Influence of platelet debris on peripheral blood mononuclear cell (PBMNC) population to induce angiogenesis markers during *in vitro* culture

A DISSERTATION SUBMITTED

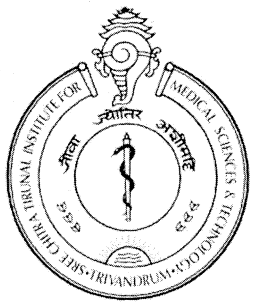
BY

ANUSHA SREESHAN

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF PHILOSOPHY



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DECLARATION

I, **Anusha Sreeshan**, hereby declare that I had personally carried out the work depicted in the dissertation entitled "**Influence of platelet debris on peripheral blood mononuclear cell (PBMNC) population to induce angiogenesis markers during *in vitro* culture**" 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 "**Influence of platelet debris on peripheral blood mononuclear cell (PBMNC) population to induce angiogenesis markers during *in vitro* culture** " submitted by **Anusha Sreeshan** in partial fulfilment for the Degree of Master of Philosophy in Biomedical Technology to be awarded by this Institute. The entire work was done by her under my supervision and guidance at **Thrombosis Research Unit**, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Thiruvananthapuram-695012.

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For

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ABBREVIATIONS

EC	Endothelial cells
SMC	Smooth muscle cell
PD	Platelet debris
PBMNCs	Peripheral blood Mononuclear cells
CD	Cluster of differentiation
PBS	Phosphate buffer saline
FITC	Fluorescein isothiocyanate
PE	Phycoerythrin
RT qPCR	Real Time quantification
DMEM/F12	Dulbecco's Modified Eagle's Medium/Nutrient F12Ham
PRP	Platelet rich plasma
ECM	Extra cellular matrix
FSC	Forward scattering
SSC	Side scattering
PAGE	Polyacrylamide gel electrophoresis
NF –kB	Nuclear factor –kB
°C	Degree Celsius
MMP	Matrix metalloprotein
PSGL-1	P-Selectin Glycoprotein Ligand-1
RANTES	Regulated on Activation Normal T-cell Expressed and Secreted
UV	Ultra Violet
bp	Base pair

cDNA	Complementary deoxy ribo nucleic acid
DEPC	Diethyl pyrocarbonate
EDTA	Ethylene diamine tetra acetic acid
BSA	Bovine serum albumin
dNTP	Deoxy nucleotide triphosphate
DTT	Dithiothreitol
PDGFR	Platelet-derived growth factor receptor
VEGFR2	Vascular endothelial growth factor receptor 2
FGF	Fibroblast growth factor
HBSS	Hank's Balanced Salt Solution
PVDF	Polyvinyl difluoride
bFGF	Basic fibroblast growth factor
PF4	Platelet factor 4
EGF	Epidermal growth factor
TGF	Transforming growth factor
PG	Prostaglandin
IL	Interleukin
DC	Dendritic cell
IGF	Insulin growth factor
vWF	von Willebrand factor
PECAM	Platelet endothelial cell adhesion molecule
VE	Vascular endothelium
eNOS	Endothelial nitric oxide synthase
VSMC	Vascular smooth muscle cell
AcLDL	Acetylated low density lipo protein
UEA	Ulex europaeus agglutinin

HSC	Hematopoietic stem cell
EMMPRIN	Extracellular matrix metallo proteinase inducer
HLA	Human Leukocyte Antigen
SDF-1	Stromal cell derived Factor-1
TCPS	Tissue culture polystyrene
nm	Nano meter
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
%	Percentage
µg	Micro gram
mM	Milli molar
µl	Micro litre
µg	Micro gram
g	gram

Table of Contents

List of Figures

List of tables

Synopsis

Angiogenesis is the process of development of new blood vessel. It is a natural process which occurs in both health and disease. The normal regulation of angiogenesis is governed by a fine balance between factors that induce the formation of blood vessels and those inhibit the process. When this balance is disturbed, it usually results in pathological angiogenesis which causes increased blood-vessel formation in disease condition. Other than vascular endothelial cells, monocytic endothelial progenitor cells also contribute to new vessel formation and vascular repair through angiogenesis. Various growth factors can stimulate the differentiation of progenitor cells to promote angiogenesis.

Platelets play a major role to arrest bleeding post vascular injury. They are involved in tissue regeneration and release pro/anti angiogenic factors from α-granules and the soluble growth factors released play major roles in angiogenesis. Other than the platelets that are recruited into blood clot, activated platelets and their fragments are found in the circulation. The circulating membrane microparticles are considered as risk factor for atherosclerosis progression. Other than the light membrane particles, insoluble platelet debris also circulates which probably has integrin though which they get bound to minor fibrin clots that are formed at the site of vascular injury. Various integrins are present on these insoluble fragments which may attract circulating monocytes. Thus platelets present soluble growth factors and integrins containing insoluble fragments which may induce cellular activity. Studies on the effect of integrins on monocyte population subsequent to such platelet-monocyte interaction are limited.

The present study hypothesizes the influence of platelet debris on PBMNCs in expressing angiogenic markers when cells are culture on 2- and 3-dimensional culture matrices composed of biomimetic fibrin matrix incorporated with the debris. The main objectives include: (i) to analyze the platelet-monocyte complex formation on activation with thrombin and the effect of dose, (ii) isolation and characterization of platelet debris from activated platelets, (iii) characterization of isolated debris for the presence of

integrins (iv) fabrication of 2- and 3- dimensional culture substrates incorporated with platelet debris, (v) isolation of CD 14+ cells, and (vi) analysis for expression of angiogenic markers like VEGFR2 and PDGFR in cultured cells.

Chapter 1 of the dissertation includes an introduction and review of the topic in which monocyte activity, the process of angiogenesis, role of platelets in angiogenesis, markers in angiogenesis, platelet-monocyte interaction, endothelial progenitors, peripheral mononuclear cells differentiation to endothelial cells and smooth muscle cells etc are reviewed with appropriate citations. Finally, it describes the gap identified, the hypothesis and the objective of the study.

Chapter II includes the materials and methods used for the study which describes the methods used staining of cells with antibodies and analysis of complex formation by flow cytometry. Isolation of PD from platelets activated with thrombin by sucrose density gradient ultracentrifugation, estimation of protein concentrations, analysis of proteins by SDS-PAGE and Western blotting to detect integrins on PD are described. Method of PBMNCs isolation by density gradient centrifugation, preparation of 2D and 3D culture substrates using fibrin and PD are explained in detail. Isolation of CD14+ cell populations from PBMNCs by magnetic activated cell sorting (MACS) and the analysis of sorted cells for purity using flow cytometry are elaborated. Culture of PBMNC and sorted CD14+ cells for 8 days on different culture substrates, morphology analysis by phase contrast microscopy, isolation & quantification of mRNA, standardization of RT PCR using differentiated endothelial and smooth muscle cell etc. are described. The expression of angiogenic markers PDGFR and VEGFR2 were analyzed by real time PCR of PBMNC and cultured monocytes.

In Chapter III the results and discussions are presented. The data on platelet activation and monocyte complex formation indicated that 0.6 IU thrombin is sufficient to produce stable complex of platelet with monocyte. Isolation of PD resulted in good yield and purity. The presence of integrins was identified using molecular weight markers and CD62P, CD41 and CD61 specific bands were developed in Western blot. PBMNCs

cultured in presence of PD showed spindle shaped morphology and in the absence of PD, macrophages were predominant. MACS sorted cells showed reduced number of macrophages even in the absence of PD. Reverse transcriptase polymerase chain reaction was standardized for PDGFR and VEGFR2 primers using mRNA from mature EC and SMC and their annealing temperature was found to be 52°C and 58°C respectively. Analysis of PDGFR and VEGFR2 using real time PCR confirmed expression of PDGFR in 2D culture but not found in 3D substrates. The VEGFR2 expression was not up regulated in test substrate as compared to either control or that in PBMNC immediately after isolation.

Chapter 1V summarizes the study and conclusions were drawn. It has been concluded that PBMNCs in presence of PD were transformed to spindle shaped cells; and in the absence of PD foam-like cells were formed on fibrin substrates. On RT PCR analysis for the angiogenic markers VEGFR2 and PDGFR, it was observed that PDGFR expression is influenced by platelet debris. More standardization of substrate properties may be required before it can be concluded, if PD has influence on angiogenesis or not when it is included with 3D- substrate. VEGFR2 was not up regulated in any of the culture substrate; therefore, PD does not induce angiogenic conversion of circulating monocytes to EC.

CHAPTER I -INTRODUCTION

1.1 Background

Angiogenesis is the growth of new capillaries by sprouting of preexisting vessels through migration and proliferation of mature endothelial cells (ECs). Blood vessels form a tubular network throughout the body that allows blood to flow from the heart to every cell and then back to the heart. The three types of blood vessels are arteries, capillaries, and veins. Each blood vessel consists of a layered wall surrounding a central blood-containing space, or lumen. When blood vessels are damaged, blood loss is prevented by clot formation in which platelets play an important role. Other than hemostasis, platelets are involved in tissue repair and maintenance of endothelium. Platelets are anucleated cellular fragments derived from bone marrow megakaryocytes, having discoid shape with 2-4μm in diameter. It has a life span of approximately 8-10 days in the blood stream.

Platelets contain variety of protein molecules, which include signaling molecules, membrane proteins, cytoskeleton regulatory proteins, cytokines, and other bioactive peptides that initiate and regulate wound healing. Platelets are rich in organelles including mitochondria, peroxisomes and secretory vesicles such as dense granules, alpha granules and lysosomes. Platelets get activated upon injury to blood vessel, adhere to the injured site and release various agonists and recruit more platelets to wound site. They have a complex cytoskeleton consisting of microtubules and dense tubular system. The immediate appearance of platelets at the site of vascular injury and wound healing reveals its importance in angiogenesis.

