD [to make FG-PMP-PD] into 1 ml of fibrin composite. Tissue culture polystyrene (TCPS) (NUNC, Rakslide, Denmark) was coated with basic composition of FG using an established procedure [Prasad CK & Krishnan LK, 2005] with modified compositions included with platelets fragments. Briefly, thrombin was adsorbed on to tissue culture polystyrene (TCPS) by incubating the culture surface with 5 IU per ml solution in 5 mM $CaCl_2$ for 30 min at 37°C. Excess thrombin was aspirated out and fibrinogen composite containing different compositions was layered on the surface and allowed to clot for 30 min at 37°C. Coated dishes were lyophilized under sterile atmosphere, in a freeze drier (*Edwards, Modulyo 4K, UK*). The matrices were then stored at 4-6°C in a refrigerator till PBMNC seeding was done.

II.4.2 Culture of PBMNC:

Peripheral blood mononuclear fraction was isolated as per the method described in *II.3.2*. Washed PBMNC fraction was resuspended in serum containing DMEM/F-12 medium (*GIBCO, USA*) and the cell suspension was added to the uncoated single-well plates. After seeding for 24 h on bare polystyrene, the supernatant was removed along with cell suspension. The lyophilized substrates in four-well plates (*II.4.1*) were washed with HBSS. A layer of settled but unattached cells from the bottom of the culture well was collected in fresh complete medium and plated in specific substrates in 4-well culture plates and were kept at 37°C, 5% CO_2 incubator (*ThermoForma 3951, Hong Kong*). Medium change was done at 24 h

intervals until 72 h and afterwards on alternate days. Cell attachment, morphology changes and survival were monitored under a phase contrast microscope (*DM IRB, Leica, Wetzlar, Germany*).

Culture medium was supplemented with serum/plasma at different proportion and in some cases platelet releasate (PR) was added to the medium at defined concentrations. Cultures were analyzed at different intervals and terminated after a defined length (usually 9 or 12 days).

II.4.3 Immunocytochemistry:

Cultured cells were stained for CD14 FITC (*Millipore, California*), CD68 PE (*Santa Cruz Biotech, USA*), CD31 PE (*Santa Cruz Biotech, USA*) and α-SMA (*Novocastra Laboratories, USA*) antibodies and analyzed using fluorescent microscope (*Leica DM IRB, Germany).*

For staining with α-smooth muscle actin (α-SMA), the cells were fixed in 3.7% formaldehyde for 20 minutes. The fixed cells were permeabilised with 0.1% Triton X-100 (*Sigma-Aldrich, Germany*). Cells were washed with $Ca^{2+/}Mg^{2+}$ containing HBSS with 1 mg/mL glucose. Cells were stained for α-SMA. The nucleus was stained using the nuclear stain, Hoechst (*Sigma,USA*). The cells were then washed, air dried and viewed under confocal microscope (*Carl Zeiss 510 Meta, Germany*).

For other antibodies, cells were stained with CD14 FITC *(Millipore, California*), CD68 PE (*Santa Cruz Biotech, USA*), and CD31 PE (*Santa Cruz Biotech, USA).* Cells were washed and fixed in 3.7% formaldehyde for 20minutes. The cells were then washed, air dried and viewed under fluorescent microscope (*Leica DM IRB, Germany).*

CHAPTER III

RESULTS AND DISCUSSION

III. A. AGONIST-INDUCED PLATELET ACTIVATION

III.A.1. Platelet activation and interaction with leukocytes:

The objective of the first experiment was to identify if the physiologically relevant concentrations of the agonists could activate platelets with significant membrane changes to influence interaction of platelets with leukocytes. In the present study, platelets were activated using 10 nM epinephrine and different concentrations of thrombin. The interaction of activated platelets with leukocytes at different time periods was analyzed using flow cytometry. Representative dot plots are shown in Fig.III.A.1. The data showed that when 10 nM epinephrine was added followed by 0.01 IU thrombin, there was an immediate synergistic action which up regulated the expression of CD62 and increased binding with leukocytes. However, this effect was found to be reversible as seen in the data obtained by analysis of samples collected from different donors (Fig.III.A.2). As the concentration of thrombin increases to 0.04 IU the interaction becomes more stable until the study was completed i.e. 1 h.

The observation is significant because in response to inflammation and thrombosis, platelets and leukocytes interact through bidirectional signal pathways that enable trans-cellular cross talk and regulate effect or responses. Leukocytes can bind to activated platelets in blood leading to the formation of circulating platelet-leukocyte aggregates [Li N *et al*, 2000]. Initial leukocyte tethering and rolling is regulated by interaction between platelet P-selectin and leukocyte PSGL-1 [Diacovo TG *et al*, 1996]. P-selectin-PSGL-1 interactions have been shown to play an important role in directing incorporation of circulating leukocytes [Palabrica T *et al*, 1992] and tissue factor-containing leukocyte microparticles into developing thrombi [Falati S *et al*, 2003].

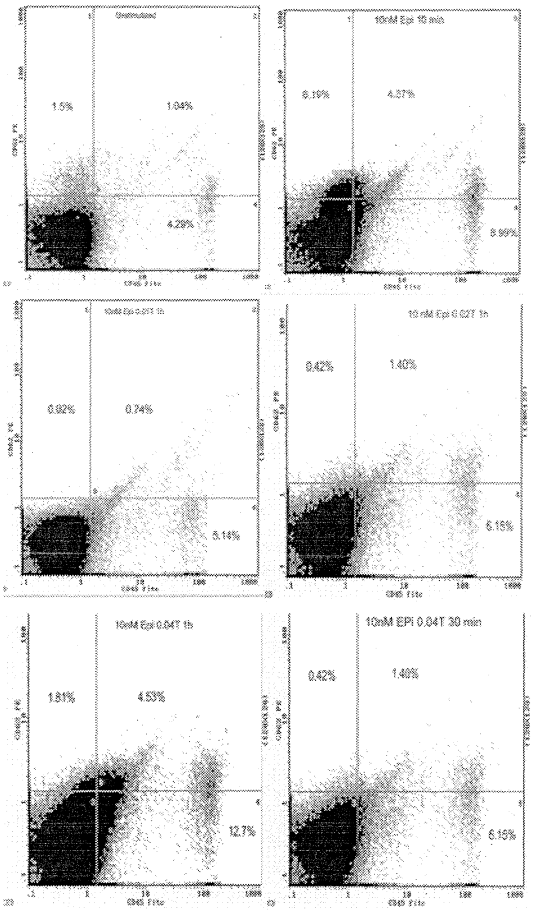

Fig.III.A.1 Dot plot representing the platelet activation and interaction with leukocytes: This flow cytometric data represents the platelet activation after adding 10 nM epinephrine and different concentrations of thrombin for a period of 1 h. The dot plot also shows the interaction of activated platelets with leukocytes.

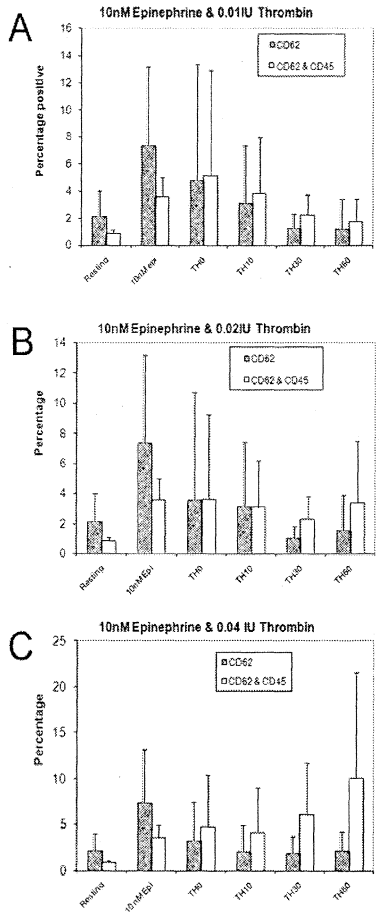

Fig.III.A.2 Graphical representation of platelet-leukocyte interaction: Platelets were activated using 10 nM epinephrine followed by different concentrations of thrombin and analyzed using flow cytometry. The graphs represent activation of platelets and their interaction with leukocytes at different time periods (A) after adding 0.01 IU thrombin; (B) after adding 0.02 IU thrombin; (C) after adding 0.04 IU thrombin.

III.A.2 Interaction of activated platelets with monocytes:

Monocytes are known to phagocytose activated platelets and get transformed to macrophages. Therefore it is important to understand the level of platelet activation

required for interaction with monocytes. Therefore, graded concentrations of agonists were used to identify the extent and stability of platelet-monocyte interaction. The platelet-monocyte interaction was high when the platelets were activated with 10 nM epinephrine alone. Along with 10 nM epinephrine, various concentrations of thrombin were used such as 0.01, 0.02, 0.04, 0.1, 0.2, and 0.4 IU. Representative dot plots are shown in Fig III.A.3.

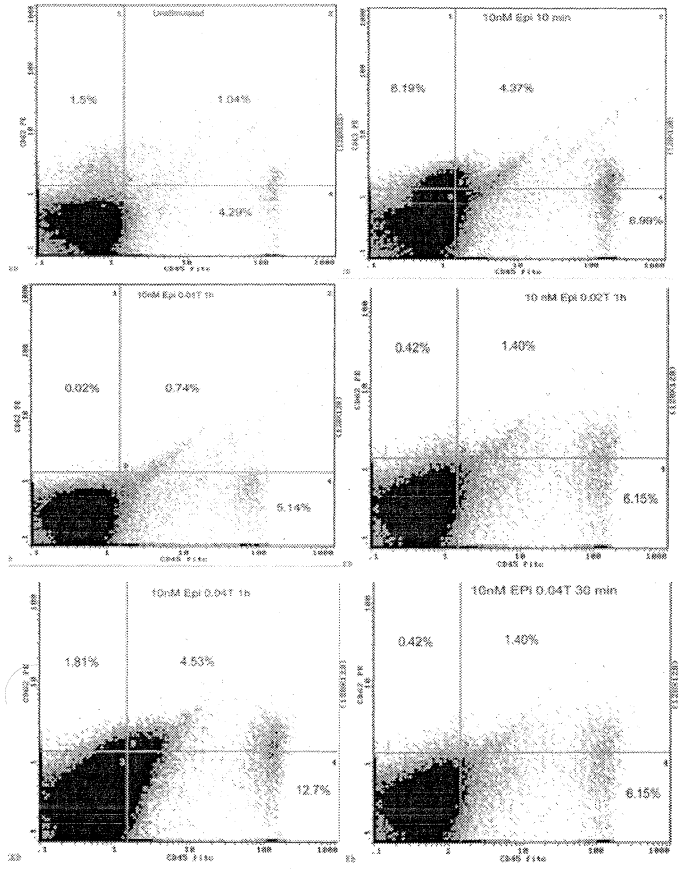

Fig III.A.3 Dot plot representing the platelet-monocyte interaction: This represents the expression of P-selectin after the activation of platelets using agonists (epinephrine and thrombin) and the interaction of activated platelets with monocytes.

First three concentrations have not made any significant increase in monocyte-platelet adhesion as compared to epinephrine alone (data of first 2 are not shown).

When the platelets were activated using 0.04 IU or 0.1 IU thrombin, the interaction of platelets and monocytes remained same as that when epinephrine alone was added and adhesion decreased with time, which indicated that the effect was reversible. But as the concentration of thrombin was increased to 0.2 IU adhesion of platelets to monocytes was increased only in 60 min of incubation. In the case of 0.4 IU thrombin, there is constant increase in the adhesion of platelets to monocytes with increase in time (60 min). The figure III.A.4 shows that platelets express more P-selectin during the first 10minutes after activation, irrespective of the concentration of thrombin, but after 10 min the stability of adhesion was dependent on the concentration of thrombin used for activation.

