Role of Integrin molecule of activated platelet membrane in differentiation of endothelial cells from PBMNCs

A DISSERTATION SUBMITTED

BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

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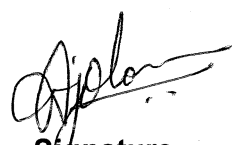
TRIVANDRUM – 695 011

DECLARATION

I, **Ajoy Aloysius**, hereby declare that I had personally carried out the work depicted in the dissertation entitled "**Role of Integrin molecule of activated platelet membrane in differentiation of endothelial cells from PBMNCs**" 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. No external help was sought.



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CERTIFICATE

This is to certify that the dissertation entitled "**Role of Integrin molecule of activated platelet membrane in differentiation of endothelial cells from PBMNCs**" is being submitted by **Ajoy Aloysius** 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 him under my supervision and guidance at **Thrombosis Research Unit,** Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Thiruvananthapuram-695012.

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ABBREVIATIONS

%	Percent
ACD	Acid Citrate Dextrose
AMS	Activated Macrophage Suspension
BrdU	Bromodeoxyuridine
CCL5	CC-chemokine ligand 5
CD	Cluster of Differentiation
CFU	Colony Formation Unit
CKD	Chronic kidney disease
CVD	Cardio Vascular Disease
CXCL4	CXC-chemokine ligand 4
Dil-Ac-LDL	Dil-labeled acetylated low-density lipoprotein
EC	Endothelial Cell
ECM	Extra Cellular Matrix
eNOS	endothelial Nitric Oxide
EPCs	Endothelial Progenitor Cells
FG	Fibrin composite
Fg	Fibrinogen
FITC	Flourescene Isothiocyanate
FMD	Flow-Mediated Dilation
fMLP	N-formyl-methionyl-leucyl-phenylalanine
FSC	Forward Scattering

GMCSF,	Granulocyte Macrophage Colony Stimulation Factor
GP	Glycoprotein
GT	Glanzmann thrombastenia
GTN	Glycerol Trinitrate
H	Hours
HbA	GLycated hemoglobin
HBSS	Hank's Balanced Salt Solution
HMG-CoA	Hydroxy-3-Methylglutaryl Coenzymea
HRP	Horse Raddish Peroxidase
ICAM	Inter Cellular Adhesion Molecule
ICAM-1	Intercellular Adhesion Molecule-1
IFN-gamma	Interferon-gamma
IL-1	Inter Leukin-1
JAM-A	Junction Adhesion Molecule A
LPS	Lipo Poly Saccharide
mAb	Mono clonal Antibody
MACS	Magnetic Assisted Cell Sorting
Mac-1	Macrophage -1 Antigen
MCP-1	Monocyte Chemoattractant Protein-1

M-CSF	Macrophage-colony stimulation factor
MDMP	Monocyte Derived MicroParticle
MMP-9	Matrix Metalloproteinase-9
MPC	Monocyte Platelet Complex
NF-kB	Nuclear Factor-kB
^{o}C	Degree Celsius
PAGE	Polyacrylamide Gel Electrophoresis
PBMNC	Peripheral Blood Mononuclear Cells
PCI	Percutaneous Coronary Intervention
PCMO	Programmable Cells Of Monocytic Origin
PD	Platelet Debri
PDMP	Platelet Derived Micro Particle
PE	Phyco Erythrin
PF4	Platelet Factor4
PLA	Platelet Leukocytic Antigen
PMA	Platelet Monocyte Aggregate
PMC	Platelet-Monocyte Complexes
PPA	Poly Morphonuclear Aggregate

PRP	Platelet Rich Plasma
PSGL-1	P-Selectin Glycoprotein Ligand-1
PTCA	Percutaneous Transluminal Coronary Angioplasty
RANTES	Regulated On Activation Normal T Cell Expressed And Secreted
SC	Stem Cell
SDF-1α	Stromal Cell-Derived Factor-1
SMCs	Smooth Muscle Cells
SSC	Side Scattering
TF	Tissue Factor
TIA	Transcent Ischemic Attack
TREM	Triggering Receptor Expressed on Myeloid Cell
UV	Ultra Violet
VCAM-1	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor
vWF	von Willebrand Factor
μM	Micromolar

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SYNOPSIS

Platelets are the primary cells that come in to contact with vessel wall during injury, and they are known to play important role in hematostasis, inflammation, wound healing, and angiogenesis. Activated platelets shed membrane fragments and secrete soluble proteins to the circulating plasma. Proteins from both groups are known to play roles in inflammation and atherosclerosis progression. The role of platelet debris, if they get adhered to vessel wall injury through adhesion molecules or through formed fibrin clot has not been well described. There fore, goal of this study was to delineate a specific role of this debris or the platelet ghost in subsequent changes after vessel injury and platelet activation.

Chapter I include background of the study, review of literature, gap analysis, hypothesis and objectives of the study for this dissertation. Current literature showed that there are enormous numbers of molecules that got released, and that remained on the activated platelets and they have varied effects on inflammatory responses, repair and regenerative mechanism. Current knowledge on the role of platelet proteins in development and progression of atherosclerosis is reviewed. It is noted that some monocytic sub population also turns into endothelial cells and participate in the vessel regeneration by the interaction with activated platelet molecules. The role of integrin molecules like, P-selectin, GPIIb and GPIIIa in the process of recruitment of $CD14^{+}$ and $CD34^{+}$ monocyte population and their transformation towards macrophage or endothelial lineage is also reviewed

After reviewing the literature, gap area was identified for exploring into this field of research. The major gap is that there is no systematic study to understand the influence of debris that forms after platelet activation on subsequent wound healing or inflammation. Therefore, the hypothesis developed for this study was that using isolated debris from thrombin activated platelets and a biomimetic culture model, effect of platelet debris on monocyte differentiation to various cell types could be identified. Also it was

hypothesized that role of specific intergrins on PD could be studied by Ab blocking experiments.

Specific objectives of the study were:

- Preparation of platelet debris from activated platelets with suitable concentration of thrombin as the agonist, characterise and confirm the presence of integrin molecules of interest on them
- Establish the effect of debris concentration on PBMNC homing and survival in culture.
- Characterization of cultured cells
  - Morphological analysis
  - Immuno-chemical staining
- Confirm the differentiation using Real Time PCR analysis.
- Study the effect of interaction of PD on monocytic population during the integrin blocking
- Study if blocking P-selectin, GPIIb and GPIIIa antigens using specific antibodies against these molecules have any effect on PBMNC and progenitor cells (CD34+) survival, proliferation and differentiation.

In chapter II methodology is described which include methods of platelet activation using various concentrations of thrombin and separation of three major fragments; platelet releasate (PR), platelet membrane particles (PMP) and platelet debris (PD) by ultracentrifigation. Yield of separated fragments by Lowry's protein assay and analysis of their MW by SDS-PAGE, identification of specific integrins by Western blot analysis etc, are described. Isolation of PBMNC by density gradient centrifugation, lyophilized culture matrix preparation, cell culture, analysis by phase contrast microscopy, and immunochemical analysis of cells to identify cell lineage are described. Blocking of integrins on PD, use of blocked PD to estimate complex formation with monocyte by flow cytometry and culture of PBMNC in presence of blocked PD and subsequent morphological and immunochemical analysis

formed the next part of the study. Finally enrichment of $CD34^+$ cells and their analysis, their culture, comparison with $CD34^-$ cell culture and identification of cell transformation in culture formed the final part of the study.

In chapter III part A, results of platelet activation and analysis of PD is presented and discussed. There were proteins which are unique on PMP and on PD. Western blot developed bands corresponding to CD62P, CD41 and CD62 on PD but not on PMP. Analytical data was sufficient to argue that PD is different from PMP in terms MW of proteins and antibody specificity, when 1IU thrombin was used for platelet activation.

In chapter III part B, results of PBMNC culture on PD incorporated fibrin matrix are presented with demonstration of morphology, specific immunochemical staining that identified macrophages and endothelial cells. Real time PCR data presented here confirmed endothelial cell generation in culture. It was obvious that if PD is present in the fibrin matrix, cells are stimulated to become EC, an effect that was dependent on the concentration of PD to certain extent. Blocking of CD41 and CD61 on PD did not affect conversion of monocyte to EC, but when CD62 was blocked, no EC specifically stained for markers, CD31 or Ulex lectin was found. The data presented in this section demonstrates that prevention of EC generation after CD62 blocking may be connected to prevention of complex formation as per flow cytometry analysis of CD41&CD61 blocked PD and monocyte mixture. The data demonstrates that $CD34^+$ cells are necessary for formation of EC from PBMNC. Magnetic sorted PBMNC enriched the $CD34^+$ cells efficiently when analysis was done using flow cytometry. In $CD34^-$ PBMNC culture, no EC could be demonstrated by immunochemical staining with EC markers, on the other hand $CD68^+$ cell demonstrated macrophage generation.

In the final chapter, results are summarized and conclusions are made. It is concluded that PD in fibrin matrix can influence differentiation of a subpopulation from PBMNC into EC. The major integrin necessary for this effect is CD62 expressed on the surface of PD. Further, the direction for future experiments is also included in this chapter.

Chapter- 1: INTRODUCTION

I.1 Back ground

Normal body physiological conditions are maintained by homeostasis, in which blood plays a crucial role. Hemostasis has a crucial role in blood homeostasis and in keeping the blood within the injured blood vessel. Upon an injury the normal hemostatic mechanism take the necessary action for the repair and regeneration, which consist of vasoconstriction, platelet adherence and activation (primary hemostasis), fibrin meshwork formation (secondary hemostasis), hemostatic plug formation and the formation of plasmin for breakdown of the clot (tertiary hemostasis). Under normal circumstances endothelial cells actively prevent thrombosis by producing factors that variously block platelet adhesion and aggregation, inhibit coagulation, and lyse clots. Platelets are the first cells to come into action at the time of hemostasis in vascular injury.

Platelets are disc-shaped, anucleate, granular fragments that are shed from megakaryocytes in the bone marrow into the blood stream. These 3.0 μm by 0.5 μm element circulate in laminar blood flow near the apical surface of the endothelium. Upon activation they undergo rapid metamorphic changes to spread and adhere to damaged endothelial surfaces, release granules, aggregate with other platelets and interact with leukocytes. They play a critical role in normal hemostasis by forming the hemostatic plug that initially seals vascular defects and by providing a surface that recruits and concentrates activated coagulation factors. These functions mainly depend on various glycoprotein receptors, a contractile cytoskeleton and two types of granules (α-granules and dense granules).

Platelets α-granules contain a variety of adhesion molecules, chemokines, coagulation and fibrinolysis proteins, growth factors, immunological molecules and other proteins. Dense granules contain ionic calcium, magnesium, phosphate and pyrophosphate as well as ATP, GTP,

ADP and GDP nucleotides and transmitter serotonin. Granule secretion therefore deposits thrombo-inflammatory mediators at the site of platelet activation, and results in expression of adhesion molecules that are not normally expressed on the platelet surface to facilitate platelet adhesion/aggregation. By changing the expression and activation status of these surface molecules, platelets are able to rapidly and markedly change their phenotype. This ability to rapidly recognize and adhere to release thrombo-inflammatory mediators at specific sites of vascular injury plays roles in both acute artherothrombosis and in chronic artherogenesis.

The platelets also play a major role in inflammation and influence the phenotype of other blood and vascular cells through cell-cell signalling. Because of this reason platelet can contribute to many other non-haematostatic disorders, like cystic fibrosis, arthritis, diabetes, cancer etc. During the abnormalities like endothelial injury, stasis or turbulent blood flow, and hypercoagulability of the blood, platelets play a major role in thrombus formation, which leads to the development of acute and further to chronic cardio vascular diseases. Atherosclerosis is a chronic inflammatory disease. Atherosclerotic lesions are characterized by an accumulation of various inflammatory cells including monocytes, macrophages/ foam cells, lymphocytes and various types of T-cells. Progressive and cumulative effect of artherosclerosis leads to the rupturing of the soft plaques to form thrombus and causes the gradual reduction of blood flow to complete arrest. Thus these will make hypoxic condition to the tissues and result in its death. Most common examples of these conditions are myocardial infraction (death of heart tissue) and stroke (death of brain tissue).

Atherosclerosis, the primary cause of cardiovascular disease, is a systemic inflammatory disease. The inflammatory nature of atherosclerosis involves chronic stimulation of the endothelial cells (EC) that line the intima, the innermost layer of the vessel wall. In addition, the inflammatory response is characterized by the accumulation of inflammatory cells in the intima, thus

initiating the atherogenic process. Atherosclerosis and cardiovascular disease involve multifactorial mechanisms with interactions among coagulation, platelets, monocytes, and EC with multiple adhesion molecules, chemokines, and receptors involved. However, the increased monocyte adhesion to and transmigration across the endothelium seem to be the most important factor in accelerating atherogenesis. Both platelets and EC can actively stimulate these processes. Platelet interaction with the monocyte in the circulation or at the vessel wall itself results in monocyte activation which subsequently becomes more adhesive, more migratory, more procoagulant (TF) and proinflammatory, and more prone to differentiate into a macrophage. Additionally, the monocytes and platelets, each individually and in bound complex form, contribute to an inflammatory phenotype of the endothelium.

Dynamic changes happen in platelet membrane during the activation which is an essential phenomenon in normal hemostasis and pathological condition. But the boundary between physiological hemostasis and pathological thrombosis is not much distinct. However, it is increasingly understood that some abnormal membrane activities and their interactions to surrounding cells is an important reason for pathological development of artherothrombosis the leading cause of death in the developing world. It is considered that platelets play an essential part in thrombus formation after the rupture of an advanced atherosclerotic plaque. Also it is understood that activation of platelets by inflammatory triggers is a critical component of artherothrombosis. But still the actual contribution of platelets in artherosclerosis is in dark. Also the interaction of platelets and the inflammatory cells like monocytes are not yet studied well.

Human peripheral blood mononuclear cells (PBMNC) contain multipotent stem cells, which can be differentiated into macrophage, lymphocyte, epithelial, endothelial, neuronal, and hepatocyte phenotypes. These cells play a critical role in vascular repair and regeneration. Such committed progenitor fraction of peripheral mononuclear cell population is

termed as endothelial progenitor cells (EPCs). A number of markers are reported to identify these populations among which CD34, CD14, CD133 etc are frequently used markers. But the correct combination and the expression levels of these markers is not established.

Platelets being the first population attached at the site of vascular injury, they play some role in vascular repair. Recent studies show that activated platelets can recruit endothelial progenitor cells (EPCs) at the site of vascular injury, which can differentiate into endothelial cells (EC) and smooth muscle cells (SMC). Adhesion molecules play a critical role in signalling for the differentiation of EPCs rather than its known adhesive function. Activated platelets expose plenty of adhering molecules for the monocyte or progenitor cells. This attachment may channelize the lineage specifications of progenitor cells. Integrins are the well known adhesion molecule for platelet-platelet and platelet-monocyte interactions. Beyond the adhesion property they have some signalling activity for the differentiation of EPCs. But this is not clearly understood yet. Also there are plenty of chemokines secreted by both platelet and progenitor cells for the necessary signalling for the differentiation.

In summary, platelet interaction with monocyte may contribute to formation of atherothrombosis and progression of vessel disease. On the other hand it may lead to vascular regeneration through endothelial repair. Avenues of research in this area are enormous and can lead to insights to prevent disease progression and accelerate organ vascularization and regeneration.

I.2 Review of literature

I.2.1 Platelet Activation in normal hemostasis

In normal circulation most platelets do not undergo significant interaction with endothelial surface. During vascular injury the subendothelial extra cellular matrix (ECM) get exposed to the blood, to which platelets promptly adhere to limit the haemorrhage and promote tissue healing. Platelets undergo dynamic membrane changes during activation and facilitate its adhesion to the ECM of the vessel wall. The ECM contains several adhesive macromolecules such as collagen, von Willebrand factor (vWF), laminin, fibronectin and thrombospondin, all of which serve as ligands for different platelet surface receptors. Also there are lots of receptors on platelet membrane for recognizing collagen in subendothelial matrix, such as Integrin $\alpha_2\beta_1$, Glycoprotein VI, CD36 (GPIV), P65 protein, P85/90 protein etc. [reviewed by Alberio and Dale, 1999]. In platelet–ECM interactions, binding of GPIbα to vWF is unique for its capability of recruiting fast flowing platelets in a high shear blood flow. Even when vWF is absent the platelet attachment to injured vascular wall and thrombus formation occur due to some alternate mechanism. Mice deficient in both vWF and Fg successfully formed thrombi with properties characteristic of both the mutations, leading to vessel occlusion in majority of the vessels. Platelets of these doubly deficient mice specifically accumulated fibronectin in their α-granules, suggesting that fibronectin could be the ligand supporting the platelet aggregation [Ni H *et al,* 2000].

The final step of platelet adhesion is their firm arrest on the ECM, a process that requires platelet activation, shifting several β1 and β3 integrins to their high-affinity, ligand binding state. The most abundant integrin on platelets is αIIbβ3 (GPIIb/IIIa, 40,000–80,000 copies/cell) on the membrane plus an exposable intracellular pool. Although this receptor is widely considered as the main platelet aggregating receptor, it also mediates firm adhesion of platelets by binding vWF and immobilized fibrinogen (Fg). The

integrin $\alpha_{IIb}\beta_3$ promotes immediate arrest onto fibrinogen but is fully efficient only at wall shear rates below 600–900 s^{-1}, perhaps because of a relatively slow rate of bond formation or low resistance to tensile stress. In contrast, glycoprotein Ibα binding to immobilized vWF appears to have fast association and dissociation rates as well as high resistance to tensile stress, supporting slow movement of platelets in continuous contact with the surface even at shear rates in excess of 6000 s^{-1}. This eventually allows activated $\alpha_{IIb}\beta_3$ to arrest platelets onto vWF under conditions not permissive of direct binding to fibrinogen [Savage B *et al,* 1996]. Platelet adhesion and thrombus growth on the exposed ECM of the injured carotid artery is not significantly altered in α2-null mice and even in mice with a Cre/loxP-mediated loss of all β1 integrins on their platelets. In contrast, inhibition of αIIbβ3 integrin on platelets in wild-type mice blocked aggregate formation and reduced platelet adhesion by 60.0%. Strikingly, αIIbβ3 inhibition had a comparable effect in α2-null mice, demonstrating that other receptors mediate shear-resistant adhesion in the absence of functional α2β1 and αIIbβ3. These were identified to be α5β1 and/or α6β1 as αIIbβ3 inhibition abrogated platelet adhesion in β1-nullmice [Gruner S *et a,l* 2003].

I.2.2. Pathology in Hemostasis

Activated platelets present at the site of injury provide both a prothrombotic surface and a procoagulant surface. Excessive platelet activation occurs in coronary bypass surgery and may result in thrombotic emboli and neurologic complications. Also many inflammatory diseases are related with platelet activation. Activated platelets enhance microparticle formation and platelet-leukocyte interaction in severe trauma and sepsis. Enhanced platelet-leukocyte interaction is dependent on P-selectin expression and may be involved in the systemic inflammatory response after severe inflammatory insult [Ogura H *et al,* 2001]. The release of mitogenic and inflammatory substances by activated platelet plays a role in the histogenesis of psoriatic lesions. It is observed in clinical studies that significantly more activated platelets were detected in patients with type 2

diabetes compared with controls. After 3 months of metabolic control, a significant decline of all platelet activation markers except CD36 was noted. Furthermore a significant correlation between CD62P, CD63 and HbA(1c) levels was observed [Eibl N *et al,* 2004].

I.2.3. Platelets in Artherosclerotic Lesions

The role of platelets in atherosclerosis was initially believed to be in thrombus formation only, upon rupture of the more developed atherosclerotic plaques. Beyond an eminent role in hemostasis and thrombosis, platelets are characterized by expert functions in assisting and modulating inflammatory reactions and immune responses. This is achieved by the regulated expression of adhesive and immune receptors on the platelet surface and by the release of a multitude of secretory products including inflammatory mediators and cytokines, which can mediate the interaction with leukocytes and enhance their recruitment [reviewed by Von Hundelshausen, P and Weber C 2007]. As monocyte adhesion to the vascular wall, its trans-endothelial migration and differentiation toward macrophages are critical for the formation of atherosclerotic lesions, it is important to realize that these events are subject to regulation by platelet adhesion molecules and platelet-derived chemokines and cytokines. Platelet-monocyte complexes (PMC) have been observed in clinical conditions.