Platelet membrane consists of phospholipids, lysophosphatidate, phosphatidic acid and sphingosine-1-phosphate, and all these serve as angiogenic phospholipids. These phospholipids have mitogenic activities and stimulate migration, proliferation, adherence, junction assembly and liberation of endothelial cells from monolayer and morphogenesis of capillary like structure. Sphingosine-1-phosphate was shown to be important in the stabilization of angiogenic vessels. Platelet activation leads to the secretion of angiogenic

phospholipids which promote endothelial migration, survival and vessel stabilization. The mechanisms by which platelets regulate angiogenesis remain unclear. Platelet acts as the initial responder to vascular changes and provides a delivery system for angiogenesis related molecules. Platelets modulate angiogenesis by releasing promoters such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), platelet derived growth factor (PDGF) and matrix metalloprotein (MMP) whereas inhibitors within platelets include endostatin, platelet factor-4 (PF4), thrombospondin, α_2-macroglobulin, plasminogen activator inhibitor-1 (PAI-1) and angiostatin. Angiogenesis regulatory proteins are localized in α-granules; it is also regulated by specific cytokines and extra cellular matrix (ECM) fragments. Upon activation with thrombin, platelets release more than 300 proteins. Platelets induce differentiation of endothelial progenitor cells to mature endothelial cells. Activated platelets release their alpha granules resulting in increased release of growth factors. These released growth factors can induce angiogenesis promoters. Thus platelets are the most abundant source of endogenous angiogenic regulators.

In a previous study (unpublished) it was observed that activated platelets release platelet debris which contains specific integrin molecules like CD 62P, CD41 and CD61. Peripheral blood mononuclear cells (PBMNC) consists of monocytes and a group of monocytes population induces angiogenesis in the presence of a suitable angiogenic stimulator. Two important growth factors involved in angiogenesis are PDGF and VEGF. PDGF is involved in the mobility of smooth muscle cells, capillary endothelial cells, etc. whereas VEGF is involved in endothelial cell growth. Endothelial and smooth muscle cells interact with each other to form new blood vessels. The presence of VEGF and PDGF, the two main growth factors involved in angiogenesis can be studied by the expression analysis of their receptors, VEGFR2 and PDGFR respectively. Influence of growth factors on angiogenesis can be studied by the expression analysis of specific markers.

It is important to understand if interaction of activated platelets with circulating monocyte can lead to angiogenesis. The integrins that are expressed on the platelet debris during platelet activation might stimulate the monocyte population to differentiate and enhance the angiogenesis. Since fibrin matrix may enhance adhesion of EPC from the monocyte population to differentiate to EC and SMC, both 2-D and 3-D culture models may be employed to study the changes on cells during PBMNC culture.

1.2. REVIEW OF LITERATURE

1.2.1. Angiogenesis

Angiogenesis is an important process occurring in both physiological and pathological conditions. The term angiogenesis describes the growth of endothelial sprouts from pre existing post capillary venules, but recently it is used to denote growth and remodeling process of primitive network into complex network. Angiogenesis is the process that leads to the sprouting out of new blood vessel from pre-existing vessels. It involves the remodeling (sprouting, bridging, folding) of preexisting endothelial cells (ECs) into a more complex network. It is a multistep process where ECs are activated by pro-angiogenic factors to degrade the basement membrane, migrate into the surrounding matrix, and form sprouting structures connecting neighboring vessels. During the process the inactive ECs are stimulated which degrade their basement membrane and invade the stroma, later these deposit new basement membrane and forms functional capillaries [Ausprunk and Folkman, 1977]. Endothelial cells initiate angiogenesis; it involves the action of many angiogenic molecules, which includes vascular endothelial growth factors (VEGF), basic fibroblast growth factors (bFGF), Ang1 and Ang 2 of which VEGF and bFGF are well characterized. Ang 1 alone and Ang 2 in the presence of VEGF are involved in angiogenesis. Adult circulating EPCs appears to play a significant role in angiogenesis. The EC migration during angiogenesis is by three mechanisms-chemotaxis, haptotaxis and mechanotaxis. VEGF plays a major role in chemotaxis. VEGF is an endothelial cell specific mitogen, which acts through two tyrosine kinase receptors, VEGFR-1/Flt [Shibuya et al., 1990] and VEGFR-2/Flk-1 [Matthews et al., 1991].VEGF,

VEGFR2 and bFGF influence the angioblast progenitor differentiation. VEGF, granulocyte monocyte colony stimulating factor, bFGF and insulin growth factor (IGF)-1 stimulate their differentiation and mobilization.

Angiogenesis begins with vasodilation, VEGF increases vascular permeability, then the EC proliferates and its migration occurs. VEGF and Ang 1 stimulates the interaction between endothelial and periendothelial cells and stabilizes the network. Endothelial cells assemble solid cords that subsequently acquire a lumen. VEGF also promotes mural cell accumulation by the release of PDGF-BB, which is a chemoattractant for smooth muscle cell (SMC) or binding to VEGF receptor. Ang1 and Tie2 affect growth and maintenance of blood vessels by stabilizing the interaction of mural cells with endothelial channels by inducing branching and remodeling.

Angiogenesis is dependent on the balance between ECM-degrading proteinases and a proteinase inhibitor which controls the action of proteinase [Pepper et al., 1996b]. Proteinases of the plasminogen activator, matrix metalloproteinase (MMP), chymase or heparinase families influence angiogenesis by degrading matrix molecules and by activating or liberating growth factors (bFGF, VEGF and IGF-1), sequestered within the ECM. Thus angiogenesis is brought about by the interaction of positive and negative regulators within the endothelial microenvironment. Angiogenesis is regulated by angiogenin, heparin binding protein, transforming growth factors (TGF), low molecular weight endothelial mitogen, chemotactic factors and lipids. Angiogenin activate endothelial and smooth muscle cell and are involved in cell migration, proliferation, invasion and formation of new tubular structure. Certain prostaglandins such as PGE1 and PGE2 are angiogenic. TGF-β acts as stimulator or inhibitor depending on the presence or absence of epidermal growth factor. Angiogenesis inhibitors suppressing the proliferation or migration of EC include angiostatin (an internal fragment of plasminogen), endostatin (a fragment of collagen XVIII), antithrombin III, interferon-β, leukemia inhibitory factor and platelet factor 4.

Many growth factors are involved in different steps of angiogenesis, like vascular endothelial growth factors (VEGF), fibroblast growth factor (FGF)-2 or platelet-derived growth factors (PDGF). Of these, VEGF and FGF-2 were extensively investigated and it was shown that they significantly contribute to the induction and progression of angiogenesis. VEGF secreted by normal and tumor cells induces angiogenesis by binding to its specific receptors, VEGFR1 and VEGFR2. Besides VEGF, other growth factors have been shown to have a significant pro angiogenic effect, like fibroblast growth factor (FGF), transforming growth factors, hepatocyte growth factor, angiopoietin-1, platelet-derived growth factors (PDGF) and others. PDGF was purified by Heldin et al [Raica & Cimpean; 2010]. PDGF signals through two cell-surface tyrosine kinase receptors, PDGFR α and PDGFR β, and induces angiogenesis by up-regulating VEGF production and modulating the proliferation and recruitment of perivascular cells. The angiogenic activity of PDGF might not only be based on the increased VEGF-A production, because PDGF-B stimulation induces an increased EC lineage commitment and restricted differentiation of hematopoietic precursors. PDGF-B is strongly expressed in the endothelium. Several angiogenic factors act directly or indirectly, depending on their action on vascular ECs. Angiogenic factors can act directly or indirectly, acidic and basic FGF and TGF-α are direct angiogenic factors where as those factors which has no effect on vascular EC in vitro are indirect angiogenic factors. Indirect angiogenic factors acts by mobilizing macrophages and activating them to secrete growth factors or chemotactic factor for vascular ECs, cause the release of endothelial mitogen stored in the ECM and the release of intracellular stores of endothelial growth factors.

1.2.2. Circulating monocytes

Cells of the monocyte /macrophage lineage derive from myelomonocytic stem cells in bone marrow. These cells give rise to monoblasts, which then develop into monocytes. Monocytes enter into the blood where they circulate with a half life of 1–3 days. They then reside in various tissues and are collectively called macrophages. Ziegler-Heitbrock introduced the use of CD14 and CD16 antibodies for blood monocytes.