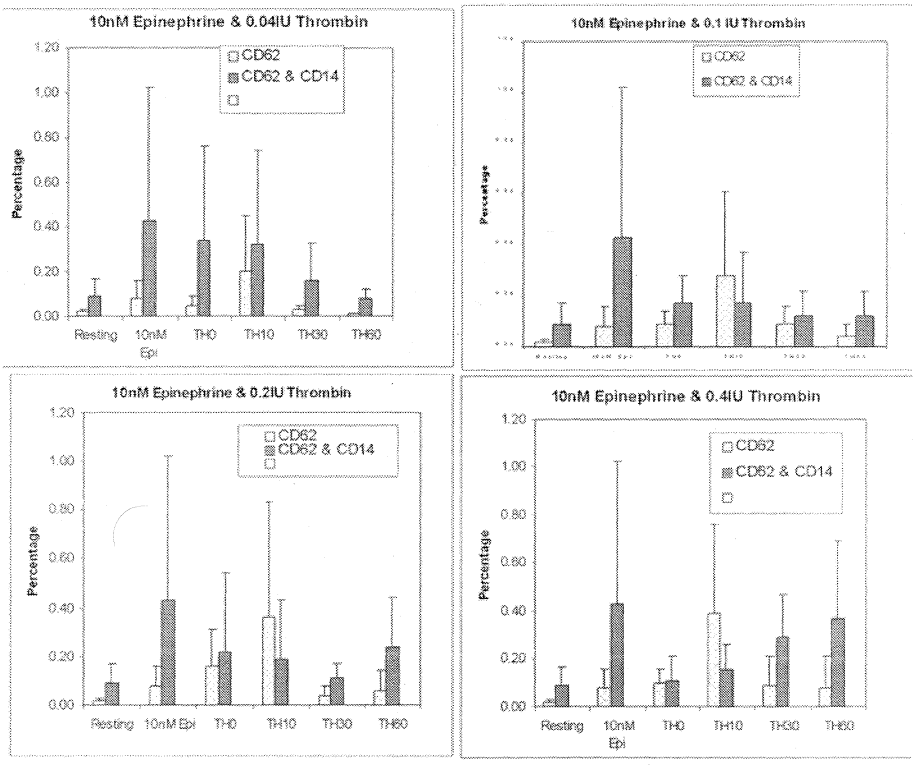

Fig III.A.4 Graphical representation of platelet-monocyte interaction: Platelets were activated with 10 nM epinephrine and different concentrations of thrombin and analyzed at different time intervals. The graph shows the expression of P-selectin on activated platelets and their interaction with monocytes during 0 min, 10 min, 30 min and 1 h.

It has been reported earlier that the P-selectin expressed on activated platelets is responsible for their interaction with monocytes which in turn enhances monocyte adhesion to endothelial cells under high shear conditions [Theilmeier G *et al*, 1999]. From the results of this study it is understood that even though thrombin is considered to be a strong agonist at very low concentration, the changes on platelet surface protein expression is reversible and may not cause any major influence on platelet-monocyte interaction. However, the physiologically relevant concentration of 0.4 IU is likely to activate platelets and cause platelets to monocyte adhesion.

In parallel to the above observation, activated platelets seem to have induced the expression of CD14 which may be seen in figure III.A.5. The CD14 expression increases significantly when 0.2 IU and 0.4 IU thrombin were used for the activation of platelets for 1 h. The observation is significant because the upregulation of CD14 on monocytes increases their survival (Heidenreich S, 1999). CD14 may operate as a multifunctional molecule standing in connection with a signal transducer that promotes survival by down-regulating apoptosis of monocytes.

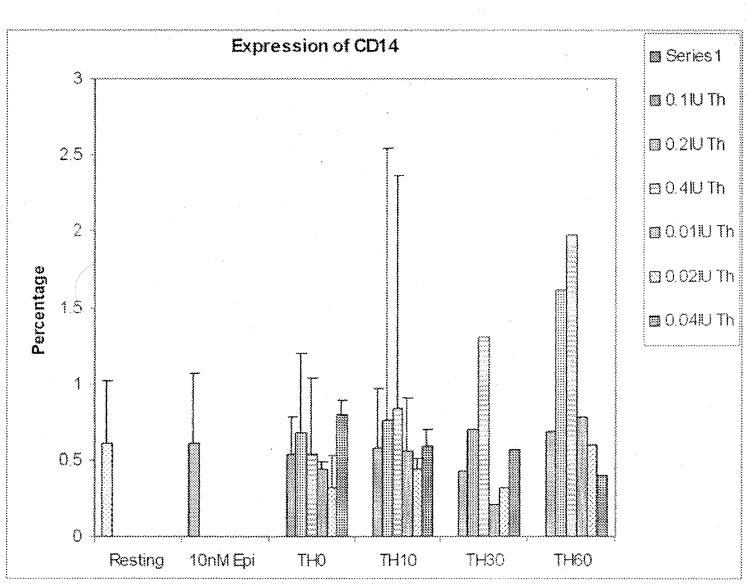

Fig III.A.5 Graphical representation of the expression of CD14 on monocytes: When the monocytes interact with activated platelets, the expression of CD14 increases with the increase in concentration of thrombin and also with time. The expression of CD14 was high when 0.4 IU thrombin was used for the activation of platelets for a period of 1 h.

III.A.3 Isolation & analysis of platelet sub cellular fractions:

Platelets from three different donors were activated using 10 nM epinephrine and 0.4 IU or 0.04 IU thrombin for 1 h at 37°C. The sub cellular fractions of activated platelets were separated by the sucrose gradient centrifugation. The platelet releasate, PMP and PD were collected (Fig.III.A.6). The PMP is present as a white layer between the releasate and the sucrose. In some cases, there was a thin narrow separation in the white layer.

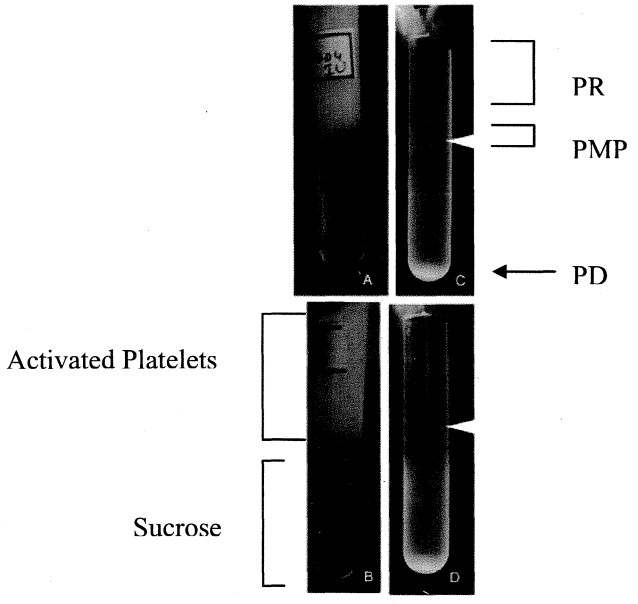

Fig III.A.6 Preparation of Platelet Sub cellular fragments: (A) and (B) represent the activated platelets (0.04 IU- and 0.4 IU- thrombin-activated platelets respectively) before centrifugation. (C) and (D) represent the sub cellular fragments of activated platelets obtained after density gradient centrifugation. PR – Platelet Releasate; PMP – Platelet Membrane Particle; PD – Platelet Debris.

III.A.3.1 Estimation of platelet fragment yield:

Platelet concentrate from three different donors were used for the preparation of platelet fragments. Total count of washed platelets used for the preparation of platelet fragments was comparable in all three donor samples ($1.53x10^{10}$, $1.32x10^{10}$ and$1.70x10^{10}$). Platelets were activated using two different concentrations of thrombin to induce release of stored granule contents and platelet membrane particles. The total

yield was normalized to get the yield per 10^{10} platelets using the starting number of washed platelets.

Table1: Yield of three platelet fragments (PR, PMP and PD) estimated by Lowry's protein assay of all three fractions.

Thrombin Concentration	Yield from $1x10^{10}$ platelets (mg)		
	PR	PMP	PD
0.4IU	1.62	0.13	13.81
0.04IU	1.63	0.06	20.11
0.4IU	1.27	0.06	19.74
0.04IU	2.13	0.10	18.8
0.4IU	1.02	0.24	12.89
0.04IU	1.14	0.19	11.28
Average	1.47	0.13	16.11
S.D	0.41	0.07	3.88

From the average S.D. given in the Table 1, it is clear that concentration of thrombin had no significant effect on the yield of any of the three fractions. Since one fold (10X) increase in thrombin concentration has not made effect on platelet release, it is apparent that mild physiological increase in thrombin concentration can induce significant platelet activation to release the granular contents and to shed the platelet membrane particles into the circulation. Such release is likely to have severe effects on the subsequent platelet-leukocyte/monocyte interaction and associated changes in the vascular wall.

If the PMP and PD released will remain in the circulation or will get adhered to the injured site is probably dependent on the extent of vascular injury, exposure of sub endothelial adhesion molecules and on the hemodynamic condition which determines the frequency and time period of contact between the PMP/PD and the injured site.

III.A.3.2 Analysis of Platelet fragments:

The proteins present in the sub cellular fractions of activated platelets were separated using 6% to 12% SDS polyacrylamide gradient gel electrophoresis and were silver stained. The concentration of proteins in all three lanes was 15 µg. The pattern of proteins in PR (lane 5), PD (lane 4) and PMP (lane 5) are shown in fig.III.A.7. Molecular weights were determined using *AlphaEaseFC* software. Lane 1 and lane 2 indicates the low molecular weight markers. Lane 3 shows four major bands present in the platelet membrane particle. The bands corresponding to 116 KDa and 94 KDa are likely to be the fibrinogen receptor (GPIIb/IIIa). Other bands seem to correspond to the collagen receptors. The lane 4 shows many different bands and some of the bands that appeared in PMP are also present in this lane. It is likely that either all platelets are not activated by the used agonist concentration or some membrane is retained on the activated platelets. The lane 5 shows low molecular weight proteins present in the platelet releasate, which corresponds to growth factors. The proteins on the membrane and the debris that interact with monocytes were not well studied. Therefore, this study focused on understanding if the PMP and PD show similar adhesion to the proliferating $CD14^{+}$ monocytes and $CD34^{+}$ circulating progenitors.

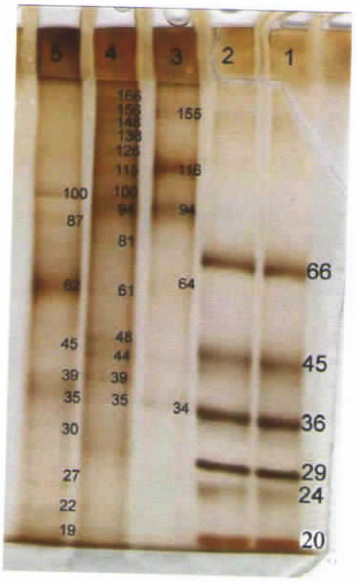

Fig III.A.7 SDS-PAGE pattern showing the proteins in platelet sub cellular fragments: Platelet sub-cellular fragments were prepared by activating the platelets using epinephrine and thrombin and centrifugation at 63000 g for 3 h at 4°C. The SDS-PAGE of the platelet sub-cellular fragments was done and silver stained. Lane 1 and lane 2 indicates the low molecular weight markers. Lane 3 has platelet microparticle (PMP). Lane 4 has platelet cell debris and lane 5 has platelet releasate from the same preparation.

III.A.4 Binding of PMP & PD to Monocytes:

The purpose of this experiment was to understand if the isolated platelet fractions (PMP and PD) can bind to the proliferating $CD14^+$ monocytes. Both washed PMP and PD were tagged with monoclonal antibodies against CD62. Excess antibodies were removed by washing the fragments at 100,000 g. The fragments were allowed to interact with isolated human monocytes tagged with CD14. Flow cytometric analysis was done to quantify the $CD14^+$ monocytes and the CD14/CD62 double positive aggregates of monocyte-PMP and monocyte-PD. The data obtained using isolated PBMNC from three separate donors is shown in Fig III.A.8 & III.A.9.

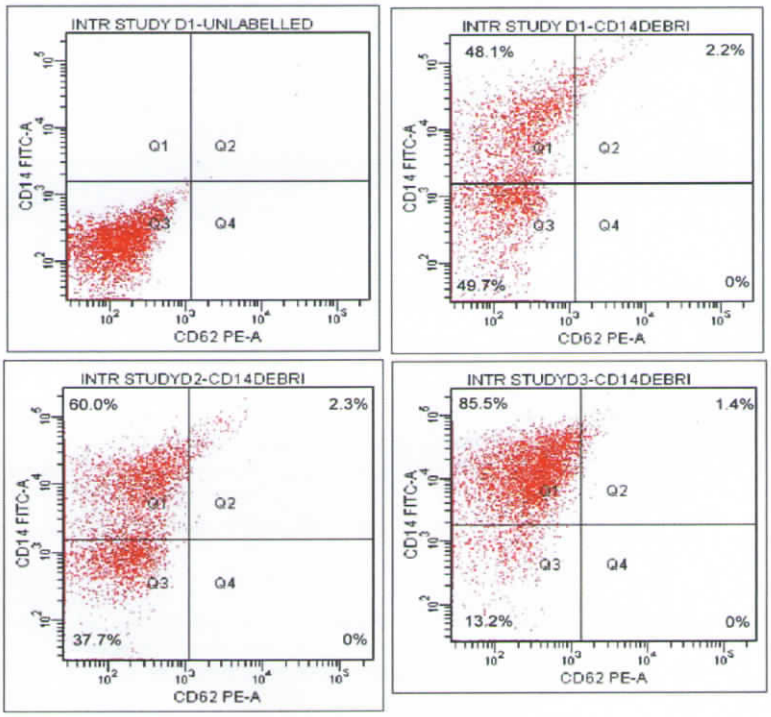

Fig III.A.8 Dot plot showing interaction of CD14 with PD: This represents the interaction of CD14 with PD in three different donors. About 2% of the $CD14^+$ cells interact with platelet debri.

It is obvious that both PD and PMP interact with $CD14^+$ monocytes. Even though 50% to 85% cells in the PBMNC isolates are $CD14^+$, only ~2% have interacted with PMP and PD. From this study there is no indication that any one of the isolated fragments interact more than the other with monocytes.