It is reported that levels of microparticles, platelet activation markers, soluble cell adhesion molecules, and soluble selectins between hypertensive patients with and without type 2 diabetes is high. Binding of anti-glycoprotein IIb/IIIa and anti-glycoprotein Ib monoclonal antibodies to platelets did not differ significantly between the hypertensive patients and controls, but expression of platelet activation markers (CD62P, CD63, PAC-1, and annexin V) was higher in the hypertensive patients. Platelet-derived microparticle (PDMP) and monocyte-derived microparticle (MDMP) levels were significantly higher in the hypertensive patients than in the controls. Soluble ICAM-1, VCAM-1, P-selectin, and E-selectin levels were also higher in the hypertensive patients,

and they were significantly higher in the hypertensive patients with diabetes. Studies using flow cytometry showed that platelets are activated in ischaemic stroke or transient ischaemic attack (TIA). Full blood count, and measured plasma levels of soluble P-selectin, soluble E-selectin, and vWF antigen (VWF:Ag) were done. The median percentage of CD62P expression and the median percentage of monocyte-platelet complexes were higher in both acute and convalescent CVD patients than controls. The mean white cell count and mean vWF:Ag levels were significantly elevated in the acute and convalescent phases after ischaemic stroke or TIA. There was no significant increase in any other marker of platelet or endothelial activation in CVD patients [McCabe D J *et al,* 2004].

It was observed that in PLA quantification using flow cytometry in both type I and type II diabetics, there is a significant increase in platelet-polymorphonuclear aggregates (PPA) and platelet-monocyte aggregates (PMA) level. Circulating PPA and PMA were significantly enhanced in diabetics with vascular lesions than in diabetics without vascular lesions. There is an increased circulating PLA level, particularly PMA, and the incidence of microvascular complications in diabetes. They reinforce the concept of pro-inflammatory cells involvement in diabetic retinopathy pathogenesis and their link with thrombotic process [Elalamy I *et al,* 2008]. The reduced platelet activation provides a potential mechanism through which fish oils confer their cardiovascular preventative benefits [Din J N *et al,* 2008].

Endothelial P-selectin is crucial for the promotion of atherosclerotic lesion growth because in its absence only relatively small lesions developed. In addition to endothelium, platelets and their P-selectin also actively promote advanced atherosclerotic lesion development [Burger P C *et al,* 2003]. The administration of a recombinant soluble PSGL-1 reduces myocardial reperfusion injury and preserves vascular endothelial function, which is largely the result of reduced PMN-endothelial cell interactions. Also rsPSGL.Ig

treatment significantly preserved endothelium-dependent vasorelaxation in ischemic-reperfused coronary arteries [Hayward R *et al,* 1999].

I.2.4. Endothelial Activation by platelet adhesion

Adhesion of platelet leads to the further activation of platelet which leads to spreading and increased surface expression of adhesion molecules and secrete potent inflammatory substances. The platelet delivered IL-1 might initiate and regulate some of the earliest phases of the inflammatory response. Also the differential induction of endothelial leucocyte adhesion molecule I on platelet depends on the functional heterogeneity on endothelial cells. In that IL-1 β is synthesized and released by platelets in significant amounts and has been identified as a key mediator of platelet-induced activation of EC, inducing MCP-1, GMCSF, and IL-6 secretion, ICAM-1 and αvβ3 integrin expression, and NF-kB activation [Gawaz M *et al,* 2000]. Infusion of activated P-selectin$^{-/-}$ platelets did not induce leukocyte rolling; indicating that platelet P-selectin was involved in the endothelial activation. The endothelial activation did not require platelet CD40L. Leukocyte rolling was mediated solely by the interaction of endothelial P-selectin and leukocyte P-selectin glycoprotein ligand 1 (PSGL-1).

I.2.5 Monocyte recruitment by platelets adhered to EC

Monocyte accumulation and its subsequent inflammatory responses including macrophage formation is one of the characteristic features of arthroscleroses. Adhered platelets efficiently mediate monocyte rolling and arrest, even at high shear. Studies with specific antibodies in flow condition showed that monocyte adhesion to platelets was mainly mediated by P-selectin and monocyte PSGL-1. Beta2-Integrin blocking CD18 and CD11b antibodies partly inhibited the arrest of rolling cells. Antibodies against other adhesion molecules such as LFA-1, PECAM-1, and β1-integrins had no effect. In totality activated platelets adhered to ECM is a powerful adhesive substrate for monocyte recruitment under flow conditions [Kuijper P H *et al,*

1998]. The initial association between platelet P-selectin and monocyte PSGL-1 leads to increased expression of the β2-integrin CD11b/CD18 [αMβ2, membrane- activated complex 1 (Mac-1)] on the monocytes, which itself supports interactions with platelets. It has been identified that counter receptor of Mac-1 on monocytes is glycoprotein (GP) Ibα, a component of the GP Ib-IX-V complex, the platelet von Willebrand factor (vWf) receptor. The adhesive interaction between platelet and monocyte is also mediated by the expression of the glycoprotein thrombospondin (TSP) on the surface of activated platelets. TSP can cross-link platelets and monocytes via an interaction with GPIV on the surface of both cells [Silverstein, R. L et al, 1989]. Additional interactions between platelets and monocytes include CD40L–CD40 and monocyte triggering receptor expressed on myeloid cell 1 (TREM-1) to platelet-expressed TREM-1 ligand. TREM-1 mediates activation of neutrophil and monocytes and in turn has a predominant role in inflammatory responses [Bouchon A *et al,* 2000]. Monocyte transmigration across stimulated ECs promotes further monocyte recruitment and inhibits monocyte apoptosis. Following the property of transmigration monocytes shows the antimicrobial property. Also the genes for apoptosis also get down regulated in these trans-migrating populations [Williams M R *et al,* 2009].

Chemokines deposited on the endothelium also facilitate recruitment of monocytes. The chemokines RANTES (regulated on activation normal T cell expressed and secreted; CC-chemokine ligand 5 [CCL5]) and platelet factor 4 (PF4; CXC-chemokine ligand 4 [CXCL4]) are both released from the α-granules of activated platelets. Chemokines are known to govern multiple biologic processes and are classified into CXC-chemokines (eg, PF4) and CC-chemokines (eg, RANTES) according to the position of N-terminal cysteine residues. RANTES secreted by thrombin-stimulated platelets is immobilized on the surface of inflamed microvascular or aortic endothelium and triggers shear-resistant monocyte arrest under flow conditions, as shown by inhibition with the RANTES receptor antagonist Met-RANTES or a blocking RANTES antibody.

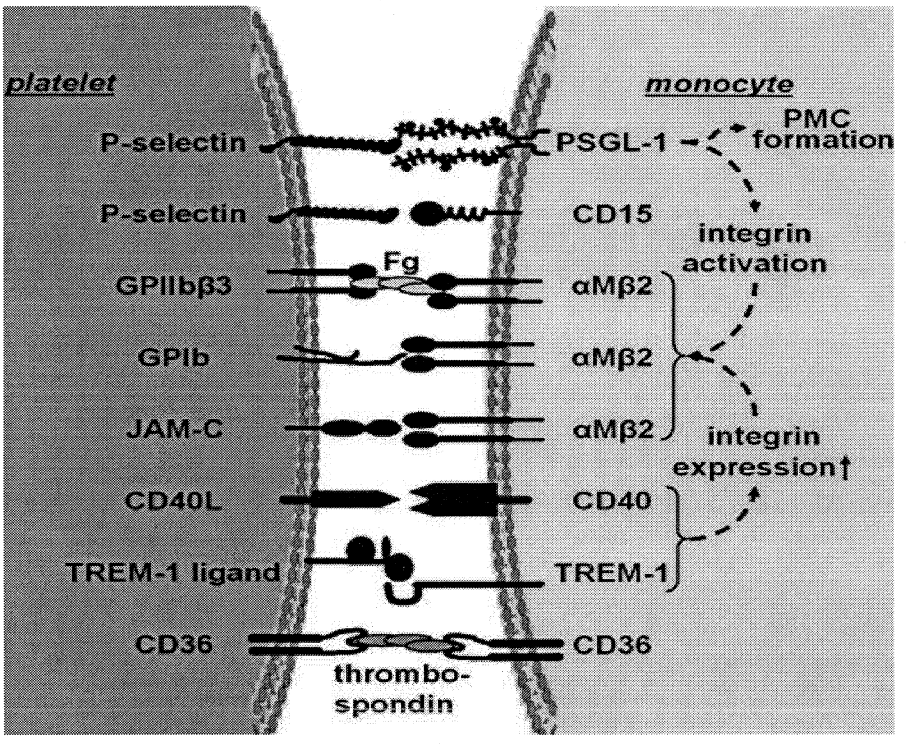


Fig I.1 : Platelet–monocyte molecular interactions (Janine M G et al. 2009).

Deposition of RANTES and its effects requires endothelial activation, eg, by interleukin-1ß, and is not supported by venous endothelium or adherent platelets. Immunohistochemistry revealed that RANTES is present on the luminal surface of carotid arteries of apolipoprotein E–deficient mice with early atherosclerotic lesions after wire-induced injury or cytokine exposure [Von Hundelshausen P *et al,* 2001]. The presence of PF4 enhanced the arrest of RANTES-stimulated monocytes and monocytic cells on activated endothelial cells under flow conditions, while binding of PF4 to the monocyte surface was increased by RANTES. Heterophilic interactions with PF4 require structural motifs important in RANTES oligomerization and amplify RANTES triggered effects on monocyte adhesion [Von Hundelshausen P *et al,* 2005].

I.2.6 Monocyte subpopulation and Cardio Vascular Events

Co expression of antigens CD14 and CD16 are identified in monocyte using two-coloured flow cytometry. $CD14^{+}/CD16^{+}$ cells account for 2.2% of the mononuclear cells and form about 13% of all cells identified by the monocyte-specific CD14 monoclonal antibody. The $CD14^{+}/CD16^{+}$ cells can be assigned to the monocyte lineage based on typical morphology, on expression of additional monocyte-associated molecules, on the ability to form reactive oxygen intermediates and on the expression of monocyte-specific NaF-sensitive esterase. Also it is characterized to have the capacity to perform adherence to plastic surfaces, as well as with ability to phagocytize antibody-coated erythrocytes. Hence the $CD14^{+}/CD16^{+}$ cells appear to represent a new monocyte subset with a distinct functional repertoire [Passlick, B et al, 1989]. Three human monocyte subsets are defined by the differential expression of the LPS receptor CD14 and the FcgIII receptor CD16, which are $CD14^{++}CD16^{-}$ cells, $CD14^{++}CD16^{+}$ cells, and $CD14^{+}CD16^{+}$ cells. In earlier studies, the latter two subsets were summarized as $CD16^{+}$ monocytes, which account for 10–20% of all circulating monocytes; whereas CD14++CD16+ monocytes were independently associated with CV events in non-dialysis CKD patients. Their results support the notion that $CD16^{+}$ monocytes rather than $CD16^{-}$ monocytes are involved in human atherosclerosis [Ziegler-Heitbrock L *et al,* 2007]. Compared with $CD14^{++}CD16^{-}$ cells, $CD14^{+}CD16^{+}$ monocytes showed elevated expression of chemokine receptors and increased adhesion to endothelial cells [MerinoA *et al,* 2011].

I.2.7 Platelet Monocyte Complex (PMC) formation and Monocyte activation

The activated platelets are able to bind to all types of leukocytes, but monocytes seem most proficient in this. Monocytes showed more and initially faster binding of activated platelets to monocytes. Hydrodynamic shear-induced collisions augment the proportion of monocytes with adherent platelets more drastically than that of neutrophils with bound platelets. These

heterotypic interactions are further potentiated by platelet activation with thrombin or to a lesser extent by monocyte stimulation with N-formyl-methionyl-leucyl-phenylalanine (fMLP). MPC formation increases with increasing shear rate and shear exposure time. Platelet P-selectin binding to monocyte P-selectin-glycoprotein-ligand-1 is solely responsible for maximal platelet adhesion to unstimulated monocytes in shear flow. However, the enhanced platelet binding to fMLP-treated monocytes involves a sequential two-step process, wherein P-selectin-PSGL-1 interactions are stabilized by CD18-integrin involvement. Blocking platelet α(IIb)β(3) or monocyte beta(1)-integrin function had no effect on this complex formation [Ahn K C *et al,* 2005]. Evidently some other studies based on the flow cytometry suggested that platelet-monocyte complex formation is mostly dependent on PSGL-1. PSGL-1 blockade, both prior to and after platelet stimulation, markedly reduced the formation of PMCs. Concomitant *ex vivo* blockade of the alphaMbeta(2) and alpha(IIb)beta(3) integrins did not result in further decreases of PMCs compared to PSGL-1 blockade alone. Antagonism of PSGL-1 also led to near elimination of leukocyte-platelet interactions under flowing conditions. Blockade of PSGL-1 alone is sufficient to inhibit and reverse the formation of PMCs following platelet stimulation [Fernandes L S *et al,* 2003]. There is evidence for a significant P-selectin-independent molecular component to the platelet-monocyte conjugation observed in peripheral blood. Patients with myocardial infarction and unstable angina demonstrate increased total binding of platelets to monocytes. Additionally, calcium-independent adhesion was significantly elevated in patients with evidence of myocardial infarction. Several adhesion receptors exhibit calcium dependence, including GP IIb/IIIa and selectins. It should be noted that a small percentage of monocytes bind platelets in the absence of divalent cations, implying that other receptors play a role in platelet adhesion. GP IIb/IIIa receptor blockade resulted in a potent inhibition of platelet–platelet aggregation but failed to inhibit monocyte–platelet interactions, suggesting that platelet GP IIb/IIIa does not play a major role in monocyte–platelet interactions and that other molecules mediate

adhesion. Blockade of P-selectin consistently produced greater inhibition than with PSGL-1 mAb, indicating involvement of other monocyte receptors. P-selectin mAb failed to inhibit monocyte-platelet binding to the same extent as divalent cation chelation, indicating involvement of other selectin receptors [Sarma J *et al,* 2002].

Clinical studies, in patients with percutaneous coronary intervention, which increases the level of activated platelets, PMC were detected much longer and only returned to baseline after 24h. Results suggested that circulating monocyte-platelet aggregates are sensitive marker of *in vivo* platelet activation [Michelson A D *et al,* 2001].

The binding of platelets to monocytes mediated via P-selectin– PSGL-1 interactions induces changes and activation process in monocytes. The binding of platelets to monocytes via P-selectin-PSGL-1 interactions was shown to increase expression and activity of α4β1 and αMβ2 integrin and increased adhesion of monocytes to activated endothelium, with a concomitant decrease in L-selectin expression. Furthermore, the binding of platelets to monocytes resulted in increased monocyte adhesion to intercellular adhesionmolecule-1, vascular cell adhesion molecule-1, and fibronectin. Platelet binding was also responsible for an increase in monocyte transendothelial migration [Da Costa Martins P A *et al,* 2006]. The studies on severe hypercholesterolemia apoE(-/-) mice gave an idea about engagement of CD40 with CD40L during monocyte activation. *In vivo,* blocking CD40L with anti-CD40L monoclonal antibody attenuated the early accumulation of neutrophils and monocytes and the late accumulation of macrophages in the denudated arteries; it also reduced the exaggerated neointima formation. *In vitro*, recombinant CD40L stimulated platelet P-selectin and neutrophil Mac-1 expression and platelet-neutrophil co-aggregation and adhesive interaction. These effects were abrogated by anti-CD40L or anti-Mac-1 monoclonal antibody. Moreover, recombinant CD40L stimulated neutrophil oxidative burst and release of matrix metalloproteinase-9 *in vitro.* Elevated sCD40L promotes

platelet-leukocyte activation and recruitment and neointima formation after arterial injury, potentially through enhancement of platelet P-selectin and leukocyte Mac-1 expression and oxidative activity [Li G *et al,* 2008].

Activated platelets regulate chemokine secretion by monocytes in inflammatory lesions *in vivo*. Thrombin-activated platelets induce the expression and secretion of monocyte chemotactic protein-1 and IL-8 by monocytes. Enhanced monokine synthesis requires engagement of P-selectin glycoprotein-1 on the leukocyte by P-selectin on the platelet. Secretion of the chemokines is not, however, directly signalled by P-selectin; instead, tethering of the monocytes by P-selectin is required for their activation by RANTES (regulated upon activation normal T cell expressed presumed secreted), a platelet chemokine not previously known to induce immediate-early gene products in monocytes. Adhesion of monocytes to activated platelets results in nuclear translocation of p65 (RelA), a component of the NF-kappaB family of transcription factors that binds kappaB sequences in the regulatory regions of monocyte chemotactic protein-1, IL-8, and other immediate-early genes. Thus, contact of monocytes with activated platelets differentially affects the expression of monocyte products [Weyrich A S *et al,* 1996]. Data of cDNA microarray analysis to determine the changes in the gene expression pattern of primary monocytes that have been attached to endothelial cells compared with monocytes that were held in suspension, they were able to identify three major groups of genes. The first group includes genes such as matrix metalloproteinase 1, monocyte chemo-attractant protein 1, and tissue trans-glutaminase 2, which are most likely to be required for monocyte extravasation. The second group consists of genes that are expressed in phagocytes such as caveolin-1 and CD74. Finally, the third group comprises genes that are expressed in cells of endothelial tissue and cartilage including E-selectin, fibronectin-1, matrix Gla protein, and aggrecanase-2 [Thomas-Ecker S *et al,* 2007]. Comparative study of transcriptome of IL-1β-stimulated ECs with untreated monocytes identified 692 differentially expressed genes. Genes up-regulated by monocyte transmigration belong to a number of

overrepresented functional groups including immune response and inhibition of apoptosis and monocyte transmigration. Additionally, quantification of Annexin V binding revealed a reduction in apoptosis following monocyte transmigration. Functional annotation of these genes showed down-regulation of antimicrobial genes (e.g., α-defens, cathelicidin, and CTSG). However, transmigrated monocytes were functional and retained intact cytokine and chemokine release upon TLR ligand exposure. Generally, it shows that the process of monocyte transmigration across stimulated ECs promotes further monocyte recruitment and inhibits monocyte apoptosis. Unexpectedly, following transmigration, monocytes displayed reduced antimicrobial protein expression [Williams M R *et al,* 2009].

I.2.8 P-selectin and PSGL-1 signaling in platelet monocyte cross talk

The adhesion molecule P-selectin (CD62P) is one of the major interest molecules because of its role in modulating interactions between blood cells and the endothelium, and also because of the possible use of the soluble form as a plasma predictor of adverse cardiovascular events. Although present on the external cell surface of both activated endothelium and activated platelets, it now seems clear that most, if not all, of the measured plasma P-selectin is of platelet origin. P-selectin is partially responsible for the adhesion of certain leukocytes and platelets to the endothelium. Animal models have also shown the important role of P-selectin in the process of atherogenesis. For example, increased P-selectin expression has been demonstrated on active atherosclerotic plaques; in contrast, fibrotic inactive plaques lack P-selectin expression, and animals lacking P-selectin have a decreased tendency to form atherosclerotic plaques. Increased levels of soluble P selectin in the plasma have also been demonstrated in a variety of cardiovascular disorders, including coronary artery disease, hypertension and atrial fibrillation, with some relationship to prognosis [Reviewed by Blann, A. D et al, 2003].

Soluble P-selectin (sP-selectin) is a biomarker for platelet/endothelial activation and is considered a risk factor for vascular disease. sP-selectin

enhances procoagulant activity by inducing leukocyte-derived microparticle production and promotes activation of leukocyte integrins. The comparative study between wild-type mice with *P-sel*$^{\Delta CT/\Delta CT}$ in which the endogenous P-selectin gene was replaced with a mutant that produces abnormally high plasma levels of sP-selectin. *P-sel*$^{\Delta CT/\Delta CT}$ mice presented several abnormalities, including higher blood brain barrier (BBB) permeability, altered social behavior with increased aggression, larger infarcts in the middle cerebral artery occlusion ischemic stroke mode and increased susceptibility to atherosclerotic, macrophage-rich lesion development [Kisucka J *et al,* 2009].