Peripheral-blood monocytes show morphological heterogeneity, such as variability of size, granularity and nuclear morphology. Monocytes were initially identified by their expression of large amounts of CD14, which is part of the receptor for lipopolysaccharide. However, the subsequent identification of differential expression of antigenic markers showed that monocytes in human peripheral blood are heterogeneous, and this provided the first clue to the differential physiological activities of monocyte subsets. Differential expression of CD14 and CD 16 classified monocyte population as strongly CD14+ monocytes ($CD14^{++}$monocytes or more precisely $CD14^{++}$ $CD16^{-}$ monocytes), called classic monocytes and the minor population of CD16+ monocytes that co express CD14 at a low level ($CD14^{+}$ $CD16^{+}$ monocytes). The $CD14^{+}$ $CD16^{+}$ monocytes expressed higher amounts of MHC class II molecules and CD32. A subpopulation of circulating $CD14^{+}$ monocytes also has EPC-like characteristics, in terms of their expression of endothelial markers upon endothelial induction, formation of tube-like structures in vitro, and incorporation into newly formed blood vessels in vivo. This EPC subset of myeloid origin, termed monocytic EPCs, is distinct from classic EPCs and may share characteristics of early outgrowth cells and circulating angiogenic cells. Distinct chemokine-receptor expression profiles were also among the phenotypic differences that were recognized between monocyte subsets: for example, $CD14^{+}$ $CD16^{+}$ monocytes expressed CC-chemokine receptor5 (CCR5), whereas $CD14^{++}CD16^{-}$ monocytes expressed CCR2. $CD14^{+}$ $CD16^{+}$ monocyte subset was found to be more likely to become dendritic cells (DCs) and reverse transmigrate than was the $CD14^{++}$ $CD16^{-}$ monocyte subset, indicating that the $CD14^{+}$ $CD16^{+}$ cells might be precursors of DCs, which can pass through tissues and then migrate to the lymph nodes through the afferent lymphatic vessels. However, these observations do not preclude an in vivo role for $CD14^{++}$ $CD16^{-}$monocytes in contributing to the DC pool. An additional monocyte subset that is defined by the expression of CD14, CD16 and CD64 (also known as FcγRI) has been reported [Heitbrock et al., 2000]. These cells seem to combine characteristics of monocytes and dendritic cells, with high expression of CD86 and HLA-DR and high T-cell-stimulatory activity. Compared with $CD14^{++}$ $CD16^{-}$ (classic) monocytes (which are

also $CD64^+$), these $CD14^+CD16^+CD64^+$ cells have a similarly high phagocytic activity and produce similarly large amounts of cytokines (such as tumor-necrosis factor (TNF) and IL-6, and these phenotypes are not shared with the $CD14^+$ $CD16^+$ $CD64^-$ subset. However, the $CD14^+$ $CD16^+$ $CD64^+$ cells share with the $CD14^+$ $CD16^+$ $CD64^-$ subset a greater stimulatory activity in mixed leukocyte reactions than $CD14^{++}CD16^-$ monocytes. Monocytic EPCs are now considered oligopotent cells that may differentiate into endothelium as well as into other elements of the vasculature, such as pericytes and SMCs, but their *in vivo* vasculogenic potential is far inferior to classic EPCs. In addition, monocytic EPCs contribute to new vessel formation and vascular repair through angiogenesis by angiogenic factor secretion and other mechanisms.

1.2.3. Endothelial progenitor cells

Endothelial progenitor cells (EPCs) were reported by Asahara et al. in 1997 from human peripheral blood through the expression of CD34 or CD133. CD34 is a human stem cell marker protein, which is a sialomucin expressed on EC and fibroblasts and on cells of epithelial lineages. EPCs express several markers, including VEGF receptors-2 (VEGFR2, KDR, Flk-1), VE-cadherin, CD34, platelet endothelial cell adhesion molecule (PECAM; CD31) and von Willebrand factor (VWF), and are also able to incorporate acetylated low-density lipoprotein (AcLDL) and to bind lectins such as BS-1 and ulex europaeus agglutinin-1 (UEA-1).Circulating EPCs are directly involved in endothelial recovery and rapidly migrate into the region of activating angiogenesis, it is important for the modulation of vessel growth. EPC population has been reported to give rise to both endothelial and mural cell types, because of their ability to differentiate into ECs, EPCs are called angioblasts. Vascular endothelial growth factor is mainly involved in the differentiation of EPCs into ECs, where as PDGF-BB play a major role in the induction of mural cell phenotype. The best characterized source of EPCs are the bone marrow hematopoietic stem cells (HSCs), which are located in the stem cell niche and released into the peripheral blood on mobilization by chemokines such as VEGF, bFGF-2,

stromal-cell-derived factor-1 (SDF-1) or granulocyte macrophage-colony-stimulating factor (GM-CSF).

Depending on the time of appearance, morphology and proliferation, EPCs are classified as early EPCs and late EPCs. Early EPCs are spindle shaped, their number increase in 2 weeks which did not replicate *in vitro* and disappeared in 4 weeks after plating. Late EPCs appears 2 to 4 weeks after plating, with smooth cytoplasmic outline and showed cobble stone appearance. They rapidly replicate and forms monolayer and showed multi population doublings without senescence. Early EPCs contribute to neovasculogenesis by secreting the angiogenic cytokines such as VEGF, FGF and G-CSF that recruit mature ECs and induce their proliferation and survival where as late EPCs enhances neovasculogenesis by providing a sufficient number of ECs. Adult bone marrow, peripheral blood, cord blood and tissue resident cells are the source of EPCs. Circulating EPCs appear to be important because they directly contribute to endothelial recovery and rapidly migrate into the region of activating angiogenesis.

Endothelial progenitor cells have the property similar to that of embryonic angioblast, which are migratory ECs that are capable of circulation, proliferation, differentiation and maturation to adult ECs. Both EPC and mature EC express similar endothelial specific marker such as VEGF receptor-2 (VEGFR-2), Tie-1, Tie-2, and vascular endothelial (VE) - cadherin. Endothelial precursor cells and circulating mature ECs are difficult to distinguish because the hematopoietic stem and progenitor cells express similar markers as those of ECs, such as VEGFR-1 (Flt-1), CD34, platelet endothelial cell adhesion molecule (PECAM), Tie-1, Tie-2, and von Willebrand's factor (vWF), and they also have the capacity to incorporate acetylated low-density lipoprotein (Ac-LDL). Endothelial precursor cells share similar characteristics with hematopoietic stem cell and they express unique surface markers, such as CD34 and early hematopoietic stem cell marker AC133 whose expression decreases with maturation and differentiation.

Endothelial precursor cells express three characteristic markers- CD34, CD133 and VEGFR-2. A combination of early progenitor and endothelial phenotype which are

localized in bone marrow are CD133/CD34/VEGFR-2 cells which do not express VE cadherin and vWF. In the peripheral circulation of adult more mature differentiated EPCs are found with CD34 and VEGFR-2 expression and decreased expression of CD133. Mature EC of large vessels highly express VEGFR-2, VE-cadherin and vWF and are negative for CD34.The loss of CD133 followed by CD34 reflects the transformation of circulating EPCs to the differentiated mature endothelial cells. Mature microvascular ECs still express CD34 [Reyes et al. 2002]. Circulating EPCs express a variety of markers that are typical for the endothelial lineage, including platelet endothelial cell adhesion molecule-1 (PECAM-1, or CD31), VE-cadherin, vWF, endothelial nitric oxide synthase (eNOS) and upon stimulation express E-selectin [Asahara et al. 1997, Kaushal et al. 2001].

EPCs in the bone marrow express CD133/CD34 /VEGFR-2/VE-cadherin, whereas circulating EPCs express CD34/ VEGFR-2/CD31/VE-cadherin, obviously lose CD133, and begin to express von Willebrand factor. No clear definition exists when an EPC turns into a mature, fully differentiated endothelial cell *in vivo*. One possibility could be the loss of CD133/CD34 and a parallel or subsequent expression of vWF together with other endothelial specific markers and characteristics. A possible mechanism of this differentiation process may be by the migration of progenitor cells from the bone marrow into the peripheral circulation. [Rehman et al. 2003] demonstrated the isolation of EPCs from the monocyte/macrophage fraction of peripheral blood. These cells did not proliferate, but secrete angiogenic growth factors. It is not known which physiologic or pathologic factors influence the homing and the differentiation of EPCs in vivo. Recent data address the question of a possible integration of EPCs into the mature endothelium [Frid et al. 2002]. These cells could trans differentiate into smooth muscle cells in vitro, and this process is dependent on transforming growth factor and cell–cell contact. A very recent study [Badorff et al. 2003] demonstrated that under certain conditions EPCs may be able to differentiate into other myogenic lineage cells, namely cardiomyocytes. Additionally, when the endothelium is injured, circulating progenitors may adhere to the underlying SMCs, thereby differentiating into SMC and contributing to

neointima formation [Sata et al. 2002]. Furthermore, EPCs seem to be involved in the regeneration of ischemic myocardium by modulation of both angiogenesis and myogenesis in the ischemic cardiac muscle [Jackson et al. 2001, Kawamoto et al. 2001, Kocher et al. 2001]. EPCs seem to participate in the regeneration and repair of vascular and possibly extra vascular tissue (e.g., myocardium).

1.2.4. Role of SMC in angiogenesis

Vascular smooth muscle cells (VSMCs) play an important role in angiogenesis, vessel maintenance, and the regulation of blood pressure. In humans, circulation, SMC progenitors have also been shown to exist [Simper D et al, 2002], and SMCs in transplant atherosclerosis are, at least in part, derived from progenitor cells [Caplice NM et al, 2003]. It has been reported that bone-marrow-cell involvement depends on the degree of arterial injury; for example, bone-marrow cells do not differentiate into mature SMCs within neointimal lesions of moderately injured arteries. Progenitor cells might migrate into the intima where they fuse with SMCs to form neo-SMCs, which might have a higher ability for proliferation. Researchers of SMCs have discovered that the normal ploidy of various fully differentiated SMCs in the vessel is tetraploid, which is related to induction of proliferation [Owens GK, 1989]. Fusion itself might also be a naturally occurring mechanism in the physiologic state such as in injury. There are three possibilities for smooth muscle cells appearing in the intima. First, circulating SMC progenitors together with blood mononuclear cells attach to neo-endothelial cells and migrate into the intima when endothelial cells are replaced. Second, EPCs replacing dead endothelium might have an ability to differentiate into SMCs, as $CD34^+$ progenitors could differentiate into SMCs [Yeh ET et al, 2003]. Finally, adventitial and medial progenitor cells can be a direct source of SMCs within the early lesions [Torsney E et al, 2005]. The molecular mechanism of mobilization, homing and differentiation of putative smooth muscle progenitors remains to be clarified.