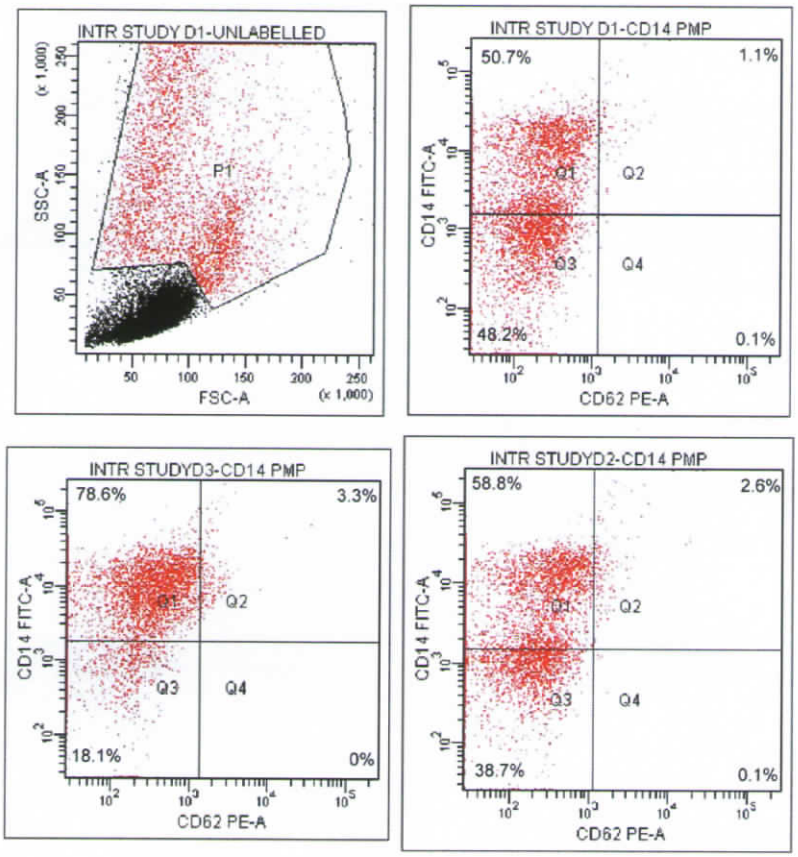

Fig III.A.9 Dot plot representing CD14-PMP interaction: This represents the interaction of CD14 with PMP in three different donors. ~2% of the $CD14^+$ cells interact with PMP.

It is not clear from the data if the monocytes that interact with platelet fragments are likely to be differentiated into macrophages in *in vitro* culture. However, in all the three experiments carried out with PBMNC isolated from three different donors, consistent results were seen.

III.A.5 Binding of PMP & PD with Circulating Progenitors:

There are reports in the literature that circulating $CD34^+$ progenitor cells which are considered to home to the injured vessel and differentiate into endothelial cells and smooth muscle cells and play a role in the vascular regeneration. It has been found that isolated $CD34^+$ cells get differentiated in to macrophages in presence of platelets in *in vitro* cultures. Therefore, the purpose of this study was to identify if

isolated fragments from activated platelets interact with $CD34^+$ progenitors in the PBMNC fraction. Study was done using CD62 tagged PMP/PD and $CD34^+$ cells to quantify dual positive cells. The data obtained using isolated PBMNC from three separate donors is shown in Fig III.A.10 & III.A. 11.

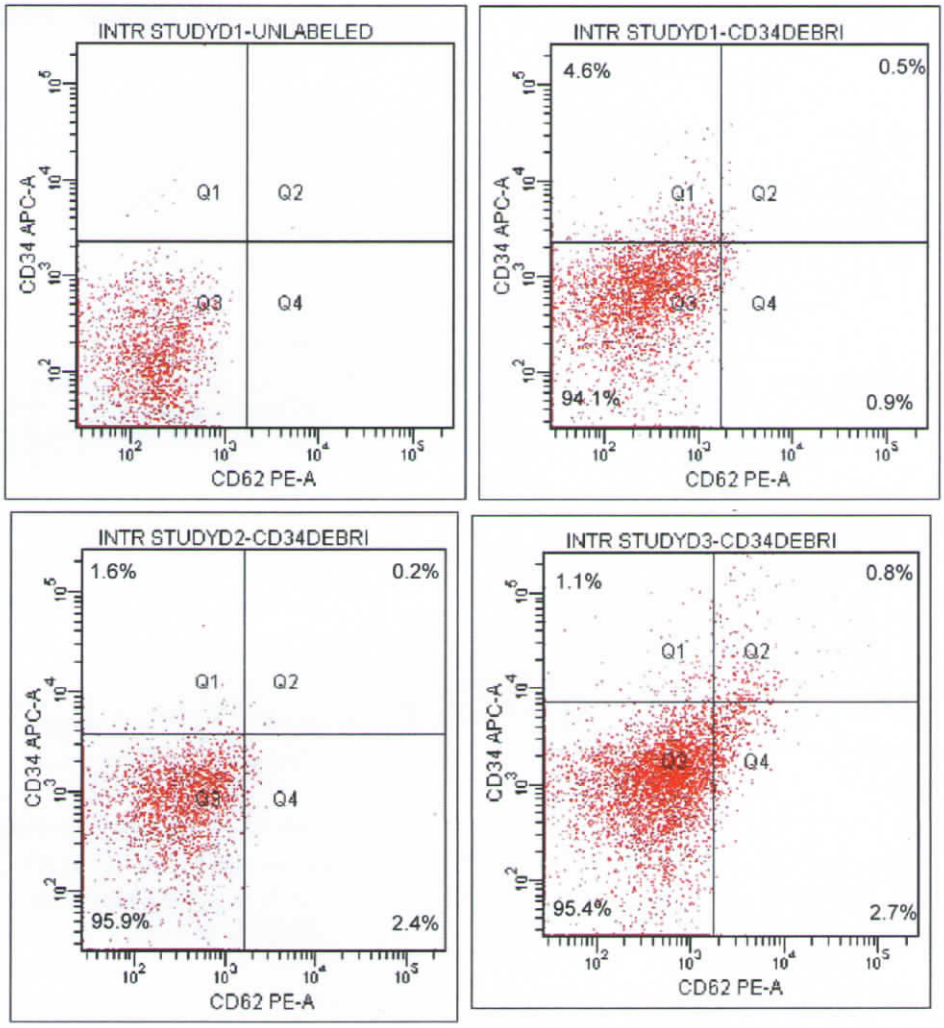

Fig III.A.10 Dot plot representing CD34-PD interaction: This represents the interaction of CD34 with PD in three different donors. About 50% to 80% of the $CD34^+$ cells interact with PD.

Only 1% to 4 % of the isolated PBMNC is positive for CD34, however, a major portion of the cells are found to interact with the PMP and the PD.

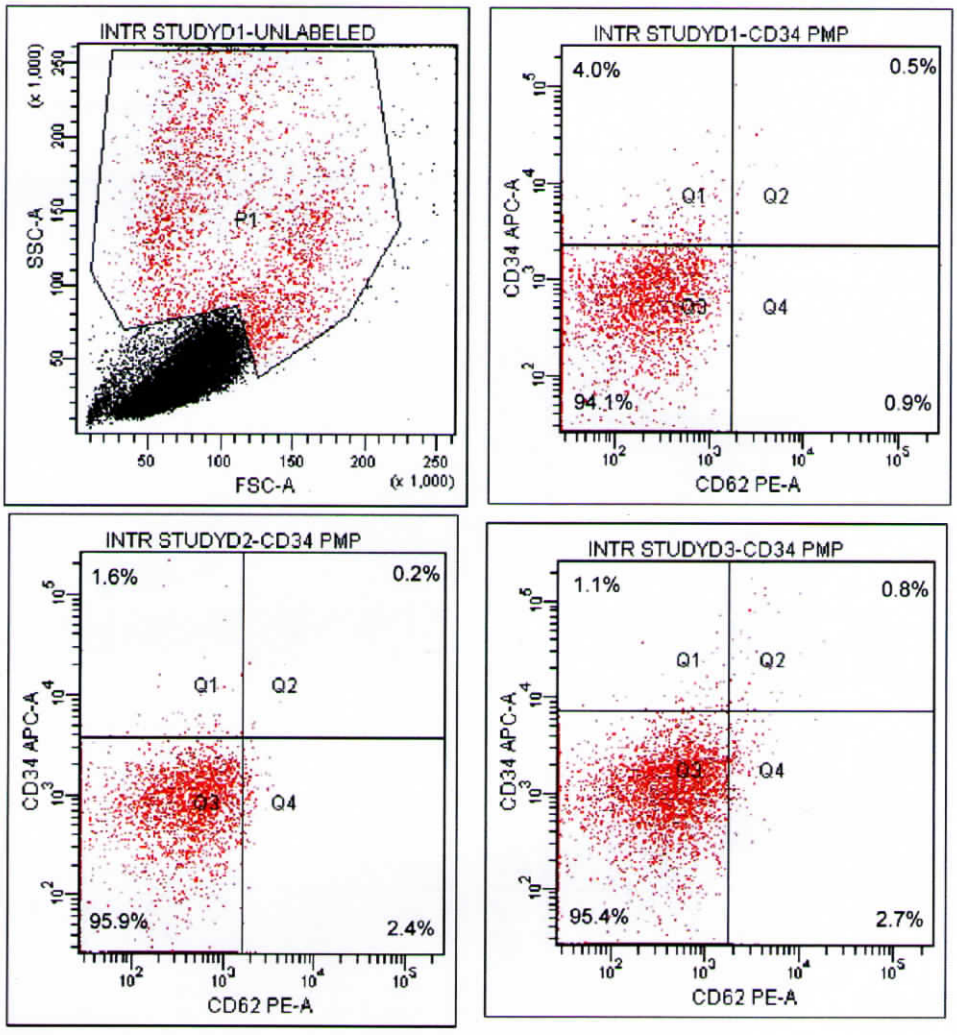

Fig III.A.11 Dot plot showing CD34-PMP interaction: This represents the interaction of CD34 with PMP in three different donors. About 50% to 80% of the $CD34^+$ cells interact with PMP.

III.A.6 Identification of PBMNCs with CD34 and CD14 expression:

It has been reported that the platelets can induce the differentiation of $CD34^+$ cells to macrophages. The present study was conducted to study whether the $CD34^+$ cells were positive for CD14 also. The study was done using monocytes tagged with monoclonal antibodies against CD14 and CD34. The analysis was done using flow cytometer. The data obtained using isolated PBMNC from three separate donors is

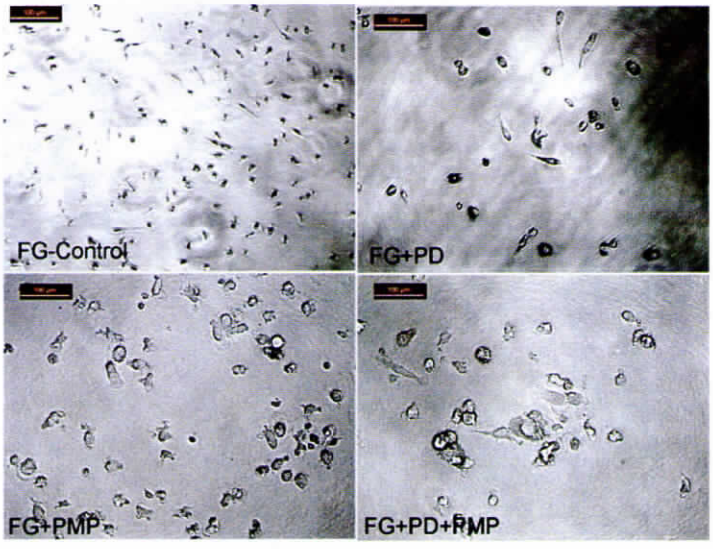

Fig.III.B.4 Phase contrast micrographs (10X) showing the effect of human serum (HS) after 120 h: Cells grown in HS show less cell density as compared to NBCS. But granulated cells were less and the cells were healthy.

Since the cultures in NBCS showed heterogeneous population with large and well spread cells, possibility of these cells being macrophages was verified using CD68 staining after a period of 216 h. Cells grown on all four substrates showed positively stained macrophage-like cells (Fig III.B.5). However, no cells grown in HS were found to be positive for CD68 on any of the four substrates (data not shown).

Fig.III.B.5 Immunocytochemical Staining: The cells grown in NBCS were stained with CD68 PE after a period of 216 h and viewed under a fluorescence microscope. The cells in all the four wells were positively stained for CD68, which indicates the presence of macrophages.

shown in Fig III.A.12. The data concludes that none of the isolated cells are positive for CD34 alone. 3% to 5% cells are found to express both CD34 and CD14. Thus it can be stated from the present study that the CD14/CD34^{+} cells may be differentiating into macrophages.