PSGL-1 is a key molecule for the attachment of monocytes to the activated platelets. Besides that they also play a role in monocyte activation signaling pathway and inflammatory process. Cytokines plays a major role in influence of signal reception leading to the platelet activation. Treatments of neutrophils with interleukin-8 (IL-8) or granulocyte colony-stimulating factor (G-CSF) potentiated the P-selectin-induced $O2^-$ production. Furthermore, interleukin-1 (IL-1) and interferon-gamma (IFN-gamma) induced surface expression of P-selectin on platelets in the presence of a low concentration of thrombin and consequently enhanced their adhesion capacity to leukocytes. These results indicated that the adhesion of activated platelets to the leukocytes through the interaction between P-selectin and its carbohydrate ligand, sialyl Lewis X (LeX), was a crucial step for the activation of leukocyte function [Tsuji T *et al,* 1994].

I.2.9 Role of αIIbβ3 (GPIIb/IIIa) in adhesion and signaling

Integrin αIIbβ3 is the most abundant integrin on the platelet surface (50,000– 80,000 copies/platelet) that has been intensively studied. Lack or dysfunction of αIIbβ3, caused by mutations in either of the two genes, gives rise to a relatively rare hereditary hemorrhagic disorder, termed Glanzmann thrombastenia (GT). GT is characterized by severe reduction of platelet aggregation in response to multiple agonists, resulting in mucocutaneous bleeding, and a normal platelet count and morphology [Nurden AT 2006].

Mouse lacking in GPIIb/IIIa motif of gama chain shows impaired platelet aggregation and continuous bleeding. The strong aggregation of platelets in thrombi formation is mediated via fibrinogen between GPIIb/IIIa molecules. So any defect in this molecule will affect the hemostasis role of platelets [Suh T T *et al,* 1995]. The use of GPIIb/IIIa in cardiovascular disease starts in early 1990's as the purpose of preventing maximum platelet aggregation. Reduction in platelet aggregation with GP IIb/IIIa inhibitors caused a reduction in major cardiovascular events (death, MI and urgent target vessel revascularization/TVR) after percutaneous coronary intervention (PCI) [Reviewed by Maia F *et al,* 2009].

Role of GPIIb/IIIa complex in monocyte platelet interaction was reported long back. This interaction was mediated through fibrinogen between monocyte and platelet. Monocytes express receptors for fibrinogen only in part related to the platelet glycoprotein IIb/IIIa complex. The binding of fibrinogen to monocytes enhances the cooperation of these cells in hemostasis [Altieri DC *et al,*1986].

Although platelet activation can occur through a number of different pathways, the common final step in platelet aggregation is the exposure of activated platelet glycoprotein (GP) IIb/IIIa on the platelet surface and its binding to fibrinogen, regardless of the initial activation pathway that triggered platelet activation. In pharmacological point of view it is a potent molecule for antithrombotic effect; mainly due to two reasons. First, the use of GPIIb/IIIa antagonists would overcome the problem of only inhibiting a single platelet activation pathway as with other drugs such as aspirin and clopidogrel. Second, GPIIb/IIIa inhibitors should not compromise platelet adhesion to wounded vessels and were thought to not induce excessive bleeding. Therefore, it is clear that blocking GPIIb/IIIa is an attractive option in the development of anti-thrombotic drugs. But it has some limitation which is the bleeding risk it induced. A large number of clinical studies are still conducted to understand the effect of Mab for blocking GPIIb/IIIa [De Meyer S F *et al,*

2006]. However, there is not much information how these integrins would affect the consequences of platelet monocyte interaction.

I.2.10 Endothelial Progenitor Cells and Vascular Repair

Endothelial cells on the arterial wall damaged by various means were initially thought to be replaced by replication of neighbouring cells. Smooth-muscle cells (SMCs) were also thought to migrate from the media into the intima, where they constituted arteriosclerotic lesions. Nowadays the concept has changed by the identification of endothelial progenitor cells (EPCs). Asahara *et al*, first reported presence of EPCs in circulating blood, which have the capacity for the regeneration of artherosclerotic injuries. EC progenitors may be useful for augmenting collateral vessel growth to ischemic tissues as well [Asahara *et al,* 1997]. EPCs are a subtype of progenitor cells of postnatal bone marrow cells that have the capacity to migrate to the peripheral circulation and to differentiate into mature endothelial cells. Therefore, these cells have been termed endothelial progenitor cells (EPCs). EPCs are characterized by the expression of 3 markers, CD133, CD34, and the vascular endothelial growth factor receptor-2. During differentiation, EPCs obviously lose CD133 and start to express CD31, vascular endothelial cadherin, and von Willebrand factor. EPCs seem to participate in endothelial repair and neovascularization of ischemic organs [Reviewed by Hristov M *et al,* 2003].The growth curve of the primary EPCs showed that the cells had little proliferative capacity. Flow cytometry analysis showed that the cells could express some of the endothelial lineage markers, while they could also express CD14, which is considered as a marker of monocyte/macrophage lineages throughout culture. In endothelial function assays, the cells demonstrated a lower level of expression of eNOS than mature endothelial cells in the reverse transcription-polymerase chain reaction and did not show an ability to develop tube-like structures in angiogenesis assay *in vitro*. In that study, they identified the monocytoid function of EPCs by the combined Dil-labeled acetylated low-density lipoprotein (Dil-Ac-LDL) and Indian ink uptake tests which gave double positive result [Zhang S J et al, 2006]. The recent *in*

vivo studies in Apo $E^{-/-}$ mouse shows that the endothelial progenitor cells are not responsible for the formation of plaque in injured epithelium [Hagensen M K *et al,* 2010].

Studies have demonstrated that atherosclerosis is a pathophysiologic process initiated by endothelial death in specific areas, such as bifurcation regions, and with subsequent replacement by endothelial progenitor cells. Differentiation of the neoendothelial cells into mature endothelium takes several days or weeks, during which LDL deposits in the intima. Blood mononuclear cells also adhere to neoendothelial cells and migrate into the subendothelial space. Meanwhile, progenitor cells from blood and the adventitia migrate into the intima, where they proliferate and differentiate into neo-SMCs. All risk factors for atherosclerosis can exert their effects on the vessel wall partly via increase in endothelial turnover, inhibition of progenitor-cell differentiation, and promotion of smooth-muscle and macrophage accumulation in lesions. Thus, progenitor cells comprise the main cell source responsible for the formation of atherosclerotic lesions, which appear in the context of inflammatory disease [Reviewed by Xu, Q, 2007].

Platelets are the first circulating cells that interact and adhere to the injured or pathological vessel wall. They have significant role in the recruitment of circulating EPCs towards the injured vessel wall. Platelets stimulate chemotaxis and migration of murine embryonic EPC (eEPCs). Further, the substantial adhesion of murine eEPCs on immobilized platelets that occurs under dynamic flow conditions is inhibited by neutralizing anti–P-selectin glycoprotein ligand-1 and anti-VLA-4 (β1-integrin) monoclonal antibodies but not by anti-CD11b (aM-integrin; macrophage antigen-1). Also the differentiation of eEPCs to matured endothelial cells occurred by the co-incubation with platelets, as verified by positive vWF and Weibel Palade bodies [Langer H *et al,* 2005]. Human platelets recruit $CD34^+$ progenitor cells via the specific adhesion receptors P-selectin/PSGL-1 and β1- and β2-integrins. Also platelets were found to induce differentiation of $CD34^+$

progenitor cells into mature foam cells and endothelial cells. Platelet-induced foam cell generation could be prevented partially by HMG coenzyme A reductase inhibitors via reduction of matrix metalloproteinase-9 (MMP-9) secretions. Agonists of peroxisome proliferator-activated receptor-α and ɣ attenuated platelet-induced foam cell generation and production of MMP-9 [Daub K *et al,* 2006].

Out comes from most of the clinical studies shows that reduced levels of circulating EPCs independently predict atherosclerotic disease progression, thus supporting an important role for endogenous vascular repair to modulate the clinical course of coronary artery disease. Patients (both stable coronary artery disease, and acute coronary syndromes) samples were analyzed by flow cytometry for circulating progenitor cells, defined by the surface markers $CD34^{+}KDR^{+}$. The follow up study for 10 months for different cardiovascular events (cardiovascular death, unstable angina, myocardial infarction, PTCA, CABG, or ischemic stroke), shows that patients suffering from cardiovascular events had significantly lower numbers of EPCs. Statistically it shows that reduced numbers of EPCs were associated with a significantly higher incidence of cardiovascular events [Schmidt-Lucke C *et al,* 2005]. Maintenance of vascular homeostasis by EPCs may be attenuated with age based on functional deficits rather than depletion of CD34/KDR or CD133/KDR cells. Study compared the healthy individuals of older age and younger age without major cardiovascular risk factors; endothelial function was analyzed for flow-mediated dilation of the brachial artery via ultrasound. Older subjects had significantly impaired endothelium-dependent dilation of brachial artery (flow-mediated dilation [FMD]. Endothelium-independent dilation after glycerol trinitrate (GTN) was not different, but the FMD/GTN ratio was significantly lower in older group suggesting endothelial dysfunction. There were no differences in the numbers of circulating EPCs, defined as $CD34^{+}/KDR^{+}$ or $CD133^{+}/KDR^{+}$ double-positive cells in peripheral blood. In contrast, lower survival, migration, and proliferation implicate functional impairment of EPCs from older subjects [Heiss C *et al,* 2005].

Therapeutically, the reduction of EPC number and the decreased functional activity in patients with coronary artery disease was counteracted by 3-hydroxy-3-methylglutaryl coenzymeA (HMG-CoA) reductase inhibitors (statins), vascular endothelial growth factor (VEGF), estrogen, or exercise. At the molecular level, these factors are well established to activate the phosphatidyl-inositol-3-kinase (PI3K)-Akt-dependent activation of the endothelial nitric oxide synthase (eNOS), suggesting that the PI3K-Akt-eNOS signalling pathway may be involved in the transduction of atheroprotective factors [Urbich C *et al*, 2005]. A clinical study with 587 patients for one year gave the significant correlation of EPCs and cardio vascular risk. The measurement of $CD34^{+}KDR^{+}$ endothelial progenitor cells is a useful tool to predict cardiovascular outcomes in patients with coronary artery disease. A significantly higher incidence of death from cardiovascular causes was observed in patients with low baseline levels of endothelial progenitor cells. The association between the EPC levels and death from cardiovascular causes was independent of the severity of coronary artery disease, a diagnosis of an acute coronary syndrome at the time of enrolment, cardiovascular risk factors, and drug therapy that are known to influence cardiovascular outcomes. Patients with high numbers of endothelial progenitor cells had a reduced risk for revascularization. These findings suggest that endothelial progenitor cells contribute to the restoration of the endothelial monolayer. Circulating endothelial progenitor cells in patients with coronary artery disease can be used to identify patients at high risk for major adverse cardiac events [Werner N *et al,* 2005].

I.2.11 Role of platelets in progenitor cell recruitment

The platelets are absolutely mandatory to recruit BM-PCs to foci of vessel injury. Platelets establish a microenvironment permissive for BM-PC proliferation and early differentiation after vascular injury *in vivo*. Platelets provide the critical signal that recruits $CD34^{+}$ bone marrow cells and c-Kit^{+} Sca-1^{+} Lin^{-} bone marrow–derived progenitor cells to sites of vascular injury.

Correspondingly, specific inhibition of platelet adhesion virtually abrogated the accumulation of both $CD34^+$ and c-Kit^+ Sca-1^+ Lin^- bone marrow–derived progenitor cells at sites of endothelial disruption. Binding of bone marrow cells to platelets involves both P-selectin and GPIIb integrin on platelets. Platelets secrete the chemokine SDF-1α, thereby supporting further primary adhesion and migration of progenitor cells [Massberg S *et al,* 2006]. Recently it was reported that the expression of junction adhesion molecule A (JAM-A) on $CD34^+$cells mediates adhesion to the vascular wall after injury and differentiation into endothelial progenitor cells, a mechanism potentially involved in vascular regeneration. Human $CD34^+$ cells express JAM-A, mediating their interaction with platelets and endothelial cells. Specifically, JAM-A expressed on human $CD34^+$ progenitor cells regulates their adhesion over immobilized platelets or inflammatory endothelium under high shear stress *in vitro* and after carotid ligation *in vivo* or ischemia/reperfusion injury in the microcirculation of mice. Moreover, it mediates differentiation of $CD34^+$ cells to endothelial progenitor cells and facilitates reendothelialization [Stellos K *et al,* 2010]. Peripheral blood mononuclear cell–derived EPCs bind platelets via CD62P and inhibit platelet activation, aggregation, adhesion to collagen, and thrombus formation, predominantly via upregulation of cyclooxygenase-2 and secretion of prostacyclin. These EPCs bind activated platelets via CD62P and inhibited its translocation, glycoprotein IIb/IIIa activation, aggregation, and adhesion to collagen, mainly via prostacyclin secretion. Indeed, this was associated with upregulation of cyclooxygenase-2 and inducible nitric oxide synthase. However, the effects on platelets *in vitro* were reversed by cyclooxygenase and cyclooxygenase-2 inhibition but not by nitric oxide or inducible nitric oxide synthase inhibition.

The activated platelets not only act as the homing site for the EPCs but also they produce some chemokines which can activate and differentiate the EPCs. Platelets provide the critical signal that recruits $CD34^+$ bone marrow cells and c-Kit^+ Sca-1^+ Lin^- bone marrow-derived progenitor cells to sites of vascular injury. Also specific inhibition of platelet adhesion virtually abrogated

the accumulation of both $CD34^+$ and c-Kit^+ Sca-1^+ Lin^- bone marrow–derived progenitor cells at sites of endothelial disruption. Binding of bone marrow cells to platelets involves both P-selectin and GPIIb integrin on platelets. Activated platelets secrete the chemokine SDF-1α, thereby supporting further primary adhesion and migration of progenitor cells [Massberg S *et al,* 2006].

I.2.12 Monocyte derived EPCs

There is some overlap between specific endothelial markers present on both ECs and hematopoietic precursors or mature blood cells, which correspond to the idea of a common embryonic precursor. Monocytes/macrophages and monocyte-derived dendritic cells, as more differentiated hematopoietic cell populations, show a wide phenotypic overlap with particularly hepatic sinusoidal, and microvascular endothelial cells within inflamed tissue, such as neovascularizised complicated atherosclerotic plaques. Furthermore, under local angiogenic growth conditions monocytes or monocyte precursors or immature dendritic cells may differentiate into endothelial like cells. It also suggests an endothelium-independent revascularization potential carried by monocyte-derived macrophages [Reviwed by Schmeisser A *et al,* 2003].

Under angiogenic conditions the monocytes lost CD14/CD45 and display a commonly accepted EPC phenotype, including LDL uptake, lectin binding, CD31/CD105/CD144 reactivity, and formation of cord-like structures. Strikingly, primary monocytes already expressed most tested endothelial genes and proteins at even higher levels than their supposed EPC progeny. Neither fresh nor cultured monocytes formed vascular networks, but CFU-EC formation was strictly dependent on monocyte presence. LDL uptake, lectin binding, and CD31/CD105/CD144 expression are inherent features of monocytes, making them phenotypically indistinguishable from putative EPCs [Rohde E *et al,* 2006]. During i*n vitro* studies suspended $CD14^+$ monocytes were found to be attached and changed their morphology to endothelial-like cells, which expressed high levels of endothelial cell markers CD31, von

Willebrand factor, and vascular endothelial growth factor receptor-1 as well as two major endothelial tight junction proteins zonula occludens -1 and occludin. Endothelial nitric oxide synthase expression was substantially increased. Endothelial-like cells were also able to uptake acetylated low-density lipoprotein and bind to Ulex europeus lectin. Also, endothelial-like cells showed a unique cytokine/chemokine profile with substantial increases of macrophage inflammatory protein-1β, IL-6, granulocyte colony-stimulating factor, and IL-8 [Zhang R *et al,* 2005]. Circulating $CD14^{+}CD34^{low}$ cells exhibit both phenotypic and functional features of stem cells. These $CD14^{+}CD34^{low}$ cells represented a variable proportion at individual level of $CD14^{+}$ cells, ranging from 0.6% to 8.5% of all peripheral-blood leukocytes. Purified circulating $CD14^{+}CD34^{low}$ cells, express highly embryonic stem cell (SC) markers Nanog and Oct-4, which are down regulated in endothelial cells (ECs). Moreover, circulating $CD14^{+}CD34^{low}$ cells, but not $CD14^{+}CD34^{-}$ cells, proliferated in response to SC growth factors, and exhibited clonogenicity and multipotency, as shown by their ability to differentiate not only into ECs, but also into osteoblasts, adipocytes, or neural cells [Romagnani P *et al,* 2005]. Although EPC exhibit endothelial-like surface markers, functional characteristics place these cells in a monocytic lineage. EPC also display antigen-presenting capacity similar to monocytes and much stronger than human vascular EC [Raemer PC et al, 2009].

Even though monocyte has the property to differentiate into ECs, not all subpopulation can do the same. A specific subpopulation of monocyte can go through the endothelial lineage. Compared to the $CD14^{+}$/VEGFR-2^{-} cells, $CD14^{+}$/VEGFR-2^{+} shows more differentiation potential towards endothelial differentiation. Also these cells show reendothelialisation of balloon-injured femoral arteries of nude mice [Elsheikh E *et al,* 2005]. A unique $CD14^{+}CD45^{+}CD34^{+}$typel collagen^{+} cell fraction derived from human circulating $CD14^{+}$ monocytes, named monocyte-derived multi-potent cells (MOMCs) has been identified. Human MOMCs can proliferate and differentiate along the endothelial lineage in a specific permissive

environment. This primitive cell population contains progenitors capable of differentiating along the mesenchymal and neuronal lineages. Treatment of MOMCs with angiogenic growth factors changed the morphology and adopted a caudate appearance with rod-shaped micro-tubular structures resembling Weibel-Palade bodies. In these cells endothelial markers are down regulated prior to the culture. Functional characteristics, including vWF release upon histamine stimulation and up regulated expression of VEGF and VEGF type 1 receptor in response to hypoxia, were indistinguishable between the MOMC-derived endothelial-like cells and cultured mature endothelial cells. The MOMCs responded to angiogenic stimuli and promoted the formation of mature endothelial cell tubules in Matrigel cultures. Angiogenesis in mouse model indicated that more than 40% of the tumor vessel sections incorporated human endothelial cells derived from MOMCs [Kuwana M et al, 2006]. An *in vivo* study on mouse for the vascular growth and wound healing in diabetes shows that freshly isolated circulating cells can dramatically impact healing and vascular growth. The $CD34^+$ PBMNCs are more effective than $CD34^+/CD14^+$cells in doing so. Nevertheless, $CD14^+$ cells could offer a therapeutic alternative for people with diabetes, the function of whose $CD34^+$ PBMNCs may be compromised. The molecular mechanism of this wound healing is mediated through the VEGF and MCP-1 pathways, and angiopoietins [Awad O *et al*, 2006].

The *in vivo* study in mouse with myo cardial infarction with a population of monocyte termed as programmable cells of monocytic origin (PCMO) succeed to restore left ventricular function. It has also shown statistically significant difference from non-modulated cell treatment. This population may be used as the autologous source for cell therapy for myocardial infarction [Dresske B *et al,* 2006]. The macrophages also can undergo endothelial phenotype development under angiogenic stimulation with the expression of specific surface markers. Also cord-like structure was observed in relatively high percentage in combination with VE-cadherin as the cell contact signal, which is same as in preformation of vascular line [Schmeisser A et al, 2001].

Activated macrophages can promote healing and repair of the infarcted myocardium. When human activated macrophage suspension (AMS) which is prepared from whole blood is administrated to mouse myocardial infarction model, it accelerates vascularization, tissue repair, and improves cardiac remodeling and function [Leor J *et al,* 2006]. Macrophage-colony stimulation factor (M-CSF) stimulates the differentiation and proliferation of macrophage lineage. M-CSF administration reduced the size of infarct in myocardial infarct model mice. M-CSF increased macrophage infiltration and neovascularization with CD31 expression in the infarct myocardium but did not increase myofibroblast (alpha-smooth muscle actin) accumulation.