1.2.5. Role of platelets in angiogenesis

Platelets modulate angiogenesis by releasing promoters such as VEGF, bFGF, EGF, PDGF, and MMPs. Platelets, in addition to their role in hemostasis, play a critical role in the modulation of angiogenesis with the capacity to release both pro- and anti-angiogenic factors, but their regulatory role is incompletely understood. Pro- and anti-angiogenic factors are stored in separate alpha-granule populations within platelets which can be released in response to specific platelet receptor stimulation. Alpha granules are 200 to500 nm in size and contain proteins that enhance the adhesive process, promote cell-cell interactions, and stimulate vascular repair. By adhering to the endothelium of injured organs and tissues and then secreting the contents of their α-granules, platelets are capable of depositing high concentrations of angiogenesis regulatory proteins in a localized manner. This segregation may allow sequential release leading to safe and effective initiation and later suppression of regenerative (proliferative and migratory) activity during wound healing and tissue regeneration.

Extra cellular purines and pyrimidines have long been known as regulators of vascular tone and permeability. Moreover, recent studies in different endothelial cell models have implicated the extra cellular nucleotides in the regulation of angiogenesis [Roedersheimer et al]. Platelets are an abundant source of endogenous ATP and ADP, which are released from dense granules in response to platelet activation with thrombin, collagen, thromboxane or ADP itself. Thus, extra cellular ATP and ADP may regulate vascular inflammation and thrombosis. Activated platelets release factors capable of promoting hematopoietic stem cell migration into a vascular injury site and their differentiation into ECs, which may favor survival via angiogenic signaling pathways.

Platelet contains angiogenic and angiostatic compounds by a regulated process. Folkman et al. showed that these proteins are organized into separate population of alpha granules. It has been reported that platelets are able to influence angiogenesis by shedding of microparticles. Integrins and selectins are involved in the interaction of platelets to the vessel wall thereby mediating angiogenesis. Microparticles released from

platelets express CD 41 integrin which are involved in endothelial cell growth and migration during angiogenesis. P-selectin secreted by α-granules of platelets brings about its adhesion to the vessel wall which enhances angiogenesis. Inhibition of integrin prevents vessel formation where as P-selectin inhibition leads to reduced interaction of platelets to endothelial cell which results in the inhibition of angiogenesis [S. Sabrkhany et al 2011].

1.2.6. Platelet-Monocyte interaction

P-selectin glycoprotein ligand-1 (PSGL-1) plays a major role in platelet- monocyte interaction. Monocyte binding to platelets is predominantly mediated by interaction between P-selectin on platelets and PSGL-1 expressed on monocytes, this interaction is dependent on the presence of divalent cations. Interaction between monocytes and activated platelets induce a proinflammatory phenotype in the monocyte characterized by the activation of the transcription factor nuclear factor-ƙB (NFƙB) and secretion of inflammatory cytokines and chemokines from monocytes. Receptor expressing carbohydrate, sialylated CD15 expressed on monocytes are able to bind P-selectin on platelets. Cell surface molecule thought to be involved in monocyte-platelet binding include CD40 expressed on monocytes and CD40 ligand (CD40L, CD154) expressed by activated platelets. Besides PSGL-1, CD15 on monocytes also been shown to bind p-selectin. The interaction between PSGL-1 and P-selectin is critical for the formation of monocyte–platelet complexes. Following initial P-selectin dependent tethering of monocytes, it appears that additional interactions involving CD40-CD40L and the immunoglobulin superfamily protein extracellular matrix metalloproteinase inducer (EMMPRIN, CD147) act together to strengthen binding by promoting firm adhesion via integrins. The adhesion of monocytes to activated platelets results in the induction of a series of signaling pathways, which regulate cytoskeletal rearrangement, activate β_2 integrins and induce the expression of inflammatory mediators such as MCP-1, IL-1β, IL-8 ,MMP 9 and the release of superoxide.

Adhesion of monocytes to activated platelets results in nuclear translocation of p65 (RelA), a component of the NF-kB family of transcription factors that binds kB sequences in the regulatory regions of monocyte chemotactic protein-1, IL-8, and other immediate early genes. Adhesion of monocytes to purified P-selectin, a tethering molecule expressed by stimulated endothelial cells and platelets, resulted in nuclear translocation of a transcription factor, NF-kB , and secretion of NF-kB–dependent cytokines when the monocytes were simultaneously exposed to platelet-activating factor (PAF). The receptor for PAF is a member of the serpentine, G-protein– linked family that can mediate NF-kB activation when it is transfected into cells. 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 directly signaled 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). P-selectin on the surfaces of platelets is relatively stable and can sustain platelet–monocyte contact for hours. P-selectin glycoprotein ligand-1 (PSGL-1) on the monocyte to P-selectin presented on activated platelets influences nuclear signaling or chemokine generation by the leukocyte. Activated platelets induce secretion of monocyte chemotactic protein-1 (MCP-1) and IL-8 by monocytes, and that adhesion via P-selectin is required for these responses. Rather than directly triggering these events, P-selectin acts in concert with RANTES (regulated upon activation normal T cell expressed presumed secreted), a chemokine that is stored and released by activated platelets that was not previously known to induce monokine synthesis. Mixing monocytes with platelets that were activated by thrombin resulted in adhesion between the two cells. Activated platelets formed rosettes around the monocytes, and platelet–monocyte rosettes were routinely observed in cell suspension. Platelets activated by thrombin induced significant release of MCP-1 than monocytes, incubated in the absence of platelets or with resting platelets. The secretion of MCP-1 is dependent on time and platelet concentration, where platelet–monocyte ratios of 100:1 (platelets/monocyte) yielded

maximal cytokine secretion. Nuclear translocation of NF-kB is required for MCP-1 expression. The p50-p65 heterodimer, commonly known as NF-kB, is located in the cytoplasm of unstimulated monocytes; with appropriate stimulation, it is rapidly translocated to the cell nucleus, where it binds to kB sequences in the promoter and enhancer regions of several immediate early genes. RANTES was present in substantial amounts in the supernatants from activated platelets. IL-8, a C-X-C chemokine, is a potent neutrophil chemoattractant released by stimulated monocytes and other cells. Adhesion of activated platelets to monocytes induces nuclear translocation of NF-kB and secretion of MCP-1, a chemokine that requires NF-kB for its synthesis. Binding of P-selectin on the platelet to PSGL-1 on the monocyte is required for MCP-1 secretion. This molecular interaction mediates rapid, stable adhesion between the two cells. P-selectin or P-selectin transfectants failed to trigger release of MCP-1. This finding indicates that one or more signaling molecules are required. In the tethering and signaling interaction between platelets and monocytes, RANTES, which is stored in platelet secretory vesicles and released upon cellular activation, provides a critical signal for MCP-1 generation by the leukocytes. P-selectin binding to PSGL-1 may facilitate monocyte activation not only by mediating close cell-to-cell contact but also by modifying intracellular signals triggered through the receptor for RANTES. Activated platelets induce MCP-1 secretion by monocytes through mechanisms that involve interactions between P-selectin and its critical ligand, PSGL-1. RANTES was released from platelets stimulated with thrombin and induced chemokine secretion from monocytes adherent to pure P-selectin. RANTES is known to be a chemoattractant for monocytes and to stimulate [Ca^{2+}] transients in these cells. High concentrations of RANTES (1 mM) can also induce IL-2 and IL-5 production by lymphocytes.

1.2.7. Differentiation of PBMNC to EC and SMC

Endothelial progenitor cells in blood play a major role in vascular repair. Monocyte population in blood is a source of endothelial precursor. In peripheral blood mononuclear cell population, there are CD34+ hematopoietic stem cells, CD14+ myeloid

cells and other progenitor cells.CD34+ cells are low in PBMNCs (0.002%) [Peichev et al.2000]. Various studies showed that these cells have the capability to differentiate into ECs. Endothelial progenitors promote vascular growth and wound healing. Human monocytes are the primary source of EPCs in circulation and under in vitro and in vivo conditions they differentiate into ECs. EPC populations can be grown from mononuclear cells, CD14+ MNCs have been used as the starting population for cultivation of EPCs. Pujol et al. [Pujol et al., 2000] showed that CD14+ monocytes from peripheral blood have the capacity to form endothelial like cells in the presence of endothelial cell growth factor. Monocytes and macrophages are in direct contact with endothelial cells of the blood vessels. Macrophages can influence angiogenesis indirectly through the release of angiogenic cytokines. Monocytes may differentiate into macrophages, dendritic cells or endothelial cells depending on the growth factors.

Cultivated EPCs grown from different starting populations, including peripheral blood MNCs, have been shown to express endothelial marker proteins such as von Willebrand factor (vWF), VEGF-receptor 2 (KDR), VE-cadherin, CD146, and CD31. Various studies demonstrated that EPCs isolated from peripheral blood is not restricted to monocytic lineage marker [Urbich etal, 2003]. Macrophages or dendritic cells derived from the mononuclear cell population were significantly less effective in improving new blood vessel formation. The EPCs generated from peripheral blood mononuclear cells exhibit a unique functional activity. EPCs derived from mononuclear cells shows monocytic characteristics. Monocytic cells are attracted by monocyte chemoattractant protein-1 are shown to enhance the diameter of existing blood vessel. Studies showed that EPCs cultured from CD14+ and CD 14- cells express endothelial marker proteins and improved neovascularisation in a hind-limb ischemia model [Urbich et al., 2003]. Endothelial cells derived from monocytes participate in wound healing, inflammation and tumor angiogenesis.

A sub population of CD14+ peripheral blood mononuclear cells expresses α-smooth muscle actin (SMA) and can differentiate into smooth muscle like cell. Simper et

al. demonstrated the presence of vascular progenitor cell that can differentiate into either endothelial or smooth muscle cell, in peripheral human blood [Simper D et al., 2002]. CD14+CD105+ PBMNC are the source of circulating human SMC, which exist in circulating human peripheral blood [Sugiyama et al., 2006]. The plasticity and heterogeneity of SMC is important for regulating vascular remodeling, and they play a key role in repairing vascular injury and damage. Thus smooth muscle progenitor cell (SMPC) provides a source of cells with reparative capacity.