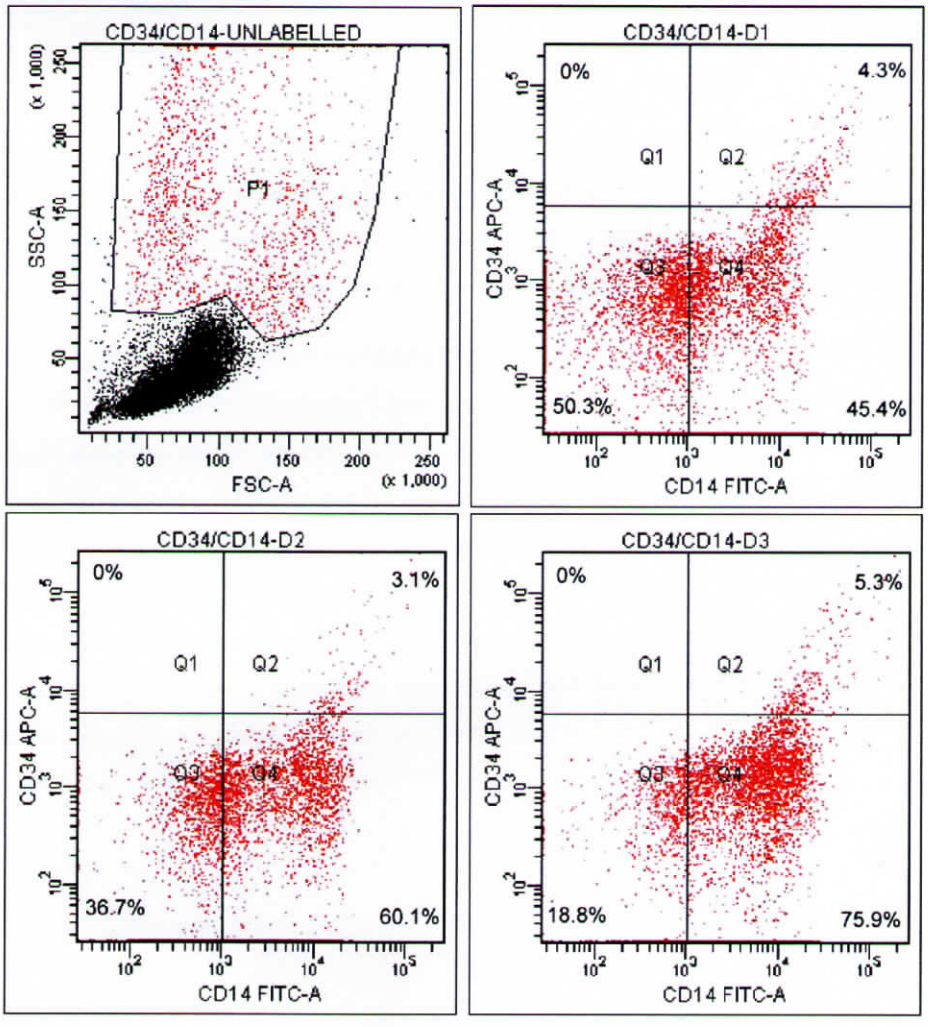

Fig III.A.12 Dot plot showing cells that are positive for CD34 and CD14: This represents the flow cytometric data obtained from three different donors. 3% to 5% cells are found to express both CD34 and CD14.

It is interesting to note that fragments of activated platelets interact with a small population of CD14^{+} monocytes and a small population of CD34^{+} population. Why the interaction between the platelet fragments and the circulating cells in the

PBMNC is selective is not understood from the current experiments. In the studies carried out by Daub K *et al* [2006], whole platelets and $CD34^+$ cells were cultured together, the latter was found to differentiate into macrophages. They have not demonstrated any adhesion of platelets to CD34 positive cells. The effects that they have observed could be due to the granule contents released from platelets. But there is no specific data to suggest if fragments from activated platelets have any specific effect on PBMNCs, more specifically the $CD34^+$ cells. Results from this study suggest that adhesion of PMP or PD with $CD14^+$ and $CD34^+$ cells may elicit signalling process for cell survival, proliferation and differentiation. To understand if PMP or PD which is bound to $CD14^+$ cells and $CD34^+$ cells influence differentiation process, culture experiments were designed and carried out.

As proteins with distinct molecular size could be obtained as demonstrated PD and PMP in the SDS-PGE pattern of both fractions, it is expected that they are likely to have different effect on cell proliferation, survival and differentiation. Since activated platelet in circulation sheds the PMP and get separated into PMP and PD if any one will preferentially adhere to the injured endothelium or both will adhere is not clear. It is also likely that they adhere to injured collagen and attract $CD14^+$ monocytes or $CD34^+$ progenitors which can contribute to atherosclerosis progression or vascular repair. Therefore, *in vitro* cultures of PBMNC in presence of PMP or PD or both together is likely to give preliminary information about the effects of $CD14^+/CD34^+$ cell interaction with PMP/PD.

III.B PBMNC Cultures in Presence of Platelet Sub cellular Fractions

III.B.1 Culture of monocytes in contact with platelet fragments:

Purpose of this experiment was to analyze if PMP and PD has any influence on monocytes culture and differentiation. In all the experiments, approximately $2.8x10^5$cells/cm^2 was used. The first experiment compared the fragments isolated from platelets activated using two different concentrations of thrombin. For each 1.75 cm^2 surface area, 10 μg PMP and 10 μg platelet debris, obtained after activation with 0.04 IU thrombin and 0.4 IU thrombin, were incorporated into the fibrin substrate to obtain FG+PD and FG+PMP substrates for culture. Fibrin (FG) served as the control and to one of the controls 10 μg/mL releasate was added to the medium. There was no difference in the effect of platelet fragments obtained from two different concentrations of thrombin. Elongated cells were found on all substrates (Fig III.B.1). The cells, which were grown in contact with the PD substrate, appeared to be more densely populated.

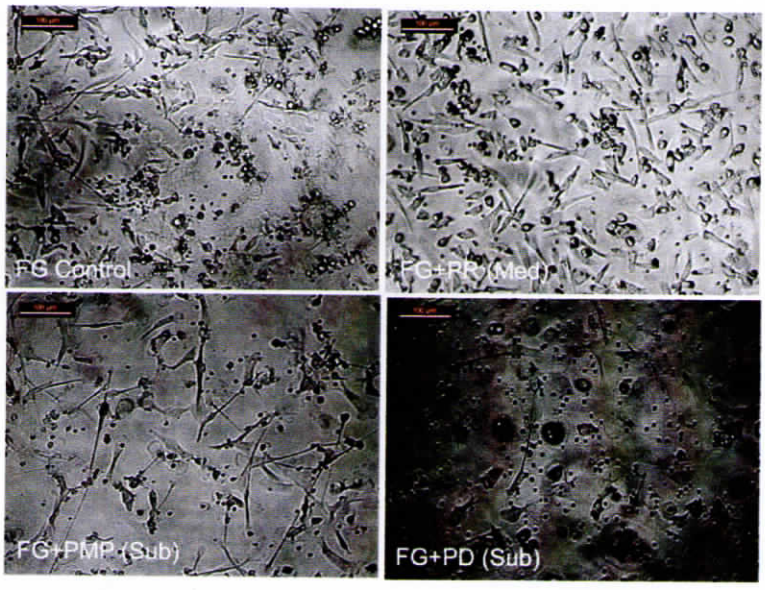

Fig.III.B.1 Phase contrast micrographs (10X) showing the effect of platelet sub cellular fractions, obtained after activation with 0.4 IU thrombin, on PBMNC culture: The cells were grown on medium containing cryopoor plasma. Elongated cells were found on all substrates. Round cells were found more in the FG control.

III.B.2 Culture on PD and PMP substrates: Effect of Serum:

Isolated monocytes were cultured in tissue culture plates containing 10 μg PD, 10 μg PMP or 10 μg PD+ 10 μg PMP along with the FG. The purpose was to identify if PMP and PD has any combinatorial effect on monocyte differentiation or survival. Three different types of supplements were added into DMEM/F-12 culture medium such as 5% new born calf serum (NBCS) or 5% human serum (HS) prepared by clotting the fresh poor plasma or cryo-poor human plasma. To each well, 10 μg/ml PR was added and FG substrate served as the control. New born calf serum is likely to contain growth factors and cytokines released from activated platelets and monocytes during blood clotting. So to avoid the effects of such cytokines, platelet poor human plasma was clotted by adding thrombin 2.0 IU per ml plasma, thus in HS no platelet releasate was expected. Human cryo-poor plasma was also used to avoid the use of thrombin.

In the case of cells grown in 5% NBCS containing medium, after 48 h of incubation, cell survival was poor in the control (Fig III.B.2). The colony of cells was more prominent when FG+PD or a combination of FG+PD+PMP was used as the substrate.

At 144 h, the granulated cells were more prominent in the control. In FG+PD coated and FG+PMP coated wells, the granulation is less compared to the FG-control and well containing both PD and PMP (Fig III.B.3).

In the case of cells grown in 5% human serum containing medium, the cells were less granulated. Cell density was less in HS-medium at 120 h, however the cells showed spindle shaped morphology when this serum was used (Fig III.B.4).

When cryopoor plasma was supplemented, cells were hardly found on the substrate after 72 h (data shown). Since the cells appeared healthy only in HS supplemented medium all subsequent experiments were done using human serum.

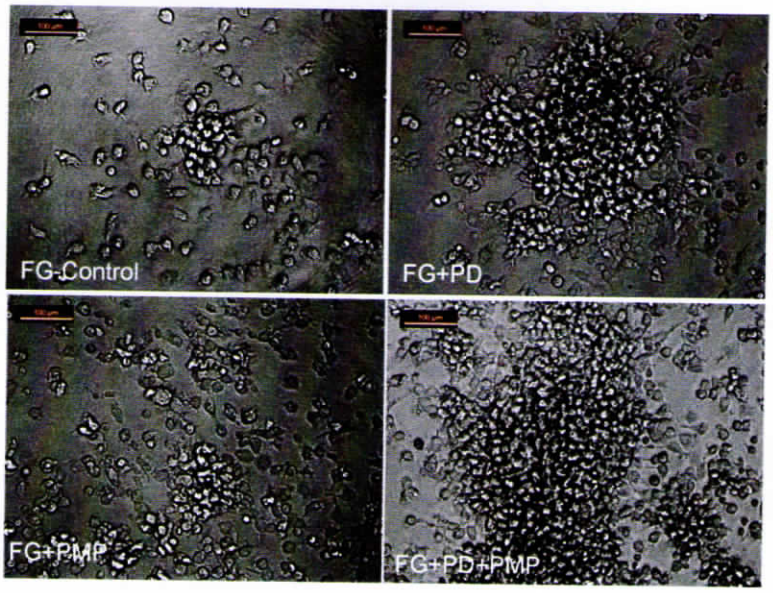

Fig.III.B.2 Phase contrast micrographs (10X) showing the effect of new born calf serum (NBCS) after 48 h: The cells were grown in medium containing NBCS and in presence of activated platelet sub cellular fractions. After 48 h, the cells in the control show poor survival, but the colony of cells were prominent in the well containing PD.

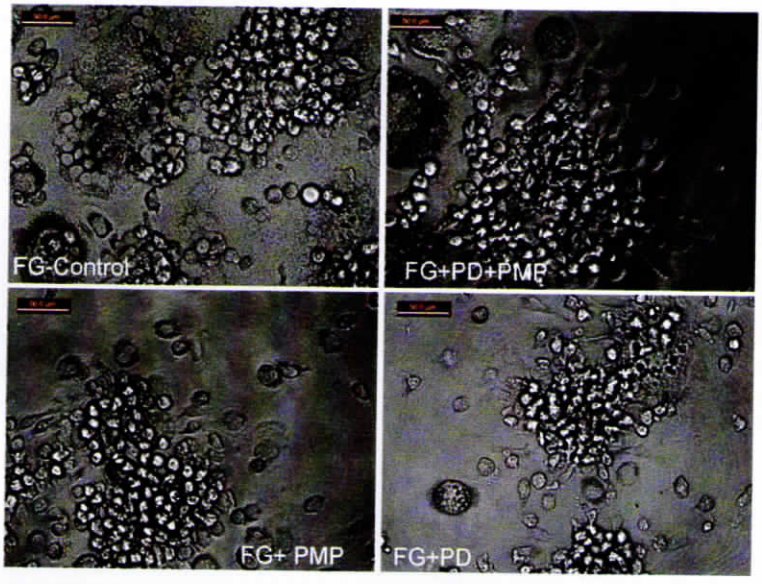

Fig.III.B.3 Phase contrast micrographs (20X) showing the effect of new born calf serum (NBCS) after 144 h: Granulated cells were more in FG-control. Granulation is very less in cells which are in contact with FG+PMP and FG+PD as compared to the control.