I.2.13 Summary of Literature Review:

Platelets play significant role in hemostasis, wound healing, inflammation, atherosclerosis progression and vascular regeneration. There is plethora of molecules that get released into circulation or that get immobilized on the dysfunctional endothelium or the injured blood vessel upon platelet activation. It is known that soluble chemokines and cytokines released from platelets are capable of inducing cell growth and wound healing whereas some other factors act negatively and induce endothelial injury. Reports suggest that insoluble fraction of the platelets contain large number of adhesive proteins that are responsible for its immobilization to the vessel wall and for linking inflammatory leukocytes and more specifically the monocyte populations. These monocytes undergo major changes in its phenotypes after they are lodged inside the vessel wall proteins. They get differentiated into macrophages and promote inflammation. Macrophages are known to have both positive and negative effects on blood vessel physiology and pathology. A subpopulation of monocytes also turns into endothelial cells and participate in the vessel regeneration. It has been demonstrated that in the presence of activated platelets, monocyte –derived EPCs differentiate into EC. It is also well established that activated platelets play an important role in monocyte recruitment to the vessel wall and can play multiple roles to produce

macrophages or endothelial cells. Each process lead to variable outcome as far as cardiovascular disease progression is concerned.

I.3 Gap Area:

The role of soluble proteins, which are released from stored platelet granules, on various vascular changes associated with CVD, is extensively studied. The role of P-selectin which is a membrane component seen in activated platelets and in the microparticles released on platelet activation is known to be involved in the adhesion of monocytes and its retention in the vessel wall. However, the role of other integrins like GPIIb and GPIIIa in monocyte adhesion is not so well understood. It is also not known whether; in the event that secreted proteins are diluted into the circulation and the activated platelet debris remain in the vessel wall what would be the effect on monocytes and the conversion of subpopulations into macrophage or endothelial cells. A previous study from this laboratory indicated that platelet debris isolated from activated platelets reduce the event of monocyte conversion to macrophage in presence of a fibrin matrix composed from cryoprecipitated plasma. Flow cytometric analysis of PBMNC identified a CD34+/CD14+ population. Therefore, this study is focused on understanding if the basic components that are present in the platelet debris influence the monocyte to macrophage conversion or monocyte to endothelial cell conversion.

I.4 Hypothesis:

The P selectin and GPIIb/IIIa molecule on the surface of activated platelet debris may help in the recruitment of monocyte subpopulations and are signalled for their transformation towards endothelial lineage. Immature monocytes ($CD34^{+}$) may act as the endothelial progenitor cells which are stimulated to EC by the signals from one or more of the platelet debris components.

I.5 Objectives:

- Isolation and characterization of activated platelet debris.
- Establish complete shedding of platelet membrane particles on activation
- Confirm the presence of CD62P and GPIIb/IIIa molecules on prepared platelet membrane using Western blot analysis.
- Isolation of PBMNCs using density gradient centrifugation from human blood.
- Establish the effect of debris concentration on PBMNC homing and survival in culture.
- Characterization of cultured cells
    - Morphological analysis
    - Immunochemical staining
- Confirm the differentiation using Real Time PCR analysis.
- Study if blocking P-selectin, GPIIb and GPIIIa antigen using antibodies have any effect on PBMNC and progenitor cells (CD34), survival, proliferation and differentiation

Chapter II- Materials and Methods

II.1Study of Platelet Integrin Molecules

II.1.1 Preparation of washed platelets:

Platelet Rich Plasma (PRP) bags were obtained with informed consent from the SCTIMST blood bank and transferred to four 50 mL tubes (*Nunc,Sweden*). One part ACD was added to nine parts PRP (by volume) and mixed gently. The cells were pelleted by spinning at 1216*g* for 20 minutes using a table-top centrifuge (*Contifuge Stratos, Heraeus, UK*) 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 1216*g* for 10 minutes for washing and the supernatant was discarded. The washing steps were repeated for two more times. 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 2.5 mM.

II.1.2 Platelet activation

The washed platelet suspension, with $CaCl_2$ was incubated at 37°C in water bath (HAAKEF4, Germany). They were then activated using desired concentration of thrombin. After 2 min of thrombin addition, 5mM EDTA was added to arrest activity of Ca^{2+} dependent proteases. .

II.1.3 Platelet membrane preparation

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 OptimaTM 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 debris was resuspended in 1 mL HBSS buffer. To the PMP and cell debris, 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. The pellets were resuspended in HBSS containing antibiotics (*GIBCO, USA*), aliquots were stored at -40°C.

II.1.4 Estimation of protein

The concentrations of protein present in the samples prepared as per the procedures described in *II.1.3* were determined using Lowry's method. Briefly, samples were diluted using 1x Phosphate Buffered Saline (PBS) 100 µL of the diluted samples were used for protein estimation at different dilutions. Absorbance at 600 nm was taken using UV-Visible Spectrophotometer (*Hewlett Packard Diode array 8453, Germany*) and calibration curve generated using known concentrations of albumin was used for determining the concentration in each preparation.

II.1.5 SDS polyacrylamide gel electrophoresis

Analysis of the isolated PMP, PD and PR obtained was done using Lammeli's method (non-reduced) on 8% SDS polyacrylamide gel electrophoresis. The samples were prepared in 1x gel loading buffer (*Sigma Chemicals, USA*). 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. Differences in protein molecular weight profile between PMP and PD were analyzed using gel doc and its software.

II.1.6 Western Blotting for CD62P, CD41 and CD61

Platelet Debris (PD) which was prepared by 1IU thrombin activation, method described as *II.1.3 and* separated on 8% SDS was transferred by Semi Dry Method. For semi dry blotting, the gel and membrane were sandwiched horizontally between two stacks of buffer-wetted filter papers that contact two closely spaced solid-plate electrodes. The proteins in the gel were transferred to the nitrocellulose membrane *(Gene Script Corporation, USA)* for 20 min at 0.8mA/cm^2 using Multiphore II *(Pharmacia Biotech, Sweden)*. After the transfer nitrocellulose membrane was cut into small strips

and washed with PBS for 3 times. One strip was taken for Amido Black staining for confirming the transfer of proteins to the membrane. Remaining strips were blocked with 3% Bovine Serum Albumin (BSA) in PBS. The strips were washed three times with PBS and dried the strips. Blots were developed for detection of CD62P, CD41 and CD61. Diluted primary antibodies raised from mouse for CD62P, CD41 and CD61 were added in three different strips and incubated at 4°C for overnight. Primary antibodies were removed and washed three times with PBS. Strips were treated with HRP-conjugated secondary antibody *(GENEI, Banglore)* against mouse and incubated for 1hour at room temperature. Prepare the substrate solution by adding 3 mg of 4-Chloro1-naphthol (4-CN) (Sigma, Germany) in to 10 mL of 50mMTris HCl (pH 7.6) and remove the white precipitate by using Whatman no.1 filter paper and 100µl of 30% H_2O_2 was added to the above solution. The strips were immersed in the substrate solution for 30minutes at room temperature under agitation. Reaction was terminated after the development of bands by the addition of PBS.

II.1.7 Platelet Debris-Monocyte Interaction

The preparations of PBMNCs, and PD (using0.4IU Thrombin) were done by method described earlier *II.2.2, II.1.3* and *II.1.2* respectively. Platelet Debris (20µg) were incubated for 1H at 37°C with monoclonal antibodies (2µg/mL) for CD62P, CD41and CD61 for blocking membrane receptors.100µl of PBMNC preparation ($1x10^6$ cells) were incubated with blocked PD along with antibodies for labelling, CD14-PE *(CALTAG Laborotories, USA)* for monocytes, CD62P-PE and CD41-PE (*Beckman Coulter, USA*) for PD and incubated for 1H. An isotype control was prepared by adding specific human IgG1-FITC antibody to PD+PBMNC preparation. For compensating fluorescence PBMNCS labelled with CD14-FITC and activated platelets (0.4IU Thrombin) labelled with CD62P-FITC were prepared. Preparations were centrifuged at 500*g* for 15 min (*Heraeus Labofuge 300, UK)* and the

cells were fixed using 3.7% formaldehyde and made up to 1 mL using PBS. The data were recorded using fluorescent activated cell sorter (*FACS-Aria, BD Biosciences, USA*).

II.2 Cell Culture- Effect of PD on PBMNC culture

II.2.1 Preparation of culture substrates:

Platelet debris was immobilized on tissue culture polystyrene (TCPS) *(NUNC, Rakslide, Denmark)* with fibrin as the matrix using an established procedure [Prasad CK & Krishnan LK, 2005]. Cryoprecipitate isolated from human plasma was made up in sterile distilled water to adjust concentration to 5 mg/mL fibrinogen, ~0.2 mg/mL fibronectin and it contains plasminogen and FXIII. To study the effect of PD different concentrations (10µg, 20µg and 40µg) were immobilized. 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. Cryoprecipitate isolated from human plasma was made up in sterile distilled water to adjust concentration to 5 mg/mL fibrinogen, ~ 0.2 mg/mL fibronectin and it contains plasminogen and FXIII (quantity not estimated). Fibrin (5mg/mL) with 2% gelatin was added to the lyophilized plates for fibrin matrix formation. Incubated the plates at 37°C for 30min in CO_2 incubator and kept for overnight incubation at -80°C. And the plates were lyophilized under sterile atmosphere, in a freeze drier (*Edwards, Modulyo 4K, UK*) for 3 hour. Coated dishes were lyophilized under sterile atmosphere, in a freeze drier (*Edwards, Modulyo 4K, UK*). The matrices were then stored at 4-6^{0}C in a refrigerator till PBMNC seeding was done.

II.2.2 Isolation of peripheral blood mononuclear cells (PBMNC):

The buffycoat was obtained from the SCTIMST blood bank and transferred to four 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 15 minutes (*Contifuge Stratos, Heraeus, UK*). The thick white layer (buffy coat) in the interface was collected which is a mixture of leukocytes and 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 450 *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 re suspended in ACD-HBSS.

II.2.3 Culture of PBMNC:

Peripheral blood mononuclear fraction was isolated as per the method described in *II.2.2*. Washed PBMNC fraction was re suspended in DMEM/F-12 medium(Gibco, USA) containing human serum. Human serum was prepared by clotting cryo-poor plasma (to avoid platelet factors) using 1U thrombin; after the clot retracted serum was separated, dialyzed extensively against HBSS, centrifuged and sterile filtered using 0.22um membrane. The lyophilized substrates in four-well plates (*II.4.1*) were washed with DMEM/F12 medium. Cells of required number in complete medium were plated in specific substrates in 4-well culture plates and were kept at 37°C, 5% CO2 incubator (*ThermoForma 3951, Hong Kong*). Medium change was done at 24 h intervals until 48 h and afterwards on alternate days. Cell attachment, morphology changes and survival were monitored under a phase contrast microscope (*DM IRB, Leica, Wetzlar, Germany*). Cultures were analyzed at different intervals and terminated after a defined length (usually 8 days).

II.3 Study of Effect of Integrins (CD62P, CD41and CD61) on PBMNC culture

II.3.1 Culture substrate preparation

The integrin molecules (CD62P, CD41and CD61) on PD were blocked using monoclonal antibodies to study the effect of those integrins on PBMNC translation towards endothelial or macrophagic lineage.

PD (20 µg/mL) were incubated with different concentrations (0.1µg, 0.2 µg, 0.4 µg and 2 µg) of monoclonal antibodies against CD62P, CD41 and CD61 *(Abcam, Cambridge)* at 37^0C for 1 hour. Tissue culture polystyrene was incubated with 5IU/mL Thrombin solution in 5 mM CaCl2 for 30 min at 37°C. Excess thrombin was aspirated out, plates were lyophilized and incubated PD-Ab preparations were layered on the thrombin treated plates. PD without antibody was used as control. Matrix coated plates were lyophilized under sterile atmosphere, in a freeze drier (*Edwards, Modulyo 4K, UK*) for 1 hour.

II.3.2 Cell culture of PBMNC

To identify the effect of PD on specific population the culture of different populations (PBMNC, $CD34^+$, $CD34^-$) were done. Peripheral blood mononuclear fraction was isolated as per the method described in *II.2.2.*

II.3.3 Magnetic-activated cell sorting (MACS) for CD34 Cells

Magnetic immune separation of CD34 cells was done. PBMNCs were isolated as per the method described in *II.2.2.* The cell count was adjusted to get $1x10^8$ cells/mL by using recommended medium (PBS+20%Human serum+5%ACD). The solution was placed in 12x75 mm polystyrene tube *(Falcon 5mL, Becton Diekenson, USA).* Easy sep positive selection cocktail *(Stem cell technologies, North America)* were added to the solution (100µl/mL) and separation was done as per manufacturer's instruction.

Analysis of CD14 and CD34 cells in $CD34^-$ and $CD34^+$ preparations were done by flow cytometry to check the purity of MACS preparation.

Medium change was done at 24 h intervals until 48 h and afterwards on alternate days. Cell attachment, morphology changes and survival were monitored under a phase contrast microscope (*DM IRB, Leica, Wetzlar, Germany*) on 5^{th} and 8^{th} day.

II.4 Immuno cytochemistry

II.4.1 Staining for Differentiation Markers

Cultured cells were stained for, CD68 PE (*Santa Cruz Biotech, USA*), CD31 PE (*Santa Cruz Biotech, USA*) antibodies and Ulex lectin FITC *(Sigma, Germany)* and analyzed using fluorescent microscope (*Leica DM IRB, Germany).* After the removal of medium, cells were washed and fixed in 3.7% formaldehyde for 20minutes. The cells were then washed, and blocked with 0.2% BSA in PBS. Cells were washed and stained with antibodies. The excess fluorochrome was removed, air dried and viewed under fluorescent microscope (*Leica DM IRB, Germany)*.

II.4.2 DiI AcLDL Uptake Assay

The medium of 8^{th} day culture were changed with fresh DMEM/F12 containing 10% human serum and 10 µg/ml of 1,1-dioctadecyl-3,3,3,3-tetramethyl Indocarbocyanine perchlorate (DiI) labelled Acetylated Low Density Lipoprotein (DiI Ac LDL) (Molecular Probes, USA) and was incubated for 4h at 37^0C in CO_2 incubator. After 4h, the culture plate was washed three times with DMEM/F12 medium and viewed under inverted fluorescent microscope (*Leica DM IRB, Germany)* using Rhodamine filter.

II.4.3 BrdU proliferation Assay

Bromodeoxyuridine (BrdU), which is an analog of the DNA precursor thymidine can incorporate into newly synthesized DNA by cells entering and progressing through the S phase of cell cycle. The incorporated BrdU is stained with specific anti-BrdU fluorescent antibodies. This is a high resolution technique which provides nature and frequency of actively proliferating cells.

Cells were cultured the in the complete medium (DMEM/F12+Human serum) incorporated with BrdU (0.01mM) *(Brdu flow kit. BD Biosciene, Canada).* Cells were fixed by cytofix/cytoperm buffer for 30min at room temperature after 24h and 8^{th} day. The cells were treated BD cytoperm plus buffer in ice and washed after 10min. A re fixation was done by BD cytofix/cytoperm buffer for 5min on ice and washed with washing buffer. Cells were treated with DNase (300µg/mL of DPBS) to expose incorporated BrdU and washed off DNase. The intracellular antigen BrdU with FITC conjugated antibody and washed off excess antibody. The cells were observed under fluorescent microscope (*Leica DM IRB, Germany).*

II.5 Analysis of expression of mRNA by Real Time PCR

The cells were harvested from Fib coated and Fib+PD (20µg) coated plate (Method described as II.2.1) at 8^{th} day of culture. RNA was isolated using Trizol method according to manufactures description. RNA was quantified by spectrophotometric measurement (Diode array spectrophotometer, Hewlett Packard 8453, Germany). Using reverse transcription cDNA was prepared from one micro gram of mRNA (Master cycler, Eppendorf) and was used for analysing the expression of specific such as vWF (vWF: F, caccattcagctaagaggagg; R,gccctggcagtagtggata) and endothelial nitric oxide synthase (eNOS: F, agctgtgctggcatacagga; R, atggtaacatcgccgcagac). To compare the mRNA expression levels of Fib and Fib+PD cultured cells, the real time PCR was performed using a Chromo4 system (MJ Research). All reactions carried out in a total volume of 20µl

containing 10µl qPCR Mastermix, 200mM forward primer, 200mM reverse primer, and 8µl template cDNA for 35 cycles. A PCR melt curve was performed after real-time PCR for ensuring specificity for each gene. The base line was set by analysing copy number of glyceraldehyde 3-phosphate dehydrogenase (F, attggctttggtccgagtcc;; R, gggggttctttggcttttac) for comparing copy number of target gene. Fold change was calculated by comparing Fib wand Fib+PD cultured extracts of RNA, respectively with PBMNC sample (extracted before seeding) after making correction for PCR efficiency.

Chapter III- Results and Discussion

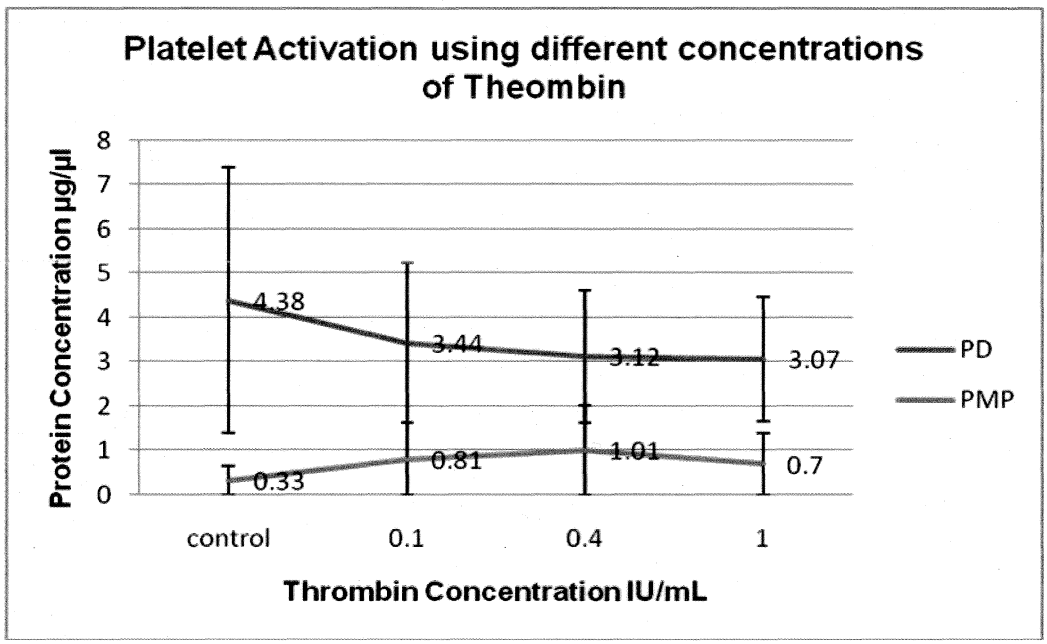
Specific objective of this study was to understand the role of platelet integrins CD62, CD61 CD41 on formation of complex between platelet debris and monocytes with subsequent changes to monocytes. Therefore, the first step in this study was to isolate PD from activated platelets and confirm that it was relatively pure and free from soluble proteins of platelets and from the light membrane particles. Initially, activated platelets with varying concentrations of thrombin were analyzed to establish the concentration at which all the PMP shedding took place. After they were found to reach a threshold, major fractions were separated, analyzed their MW and further characterized using antibodies specific to all three molecules. Results of fragment preparation and analysis are presented in III.A. Once it was proven that all the three molecules were present only on platelet debris, this fraction was exposed to PBMNC, and their complex was characterized by flow cytometry. Further, PBMNC culture and characterization of cell transformation was studied. Effect of integrins, CD62, CD41 and CD62 were tested by blocking these antigens using their specific antibodies and the blocked PD was exposed to study their role on PBMNC transformation. Results of all studies of PBMNC culture are presented in III.B.