Peripheral blood is the source of endothelial progenitor cell (EPC). Monocytic EPCs are involved in new vessel formation and vascular repair and thus they are involved in angiogenesis. Platelets act as initial responder of vascular change and provide a flexible delivery system for angiogenesis related molecules. As platelets are the first to reach the site of vascular injury and their importance in wound healing raised the hypothesis that they are involved in angiogenesis. Platelets release pro and anti angiogenic factors stored in its α-granules which play a major role in angiogenesis but its regulatory role is incompletely understood. Platelet monocyte complex represent a potential therapeutic agent for limiting cardiovascular diseases. Targeting inhibition of proinflammatory platelet activation, in contrast to targeting platelet aggregation is a good source for future drug.

1.2.8. Platelet endothelial cell interaction

Although stem cell biology in general is not well-understood currently, and the exact role of endothelial progenitor cells (EPCs) for atherosclerosis in particular, there is a consensus that circulating EPCs derived from bone marrow, typically surface express CD34 or CD133 and have the capability to differentiate to endothelial cells and, therefore, to repair vascular damage. A variety of factors have the potential to mobilize EPCs from bone marrow, including SDF-1. A variety of physical or clinical conditions appear to influence the number and function of circulating EPCs, including exercise, statin use, age, smoking, diabetes, chronic heart failure, and acute coronary syndromes. Although EPCs can repair vascular damage by differentiation to an endothelial cell

phenotype, they also may contribute to atheroprogression or restenosis, because they can also differentiate to SMCs or foam cells.

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

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

1.2.9. Receptor expressions in angiogenesis

There are cell surface proteins that are expressed on angiogenic endothelial cells, but not on quiescent endothelial or other cells. These proteins include the receptors for VEGF [Vaisman et al., 1990]. The expression of VEGFR correlates both temporally and spatially with the onset of neovascularization [Ferrara and Davis-Smyth, 1997]. Expression of both VEGF receptor types occurs in adult endothelial cells, including human umbilical vein endothelial cells. The angiogenic response involves changes that occur in endothelial cell interactions with the extra cellular matrix, as well as changes in

cell-to-cell interactions. It has been shown that circulating human non-adherent CD34 cells co-expressing vascular endothelial growth factor (VEGF)-R2 and AC133 have the capacity to differentiate into adherent mature endothelial cells. Under angiogenic stimulation macrophages develop an endothelial phenotype with the expression of specific surface markers and even form cord- and tubular-like structures *in vitro*, suggesting that this leukocyte cell population may be recruited for vasculogenesis. Mammalian PDGFs and VEGFs separate into four distinguishable classes of proteins. Classification into PDGFs or VEGFs is based on receptor binding. It has been generally assumed that PDGFs and VEGFs are selective for their own receptors.

Huge amounts of information on PDGFR signaling have accumulated over the past decade, but the understanding about how signaling specificity is obtained downstream from the individual PDGFRs, as well as in selected cell types at specific stages of development, is still limited. To what extent the cell systems commonly used for *in vitro* studies represent and reflect the critical PDGF signaling pathways *in vivo* remains largely unclear. It was demonstrated that pharmacological inhibition of PDGFR-β signaling in tumor pericytes synergized with the inhibition of VEGFR signaling in endothelial cells in suppressing tumor growth [Bergers et al. 2003]. In the response-to-injury hypothesis of atherosclerosis pathogenesis, PDGF released from aggregating platelets at sites of endothelial injury was assigned a key role in the migration of vSMC from the media into the intima, as well as in the subsequent proliferation of the vSMCs at this site [Ross 1993].

Quantitative real –time PCR is a method to monitor low abundance mRNA expression and to analyze changes in gene expression in presence of specific inducers. The expression analysis of these growth factor receptors in the cells which were induced to endothelial cells by CD34+/CD14+ stem cells , grown in presence of platelet debris gives the idea about the role of integrin molecules present in platelet debris in inducing angiogenesis. The vessel formation is usually studied in a three dimensional culture system within a suitable matrix.

1.3 Gap

Several studies have reported that soluble proteins which include various growth factors act on EC or EPC to induce angiogenesis. Growth factor receptors are present on differentiated endothelial cells and also on circulating EPCs. Monocytes are also known to express receptors for VEGF and PDGF. On activation, platelets shed membrane microparticle and platelet debris expose various integrins through which they interact with endothelial cells and circulating monocytes. Upon binding to monocytes, platelet integrins are known to activate monocytes. However, it is not understood, if the activated platelet debris plays any role in the differentiation of monocytes to either ECs or SMCs. If any such lineage commitment to EC or SMC takes place, such changes could be picked up by analyzing the expression of VEGFR and PDGFR. But it has not been studied if the platelet debris that is retained within the fibrin clot has any influence on the monocytes attracted to the vessel wall by the integrins.

1.4 Hypothesis

This study hypothesized that platelet debris may stimulate circulating progenitors to promote angiogenesis and subsequent wound healing. Such influence may be studied using *in vitro* culture systems in which PD is immobilized in fibrin matrix and thus mimicking an *in vivo* clot.

The mRNA isolated from PBMNC on the day of their collection and after 8 days of culture on the matrix may be the material for analysis of molecular changes on cells by reverse transcriptase polymerase chain reaction (RT PCR). By analyzing the expression of VEGFR and PDGFR respectively, using real time PCR, differentiation may be tracked. In order to test the hypothesis, following specific objectives are defined.

1.5 Objectives

- To analyze the interaction of activated platelets with monocytes.
- To isolate and characterize platelet debris from thrombin activated platelets.
- To establish the presence of various integrins on platelet debris.
- To isolate peripheral blood mononuclear cells using histopaque-1077.
- To incorporate platelet debris in to fibrin matrix on 2-D culture surface and to study its influence on PBMNC in culture.
- To simulate *in vivo* conditions by making 3D fibrin substrate incorporated with platelet debris and to study the influence on PBMNC in culture.
- To carryout expression analysis of angiogenesis specific markers, VEGFR2 and PDGFR in cells grown in presence of platelet debris.

CHAPTER II MATERIALS AND METHODS

II.1 Analysis of platelet-monocyte interaction

II.1.1 Isolation of PBMNC and staining

Blood samples were collected and buffy coat was separated by centrifugation at 1216g for 10 minutes. Separation of PBMNCs was achieved by centrifugation of buffy coat that was layered over histopaque-1077 (Sigma Aldrich, USA) at 400g for 30 minutes. The PBMNCs layer was resuspended in buffer and pelleted by centrifugation at 150g for 10 minutes. The cell pellet was resupended in ACD-Tyrode's buffer, 2mM (final concentration) calcium chloride was added. Platelets were activated using 0 IU, 0.1IU and 0.6IU thrombin.The samples were taken into two tubes, one part was incubated for 10 min and the other part for 60 min at 37°C. To 100µl of cell suspension, 2µL of fluorescent labelled antibodies [CD62-PE (Beckman Coulter, USA), CD14 FITC (Millipore, California)] were added. The samples were kept at dark for 1h and were made up to 1mL using 650µL phosphate buffered saline (PBS) and 250µL, 1% paraformaldehyde and kept under dark till the analysis was done.

II.1.2 Analysis of cell suspension using flow cytometer

Analysis was done using a single laser (488nm) 3-color flow cytometer (Epics XL Beckman coulter, USA). Unlabelled resting platelets were used for adjusting the gate position based on FSC and SSC. Protocol was developed for analysis of $CD62^+$ platelets, $CD14^+$ monocytes and dual labelled CD62+/$CD14^+$ cells in one set of experiments. The position of gate was adjusted in control to include almost all leukocytes and <0.1% positive in all three quadrants (Q1 [$CD62^+$], Q2 [$CD62^+$/$CD14^+$] and Q4 [$CD14^+$] signals. As the target population was monocyte, gate position was adjusted to get almost all monocytes within the gate. Study was repeated using samples isolated from at least 3

donors. Samples were acquired and analyzed for 60 s for analysis of each samples. Percentage of positive cells in each quadrant was noted for comparison between different experimental conditions used and for determining statistical significance.

II.2 Preparation of Platelet Debris

Bags of platelet rich plasma (PRP) were obtained from SCTIMST blood bank after getting consent. The samples were transferred to 50ml falcon tubes. One part ACD was added to 9 part PRP (v/v). Platelets were pelleted by centrifugation at 1216g for 20 minutes (Biofuge Stratos). The pellet was resuspended in Tyrode's –ACD (9:1 ratio) and centrifuged for 10 min at 1216 g for 10 min. The washing step was repeated for two more times by resuspension and centrifugation in Tyrode's-ACD. Final pellet was resuspended in Tyrode's-ACD and 25mM $CaCl_2$ was added to make final concentration of $CaCl_2$ to 5 mM. The suspension was incubated for 30 min and at 37°C, and platelets were activated using 1 IU concentration of thrombin at 37°C. The activated platelets were layered over 27% sucrose and centrifuged at 63000g for 3 hours at 4°C in an ultracentrifuge (Beckman Coulter, Optima L-90K).The top layer containing proteins released from platelets, middle layer containing platelet membrane and the sucrose solution were discarded and the debris which settled at the bottom was collected. The collected platelet debris (PD) was resuspended in HBSS containing antibiotics. The PD was then washed to remove sucrose remnants by centrifugation at 10,000g for 30 minutes. Decanted the supernatant and PD was resuspended in HBSS containing antibiotics.

II.3 Analysis of protein content and SDS-PAGE

Protein in the isolated PD was estimated by Lowry's method [Lowry et al., 1951] using spectrophotometer (Hewlett Packard Diode array 8453, USA). Analysis of the isolated PD 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) and denatured at 94°C.The samples were loaded into wells and

subjected to electrophoresis at 100V(Amersham pharmacia biotec). After electrophoresis the gel was separated from glass plate. The gel was stained with Coomassie Brilliant Blue R 250 (Sigma Chemicals, USA).