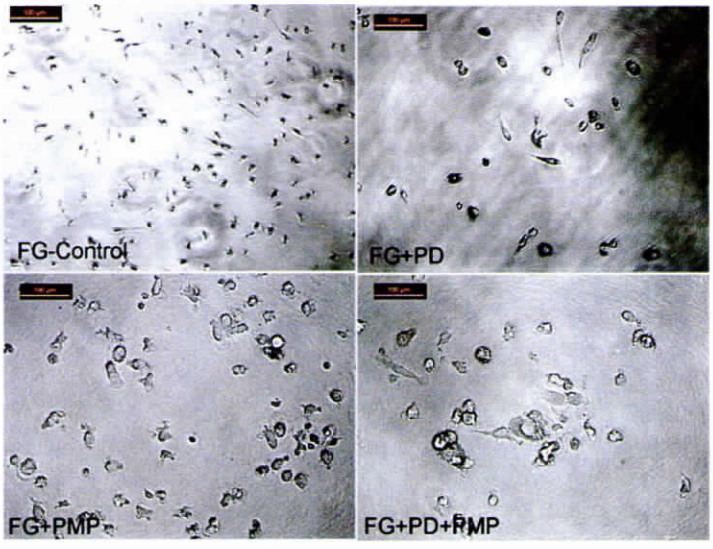

Fig.III.B.4 Phase contrast micrographs (10X) showing the effect of human serum (HS) after 120 h: Cells grown in HS show less cell density as compared to NBCS. But granulated cells were less and the cells were healthy.

Since the cultures in NBCS showed heterogeneous population with large and well spread cells, possibility of these cells being macrophages was verified using CD68 staining after a period of 216 h. Cells grown on all four substrates showed positively stained macrophage-like cells (Fig III.B.5). However, no cells grown in HS were found to be positive for CD68 on any of the four substrates (data not shown).

Fig.III.B.5 Immunocytochemical Staining: The cells grown in NBCS were stained with CD68 PE after a period of 216 h and viewed under a fluorescence microscope. The cells in all the four wells were positively stained for CD68, which indicates the presence of macrophages.

III.B.3 Effect of PD concentration on cell survival and differentiation:

This experiment was done under the impression that usually in physiological condition PD may adhere to the injured endothelium and PMP will remain in the circulation. It has been already seen that FG+PD support good cell survival, spreading and morphological changes to get spindle shaped cells when PBMNCs were grown in medium containing HS. In order to determine if the PD concentration play a role in cell attachment and differentiation, three different concentrations were included with FG. The quantities used per well (1.75 cm^2) were 2 μg, 10 μg or 20 μg. Similar number of PBMNC was cultured on these substrates. After 24h of incubation it was found that the cell density on FG+20 μg PD was more (Fig III.B.6); after 72h of incubation, groups of cells were found to be aligned in parallel (Fig III.B.7). The cell density was found to be better on FG+10 μg PD coated wells as compared to FG+2 μg PD substrate. After 192 h of incubation, elongated cells were found to be more prominent in all the FG+PD-coated wells (Fig III.B.8).

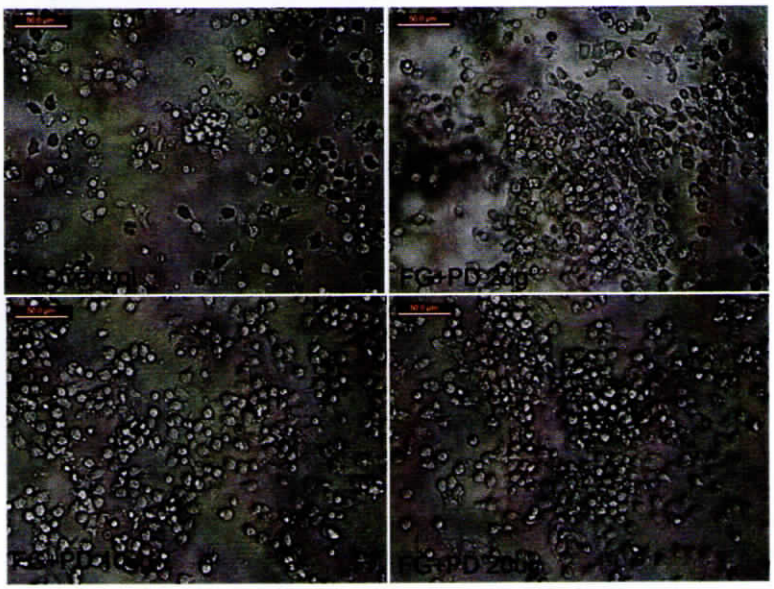

Fig III.B.6 Phase contrast micrographs (20X) of cells grown on different concentrations of PD substrates after 24 h of incubation: The cell density was found to be more in FG+20 μg PD as substrate.

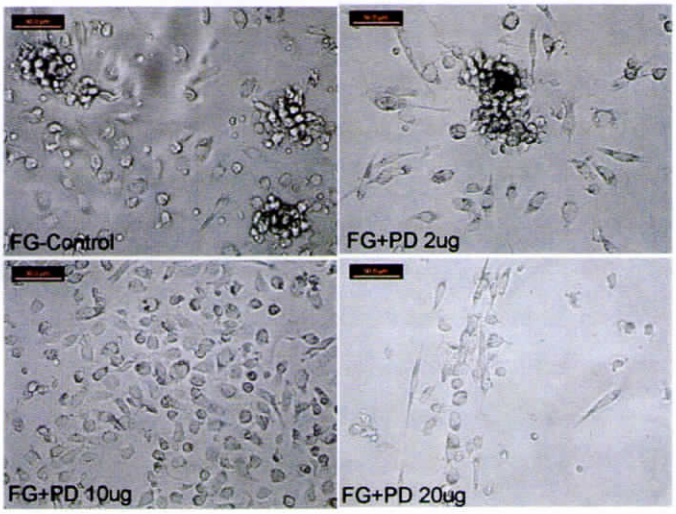

Fig III.B.7 Phase contrast micrographs (20X) of cells grown on different concentrations of PD substrates after 72 h of incubation: The cells in FG+20 µg PD-coated wells were aligned parallel. In control colony of cells can be seen.

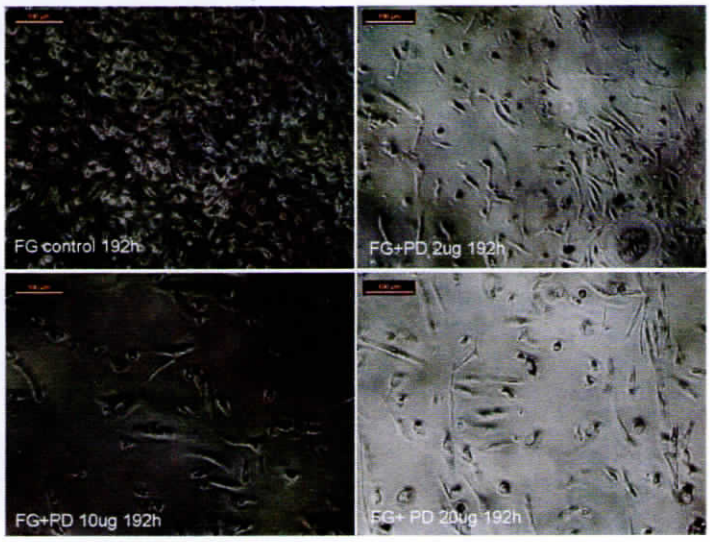

Fig III.B.8 Phase contrast micrographs (10X) of cells grown on different concentrations of PD substrates after 192 h of incubation: Elongated cells were likely to be more prominent in FG+PD coated wells than in control.

In order to bring a bio-mimetic condition, to one set of cultures, PD was included into the substrate and PMP (26 µg/ml) and releasate (20 µg/ml) were added

into the medium. In this experiment, many cells did not survive after 72 h. Towards the end of culture period (216 h) hardly any cells were found on the substrates.

After a period of 216 h, the cells grown on substrates without PMP and PR supplements were immunostained for CD68. The cells in the FG-control plates were positive for CD68. Some of the cells in the well containing FG+20 μg were found to express CD68 (Fig III.B.9).

It has been already found that PD contains traces of the PMP proteins as well. So it is probable that once the concentration increases, there may be an increase in the quantity of one or more proteins that influence differentiation of monocytes into macrophages. Daub *et al* had already reported that co culture of PBMNC with whole platelets resulted in monocyte to macrophage conversion.

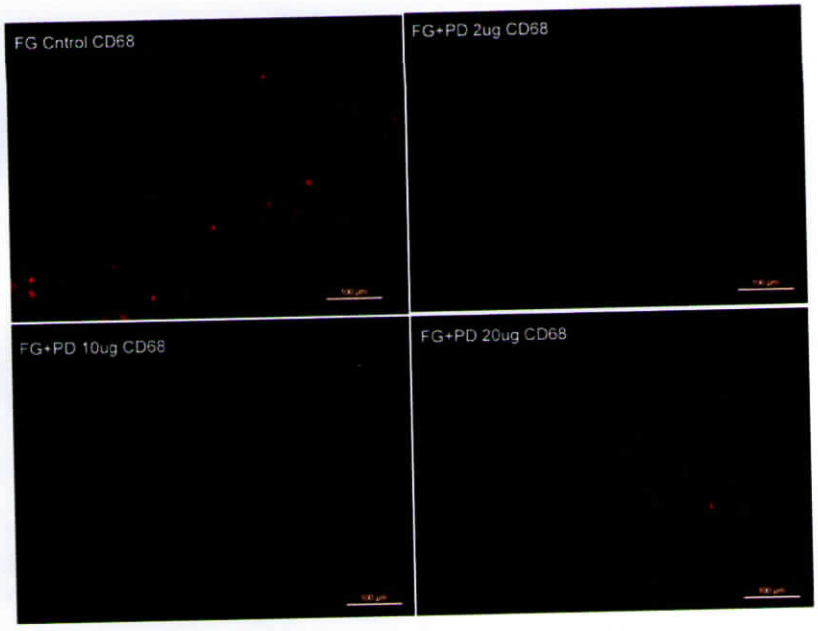

Fig.III.B.9 Immunocytochemical Staining: The fluorescent micrographs (10X) show CD68 positive cells in the control. The cells in the FG+20 μg also expressed CD68. All other wells did not express CD68.

III.B.4 Effect of PD or PMP, obtained from $1X10^{10}$ platelets, on PBMNC culture:

In this experiment the effect of platelet membrane particles and platelet debris obtained from $1x10^{10}$ platelets were studied. From Table 1 it may be seen that the

yield of PD and PMP from 10^{10} platelets is 16 μg and 0.13 μg, respectively. So the purpose was to determine if all the PMP and PD obtained from 10^{10} platelets when put together into the substrate, the cells in culture will be influenced for differentiation. The tissue culture plates were coated with fibrin-gelatin composite containing 16 μg PD and 0.15 μg PMP. There were two types of controls, uncoated polystyrene and FG-coated polystyrene. The cells in the uncoated well show granularity whereas the cells in presence of PD show elongated morphology after the culture period of 216 h (Fig.III.B.10).

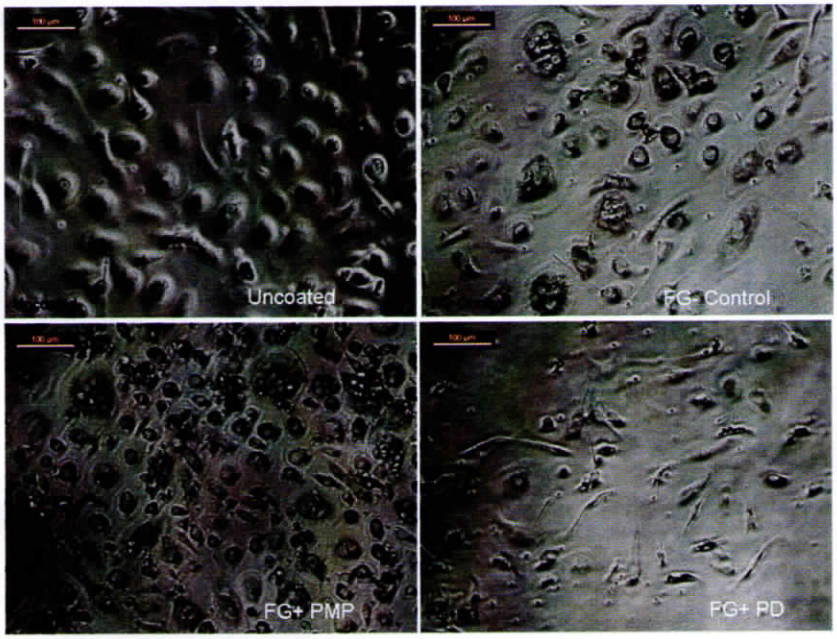

Fig.III.B.10 Phase contrast micrographs (10X) showing the effect of PD or PMP, obtained from 1x10^{10} platelets, on PBMNC culture after 216 h: The cells in the uncoated control and FG-control show more granularity whereas cells in the PMP or PD coated wells show spindle-shaped morphology.

Four similar cultures were used for the identification of cells based on marker expression at different culture intervals; 72 h, 144 h and 216 h. The cells were stained for CD14 (monocyte marker), CD68 (macrophage marker), CD31 (endothelial marker) and α-SMA (Smooth muscle cell marker). The cells that were stained with α-SMA were also stained with the nuclear stain, Hoechst. After 72 h of culture the cells in the uncoated control and FG-control were more prominently CD14 positive (data

not shown). At 216 h, the cells in the uncoated plates and fibrin coated dishes were positive for CD14 and CD68, whereas the cells in presence of PMP and PD show positive for CD31 and α-SMA (Figs: III.B.11, III.B12, III.B.13 and III.B.14).