III. A. Preparation of Platelet Integrins

III. A.1 Platelet Activation and Separation of Subcellular Fragments

Platelets from seven different donors were activated using different concentrations of thrombin (0.1, 0.2, 0.4 and 1 IU) for 1 h at 37°C. The sub cellular fractions of activated platelets were separated by the sucrose gradient centrifugation into three groups. Using ultracentrifugation of activated cell suspension on sucrose gradient, efficient segregation of activated platelet suspension into the soluble platelet releasate (PR), less dense platelet microparticles (PMP) that settled on sucrose bed and denser platelet debris (PD) was achieved. The insoluble fragments were further washed to remove contaminating sucrose completely. The yield of PMP and PR varied from

donor to donor as found in Lowry's protein assay (table 1). A major difference was observed when blood bank stored (>72h at 22°C) PRP was used for separating fragments (data not shown). Clearly, the best yield was from fresh platelets activated with 1U thrombin. The observation was consistent and indicated that when platelets were stored under standard conditions for transfusion, platelet activation caused PMP shedding and they probably mix with plasma in which platelets were stored.



FigIII.A.1 Graphical representation of PMP shedding from activated platelet: Platelets from 3 donors were activated using different concentrations of thrombin (0.1, 0.4 and 1 IU/mL).

The yield of PD and PMP proteins was calculated from Lowry's estimation. Graph represents estimated shed PMP of platelets (FigIII.A.1) from 3 donors activated using different concentrations of thrombin (0.1, 0.4 and 1 IU/mL). There is trend toward increased PMP and decreased PD concentration with thrombin dose up to 0.4 IU. But the 1IU thrombin did not show any proportionate increase in PMP with thrombin and PD concentration also remained steady with not much reduction. Thrombin is a strong agonist which actively accumulates at sites of vascular injury and provides one of the

chief means for recruiting platelets into a growing hemostatic plug. Recent studies showed that the higher concentrations of thrombin induce apoptosis rather than the blood coagulation and platelet activation. At high thrombin concentrations 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]. In the experiment presented here, active shedding of PMP may have decreased due to the initiation of apoptosis. No experiment was done to prove that there was any apoptosis.

Shedding of PMP on platelet activation is well described in literature. They consist of membrane vesicles and some of the adhesion receptors. Therefore, it has been reported that activated platelets are found as round cells which are not capable of recruiting into aggregates causing bleeding disorders in patients who undergo cardiopulmonary bypass and hemodialysis. In these cases platelet activation is due to contact with extracorporeal devices and mechanical activation due to the use of pumps. A similar activation and shedding of membrane takes place in patho-physiological condition due to generation and exposure to mild dose of agonists such as thrombin and ADP. Here we have seen that PMP shedding takes place even with very low thrombin dose.

III.A.2 Analysis proteomic pattern of isolated platelet fractions

The proteins of PMP and PD were separated using 8% SDS polyacrylamide gradient gel electrophoresis and were silver stained. The loaded concentrations of proteins were adjusted for PD (20μg) and PMP (10μg). Molecular weights were determined using *AlphaEaseFC* software. The patterns of separated protein bands are shown in Fig III.A.2.

The figure shows that the PMP shedding increases with the concentration of thrombin. The number of bands in 0.1 IU thrombin activated PMP was visibly less as compared to PMP collected after activation with IU

thrombin. At 1IU almost all PMP fractions actively released from the platelet with prominent protein bands in SDS-PAGE. In case of PD both the concentrations show almost same number and pattern of bands, which may be due to the large quantity of PD compared to PMP. Here it was clearly demonstrated that a minor increase of thrombin concentration reduce the chance of PMP retention with PD.

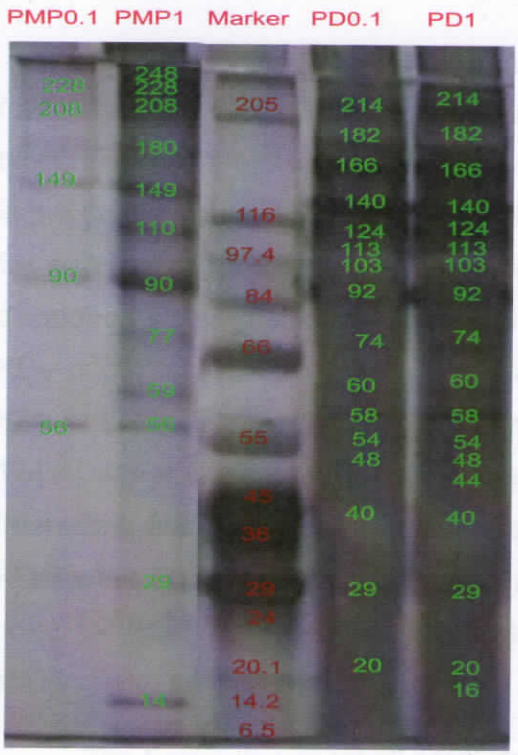


Fig III.A.2 SDS-PAGE pattern showing the proteins in platelet sub cellular fragments. Estimated quantity of PD and PMP were digested with loading buffer. The SDS-PAGE of the platelet sub-cellular fragments was done using 8% gel and silver stained. First two lanes are PMP from 0.1 and 1 IU thrombin activated platelet and lane 4 and 5 are associated PD from the same sample as PMP. Marker loaded was high molecular weight mixture from Sigma.

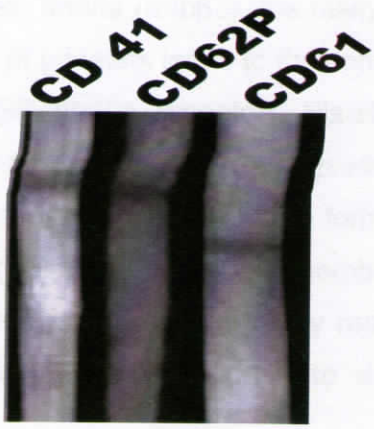


Fig III.A.3 Western blot for CD41, CD62P and CD61 of PD (prepared by 1 IU thrombin activation)

CD62P (P selectin) – 140 KDa

CD41 (Alpha2b) – 135 KDa

CD61 (Beta 3) – 87KDa

So, 1 IU thrombin was used to insure that all PMP was removed and PD with relatively no PMP component could be isolated for specific study purpose. There is difference in MW of proteins between PMP and PD; molecular characterization of 3 different proteins was done in PD using Western blot analysis. Moreover, focus of this study was to understand the effect of integrin molecules of PD for transformation of progenitor population in to endothelial cells. Western blot analysis confirmed that integrin molecules of interest were present on PD.

All three molecules of interest in this study, CD41 (MW 140KDa), CD61 (135KDa), and CD61(87 KDa) were developed as strong bands on Western blots. For all the blots SDS-PAGE was done by loading digested PD protein into a single trough and after the transfer to nitrocellulose, slices were made for each staining. Therefore, difference in MW is appreciable even in the developed blots. Position of CD62 in the SDS PAGE (Fig IIIA2) might be 140 KDa band; for CD41 the 135 KDa MW proteins might be merged with 140 KDa proteins. There was no other band close to 135 KDa and the next was 124 KDa. For CD61 (87Kda) the closest band in SDS-PAGE might be 90KDa. Interesting finding is none of these protein bands (140 KDa, 135 KDa, or 87 KDa) were there in the PMP fraction (FigIII.A2). Western blot analysis was done for PMP but no band developed for any of these three proteins on staining with antibodies.

The final pathway for all agonists is the activation of the platelet integrin glycoprotein IIb/IIIa (αIIbβ3), the main receptor for adhesion and aggregation. Activation of platelets leads to the conformational changes in the platelet $\alpha_{IIb}\beta_3$ integrin (GP IIb/IIIa receptor). Platelet aggregation, mediated primarily by interaction between the activated platelet GP IIb/IIIa receptor and its ligands, fibrinogen and vWF, results in the formation of a platelet-rich thrombus. Also P selectin (CD 62P) on platelet membrane actively participate in recruitment of monocytes for the inflammatory reaction [Tsuji T *et al,* 1994]. Therefore, further experiments were done to delineate role of these three integrin

molecules GPIIb (CD41), GPIIIa (CD 61) and P-selelctin (CD62P) for interaction with monocytes.

III.B. Cell Culture Results

Objective of the second phase of the study was to identify effect of PD on PBMNC when they were cultured on a biomimetic matrix which includes a fibrin matrix, and the PD immobilized within the fibrin network. Human serum devoid of any soluble platelet factors was used as culture supplement. Thus out of various components of activated platelets, the more dense and insoluble fragments only were exposed to PBMNC. Incorporation PD within the fibrin matrix was done by adsorbing thrombin on the surface and clotting the mixture of cryoprecipitate and PD on the surface, as a very thin uniform layer. Various experiments were designed to address the questions such as: (i) Is there a difference if quantity of PD incorporated in a unit area is increased? (ii) What is the effect on cell morphology? (iii) How long it takes to attain stable morphology? (iv) Is the cell population immunochemically distinguishable? (v) Is there a difference in the expression of cell specific mRNA? (vi) What may be the influence of surface integrins CD61, CD41 and CD62 on complex formation of PD with monocytes? (vii) What may be the effect of the three integrins on cell transformation? and (viii) What could be the role of CD34 population for conversion to endothelial lineage in the designed matrix? The systematic approach employed in this study has revealed a difference in monocyte transformation to macrophage or endothelial cells, depending on the nature of the culture matrix used. The result of each specific experiment is illustrated below.

III.B.1 PBMNC culture: effect of PD concentration

Concentration of PD was adjusted in each culture well based on the Lowry's protein. Each well with surface area 1.75 cm^2 in 4-well culture plates were coated with fibrin as the control; in each case and other three wells were coated with PD incorporated in different concentrations, like 10μg, 20μg and 40μg per 1.75cm^2 per well with no change in fibrin concentration (details in *II.2.1*). Number of monocytes in each well was adjusted by counting the PBMNC suspension using Sysmex Hematology counter and was maintained

same in all 4 wells for each experiment. Cells added in all 4 wells for each experiment was from the same donor and at least 3 replicate experiments were done using 3 separate donor's PBMNC. Culture medium used (DMEM/F12) and the supplement (10% human serum) added were also kept the same in all replicate experiments. Thus keeping all conditions same in culture wells except for the PD and its dose, morphology of cells was monitored closely at different time intervals. It was observed that the morphology attained in 8 days remained the same on the 10^{th} day as well. Therefore, for further experiments, period of final analysis was kept as 8 days after starting the culture.

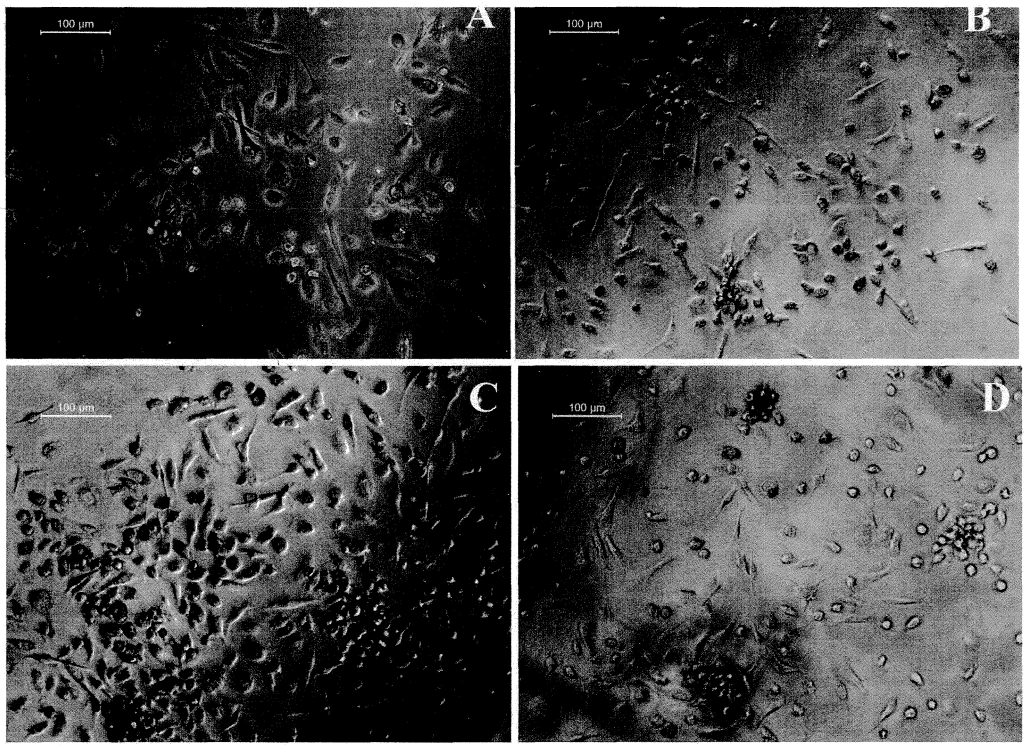


Fig III.B.1 Phase contrast micrographs (20X) showing the effect of PD, on PBMNC culture viewed on 8^{th} day: A, FG control; B, 10ug PD+FG; C, 20ug PD+FG; D, 40ug PD+FG. Elongated cells were found on all substrates. Round and foamy appearance for cells were found more in the FG control.

Within the heterogenous population of culture there were two major types of cells on which the analysis was focused: (i) Round or slightly elongated cell with a foamy appearance; (ii) elongated and spindle-shaped cell with a cobble stone appearance. There were many cells which did not fall

into both groups with no major change in shape or size. Focus of our analysis was foamy cells and spindle-shaped cells. The most significant observation was that in almost all replicate cultures of fibrin control, appearance of foamy cell was predominant (FigIIIB.I.A). They also had spindle shaped cells but with large number of fenestrations which is a characteristic feature of foam cells. Clearly, the presence of PD in culture matrix showed appreciable reduction in round cells with foamy appearance (III.B.1. B, C& D). As the concentration was increased from 10 ug to 20 ug, spindle shaped cell with cobblestone morphology was dense, and the shape is a characteristic feature of endothelial cells. Between the well of fibrin control and 10 ug PD, the major difference was presence of elongated cells in the latter, even though round foamy cell was also present in these, in relatively low number. Increase in PD concentration from 20 μg to 40 μg did not show significant increase in the density of spindle-shaped cells.

Morphological observation thus indicated differentiation of monocytes into macrophages or endothelial cells. Therefore, next approach was to identify if these cells turned to macrophages or endothelial cells, with the influence of specific matrix composition or concentration. So as a preliminary step, cells in all 4 culture wells were stained using antibodies against CD68 and CD 31, separately.

In fibrin control, cells showed strong positivity for CD68 (Fig III.B.2.A) and the signals were much lighter in PD coated plates (Fig.III.B.2.B,C,D). This observation substantiates the morphological feature that PD in the matrix reduced the incidence of macrophages or foam cell formation from PBMNC. Staining with CD31 showed no positive signals in fibrin control plate (Fig III.B.3 A) in spite of many elongated cells that showed up during morphological analysis. In cultures with 10μg and 20μg PD, weak $CD68^{+}$ signals were seen infrequently (Fig III.B.3, B, C). On the other hand, the plate with 40μg PD ((Fig III.B.3D) showed significantly high positivity for CD31 marker, well distributed in the entire culture surface.

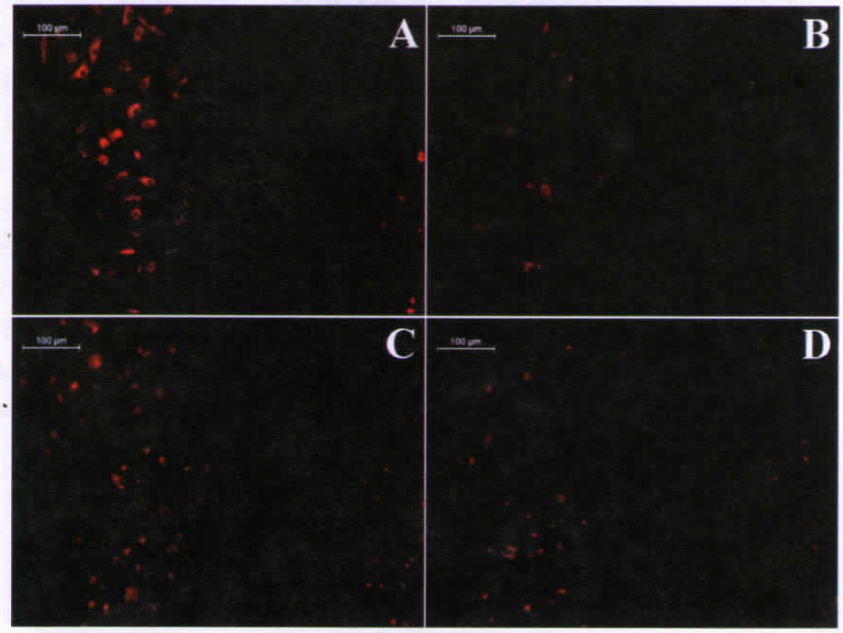


Fig.III.B.2 The fluorescent micrographs (20X) for CD68$^+$ on 8th day: A, FG control; B, 10ug PD+FG; C, 20ug PD+FG; D, 40ug PD+FG. . The micrographs (20X) show CD68 positive bright cells in the control.

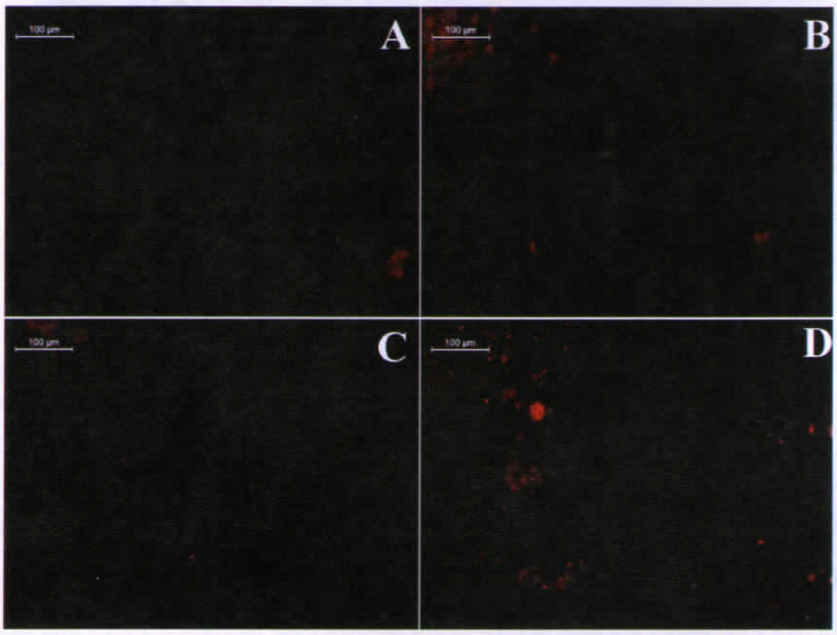


Fig.III.B.3 The fluorescent micrographs (20X) for CD31$^+$ on 8th day: A, FG control; B, 10ug PD+FG; C, 20ug PD+FG; D, 40ug PD+FG. . The micrographs (20X) show CD31positive cells PD coated plates and most prominently with 40ug.

Clearly, more concentration of PD betters the expression of CD31, the endothelial marker. On the contrary, more the concentration of PD less was the expression of CD68, the macrophage marker.

It is likely that the increased generation of CD31$^+$ cells in presence of PD, is either due to cell proliferation or due to increased cell attachment, and

needs evaluation. This aspect is not well addressed in this study. However, gross observation suggested that there was increased attachment of PBMNC population to the matrix which had more PD. The effect on cell attachment was not quantified because there were too many cells after seeding and they were a heterogenous population. The proliferation capacity of PBMNC population was analyzed by BrdU uptake assay. By the time the culture reached 6 days old, by which time about 4 medium changes were done, cell number was reduced, probably because most of the nonspecific lymphocyte contamination was eliminated. The BrdU incorporation was observed after 8 days of culture which only might indicate if one or more population has proliferated.