II.4 Identification of integrins on PD

To analyze for the presence of CD61, CD62 and CD41 in PD, semidry Western blotting was employed. For semidry blotting, the gel after SDS-PAGE 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 PVDF membrane in a semidry blotting apparatus (Pharmacia LKB Novablot) for 20 min at 0.8mA/cm^2 using Multiphor (Pharmacia Biotech, Sweden). After the transfer, 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 (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. Prepared 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 continuous agitation. Reaction terminated after the development of bands by the addition of PBS.

II.5 Cell Culture

II.5.1. Preparation of culture substrates

Tissue culture polystyrene (TCPS) dishes were incubated with 5 IU/ml thrombin in 5 mM $CaCl_2$ for 30 min at 37°C. Excess thrombin was aspirated out, wells were smeared with 10mg/ml fibrinogen and platelet debris at a concentration of 8μg, 16μg and 24μg per 1.9 cm^2 well. Fibrin coated wells without platelet debris was used as control. Plates were lyophilized before cell seeding.

II.5.2 Isolation of PBMNC for culture

Discarded buffy coat was collected from SCTIMST blood bank, diluted with Hank's Balanced Salt Solution (HBSS) and layered over Histopaque -1077 (Sigma Aldrich) and centrifuged at 400 g for 30 minutes. 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 (Biofuge Stratos, Heraeus, UK). The thick white layer (buffy coat) in the interface was collected which is a mixture of leukocytes, platelets contaminated with RBCs. Buffy coat was diluted to make 15 ml cell suspension using ACD-HBSS and was layered over to 15 ml tubes containing 7.5 ml Histopaque-1077 (Sigma-Aldrich, USA) 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 resuspended in ACD-HBSS. PBMNCs were pelleted by centrifugation at 150g for 10minutes. Cells were resuspended in DMEM/F12 medium containing human serum, and the cells were counted using hematological analyzer (Sysmex K-4500, Japan). Same numbers (10^6cells/well) of PBMNC were seeded into each well. Culture was allowed to grow in DMEM/F12 media with 10% human serum. Media was changed every day for three days and cells were visualized through phase contrast microscope (DMIRB Leica, Wetzlar, Germany).

II.5.3. Magnetic sorting to isolate CD14+ population

Isolated PBMNCs as described in #II.5.2 were seeded in uncoated wells and kept overnight at 37°C. After incubation, plates were mixed gently and kept for 5 minutes. The cells were flushed out from the wells using DMEM/F12 media. The supernatant was centrifuged to pellet the cells and was re suspended in serum-free media. Whole blood CD14 microbeads (MACS Miltenyi Biotec) were added to the samples and incubated for 15 minutes at 4°C and centrifuged at 150g for 10 minutes. Supernatant was removed and separation buffer (PBS, 0.5%BSA, 2mM EDTA) was added to pellet, resuspended and kept in Easysep magnetic separator for 5 minutes. Remove the supernatant, media was added to the remaining cells in the tube and used for culture.

II.5.4 Cell culture on 2-D substrate

The culture substrate for 2-D culture was prepared as described in #II.5.1. For whole PBMNC culture, the control surface without PD and to understand the effect of PD, substrates in which 3 different concentrations such as 8ug, 16ug, and 24ug (per 1.9cm^2 area) of PD was included and was used separately to compare growth pattern of cells from each donor. For culture of sorted CD14+ cells, only one concentration of PD i.e. 8ug/1.9cm^2 was used.

II.5.5 Cell culture on 3-D substrate

Cells isolated by MACS were mixed with fibrinogen (30mg/ml), 10% gelatin and 2IU aprotinin. Using fibrin glue applicator, fibrinogen was mixed with thrombin (3.75 IU/ml) and drop of liquid mixture was delivered to 1.9 cm^2 well. In test clot, platelet debris (8μg) was mixed to the cell suspension in fibrinogen. The drops were allowed to clot for 10 min and DMEM/F12 media was added and kept in incubator. After 8 days of culture the clot was fixed in 10% formaldehyde, processed, sectioned and stained by standardized Hematoxylin –eosin (H&E) staining for histological evaluation.

II.6. Analysis of receptor expression

II.6.1. RNA isolation

Cultured cells were harvested from plates at 8th day using TRIzol reagent. The homogenized samples were incubated for 5 minutes at room temperature. Chloroform (0.2 ml) was added per 1ml of TRIzol (Invitrogen) used for harvesting, mixed vigorously for 15 seconds and incubated for 2-5 minutes at room temperature. The samples were centrifuged at 12,000g for 15 minutes at 4°C. Aqueous phase was transferred to a fresh tube and mixed with isopropanol, incubated at room temperature for 10 minutes, and centrifuged at 12,000g for 15 min at 4°C. The supernatant was discarded and washed the RNA pellet with 75% ethanol, centrifuged at 7500g for 5 minutes at 4°C, discarded the supernatant, air dried the pellet and dissolved in DEPC water.

II.6.2. RNA quantification

RNA was quantified using Qubit fluorometer (Invitrogen, USA). The instrument was calibrated using Qubit standards. For Qubit standards load 190 µl of qubit working solution (Qubit RNA reagent 1:200 in qubit RNA buffer), add 10 µl of Qubit standards. 1-20µl of samples was mixed with Qubit working solution to make a final volume of 200 µl and mixed by vortexing. Tubes were incubated at room temperature for 2 minutes and read the concentration in Qubit 2.0 flurometer.

II.6.3 cDNA Synthesis

cDNA was synthesized using Oligo (dT) primer and superscript III reverse transcriptase. To prepare cDNA, approximately 1µg of total RNA was mixed with 1µl of Oligo (dT) primer and 1µl dNTP mix (10mM) and made up to 18µl with DEPC water and incubated at 65°C for 5 minutes. The reaction mix was immediately chilled on ice and cDNA synthesis was undertaken by the addition of 5µl of 5x first strand buffer, 1µl of 0.1M DTT, 0.5 µl of RNase OUT and superscript III reverse transcriptase. The reaction

was adjusted to a total volume of 25µl with DEPC water and incubated at 50°C for 60 minutes, then the reaction was inactivated at 90°C for 10 minutes.

II.6.4. Primer standardization

cDNA was synthesized using Oligo dT primers from human umbilical vein EC and human SMC. Optimum annealing temperature for forward and reverse primer: PDGFR (F:CAGTAAGGAGGACTTCCTGGAG,R:CCTGAGAGATCTGTGGTTCCAG)and VEGFR2(F:CAAGACAGGAAGACCAAGAAAAGAC,R:GGTGCCACACGCTCTAG GA) genes was determined from a set of forward and reverse primers at a concentration of 10 pico moles. Standard PCR conditions were 94°C for 5 min followed by 40 cycles of 95 °C for 30sec,annealing temperature of 50-60 °C for 30sec,72 °C for 30 sec and 72 °C for 5 minutes. Annealing temperature which gave good amplicon of the expected size without non specific products and primer dimmers were selected.

II.6.5 Qualitative analysis of PCR product:

PCR products were checked by electrophoresis on 1.5% agarose gel. Agarose (Sigma) was weighed and melted by adding 1X TBE (Tris Borate EDTA) buffer and allowed to cool. After cooling ethidium bromide (10mg/ml) (Biogene,USA) was added and mixed well. Comb was placed in correct positon in the gel casting tray and molten agarose gel was poured into it. After the gel got solidified, comb was removed and the gel was placed in electrophoresis tank filled with 1X TBE. The samples were loaded by mixing with gel loading dye (bromophenol blue and xylene cyanol FF).100 bp DNA ladder (Invitrogen) was loaded as the marker. Electrphoresis was carried at 100V in an electrophoresis power supply (Amersham pharmacia biotec).The DNA bands were visualized under UV trans illuminator (Spectroline). Gel was imaged in gel documentation unit (Alpha Imager).

II.6.6. Real Time PCR

The expression analysis of reference and target gene in cDNA samples were performed in Chromo4 systems. The PCR reaction contained 6µl of template cDNA, 12.5µl of qPCR mastermix for SYBR green 1, 0.5µl of forward and reverse primers and the total volume was made up to 25µl with DEPC water. GAPDH was kept as internal control. Samples were assayed under following conditions 94°C for 5 min followed by 40 cycles of 95 °C for 30sec,50-60 °C for 30sec,72 °C for 30 sec and 72 °C for 5 minutes. The PCR products were then subjected to melt curve analysis. The melt curve analysis was done from 60-90°C to ensure that the resulting fluorescence originate from single PCR product and did not represent primer dimmers formed during the PCR or a non-specific product.

CHAPTER III – RESULT AND DISCUSSION

The integrins play a major role in interaction of platelets with PBMNCs. Upon activation, but in the absence of aggregation cell fragmentation may result in release of platelet microparticles (PMP) and platelet debris (PD). Some of the integrins may shed away with PMP whereas others may remain with PD. The objective of this study was to establish that the isolated PD contains integrins and to further analyze if they have any effect on PBMNC culture to induce angiogenic marker expression. The first step was to study the interaction of monocytes to activated platelets which was analyzed by flow cytometry and the results are presented in III A. PD was isolated after activating platelets with 1.0 IU thrombin, which was found to be the best concentration for a better yield. Subsequently, PD was characterized for the presence of integrins such as CD62, CD61 and CD41 and the results are presented in IIIB. Following that, PBMNCs were isolated and further purified by MACS to obtain only the CD14+ cells. Both isolated CD14+ cells and crude PBMNCs were used for culture in presence and absence of PD. Two dimensional substrates used were fibrin network that was deposited on tissue culture polystyrene (TCPS) as control and fibrin incorporated with PD as test in all cases. For 3-D culture, fibrin clot incorporated with cells was prepared as control; whereas cells and PD incorporated fibrin clot was used as test. The morphology of cells was analyzed by phase contrast microscopy; expression of angiogenic markers was analyzed by RT-PCR and the results are presented in III C.