It has been previously reported that platelet-monocyte aggregates are formed during atherosclerosis. The interaction of platelets with circulating monocytes results in the differentiation of monocytes to macrophages. But in the present study, it has been found that when the peripheral blood mononuclear cells were cultured in presence of sub cellular fractions of activated platelets for a period of 216 h, they express the markers for endothelial cells and smooth muscle cells.

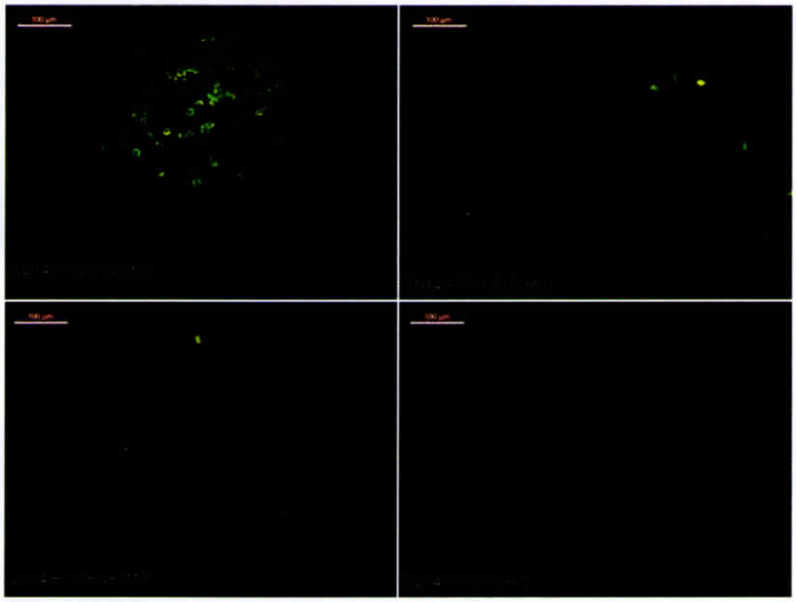

Fig.III.B.11 The fluorescent micrographs (10X) showing the immunostaining for CD14 after 216 h of culture: Only the cells in the uncoated and FG-control showed positivity for CD14 whereas the cells in the FG+PMP or FG+PD coated wells were negative for the marker.

not shown). At 216 h, the cells in the uncoated plates and fibrin coated dishes were positive for CD14 and CD68, whereas the cells in presence of PMP and PD show positive for CD31 and α-SMA (Figs: III.B.11, III.B12, III.B.13 and III.B.14).

It has been previously reported that platelet-monocyte aggregates are formed during atherosclerosis. The interaction of platelets with circulating monocytes results in the differentiation of monocytes to macrophages. But in the present study, it has been found that when the peripheral blood mononuclear cells were cultured in presence of sub cellular fractions of activated platelets for a period of 216 h, they express the markers for endothelial cells and smooth muscle cells.

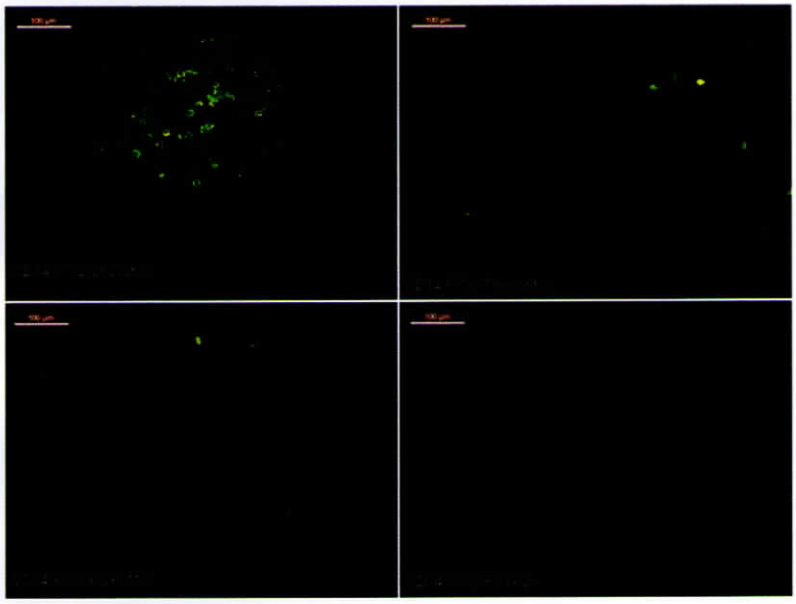

Fig.III.B.11 The fluorescent micrographs (10X) showing the immunostaining for CD14 after 216 h of culture: Only the cells in the uncoated and FG-control showed positivity for CD14 whereas the cells in the FG+PMP or FG+PD coated wells were negative for the marker.

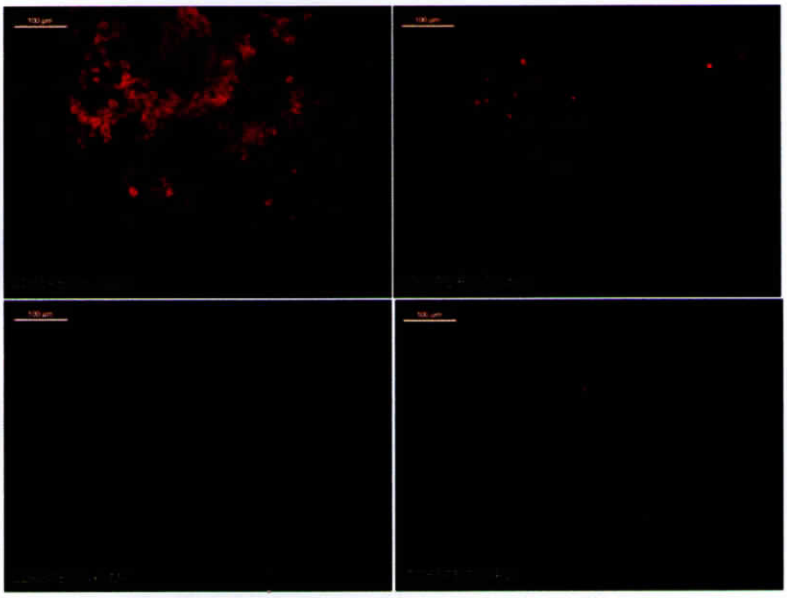

Fig.III.B.12 The fluorescent micrographs (10X) showing the immunostaining for CD68 after 216 h: Only the cells in the uncoated well and FG-control express CD68. Cells in the PMP/PD substrate were negative for CD68.

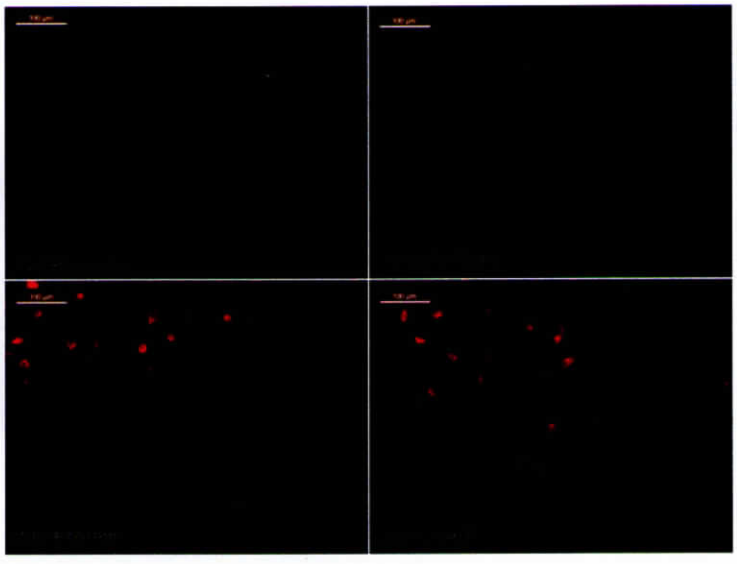

Fig.III.B. The fluorescent micrographs (10X) showing the immunostaining for CD31 after 216 h: The cells in the uncoated well and FG-control were not stained for CD31. But the cells in the wells coated with FG+PMP and FG+PD show CD31 positivity.

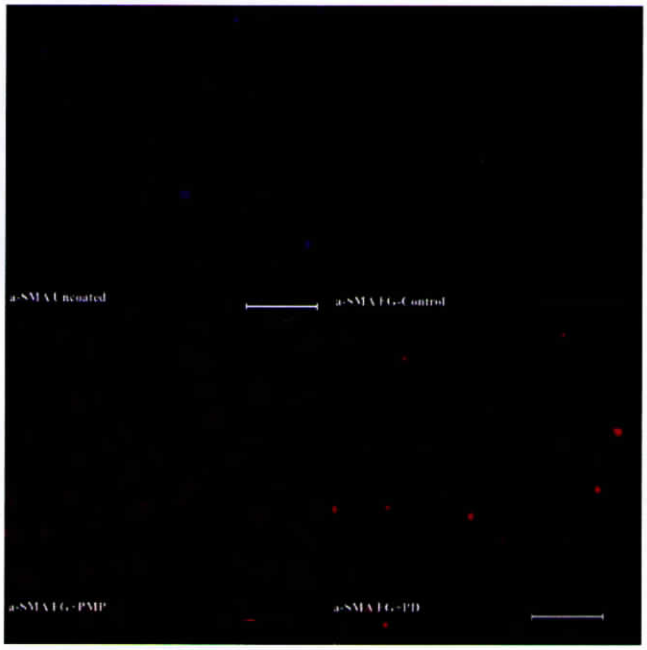

Fig.III.B.14 The fluorescent micrographs (10X) showing the immunostaining for α-SMA after 216 h: The cells in the uncoated well and FG-control were not stained for α-SMA. But the cells in the wells coated with FG+PMP and FG+PD show positive for *α-SMA.*

Accumulating evidences indicate that bone marrow–derived cells contribute to postnatal vasculogenesis in tumor growth, wound healing, post myocardial ischemia, cerebral ischemia, limb ischemia, endothelialization of vascular grafts, atherosclerosis, and occular neovascularization (Rafii DC *et al*, 2008). The majority of studies have focused on the role of endothelial progenitor cells (EPCs) in vascular remodelling. In addition to EPCs, a subset of hematopoietic progenitor cells (HPCs) are mobilized from the bone marrow and are thought to contribute to the early initiation and stabilization of newly-forming vessels, however their precise role is not yet clear. Platelets are integral to vasculogenesis, delivering angio-active growth factors and cytokines that direct the site-specific recruitment and differentiation of bone marrow–derived progenitor cells at vasculogenic sites. The extent to which new endothelium in postnatal neovascularization is dependent on bone marrow–derived progenitor cells versus mature or locally-derived cells is still a matter of controversy. As yet, there is no clear definition of either an EPC or an HPC, and the precise roles of these subpopulations remain incompletely understood. The identifying cell surface markers

used for EPCs are also present on primitive HPCs, and it has been reported that human $CD34^+AC133^+VEGFR2^+$ cells isolated from umbilical cord blood or adult peripheral blood yield HPCs *in vitro* with no vessel-forming activity. Secondly, *in vitro* studies have indicated that with angiogenic stimulation, isolated $CD34^-/CD14^+$ myeloid cells may develop an endothelial phenotype, expressing endothelial surface markers including von Willebrand factor (vWF) and VE-cadherin and forming tubular-like structures. Further clarification of the expression profiles, differentiation potentials, and *in vivo* phenotypes of bone marrow–derived cell subpopulations in human pathophysiology is likely to be forthcoming.

Results presented here indicate that platelet membrane particles and platelet debris released from moderately activated platelets induce circulating progenitors into vascular endothelial and smooth muscle lineages. The PD or PMP were immobilized with fibrin matrix which is a natural scaffold. Most striking observation is that the lineage commitment of the progenitors has taken place without the addition of any exogenous growth factors. Therefore, it is likely that paracrine or autocrine growth factors may have acted on the progenitors along with the PMP or PD immobilized with the substrate. It is not understood if the PMP/PD plays any stimulatory role on the progenitors or if they only support cell adhesion and spreading on the substrate. It is also not clear which population of PBMNCs have differentiated into SMC and EC lineage observed in this study. As per the flow cytometric analysis data presented in the section III.A.4, majority of cells in the PBMNC isolate in this study was $CD14^+/CD34^-$ population and all $CD34^+$ cells are also $CD14^+$. Therefore, it could be $CD34^+/CD14^+$ or $CD14^+/CD34^-$ population which got differentiated.

CHAPTER IV

SUMMARY AND CONCLUSION

SUMMARY

Bone marrow derived progenitor cells are increasingly recognized to play a critical role in vascular repair mechanisms and atherogenesis. Recruitment of human $CD34^+$ progenitor cells towards vascular lesions and differentiation into vascular cells has been regarded as a critical initial step in atherosclerosis. Furthermore, the progenitors of haematopoetic origin are regarded to have a key role in the maintenance of vascular integrity and to act as "repair" cells in response to endothelial injury. There is increasing evidence that bone marrow-derived progenitor cells play a critical role in vascular repair mechanisms at the site of vascular lesions.