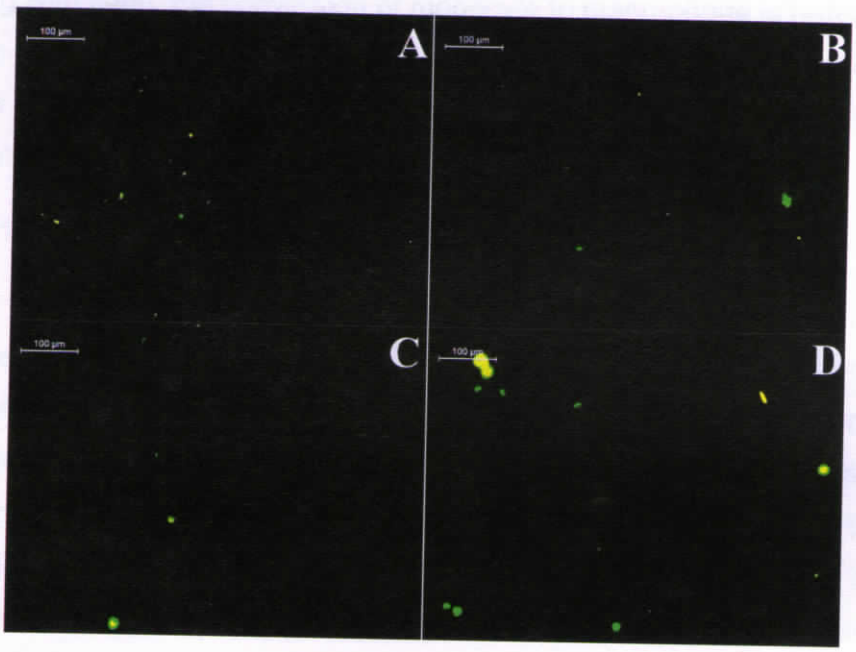


Fig III.B.4 The fluorescent micrographs (20X) showing the BrdU staining for cultured cells up to 8th day: T A, FG control; B, 10ug PD+FG; C, 20ug PD+FG; D, 40ug PD+FG.

Analysis for BrdU positive cells revealed that (Fig III.B.4) the number of proliferative cells in fibrin control and other three PD coated plates were almost similar. So incorporation of PD in culture did not seem to induce any significant acceleration of proliferation potential of cells in culture. The signal seen may reside on mixed population. Unless dual labeling is done it is difficult to pinpoint which of the specific cell in the mixture has proliferated.

During early atherosclerosis, monocytes migrate into the subendothelial layer of the intima where they differentiate into macrophages or dendritic cells. In the subendothelial space, enriched with atherogenic

lipoproteins, most macrophages transform into foam cells. Foam cells aggregate to form the atheromatous core and as this process progresses, the atheromatous centres of plaques become necrotic, consisting of lipids, cholesterol crystals and cell debris (Bobryshev YV 2006). From the current study results, it is suggested that if monocytes lodge into fibrin clots with not many platelets around, they are likely to differentiate in to macrophages. Whereas in the presence of platelet debris, which is part of activated platelets, along with fibrin, the conversion of monocyte to macrophage is reduced and in such cases more monocyte to endothelial conversion becomes effective. In such an event, most likely regenerative role of platelets may develop and not the inflammatory role.

III.B.1.1 Real Time PCR for endothelial markers

This real time PCR analysis was conducted to verify the endothelial gene expression levels in transformed PBMNCs in fibrin control and PD coated plates. In order to facilitate good mRNA yield culture was done on a larger surface area (9.6cm^2 dishes). Concentration of PD in the coating was proportionately increased as the surface area was different. Since there was no significant difference between 20 ug dose and 40 ug dose in the initial experiment that analysed the dose effect on cell morphology and immunochemistry; only 20ug was used in this experiment which analysed gene expression. Cells were harvested after 8 days of PBMNC culture and RNA isolation was done. A fraction of PBNMC from the same donor was used for RNA isolation on the day of blood collection to demonstrate increase in mRNA expression as an effect of culture on various study matrices. After converting known quantity (same in each case) of RNA to cDNA, fixed volume was used for Real Time PCR analysis for the endothelial genes, vWF and eNOS and GAPDH was set as the house- keeping gene.

The gene expression was calculated by ddCt (delta delta Ct) method using the software. In Real Time PCR analysis Ct number is defined as the threshold level of log-based fluorescence, which is the observed value in most of the real-time PCR experiments. Delta Ct (dCt) for each gene (target or reference) is calculated by subtracting the Ct number of target sample from that of control sample.

Here it is, $\mathbf{dCt = Ct_{\text{sample gene}} - Ct_{\text{GAPDH}}}$

ddct is calculation by substraction dCt value of control sample from dCt value of treatment.

Here it is, $\mathbf{ddCt = dCt_{\text{(PD/Fibrin)}} - dCt_{\text{PBMNC}}}$

And the ratio of target gene expression in treatment (PD/Fibrin) versus control (PBMNC) can be calculated by 2^{-ddCt}

Interestingly, only cDNA from cells on PD coated plate showed a two fold increase in eNOS (2.492) and vWF (1.986). At the same time cells grown on fibrin coated plates showed quiescence of eNOS expression (0.7022) whereas vWF did not amplify in the case of original PBMNC or those cultured on fibrin.

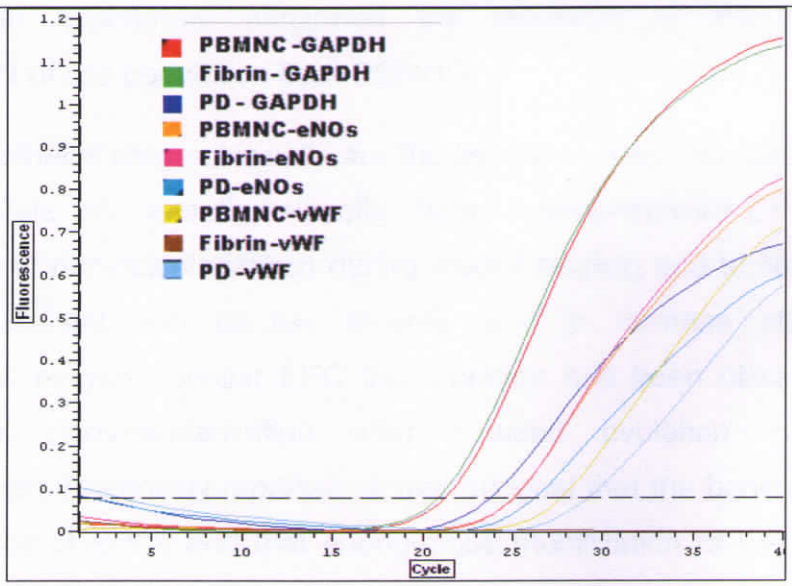


Fig.IIIB.5 Real time PCR amplification plot

Well / Set	Description	C(t)	dc(t)	ddc(t)	2-ddc(t)
A1	PBMNC GAPDH	21.25	NA	NA	
A2	Fibrin GAPDH	20.79	NA	NA	
A3	PD GAPDH	23.73	NA	NA	
B1	PBMNC eNOs	25.03	3.78	NA	
B2	Fibrin eNOs	25.08	4.29	0.51	0.7022
B3	PD eNOs	26.19	2.46	-1.32	2.492
C1	PBMNC vWF	27.68	6.43	NA	NA
C2	Fibrin vWF	N/A	NA	NA	NA
C3	PD vWF	29.17	5.44	-0.99	1.986

Tabele I: C(t) values obtained from Real Time PCR analysis and calculation of $2^{-ddc(t)}$

The data showed that even though elongated morphology was seen in both cultures (fibrin alone and fibrin+PD) only the cells grown on matrix containing fibrin+PD, expressed genes specific for endothelial lineage. The RNA amplification experiment confirmed the influence of PD on lineage commitment of cell population from PBMNC.

Endothelial progenitor cells are the immature cells that have the ability to differentiate into endothelial cells. Bone marrow-derived EPCs have a critical role in neovascularization during wound healing and tumor growth, in ischemic skeletal and cardiac muscle, and in corneas after corneal micropocket surgery. Similar EPC incorporation has been observed during endometrial neovascularization after induced ovulation or estrogen administration. Previously reported studies suggest that the bone marrow is a major source of EPCs and that endogenous mobilization of EPCs from the bone marrow into the peripheral blood occurs in response to a physiological need for neovascularization. The mobilized EPCs are then recruited into the foci of neovascularization and contribute to new blood vessel formation. This mechanism is also supported by clinical observations of EPC mobilization in

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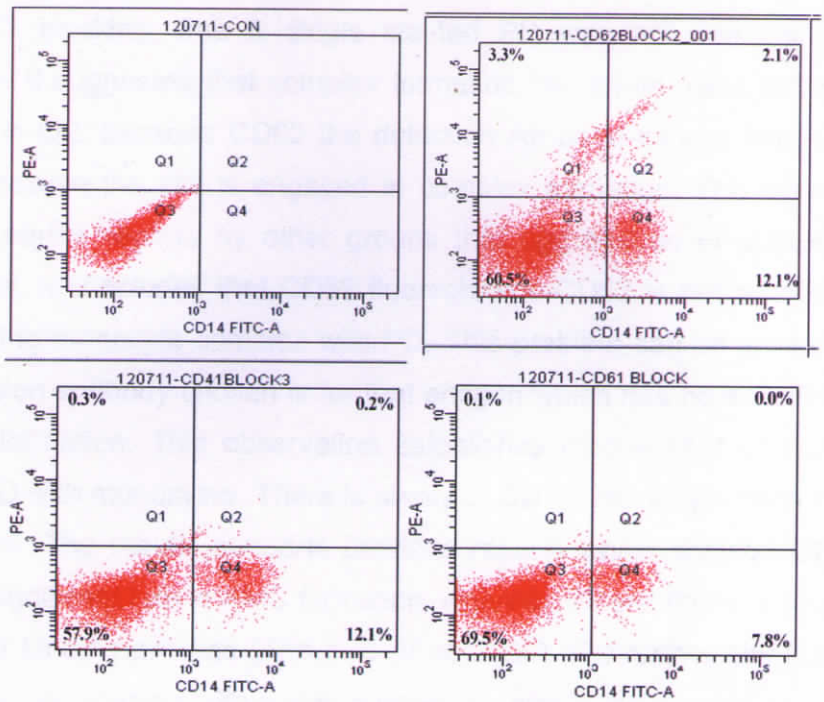


Fig.IIIB.6 Representative dot plots of dual stained PD-monocyte complex: When CD62 on PD was blocked with antibody, staining was done using PE-CD41 antibodies (B). When PD was blocked with CD41 (C) or CD61antibody (D) complex was stained with CD62 antibody.

Antibody bloked	Q1 (PD)	Q2 (PD+Mono.)	Q4 (Mono.)
CD62P	3.5±0.2	2.7±0.6	12.6±2.9
CD41	0.4±0.2	0.4±0.2	11.9±0.2
CD61	0.2±0.1	0.13±0.11	9.6±1.6

Tabele 2 : Flow cytometry data showing % of PD, PD+Monocyte and Monocyte in antibody blocked PD- Monocyte interaction

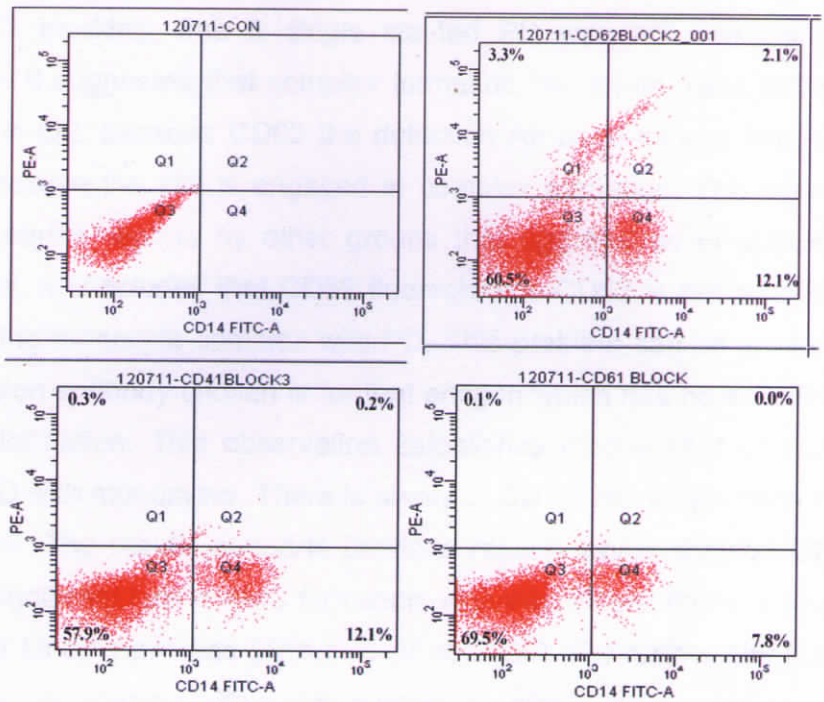
Most significant observation was that single stained PD was maximal in the suspension when CD62P was blocked using antibody. Most of PD remained single stained probably because monocyte-PD complex formation did not take place. It may be interesting to see if consequence of blocking the

patients with burns or acute myocardial infarction and in patients undergoing coronary artery bypass graft surgery. Mononuclear cells other than EPCs (e.g., $CD34^-$ cells) may contribute to ischemic neovascularization by secreting angiogenic cytokines. The preclinical and clinical evaluation of EPCs for neovascularisation is a dynamic field in cardio vascular research [reviewed by Kawamoto A and Losordo DW, 2008]. Present result suggests that conversion of progenitor cells in to endothelial cells may take place in the vascular injury environment, in presence of activated platelet debris.

III.B.2 Influence of integrins on complex formation of PD with monocyte

Prolonged inhibition of platelet adhesion to the artery wall by infusion of antibodies against GPIb or GPIIb/IIIa dramatically reduced atheroma formation, while the infusion of activated platelets significantly increased plaque burden (Huo A 2003, Massberg s 2002). Platelet expressed P-selectin has been reported to play a major role in atherosclerotic lesion development (Buger PC 2003). There are various reports which suggest role of integrins on atheroma formation. In this context, experiments were done to understand if platelet integrins CD62P, CD41 or CD61 play any role in forming PD-monocyte complexes which in turn, may influence further changes in monocyte during long term culture. For enabling this, antibody against each integrin was incubated with separate aliquots of PD and then each PD-antibody complex was mixed with isolated PBMNC. A different antibody, which was not added for blocking antigen site, was used to analyze presence of PD in monocyte-PD aggregates. The aggregates were also labeled with monocyte marker CD14. The dual stained monocyte-PD complex was then analyzed using flowcytometry and fluorescence from both fluorochromes, PE and FITC was compensated for possible spectral overlap.

Flow cytometry analysis showed significant reduction in interaction of PD with monocyte when blocked with three antibodies (CD62P, CD41 and CD61). In the dot plots, Q1 represent PD, Q2 represent PD-monocyte complex and Q4 represent CD14+ monocyte.



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Most significant observation was that single stained PD was maximal in the suspension when CD62P was blocked using antibody. Most of PD remained single stained probably because monocyte-PD complex formation did not take place. It may be interesting to see if consequence of blocking the

binding site has reflected on long culture too. In the case of CD41 blocking and CD61 blocking, free & single stained PD was not detected in flow cytometry. It suggested that complex formation has taken place but was not detected in Q2, because CD62 the detection Ab used cannot bind with the coplex because the site is engaged in complex formation. This observation supports earlier reports by other groups that aggregation of platelets with monocytes. It concludes that CD62 fluorochrome-CD62 is not a good probe for detecting monocyte complex with PD. This problem can be solved only if the detection antibody chosen is for that antigen which has no specific role in complex formation. This observation establishes involvement of CD62P in binding PD with monocytes. There is plenty of $CD14^+$ monocyte seen in Q4 in both cases. The results supports previous reports which showed GPIIb/IIIa have no significant role in PMC formation, whereas P-selectin have significant role in the binding process [Ahn K C *et al,* 2005]. For further clarification of this data, more analysis with higher number of sample are needed.

III.B.3. Study of Integrin molecules on PBMNC culture

This experiment was designed for elucidating role of three major integrin molecules of PD, such as CD62P (P-selectin), CD41(α2b), CD61(β3) in transformation of PBMNC population. How each one may have effect on monocyte-macrophage conversion or monocyte-endothelial conversion was the focus.

Monoclonal antibodies against each of these three antigens were separately incubated with PD to block their activity, and then the blocked PD was incorporated into fibrin matrix that was used as culture surface. Here the control well had unblocked PD and in each case 20 µg PD concentration was used. The culture was done as described in II.3.1 and II.3.2. Significant morphology changes were observed in phase contrast microscopy at 20x resolution (Fig III.B.7) among 4 culture matrices. All cultures were heterogenous on the 8^{th} day when analysis was done. Most significant observation was that when CD62P was blocked; higher number

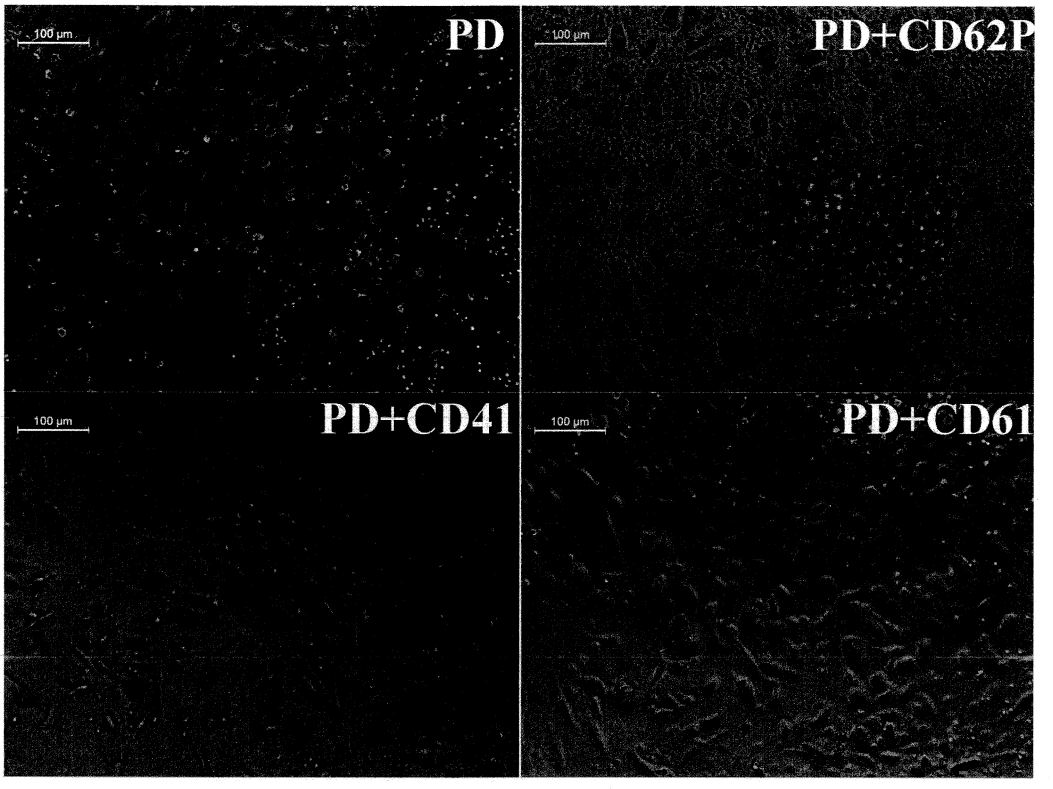

Fig III.B.7 Phase contrast micrographs (20X) for effect of PD blocked with antibodies on PBMNC culture viewed on 8th day: The type of Ab used for blocking is marked. FG used was same in all wells.

Spindle shaped cells were seen infrequently (Fig IIIB7 B), but even when the morphology was endothelial-like, cells showed foamy appearance. The results of binding experiments using flow cytometry may be connected to the cell culture results. It was found that blocking of CD62 on PD with antibodies left unbound PD, and the monocytes turned into macrophages as it appeared on the fibrin control in the first experiment. On unblocked PD surface spindle-shaped cells were present but few round foamy cells were also seen. Blocking the CD41 and CD61 sites distinctly produced more spindle shaped cells with cobblestone morphology (Fig.IIIB7.C &D), which is a characteristic feature of endothelial cells. However, round and foamy cells were also present on such blocked matrices as well. Out of these CD61 blocked plates showed higher number of endothelial-like cells compared to that on CD41 blocked or the unblocked control culture matrix. To confirm these observations, immunocytochemistry for CD68, CD31, Ulex lectin were done.