III.A Platelet –Monocyte interaction analysis

III.A.1 Antibody titration for CD14+ staining

In order to show that activated platelets interact with monocytes and that the extent of activation is sufficient to produce stable platelet monocyte complexes. So on activation of a mixture of platelets and monocytes with thrombin, complex of platelet and monocyte that is formed was analyzed. Markers chosen were activation dependent CD62 exposure and formation of complex that are positive for CD62 and CD14. In order to avoid false

positive results by adding excess antibody during flow cytometry analysis, optimum antibody concentration was determined by staining PBMNCs using two different concentrations of antibody. The cells were stained using 1µl and 2 µl CD14 FITC conjugated antibody and analyzed by flow cytometry. The analysis showed that 9.1% of cells were CD14+ when 1µl antibody (Ab) was used and 9.9% was CD14+ when 2 µl Ab was used for staining (Fig.III.A.1).There is no significant increase in the percentage of positive cells in spite of doubling antibody concentration, so at this concentration no false positive signals are likely to occur. So 2 µl was used for further analysis to ensure that sufficient quantity of antibody is added to stain all CD14 sites on the free cells and the complex. The staining of activated platelets using CD62 Ab was performed using the standardized protocol being followed in our laboratory (WPTRU058). Since the protocol is well-standardized no antibody titration was carried out for CD62P staining.

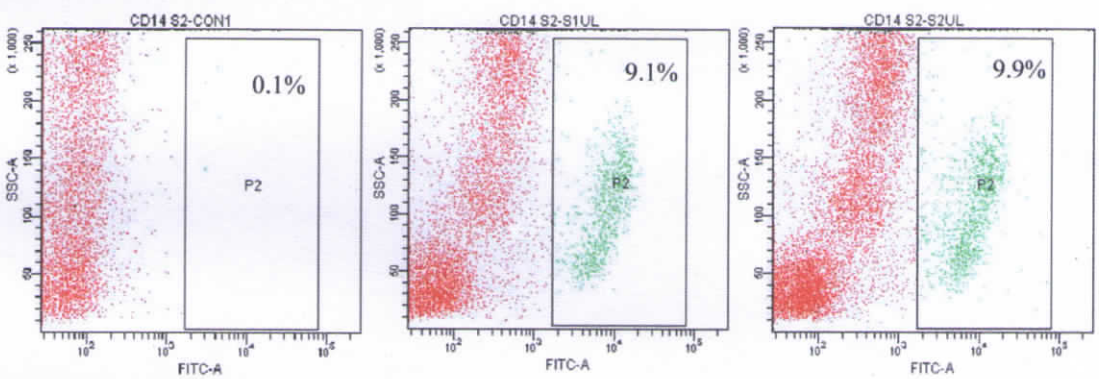

Fig.III.A.1.CD14 antibody staining of PBMNC. Flow cytometry analysis of CD14 staining of PBMNC using two concentration of Ab. First dot plot represent the unstained control, 2nd represent cells stained with 1µl CD 14 Ab and 3rd represent cells stained with 2µl Ab. The percentage of cells is as given in dot plots.

III.A.2 Activated platelet-monocyte interaction

Monocyte-platelet aggregates are heterotypic complexes detectable in the peripheral blood which form in response to platelet activation [Passacquale et al, 2011]. This study was to analyze the platelet-monocyte complex in normal condition, upon *in vitro* platelet

activation using a lower concentration (0.1IU) of thrombin which is similar to physiological concentration and a higher concentration (0.6 IU) of thrombin, which mimics pathological concentration of thrombin. Platelets and PBMNCs were separated from whole blood. Platelets that activated using both the dose of thrombin were incubated for a time period of 10 minutes and 60 minutes. Non activated samples were kept as control. After incubation, the samples were stained using fluorochrome conjugated antibodies such as PE for CD62P and FITC for CD14. The dual stained CD62+CD14+ complexes and single stained CD14+ cells were analyzed by flow cytometry, the fluorescence of PE and FITC was compensated using single stained cells to avoid spectral overlap.

The analysis showed that in non activated samples, the percentage of platelet – monocyte complex and CD14+ monocyte after 10 and 60 minutes remains almost same and it was ~ 0.5% of the gated population. As the platelets were activated with 0.1 IU thrombin the percentage of platelet-monocytes complex was increased to ~2.2% and CD14+ cells also increased significantly from 0.5% to 2.6%. But after 1 h, percentage of single stained and double stained cells decreased when cells were activated with 0.1 IU thrombin. Immediately after activation with 0.6 IU thrombin, the percentage of double positive and CD14+ cells were lower (~1%) as compared to the cells activated with 0.1 IU thrombin. But after 60 min, unlike the previous experiment, percentage of positive cell did not decrease (Fig.III.A.2). On the other hand there was significant increase in the percentage of CD14+ cells within 60 min of activation in the case of both 0.1IU thrombin and 0.6IU thrombin added for activation. This observation indicates that prolonged period of exposure to thrombin can activate monocyte and expose more CD14 sites on the cells. Such activation may have implication in inflammatory response mediated by monocytes. However, formed complex is stable only when higher concentration of thrombin is added to the mixture of cells. This observation has relevance in the *in vivo* situation because if more thrombin is generated due to pathological reasons, monocytes may more activated and platelet-monocyte complex may be stable in circulation.

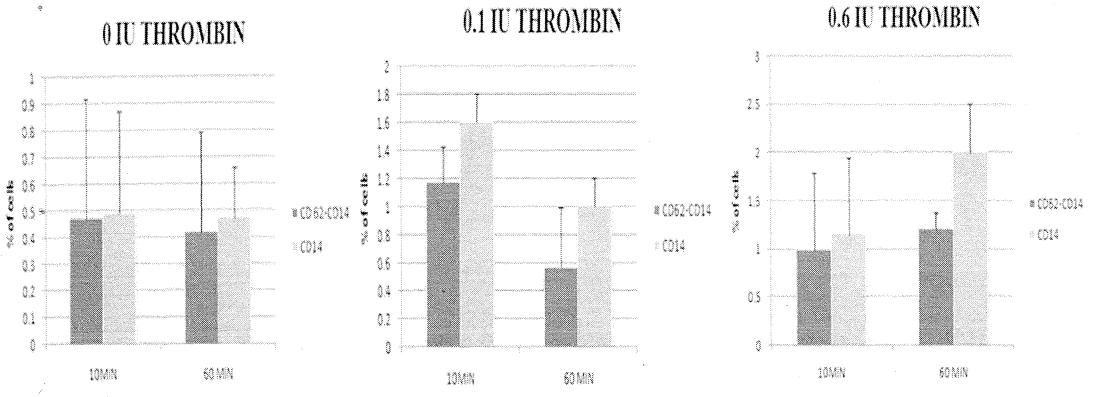

Fig.III.A.2.Activated platelet-monocyte interaction analysis. Histogram representing the percentage of cells after flow cytometry analysis of CD62-CD14 and CD-14 cells in 10 min and 60 min after thrombin activation of platelet.

Thrombin plays a major role in the initiation and maintenance of thrombosis [Hamon et al. 2006]. The activation of monocytes induces a pro coagulant and pro adhesive inflammatory response which leads to thrombotic complications. It acts by activating clotting factors and by stimulating platelets. Platelet monocyte interaction occurs by P-selectin and PSGL-1 interaction. Platelet surface P-selectin also induces the expression of tissue factor on monocytes, which in turn interact with P-selectin to accelerate fibrin formation and deposition. Sarma et al [Sarma et al., 2002] suggest that in addition to the P-selectin & PSGL-1 interaction, cation dependent interactions are also involved. PSGL-1 blockade inhibits majority of platelet binding to monocytes, indicating that P-selectin / PSGL-1 mediated adhesion occur physiologically. Furman and colleagues showed that circulating platelet-monocyte aggregates are an early marker of acute myocardial infarction. Studies reported that platelet-monocyte aggregate alters the phenotype of circulating monocytes and this causes the adhesion of monocytes to endothelium.

Crosstalk between platelets and monocytes is regarded as a crucial pathophysiological mechanism linking thrombosis and inflammation. The importance of monocyte platelet interaction in human inflammatory pathophysiology as well as the mechanism by which such interaction modulate monocyte function remains unclear [Passacquale et al, 2011].

Platelet –monocyte aggregate can be used as a diagnostic tool and a possible therapeutic consequence of inhibiting platelet-monocyte complex formation.

III.B Isolation of PD

III.B.1 Purity, identity and protein content estimation

PRP bags were collected and the platelets were activated using 1IU thrombin at 37°C. The activated platelet suspension was layered over sucrose and the platelet debris was separated by ultracentrifugation. The debris was collected and washed to remove the excess sucrose and other contaminants. The yield of PD was determined by Lowry's estimation. The protein samples were diluted using PBS and the color developed by the addition of Lowry's reagent and Folin's ciocalteau reagent was read at 600nm in a spectrophotometer. The protein in PD was found to be 25µg/µl. The total PD yield was 2.5×10^{4} µg/ml from 3.8×10^{10} platelets.

Since the protein released from activated platelets remained on the top after sucrose gradient centrifugation, it can be confirmed that all growth factors that were released from platelet granules were removed from the debris fraction. Also the membrane micro particles were settled above the sucrose layer and were discarded. Therefore the preparation is pure debris and no membrane vesicles or soluble proteins are contaminating the preparation. It can be assured that the molecules integrated within PD are solely responsible for the protein content.