Platelets are the first circulating blood cells that interact and adhere to vascular lesions. Thus, platelets may be involved in recruitment of circulating progenitor cells towards the injured vessel wall. Platelet activation results in an increased number of circulating leukocyte-platelet aggregates. In particular, platelet-monocyte complexes (PMC) have been observed in clinical conditions such as peripheral vascular disease, hypertension, acute or stable coronary syndromes, stroke, or diabetes.

The present *in vitro* study evaluated the effect of activated platelets on the recruitment and differentiation of human peripheral blood mononuclear cells. Attention was given to study the effect of platelets activated using physiologically relevant concentrations of epinephrine and thrombin. The markers used to identify monocytes, progenitors, platelets and macrophages were also appropriate to evaluate cell-cell interaction by dual staining and flow cytometric analysis. Markers for cytochemical staining were suitably selected to distinguish smooth muscle cells, endothelial cells and macrophages.

Activated platelets were found to express CD62 and the extent of expression was dependent on agonist strength and duration of incubation. Stimulation of isolated human platelets with 10nM epinephrine induced instantaneous effect on expression of P-selectin (CD62). This being the protein responsible for adhesion of platelets to leukocytes, as per the expectation, complexes of $CD45^+/CD14^+$ cells with $CD62^+$ increased when the agonist strength was increased. In physiological condition, platelet

activation is a synergistic process which means very low concentration of two or more agonists can strengthen the effects of platelet activation. With time more and more platelet granular contents are expected to release which in turn increase the activation status. Accordingly, here it is seen that the expression of CD62 increased with time and as a result complexes with leukocyte also increased. At low agonist concentration platelet activation and complex formation or both were found to be reversible. But as the strength of agonist increased interaction was more stable.

Activated platelets were fractionated into three main parts; the heaviest platelet debris which settled at the bottom of the tube, lipid rich and light platelet membrane particles which remained on top of the 27% sucrose as a white band and the platelet releasate which was located as supernatant above the membrane. All three fractions showed similar yield irrespective of the agonist strength (10 nM epi + 0.04/0.4 IU thrombin) used for activation of platelets. The results were consistent in all three donor platelets used. Protein profile in PMP showed very few bands as compared to PD or PR. The bands in PMP indicated that collagen receptors and fibrinogen receptors are retained with the membrane even though more experiments are required to confirm it. In addition, it is found that most of the protein bands found in PMP is also found with PD but at much less band intensity. It is likely that the entire membrane is not shed or some whole platelets are included in the debris resulting in the spill over of PMP protein bands into the PD fraction.

In order to establish that both PMP and PD interact with $CD14^+$ and $CD34^+$ cells the isolated PMP and PD were tagged with CD62. The monocytes isolated from human blood was labelled with CD14/CD34 and allowed to interact with CD62 labelled PMP/PD. Both fractions of platelets were found to interact with $CD14^+$ and $CD34^+$ cells. In addition it was also found that all $CD34^+$ cells were also $CD14^+$ cells. Since it is known that $CD34^-/CD14^+$ cells are proliferating population and can get differentiated to promote repair of vessel injury, the finding is significant. If the binding of platelet membrane in suspension has any relevance to adhesion of platelet/leukocyte complex to injured site is not clear from this study.

The platelet fractions were incorporated with fibrin matrix thereby mimicking patho-physiological condition where activated platelets or its fragments are adhered to the fibrin clot in the injured vessel. A well standardized procedure was used for coating of tissue culture polystyrene with fibrin. Addition of PMP, PD or both together at varying proportions were effective in supporting cell adhesion, spreading and survival. While fibrin coated polystyrene served as control in most of the experiments, uncoated controls were also included in some cases as control. The culture medium was also of different composition. To avoid the effects of platelet growth factors and cytokines, human serum prepared from platelet poor plasma was used as the medium supplement. When new born calf serum and the PR/PMP supplemented in the culture medium was used for monocytes cultured on PD/PMP/FG substrates, the cells were found to granulate and die within 96h. So it is evident that there is some unidentified effect of one more components that caused cell death. When human serum was used cells survived and differentiated into various cell types depending on the type of substrate used and the length of culture. Cell attachment and spreading was supported by PMP and PD and in both cases EC-like and/or SMC-like morphology was seen. Specific staining recognized that some cells were of SMC lineage and some were EC lineage after 6 days of culture. During the early period of culture (72h) cells were not positive for either CD31 or SMA but were positive for CD14. On the bare polystyrene and fibrin substrate cells were prone to remain $CD14^+/CD68^+$ monocytes or macrophages after 9 days in culture.

CONCLUSION

The present study can be concluded that the interaction of activated platelets with leukocytes, especially monocytes depends on time and concentration of agonists. The CD14 expression significantly increases when monocytes interact with platelets. The sub cellular fractions of activated platelets are able to interact with monocytes. All $CD34^+$ cells express CD14 also. When the PBMNC were cultured in contact with platelet membrane or debris for a period of 9 days, the cells express CD31 and α-SMA which are characteristic of endothelial cells and smooth muscle cells, respectively. Platelet fragments embedded in the fibrin matrix do not induce differentiation of progenitor cells into macrophages as reported by other authors.

FUTURE PROSPECTS

There are many finding in this study which needs to be confirmed through more experiments.

⇒ Evaluate if further increase in agonist strength will remove all membranes and eliminate spill out of proteins found in PMP into PD.

⇒ Characterize EC and SMC using more markers to confirm the differentiation.

⇒ Confirm if the $CD68^+$ cells are macrophages by carrying out LDL uptake assay.

⇒ Use dual staining of CD14 and CD68 to distinguish the cell population and quantify if there are single or double positive cells.

⇒ More cell culture experiments to understand if PMP or PD is involved only in cell adhesion and spreading or do they have any role in cell signalling and differentiation process.

REFERENCES

- Asahara T, Murohara T, Sullivan A, Silver M, van der ZR, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. Science.1997; 275:964 –967.

- Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K. Immunobiology of dendritic cells. Annu. Rev. Immunol. 2000; 18, 767–811.

- Banchereau J and Steinman RM. Dendritic cells and the control of immunity. Nature. 1998; 392, 245–252. 47.

- Barry OP, Praticò D, Savani RC, FitzGerald GA. Modulation of monocyte-endothelial cell interactions by platelet microparticles. J Clin Invest. 1998; 102(1):136-44.

- Brass LF. Thrombin and platelet activation. Chest. 2003; 124(3 Suppl):18S-25S.

- Caplice NM, Bunch JT, Stalboerger PG, Wang S *et al.* Smooth muscle cells in human coronary atherosclerosis can originate from cells administered at marrow transplantation. Proc Natl Acad Sci USA. 2003; 100: 4754–4759.

- Carlos TM and Harlan JM. Leukocyte-endothelial adhesion molecules. Blood. 1994; 84:2068–2101.

- Chennazhy KP and Krishnan LK. Effect of passage number and matrix characteristics on differentiation of endothelial cells cultured for tissue engineering. Biomaterials. 2005; 26:5658-5667.

- Clanchy FIL, Holloway AC, Lari R, Cameron PU, Hamilton JA. Detection and properties of the human proliferative monocyte subpopulation. J. Leukoc.Biol. 2006; 79: 757–766.

- Daub K, Langer H, Seizer P, Stellos K, May AE *et al.* Platelets induce differentiation of human $CD34^+$ progenitor cells into foam cells and endothelial cells. FASEB J. 2006; 20:1935-1944.

- Dav G and Patrono C. Platelet Activation and Atherothrombosis. N Engl J Med 2007; 357:2482-94.

- Diacovo TG, Roth SJ, Buccola JM, Bainton DF, Springer TA. Neutrophil rolling, arrest and transmigration across activated, surface-adherent platelets via sequential action of P-selectin and beta2-integrin CD11b/CD18. Blood. 1996; 88:146-57.

- Distler JHW, Pisetsky DS, Huber LC, Kalden JR, Gay S, Distler O. Microparticles as regulators of inflammation. Arthritis Rheum. 2005; 52:3337–48.

- Dixon DA, Tolley ND, Bemis-Standoli K *et al.* Expression of COX-2 in platelet-monocyte interactions occurs via combinatorial regulation involving adhesion and cytokine signaling. J Clin Invest 2006; 116:2727-38.

- Drake TA, Ruf W, Morrissey JH, Edgington TS. Functional tissue factor is entirely cell surface expressed on lipopolysaccharide-stimulated human blood monocytes and a constitutively tissue factor-producing neoplastic cell line. J Cell Biol. 1989; 109: 389-95.

- Falati S, Liu Q, Gross P *et al.* Accumulation of tissue factor into developing thrombi in vivo is dependent upon microparticle P-selectin glycoprotein ligand 1 and platelet P-selectin. J Exp Med. 2003; 197:1585-98.

- Farb A, Burke AP, Tang AL, Liang Y, Mannan P *et al.* Coronary plaque erosion without rupture into a lipid core: A frequent cause of coronary thrombosis in sudden coronary death. Circulation 1996; 93: 1354–1363.

- Garcia AB, Smalley DM, Cho HJ, Shabanowitz J, Ley K, and Hunt DF. The Platelet Microparticle Proteome. J Proteome Res. 2005; 4:1516-1521.

- Gershlick A H and Baron J. Dealing with in stent restenosis. Heart. 1998; 79: 319-323.

- Glagov S, Weisenberg E, Zarins CK, Stankunavicius R, Kolettis GJ. Compensatory Enlargement of Human Atherosclerotic Coronary Arteries. N Engl J Med. 1987; 316:1371-1375.

- Goy JJ, Eeckhout E. Intracoronary stenting. Lancet. 1998; 351:1943-1949.

- Gresele P, Momi S, Migliacci R. Endothelium, venous thromboembolism and ischaemic cardiovascular events. Thromb Haemost. 2010; 103: 56–61.

- Han CI, Campbell GR, Campbell JH. Circulating bone marrow cells can contribute to neointimal formation. J Vasc Res. 2001; 38:113–119.

- Hansson GK, Holm J, Jonasson L. Detection of activated T lymphocytes in the human atherosclerotic plaque. Am J Pathol. 1989; 135:169-175.

- Heidenreich S. Monocyte CD14: a multifunctional receptor engaged in apoptosis from both sides. J. Leukoc. Biol. 1999; 65: 737–743.

- Hjemdahl P, Chronos NA, Wilson DJ, Bouloux P and Goodall AH. Fibrinogen binding and P-selectin expression. Arterioscler Thromb Vasc Biol. 1994; 14:77-84.

- Hu Y, Davison F, Zhang Z, Xu Q. Endothelial replacement and angiogenesis in arteriosclerotic lesions of allografts are contributed by circulating progenitor cells. Circulation. 2003; 108:3122–3127.

- Hunting CB, Noort WA & Zwaginga JJ. Circulating endothelial (progenitor) cells reflect the state of the endothelium: vascular injury, repair and neovascularization. Vox Sanguinis. 2005; 88: 1–9.

- Jawien A, Bowen-Pope DF, Lindner V, Schwartz SM, and Clowes AW. Platelet-derived growth factor promotes smooth muscle migration and intimal thickening in a rat model of balloon angioplasty. J Clin Invest. 1992; 89(2): 507–511

- Johnston, R. B, Zucker-Franklin, D. The mononuclear phagocyte system In *Atlas of Blood Cells: Function and Pathology.* (D. Zucker- Franklin, M. F. Greaves, C. E. Gross, A. M. Marmont, eds.), Stuttgart, Germany: G. Fischer Verlag. 1988.

- Jonasson L, Holm J, Skalli O, Gabbiani G, Hansson GK. Expression of class II transplantation antigen on vascular smooth muscle cells in human atherosclerosis. J Clin Invest. 1985; 76: 125–131.

- Kawamoto A, Gwon HC, Iwaguro H, Yamaguchi JI, Uchida S, Masuda H, Silver M, Ma H, Kearney M, Isner JM, Asahara T. Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. Circulation. 2001;103: 634–637.

- Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, Homma S, Edwards NM, Itescu S. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. Nat Med. 2001; 7:430–436.

- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970; 227(5259):680-685.

- Lauri D, Cerletti C, de Gaetano G. Amplification of primary response of human platelets to platelet-activating factor: aspirin-sensitive and aspirin-insensitive pathways. J Lab Clin Med. 1985; 105(6):653-8.

- Leytin V, Allen DJ, Lyubimov E and Freedman J. Higher thrombin concentrations are required to induce platelet apoptosis than to induce platelet activation. British Journal of Haematology. 2007; 136 (5), 762-764.

- Li AC, Glass CK. The macrophage foam cell as a target for therapeutic intervention. Nat Med. 2002; 8: 1235 – 1242.

- Li N, Hu H, Lindqvist M, Wikstrom-Jonsson E, Goodall AH, Hjemdahl P. Platelet-leukocyte cross talk in whole blood. Arterioscler Thromb Vasc Biol. 2000; 20:2702-8.

- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J.Biol.Chem. 1951; 193: 265.

- Mannucci PM. Treatment of von Willebrand's disease. N Engl J Med. 2004; 351: 683-94.

- Morrissey JH. Silver staining of proteins in Polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal Biochem. 1981; 117:307-10.

- Muller WA. Leukocyte-endothelial cell interactions in the inflammatory response. Lab Invest. 2002; 82:521–533.

- Nozawa N, Hibi K, Endo M, Sugano T, Ebina T, Kosuge M, Tsukahara K, Okuda J, Umemura S, Kimura K. Association Between Circulating Monocytes and Coronary

Plaque Progression in Patients With Acute Myocardial Infarction. Circulation. 2010; 74(7):1292-3.

- Otsuka G, Agah R, Frutkin AD, Wight TN, Dichek DA. Transforming Growth Factor Beta 1 Induces Neointima Formation Through Plasminogen Activator Inhibitor-1–Dependent Pathways. Arterioscler Thromb Vasc Biol. 2006; 26:737.

- Owens GK. Growth response of tetraploid smooth muscle cells to balloon embolectomy-induced vascular injury in the spontaneously hypertensive rat. Am Rev Respir Dis. 1989; 140: 1467–1470.

- Palabrica T, Lobb R, Furie BC *et al.* Leukocyte accumulation promoting fibrin deposition is mediated in vivo by P-selectin on adherent platelets. Nature. 1992; 359:848-51.

- Rafii DC, Psaila B, Butler J, Jin DK, Lyden D. Regulation of Vasculogenesis by Platelet-Mediated Recruitment of Bone Marrow–Derived Cells. Arterioscler Thromb Vasc Biol. 2008; 28:217.

- Raiesdana A, Loscalzo J. Vessel wall-derived substances affecting platelets. In: Platelets in Hematological and Cardiovascular Disorders. A Clinical Handbook. Cambridge University Press. 2008: 92–105.

- Ross R, Glomset H, Harker L . Response to injury and atherogenesis. *Am J Pathol.* 1977; 86: 675–684.

- Ross R. The pathogenesis of atherosclerosis: A perspective for the 1990s. *Nature* 1993; 362: 801–809.

- Ruggeri ZM. Platelets in atherothrombosis. Nat Med 2002; 8:1227-34.

- Sata M, Saiura A, Kunisato A, Tojo A *et al.* Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. Nat Med. 2002; 8: 403–409.

- Scott LM, Priestley GV, Papayannopoulou T. Deletion of alpha4 integrins from [illegible] hematopoietic cells reveals roles in homeostasis, regeneration, and homing. *Mol* [illegible] *Biol.* 2003; 23:9349–9360.

- Shalhoub V, Elliott G, Chiu L, Manoukian R, Kelley M, Hawkins N, Davy E, Shimamoto G, Beck J, Kaufman SA, Van G, Scully S, Qi M, Grisanti M, Dunstan C, Boyle WJ, Lacey, DL. Characterization of osteoclast precursors in human blood. Br. J. Haematol. 2000; 111: 501–512.

- Shantsila E, Lip GYH. Monocytes in Acute Coronary Syndromes. Arterioscler Thromb Vasc Biol. 2009; 29: 1433-1438.

- Shimizu K *et al.* Host bone-marrow cells are a source of donor intimal smooth- muscle-like cells in murine aortic transplant arteriopathy. Nat Med. 2001; 7:738–741.

- Simionescu M. Implications of early structural-functional changes in the endothelium for vascular disease. Arterioscler Thromb Vasc Biol. 2007; 27:266–274.

- Simper D, Stalboerger PG, Panetta CJ, Wang S *et al.* Smooth muscle progenitor cells in human blood. Circulation. 2002; 106: 1199–1204.

- Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell. 1994; 76:301–314.

- Sreerekha PR, Divya P and Krishnan LK. Adult Stem Cell Homing and Differentiation in vitro on Composite Fibrin Matrix. Cell Prolif. 2006; 39:301-312.

- Steinberg D. Low density lipoprotein oxidation and its pathobiological significance. J Biol Chem 1997; 272:20963-6.

- Swirski FK, Libby P, Aikawa E, Alcaide P, Luscinskas FW, Weissleder R, *et al.* Ly-6C monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata. *J Clin Invest* 2007; 117: 195 – 205.

- Tanaka K *et al.* Diverse contribution of bone marrow cells to neointimal hyperplasia after mechanical vascular injuries. Circ Res. 2003; 93: 783–790.

- Theilmeier G, Lenaerts T, Remacle C *et al.* Circulating activated platelets assist [illegible]P-1 monocytoid/endothelial cell interaction under shear stress. Blood.1999; 94:2725-34.

- Topol EJ, Serruys PW. Frontiers in interventional cardiology. Circulation. 1998;98:1802-1820

- Torsney E, Hu Y, Xu Q. Adventitial progenitor cells contribute to arteriosclerosis. Trends Cardiovasc Med. 2005; 15: 64–68.

- Trial J, Baughn RE, Wygant JN, McIntyre BW, Birdsall HH, Youker KA, Evans A, Entman ML, Rossen RD. Fibronectin fragments modulate monocyte VLA-5 expression and monocyte migration. *J Clin Invest.* 1999; 104: 419–430.

- Vajkoczy P, Blum S, Lamparter M, Mailhammer R, Erber R, Engelhardt B, Vestweber D, Hatzopoulos AK. Multistep nature of microvascular recruitment of ex vivo-expanded embryonic endothelial progenitor cells during tumor angiogenesis. J Exp Med. 2003; 197:1755–1765.

- van der Wal AC, Piek JJ, de Boer OJ, *et al.* Recent activation of the plaque immune response in coronary lesions underlying acute coronary syndromes. Heart 1998; 80: 14-18.

- Verma S, Anderson TJ. Fundamentals of endothelial function for the clinical cardiologist. Circulation. 2002; 105: 546–549.

- Waller BF. "Crackers, breakers, stretchers, drillers, scrapers, shavers, burners, welders and melters" - the future treatment of atherosclerotic coronary artery disease? A clinical-morphologic assessment. J Am Coll Cardiol. 1989; 13:969-987.

- Wenche Jy , Wei-Wei Mao, Lawrence L, Horstman, Jianguo Tao, Yeon S. Ahn. Platelet Microparticles Bind, Activate and Aggregate Neutrophils *in vitro.* Blood Cells, Molecules, and Diseases. 1995; 21(22) 30: 217-231.

- Windecker S and Meier B. Coronary disease: intervention in coronary artery disease. Heart. 2000; 83: 481-490.

- Xu Y, Arai H, Zhuge X, Sano H *et al.* Role of bone marrow-derived progenitor cells in cuff-induced vascular injury in mice. Arterioscler Thromb Vasc Biol. 2004; 24: 477–482.

- Yan ZQ and Hansson GK. Innate immunity, macrophage activation, and athero[illegible]rosis. Immunol Rev. 2007; 219: 187 – 203.

- Yeh ET, Zhang S, Wu HD, Korbling M *et al.* Transdifferentiation of human peripheral blood CD34+-enriched cell population into cardiomyocytes, endothelial cells, and smooth muscle cells *in vivo*. Circulation. 2003; 108: 2070–2073.

- Zeiffer U, Schober A, Lietz M, Liehn EA *et al.* Neointimal smooth muscle cells display a proinflammatory phenotype resulting in increased leukocyte recruitment mediated by P-selectin and chemokines. Circ Res. 2004; 94: 776–784.

- Zhao Y, Glesne D, Huberman E. A human peripheral blood monocyte-derived subset acts as pluripotent stem cells. Proc. Natl. Acad.Sci. USA. 2003; 100, 2426–2431.

APPENDIX

1. **Acid Citrate Dextrose**

   For 100mL

   Trisodium citrate - 2.20g

   Citric acid - 0.80g

   Dextrose - 2.50g

   Made upto 1L using deionized water and filter

   Stored at 4-8°C

2. **Tyrode's Buffer**

   For 1L

   Dextrose - 1g

   $MgCl_2$ - 0.199g

   KCl - 0.402g

   NaCl - 8.12g

   Tris base - 1.756g

   Made upto 1L using deionized water

   pH was adjusted to 7.4

   Filtered and stored at 4-8°C

3. **Phosphate Buffered Saline (1X PBS)**

   For 1L

   NaCl - 8.166g

   Na_2HPO_4 - 1.419g

   KH_2PO_4 - 0.204g

   Made upto 1L using deionized water

   Filtered and stored at room temperature

4. **Ca^{2+}/Mg^{2+} free Hank's Balanced Salt Solution**

For 1L

KCl	-	0.4g
KH_2PO_4	-	0.06g
NaCl	-	8g
Na_2HPO_4	-	0.0482g

Made upto 1L using deionized water

pH was adjusted to 7.4

Filtered and stored at 4-8°C

5. **Hank's Balanced Salt Solution with Ca^{2+}/Mg^{2+}**

For 100mL

$CaCl_2.2H_2O$	-	0.0185g
KCl	-	0.04g
KH_2PO_4	-	0.006g
$MgCl_2.2H_2O$	-	0.01g
NaCl	-	0.8g
Na_2HPO_4	-	0.00482g

Made upto 100mL using deionized water

pH was adjusted to 7.4

1mg/mL D-glucose was added

Filtered and stored at 4-8°C

6. **Paraformaldehyde (1%)**

Weighed out 100mg paraformaldehyde.

Dissolved in 5mL deionized water by heating.

2-3 drops of 1M NaOH was added while heating.

Made upto 10mL using 2X PBS.

7. **1mM Epinephrine(-/-)**

Weighed out 1.832mg epinephrine(-/-).

Dissolved in 8mL deionized water.

40µL, 1M NaOH was added.

Made upto 10mL using deionized water.

8. 27% Sucrose

27g sucrose was dissolved in 100mL Tyrode's buffer

0.02% sodium azide was added

Filtered and stored at 2-8°C

9. Reagents for the Estimation of Protein

Reagent A

2% Na_2CO_3 in 0.1N NaOH

Reagent B

0.5% $CuSO_4.5H_2O$ in 1% potassium tartarate

Reagent C

10mL Reagent A + 0.2mL Reagent B

Reagent D

1mL Folin-Ciocalteau Reagent + 2mL deionized water

10. Reagents for SDS PAGE (Gradient - 6% to 12%)

Preparation of Resolving gel – For 15mL

	6%	**12%**
Deionized Water	8mL	5mL
30% Acrylamide mix	3mL	6mL
1.5M Tris HCl (pH 8.8)	3.8mL	3.8mL
10% SDS	0.15mL	0.15mL
10% APS	0.15mL	0.15mL
TEMED	0.004mL	0.004mL

Preparation of Stacking gel – For 10mL

Deionized Water	6.8mL
30% Acrylamide mix	1.7mL
Tris HCl (pH 6.8)	1.25mL

10% SDS	0.1mL
10% APS	0.1mL
TEMED	0.004mL

Gel Loading Buffer (1X) – For 10mL

Deionized Water	3.55mL
0.5M Tris HCl (pH 6.8)	1.25mL
Glycerol	2.5mL
10% SDS	2mL
0.5% Bromophenol blue	0.2mL

(a) Coomassie Brilliant Blue Solution

For 100mL

Brilliant Blue R-250	-	0.1g (0.1%)
Acetic acid	-	10mL (10%)
Methanol	-	40mL (40%)
Distilled Water	-	50mL

Destaining Solution

For 1L

Methanol	-	250mL (25%)
Acetic acid	-	70mL (7%)
Distilled Water	-	680mL

(b) Silver Staining

Fixative

Methanol	-	50mL
Acetic acid	-	10mL
Glycerol	-	250µL
Deionized Water	-	30mL

Glutaraldehyde (10%)

40mL, 25% glutaraldehyde was made upto 100mL using deion[illegible] water

Developer

3% Na_2CO_3

0.02% Formaldehyde

1M Citric acid

2.1014g citric acid was made upto 10mL using deionized water.

11. Culture Medium

DMEM/F-12

5% Human Serum

1X Antibiotics

Preparation of Human Serum from Fresh Frozen Plasma

Fresh Frozen plasma was thawed at 4°C and clotted using 2IU/mL thrombin.

The serum was collected by centrifuging at 3500rpm for 20minutes at 4°C.

Dialysed against Ca^{2+}/Mg^{2+} free HBSS for 48h and centrifuged at 13000rpm for 15min at 4°C.

Heat inactivated at 56°C for 30minutes and centrifuged at 3500rpm.

Syringe filtered aseptically using 0.22µm filter and stored at -80°C.

Preparation of Cryopoor Plasma from Fresh Frozen Plasma

Fresh Frozen plasma was thawed at 4°C and centrifuged at 12000rpm for 20min at 4°C to remove fibrinogen.

The supernatant was collected, dialysed against Ca^{2+}/Mg^{2+} free HBSS for 48h and centrifuged at 13000rpm for 15min at 4°C.

Syringe filtered aseptically using 0.22µm filter and stored at -80°C.