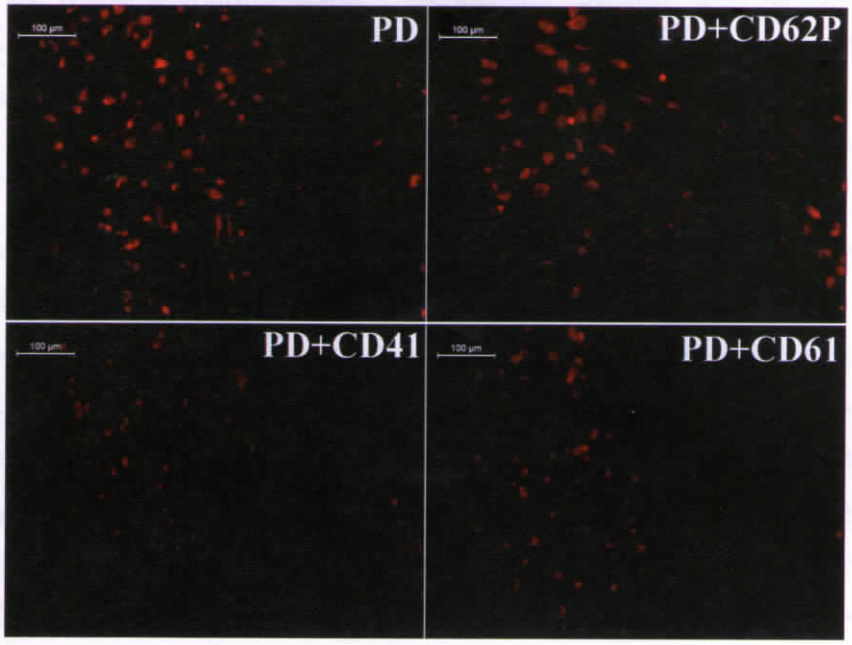

Fig.III.B.8 Fluorescent micrographs (20X) for effect of blocking antigens of PD, on CD68 expression. The type of Ab used for blocking is marked. FG used was same in all wells. Ab concentration was 0.4 ug/well

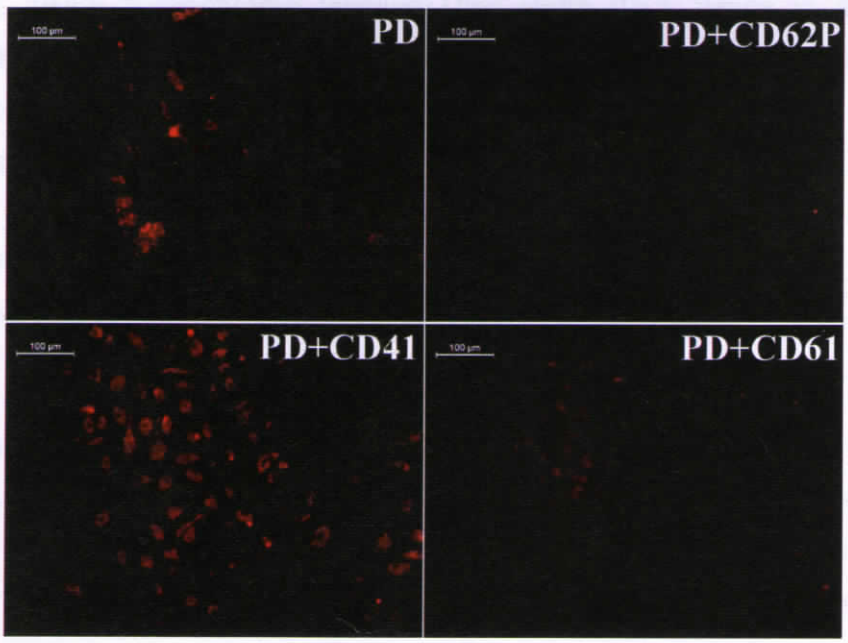

Fig.III.B.9. Fluorescent micrographs (20X) for effect of blocking antigens of PD, on CD31 expression. The type of Ab used for blocking is marked. FG used was same in all wells. Ab concentration was 0.4 ug/well.

By CD68 immunostaining, macrophage lineage committed cell with varied expression of CD68 antigen were found in PD control and CD62 P blocked cultures (Fig III.B.8 A &B). Cells on CD62P blocked plates showed higher frequency of $CD68^{+}$ population compared to the PD control plates. There is a visible reduction in CD68 intensity in CD41 and CD61 blocked cultures. No flow cytometry was done with these cells to quantify the percentage of positive cells or intensity of fluorescence. Only if such expression is quantified *we* may conclude what is the proportion of each cell type. The lineage committed cells may be detected as bright positives, weak positives or negative and the percentage of each would be known by using flow cytometry. On the other hand, there was not a single cell positive for CD31 in culture on CD62-blocked PD (Fig III.B.9). On the culture matrices with CD41 and CD61 blocked PD; cells were positive for CD31 and so were on unblocked PD-fibrin surface as well. This observation confirmed that interaction of monocytes with platelets through CD62 is needed for transformation of the cells to endothelial cells.

An additional staining that was done to ascertain the CD31 staining profile, using fluorochrome labelled Ulex lectin (UL), which is a specific endothelial marker. The micrographs show that (Fig.III.B.10) UL binding profile followed exactly similar pattern like CD31 staining. No cell on CD62 blocked surface stained positive for UL. On all the other matrices; CD41 and CD61 blocked or unblocked PD, UL positive endothelial cells were found. This finding further substantiated the data and confirmed that if CD62 is blocked there is no monocyte conversion to endothelial cells.

Also a Dil AcLDL uptake assay was performed (described in II.3.2) for confirming presence of macrophage or endothelial cells in all cultures. This test is not sufficient to distinguish between EC and macrophage because both types of cells are known to take up LDL through the receptors. The fig III.B.11 show that all the evolved populations have the ability to uptake tagged LDL,

so, confirming that mixture of endothelial and macrophagic lineage co-exists. In the long term cultures if there is a mutual effect is not known.

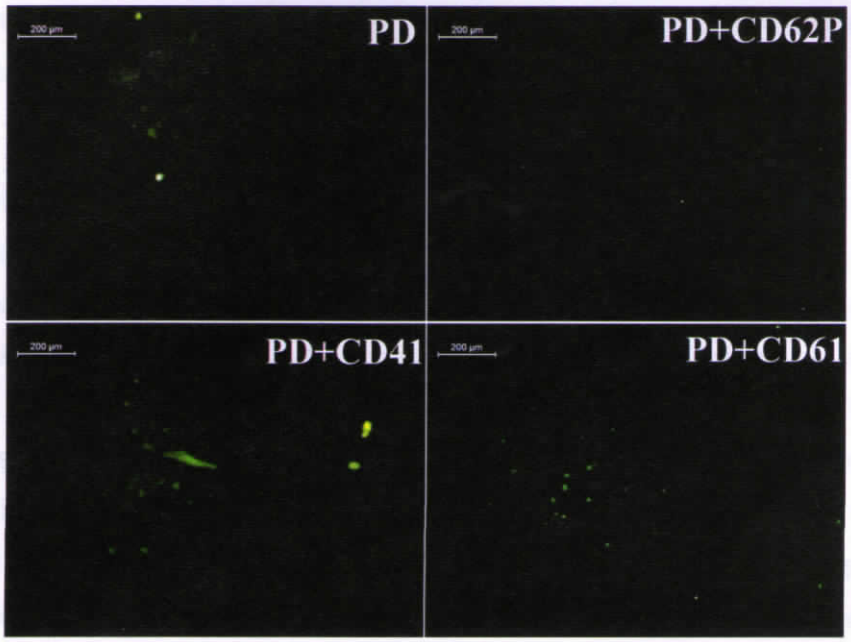

Fig.III.B.10 Fluorescent micrographs (20X) for effect of blocking antigens of PD, on Ulex lectin binding. The type of Ab used for blocking is marked. FG used was same in all wells. Ab concentration was 0.4 ug/well.

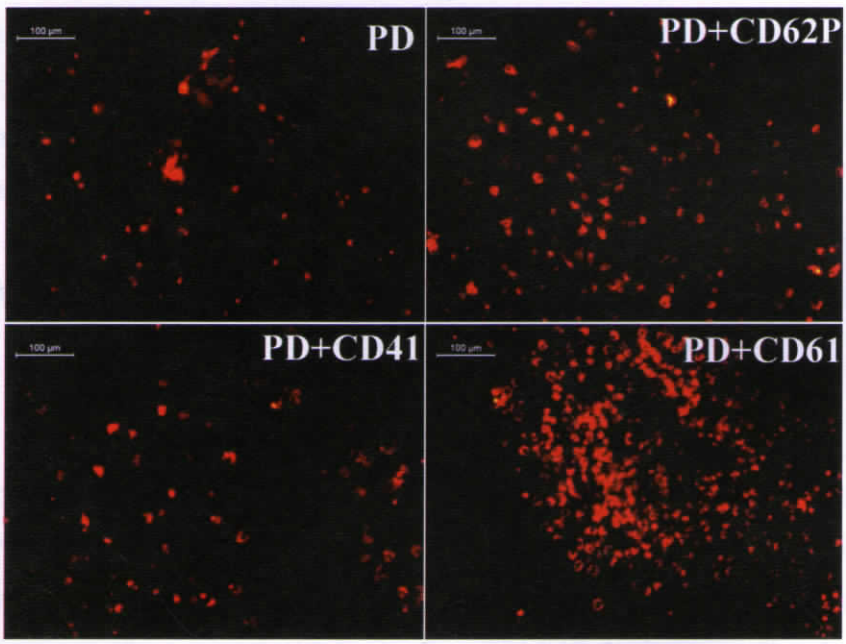

Fig.III.B.11 Fluorescent micrographs (20X) for effect of blocking antigens of PD, on DiI-AcLDL uptake. The type of Ab used for blocking is marked. FG used was same in all wells. Ab concentration was 0.4 ug/well.

There is appreciable density in culture wells that were already transformed to either of the cell type. Phase contrast microscopy had already shown the presence of heterogenous cell population which was further confirmed to be either macrophage or EC and cells are viable because LDL was endocytosed.

It has been already reported that GP IIb/IIIa receptor block resulted in a potent inhibition of platelet–platelet aggregation but failed to inhibit monocyte–platelet interactions, suggesting that platelet GP IIb/IIIa does not play a role in monocyte–platelet interactions and that other molecules mediate adhesion. Blockade of P-selectin consistently produced greater inhibition than with PSGL-1 mAb, indicating involvement of other monocyte receptors. The results of the blocking experiments in the current study suggest importance of CD62P in combining with PD to influence angiogenesis, via endothelial transformation of PBMNC population. Also, it is noted that absence of P-selectin interaction may lead to inflammatory pathways by enhancing macrophagic lineage commitment of monocytes. So inefficiency of activated platelet-selectin binding to monocyte may be one of the reasons for atherosclerosis progression. Also integrins like CD41 and CD61 did not show any significant role in endothelial formation. Their block helped to reduce the macrophagic conversion of monocyte culture, suggesting that platelet GPIIb/IIIa binding to monocytes may be necessary for inflammatory process in atherosclerosis. Further studies are needed to confirm this result, and it can be a potential reason for effectiveness of these molecules as therapeutic targets.

III.B.3 Effect of integrin blocked membrane on CD34 progenitor cell

The major objective of this experiment was to identify the subpopulation which gets transformed to endothelial lineage and the effect of integrins on the differentiation. In order to test if $CD34^+$ cells (endothelial progenitor cells?) were the major cell type in PBMNC population that got transformed; they were enriched after PBMNC isolation. For isolation of

$CD34^+$ cells, magnetic cell sorting method was used (method II.3.3) and the efficiency of isolation was analyzed using flow cytometry after staining the MACS selected/unselected cell isolates. MACS sorted cells were too few to be used as negative control in FACS analysis. The negative gate for fluorochrome analysis was chosen using whole PBMNC fraction. Enrichment was effective and resulted in ~30% $CD34^+$ cells in the magnetic sorted PBMNC. Flow cytometry data shows that (Fig III.B.12) the sorted population contains good population of $CD34^+$ cells in MAC sorted population. And there were no $CD34^+$ cells in the depleted population. Since there was no $CD34^+$ cell in the magnetic field washout cells, it is a good population for testing the effect of PD-fibrin matrix on $CD34^+$ culture and $CD34^-$PBMNC culture.

The larger and denser $CD34^{+low}$ cells are the more mature progenitor cells, already committed to myeloid, lymphoid or erythroid differentiation. They are $CD34^{-dim}$ and were responsible for short-term colony growth *in vitro* (Herbein, G *et al,* 1994). So the entire CD34 cells from a donor was isolated my this method. The culture of PBMNC, $CD34^+$ and $CD34^-$ cells from a single donor were set in PD and integrin blocked PD matrices. Phase contrast image of PBMNC was used as the control for $CD34^+$ and $CD34^-$ culture. PBMNC culture showed elongated morphology in all the plates with PD alone and integrin blocked PD (Fig III.B.13). However, the cell on CD62P blocked PD showed foamy appearance, in spite of the elongated morphology. In CD34+ cell culture large round cells were infrequent or absent. Cells were transformed into spindle shape cells and the change was prominent by 8^{th} day of culture (Fig III.B.14).

Here also the CD62P blocked plate gave foamy elongated cell and the other two integrin blocked plates showed few cobble stone morphology. The $CD34^-$ cells do not show any elongated cells even at the end of 8^{th} day. The CD62P blocked plate showed presence of large round population with foamy appearance, which is a typical characteristic of macrophages (Fig III.B.15). So this illustrated that $CD34^+$ population is not prone to macrophagic lineage.

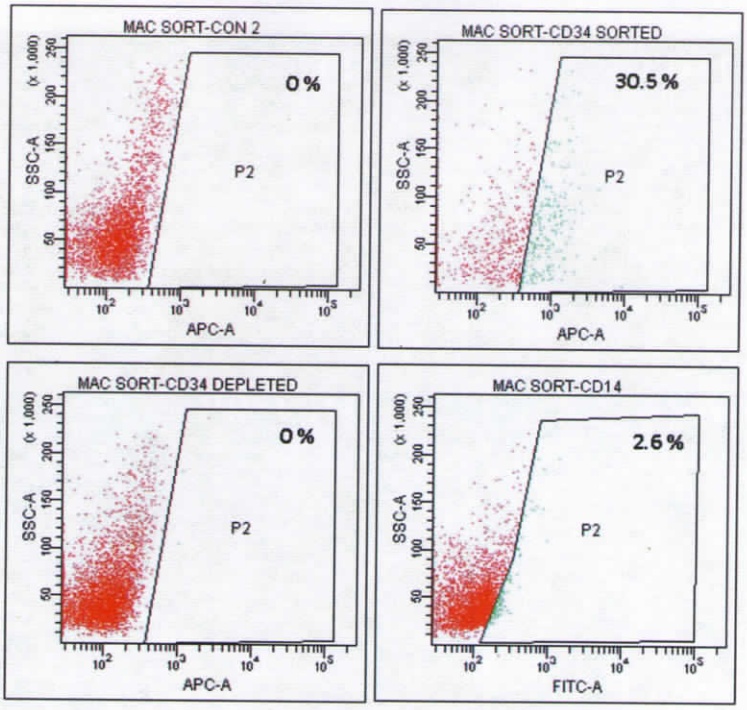

Fig III.B.12 Dot blot for CD34+ in MAC sorted and depleted population: This represents ~ 30% of $CD34^+$ population in the sorted sample. And the depleted population do not contain any $CD34^+$ population but contains 2.6% of CD14 cells

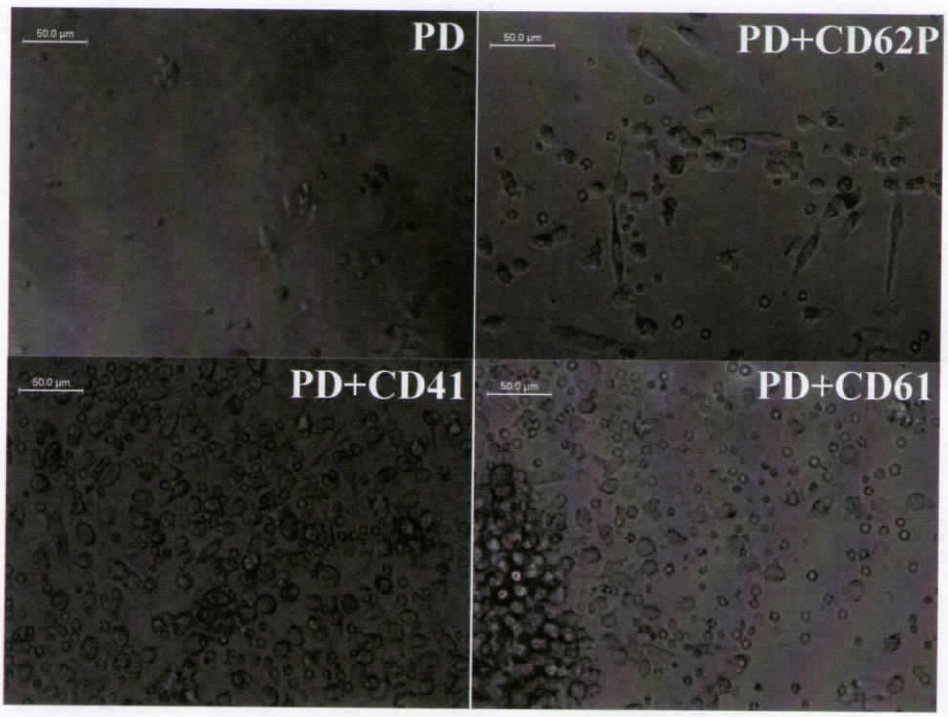

Fig III.B.13 Phase contrast micrographs (20X) for effect of PD blocked with antibodies (2μg each) on PBMNC culture viewed on 8th day: The type of Ab used for blocking is marked. FG used was same in all wells.

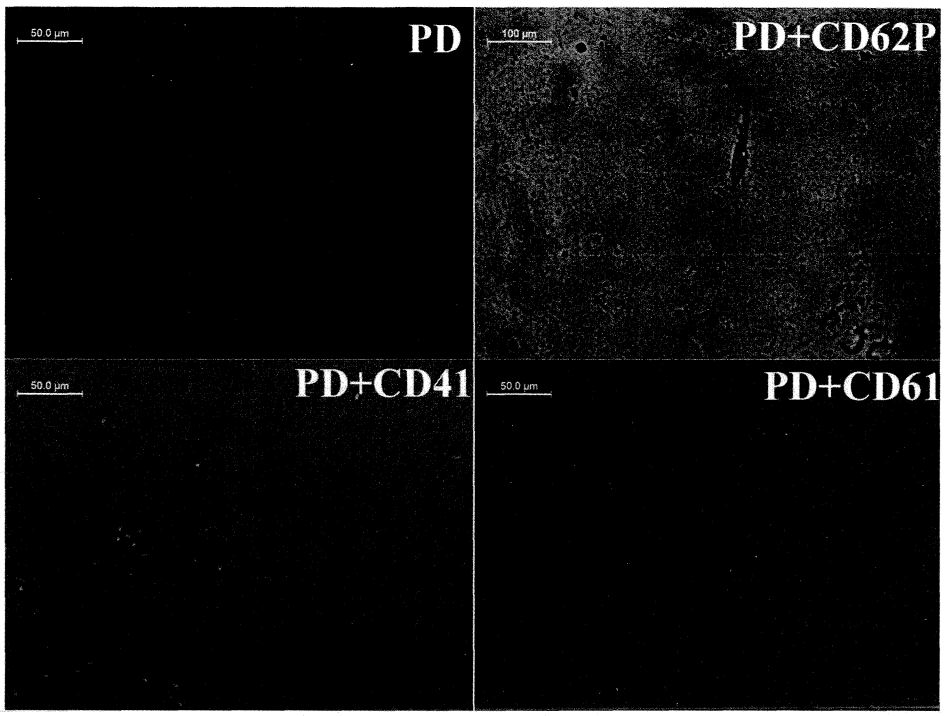

Fig III.B.14 Phase contrast micrographs (20X) for effect of PD blocked with antibodies (2ug each) on $CD34^+$ population culture viewed on 8^{th} day: The type of Ab used for blocking is marked. FG used was same in all wells.

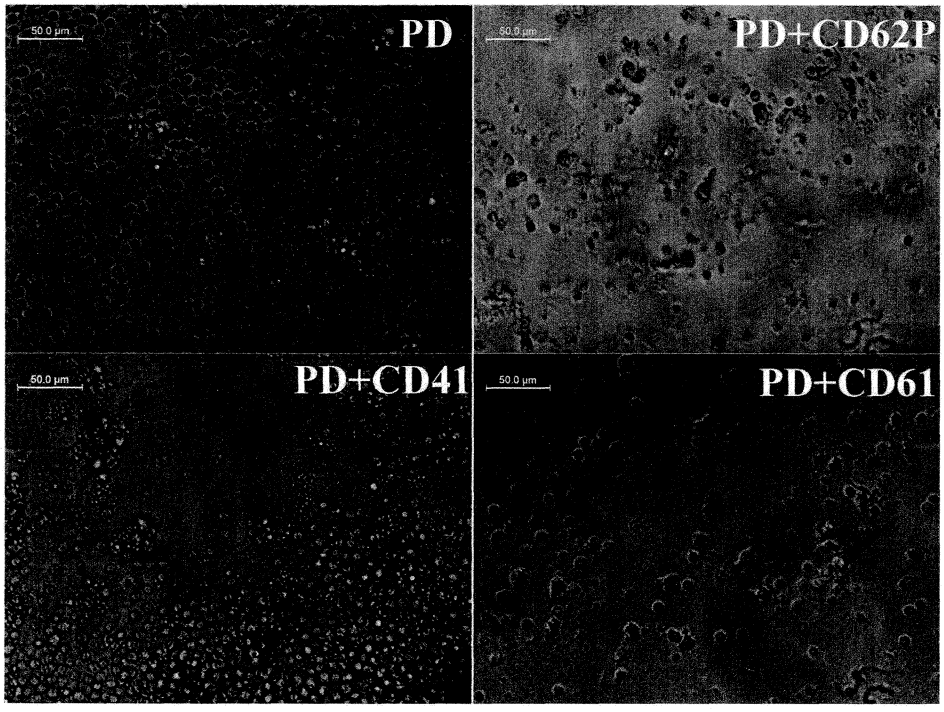

Fig III.B.15 Phase contrast micrographs (20X) for effect of PD blocked with antibodies (2ug each) on $CD34^-$ population culture viewed on 8^{th} day: The type of Ab used for blocking is marked. FG used was same in all wells

On the other hand for $CD34^+$ population appeared to commit to endothelial lineage when CD62P was blocked. It appeared that the cell number remained steady when $CD34^+$ cells were put in culture. The number of cells seeded in each dish was very few because multiple cultures were done with a single donor isolate. Whether lack of sufficient cell number is the reason reduced proliferation is not understood from this study. Another possibility is that for $CD34^+$ cell expansion, paracrine effect from other cell types in PBMNC isolates may be necessary. In $CD34^-$ cell isolate culture, seeding density was high and a dense population was observed at the end of culture period as well.

These observations clearly suggested that unless $CD34^+$ cells were that were present in PBMNC did not differentiate into endothelial like cells. The observation is strongly supported by the immunocytochemical data of CD68 for macrophage. This is necessary to substantiate a balanced transformation of $CD34^+$ (FigIII.B.17) and $CD34^-$ (FigIII.B.18) cells due to the integrin blocking or on the other hand, the role of these integrins for transformation of these cells. The $CD34^+$ cell culture did not show any positive signals for CD68 that can be interpreted as macrophage conversion. But $CD34^-$ culture showed almost similar pattern of CD68 positivity that was seen in PBMNC culture (FigIII.B.13). This data is a clear evidence to suggest that among all cells in PBMNC $CD34^-$ monocytes are responsible for macrophagic conversion.

The difference between this experiment and the earlier reported (in this section) PBMNC culture, stained with CD68 on blocked surfaces is that here 10 times higher concentration (2ug) was used for blocking antigens. It is evident that with more efficient blocking with CD41 and CD61 antibodies, hardly any macrophage conversion was seen. However, efficient CD62 antigen blocking has not reduced $CD68^+$ cell generation.

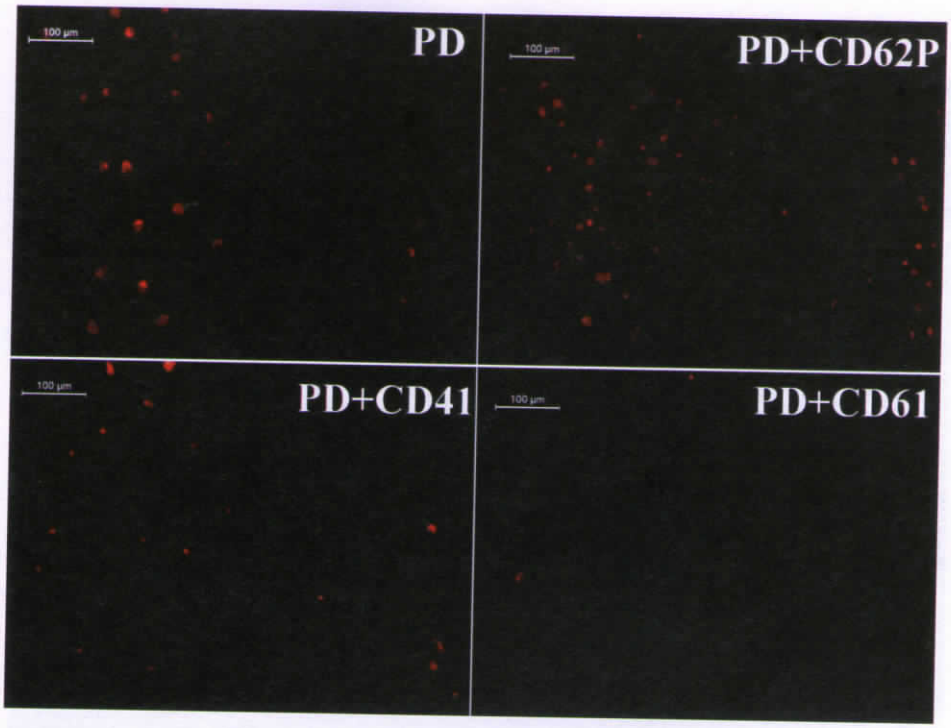

Fig.III.B.16 Fluorescent micrographs (20X) for effect of PD blocked with antibodies (2ug each) on PBMNC culture CD68 expression: The type of Ab used for blocking is marked. FG used was same in all wells

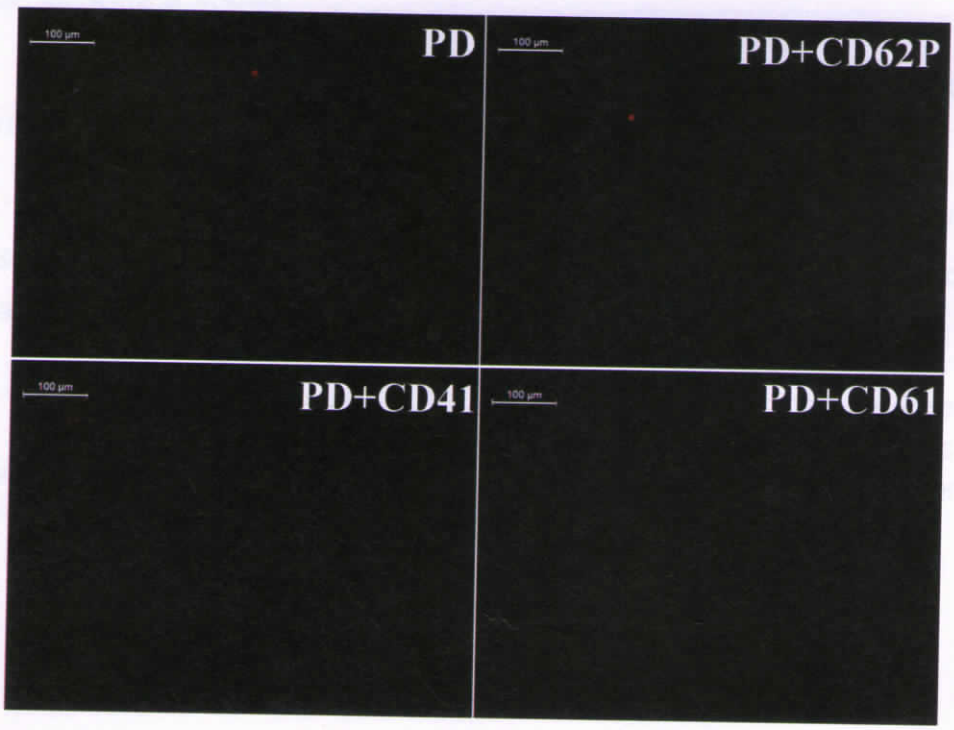

Fig.III.B.17 The fluorescent micrographs (20X) of CD68 on 8th day integrin blocked: Starting cells were CD34^{+} population.: The type of Ab used for blocking is marked. FG used was same in all wells

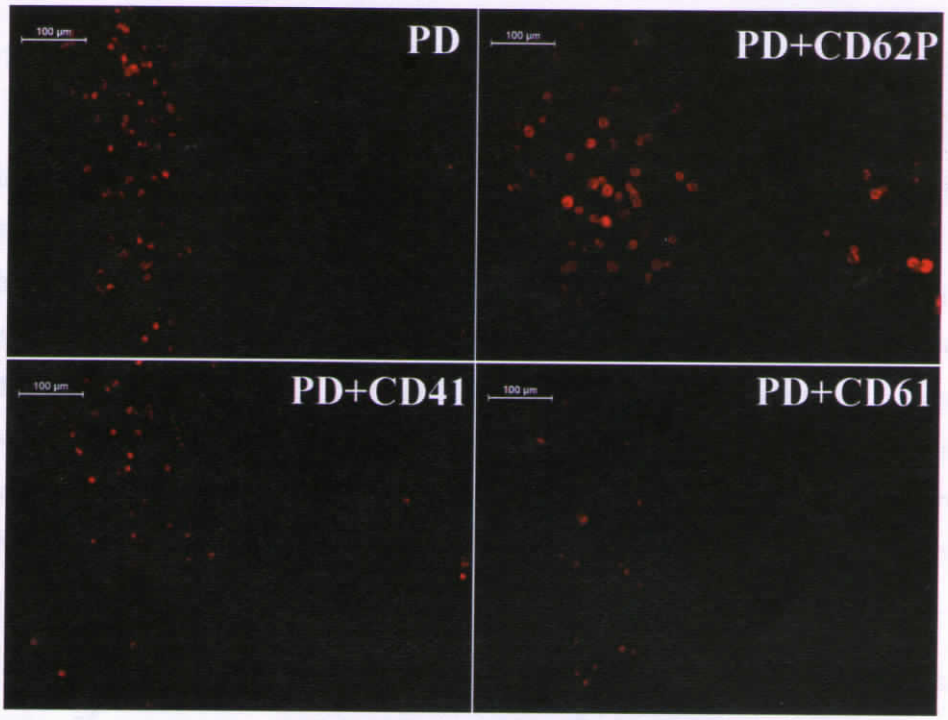

Fig.III.B.18 The fluorescent micrographs (20X) of CD68 on 8th day integrin blocked: Starting cells were $CD34^-$ population. The type of Ab used for blocking is marked. FG used was same in all wells

None of the $CD34^+$ cell got converted to macrophage on any of the culture matrix; whether it was blocked or not blocked.

Here also, blocking with a higher antibody concentration (2 ug/well) $CD34^-$ cells showed a similar behavior as in the case of PBMNC (blocking experiment).

The results MACS selected unselected culture experiments suggested that a subpopulation in $CD34^+$ cells give rise to endothelial cells for angiogenesis but they do not have a possible role in macrophagic conversion for inflammatory reaction.

Chapter 4- Summary and Conclusion

IV.1 Summary

Platelets are one of the important fractions of blood cells which actively participate in hematostasis and blood clotting. Platelet activation is essential for the blood clotting cascade to maintain normal physiological condition and protection at the time of injury. But platelet activation in unwanted place may become one of the causes of artherosclerosis, which is the leading cause of death in developing world. Recent evidence points out different roles of activated platelet in the regeneration process of vascular wall. Platelets activated during vascular injury significantly support recruitment of progenitor cells to the vascular wall, which can be further transformed to endothelial or smooth muscle lineage.

Activated platelet contains different subcellular fractions which include proteins of releasate, membrane vesicles which shed and circulate and platelet debris. After activation most probable fraction that may remain with vascular wall through adhesion to injured endothelium or fibrin clot, is platelet debris. Therefore, these particles may have a role in switching processes between angiogenesis or the inflammation at the vascular site activity. The integrin molecules present on these debris have a significant role in the recruitment of progenitor cells and the further lineage commitment towards endothelial or smooth muscle cells.

In the first part of this study which mainly focussed on the activation of washed platelets using different concentration of agonist, thrombin, it was found that even with physiological level of activation platelet debris get dissociated with a unique feature. The protein estimation study by Lowry's method showed significant increase in concentration of thrombin (0.1, 0.2 1and 0.4) resulted in active shedding of PMP, and there is a corresponding decrease in PD amount. But at higher concentration (1IU) shedding is complete and in parallel PD concentration decreased. So for further

experiments were done with PD prepared by 1IU thrombin, for complete elimination of PMP contaminates.

SDS -PAGE analysis of PD and PMP after separation each fraction showed clear differences in MW of the proteins in each fraction. It was evident that there are protein bands dedicated to each fraction and very few numbers of proteins were seen on both fractions. Therefore, when the PD separated from 1IU thrombin-activated platelets were used for further studies, it was sure that only PD components were present on them and there was no PMP component that may act in further studies. Western blot analysis of PD for three important integrin molecules -CD62P (P-selectin), CD41 (GP2b) and CD61 (GP3a) developed strong bands for all the three molecules of interest CD41, CD61 and CD62. It strongly suggested that the PD preparation contains significant amount of molecules of interest for study of their specific effect on monocytes.

The culture model used in the study mimicked patho-physiological condition by incorporating PD in fibrin matrix. Human serum that has no platelet factor was used as culture supplement. There was no exogenous growth factor added into cultures so the the effect seen is exclusively that of fibrin matrix, PD or both in combination. First experiment evaluated difference between having no PD and dose response of PD on monocyte by keeping all other conditions same. Fibrin without PD produced macrophages whereas with PD more cells of endothelial lineage were seen, Even with PD, culture was heterogenous with both macrophages and EC together. But more cells turned positive for CD31 which is marker for endothelial cells. Clearly, there is effect is concentration dependent for conversion of monocytes to endothelial cells. But after certain concentration there was no further increase in EC formation in terms of cell density or maintaining homogeneity of differentiated cell population. On 8^{th} day analysis of cells on PD coated plates established cobble stone morphology and the reduced level of macrophagic effect. This observation was confirmed by CD68 for macrophages and CD31 for

endothelial immunocytochemistry. Real time PCR analysis showed that only in presence of PD, cells expressed endothelial markers (eNOS and vWF). This information confirms effect of PD for commitment of monocyte to endothelial lineage. BrdU incorporation demonstrated that cells proliferate but no specific effect of PD for cell proliferation was identified.

Ability of monocyte-PD complex formation could be prevented by blocking CD62 antigen using specific antibodies. There is no free PD in the mixture of PD blocked with CD41 and CD61 antibodies; therefore, these two antigens have no role in PD complex formation with monocytes. On the 8^{th} day, culture in presence of integrin-blocked PD also showed significant morphological changes. Most of the cells in CD62P blocked plates showed macrophage- and foam cell characteristic rather than endothelial morphology. Other two integrins blocking did not show any significant change compared to unblocked PD control. Results from CD62 blocked plates were further confirmed by absence of endothelial markers (CD31 and Ulex lectin) and expression of macrophagic marker (CD68). A common marker for macrophage and EC, DilAcLDL uptake was found in majority of cells in culture. Therefore, phenotype conversion of PBMNC to either macrophage or endothelial cells is evident.

To know the specific population which got committed to endothelium magnetic sorting for $CD34^{+}$ cell which is a sub population of PBMNC turned out to be an effective experiment. Sorting produced a CD34+ population with 30% purity and in the unselected population no CD34+ cell was found on flow cytometric analysis. No EC-like morphology was seen in CD34-population and in the positive population, cells differentiated into EC with appropriate marker expressions. Again, blocking with CD62P of PD prevented CD34+ cell differentiation to endothelial lineage. None of the cells expressed EC markers inspite of the appearance of spindle-shaped EC-like cell when CD62 was blocked

IV.2 Conclusions

- ✓ From present study it can be concluded that activated platelet debris could play a significant role in repair of vascular injury and angiogenesis if they get lodged at the site of vascular injury along with fibrin clot.
- ✓ Effect of PD on differentiation of moncyte to macrophage takes place through CD62P interaction with monocytes.
- ✓ Antibodies specific for CD62P added with PD could block the activity of PD on monocytes.
- ✓ CD34+ population PBMNC is required for differentiation into EC.
- ✓ With no PD present when PBMNC bind to fibrin, they are more prone to become macrophages.

IV.3 Future Prospects

Study may be improved by

- ➢ Quantitative analysis od CD68 expression and CD31 expression to identify regulatory role of PD in transforming PBMNC to both macrophages and EC.
- ➢ Real time PCR analysis should also address CD68 expression
- ➢ MAC sorting of CD14 cells and culture to verify the role of this population in angiogenesis and inflammatory lineage by the effect of PD
- ➢ Study the synergic effect of CD62P, CD41 and C61 integrin molecules in culture
- ➢ Analyze role of other integrins on platelets in monocyte recruitment and angiogenesis.

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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

MgCl2 - 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

Na2HPO4 - 1.419g

KH2PO4 - 0.204g

Made upto 1L using deionized water

Filtered and stored at room temperature

4.Ca2+/Mg2+ free Hank's Balanced Salt Solution

For 1L

KCl - 0.4g

KH2PO4 - 0.06g

NaCl - 8g

Na2HPO4 - 0.0482g

Made upto 1L using deionized water

pH was adjusted to 7.4

Filtered and stored at 4-8°C

5. 27% Sucrose

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

0.2% sodium azide was added

Filtered and stored at 2-8°C

6. Reagents for the Estimation of Protein

Reagent A

2% Na2CO3 in 0.1N NaOH

Reagent B

0.5% CuSO4.5H2O in 1% potassium tartarate

Reagent C

10mL Reagent A + 0.2mL Reagent B

Reagent D

1mL Folin-Ciocalteau Reagent + 2mL deionized water

7. **Reagents for SDS PAGE (8%)**

Preparation of Resolving gel (For 15mL)

Deionized Water	-	7mL
30% Acrylamide mix	-	4mL
1.5M Tris HCl (pH 8.8)	-	3.8mL
10% SDS	-	0.15mL
10% APS	-	0.15mL
TEMED	-	0.009mL

Preparation of Stacking gel (For 6mL)

Deionized Water	-	4.1mL
30% Acrylamide mix	-	1.0mL
Tris HCl (pH 6.8)	-	0.75mL
10% SDS	-	0.06mL

10% APS	-	0.06mL
TEMED	-	0.006mL

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

Electrophoretic Buffer (5X)

For 1L

Tris base	-	15.1g
Glycein	-	94g
10% SDS	-	50mL

pH was adjusted to 8.3

Staining solutions

(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 deionized water

Developer

3% Na2CO3

0.02% Formaldehyde

1M Citric acid

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

8. Reagents for Western Blotting

Transfer buffer

For 1L

Tris	-	5.8g
Glycein	-	2.9g
SDS	-	0.37g
Methanol	-	200mL

Amido black stain

0.1% Amido black

45% Methanol

10% Acetic acid

Destain solution

90% Methanol

2% Acetic acid

8% water

50mM Tris

60.5 mg in 10 mL distilled water

Adjust the pH for 7.6

9.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 Ca2+/Mg2+ 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.

10. Reagent for MACS

Recommended Medium

PBS containing 2% Human serum and 1mM EDTA (Ca^{2+} and Mg^{2+})