III.B.2 Analysis for integrins in platelet debris

The PD samples were loaded on 8% SDS-PAGE at a concentration of 20 µg. The presence of CD61 Integrin β, CD62 P and CD41 Integrin α- 2b in PD was determined by the molecular weight characteristic of protein bands such as 87 KDa,140 KDa and 137 KDa, respectively, in the stained gel (Fig. III .B.1). Considering that there are more than 300 different types of proteins in platelets, numbers of prominent bands of debris is low. Since there are protein bands corresponding to major integrins, it is worthwhile to test if

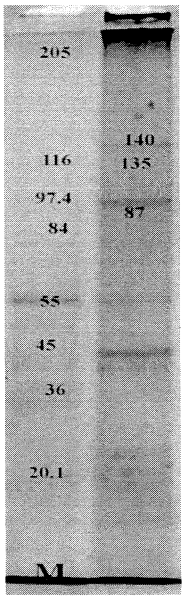

Fig.III.B.1.SDS PAGE analysis of PD proteins.SDS polyacrylamide gel stained by coomasie staining .First lane represent high molecular weight marker and 2nd lane represent PD sample.

the important integrins that are involved in platelet-monocyte interaction is present or not in the PD fraction.

Thrombin causes platelets to change shape, stick to each other, and secrete the contents of their storage granules. Platelet activation leads to the release of active factors from their α-granules. Platelet glycoproteins αIIb and ß3 (CD41/CD61) are membrane proteins which are involved in platelet aggregation and haemostasis and these integrins are more expressed during platelet activation. P-selectins are the most important biological molecule that appears on platelet surface after activation. P-selectin has a key role in inflammation and inflammation-related diseases [Alan T. Nurden, 2011]. Further, the role of CD62P on activated platelets and their interactions with monocytes were analyzed.

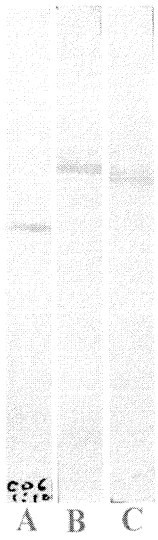

Fig.III.B.2.Western blot analysis. Blot developed for CD61, CD41 and CD62 in PD.A, CD61 (Beta 3)-87KDa, B, CD41 (Alpha 2b)-135KDa and C, CD 62 (P-selectin)-140 KDa

Specific bands for CD61, CD62P and CD41 were clearly seen in Western blot after the membranes were developed using antibodies (Fig.III .B.2). So it was confirmed that important integrins of platelets remained with PD. So it is likely that the PD can interact with monocytes through CD62P. Other integrins present on PD are CD61 and CD41. These two integrins are responsible for binding of platelets to fibrinogen. Therefore, when PD is mixed with fibrinogen to formulate a fibrin based culture matrix, the immobilization of PD to fibrin can be assured. In this study no effort was taken to demonstrate binding of PD to fibrinogen/fibrin, the presence of integrins on PD, which is also responsible for platelet aggregation through fibrinogen cross-linking is an indirect evidence for strong interaction between PD and fibrin when the cell culture matrices are formulated. So, while CD62 would attract monocytes to PD, CD41 and CD61 will facilitate immobilization of PD to fibrin.

III.C. Cell Culture results

III.C.1.The effect of PD on PBMNCs in 2D culture

PBMNCs were isolated and seeded in TCPS wells coated with fibrin composite without PD which was kept as control and fibrin incorporated with PD in different concentration (8μg, 16 μg and 24 μg) were the tests. Same seeding density was maintained in control and tests when culture was started. Cultures were grown in DMEM/F12 media, kept in 5% CO_2 incubator at 37°C. After 8 days the cells were visualized under phase contrast microscope. Macrophages were prominent in control wells which showed a foam-like appearance. In test wells spindle shaped cells were observed. There were other cells with elongated morphology (Fig.III.C.1). This observation suggested that in the presence of platelet debris there was less number of macrophages. No relation was found to the concentration of PD that was added in each well. So for further experiments, only 8 μg PD was used.

It has been reported that the released proteins of platelets can stimulate endothelial cell growth. But no study has reported that platelet debris may have angiogenic potential and induce PBMNCs to spindle like cells. Brill et al [Brill et al., 2004] showed that platelet releasate is able to support angiogenesis in vitro and in vivo. Studies by Kisucka et al [Kisucka et al., 2006] showed that platelets are involved in early stages of neovascularization. It is known that platelet derived microparticle as well as adhesion molecules which shed from the activated platelets are capable of activating leukocytes and endothelial cells. Evidence has also suggested that incorporation of bone marrow-derived endothelial precursor cells that circulate in blood contributes to the growth of vessels, complementing the sprouting of resident endothelial cells [Asahara et al,1997].

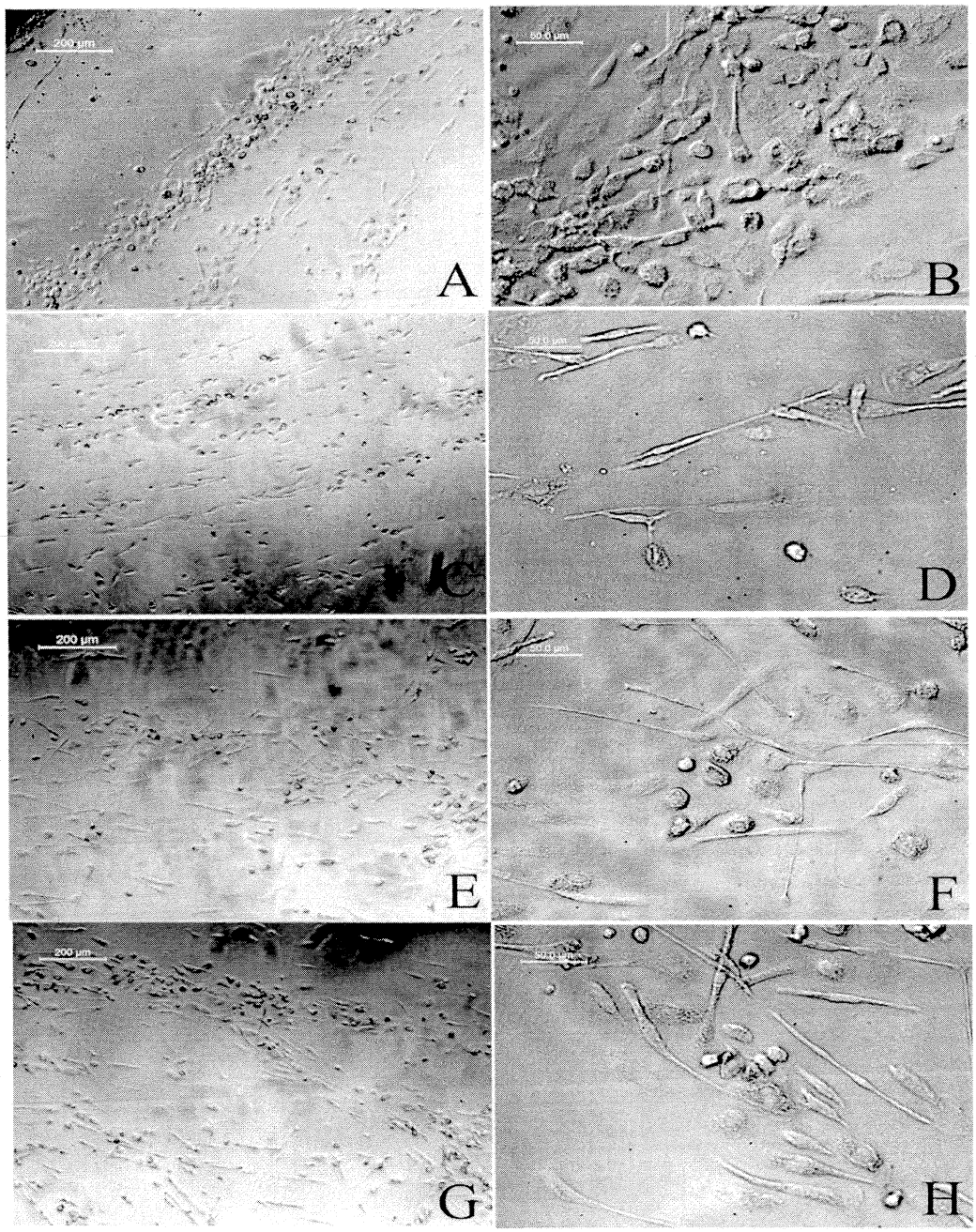

Fig.III.C.1 Photomicrographs of PBMNC in culture showing the effect of PD dose. Representative phase contrast images showing cells on 8th day of starting the culture: A & B, cells on control matrix; C&D, cells on test matrix with 8ug PD, E&F, cells on test matrix with 16ug PD, G&H, cells on test matrix with 24ug PD. Magnification of all figures is as marked in the image. Culture detail is described in II.5.1

In PBMNCs there are heterogenous cell populations and how those cells might influence cell transformation is not known. There may be cytokines, chemokines or death inducing factors might be released to the medium and multiple effects may be elicited. So CD14+ cells were sorted using MACS technique to separate them from other subsets such as lymphocytes, poly nucleated cells and platelets.

III.C.2 MACS separation of CD14 + cells and culture

The sorted cells were stained using a secondary Ab tagged with a fluorochrome and analyzed by flow cytometry to determine the percentage of CD14+ cells after MACS and was found that above 90% cells were positive(Fig.III.C.2). Such isolation could have eliminated the cytokines released from other types of leukocytes which might influence the differentiation of monocytes. And also when leukocytes and lymphocytes undergo death in culture wells, it is likely to cause the release of various proteases that could affect the monocyte growth and differentiation adversely. Therefore, the cells that were obtained after 8 day culture starting from PBMNC and that were obtained by growing MAC sorted cells could have different characteristics.

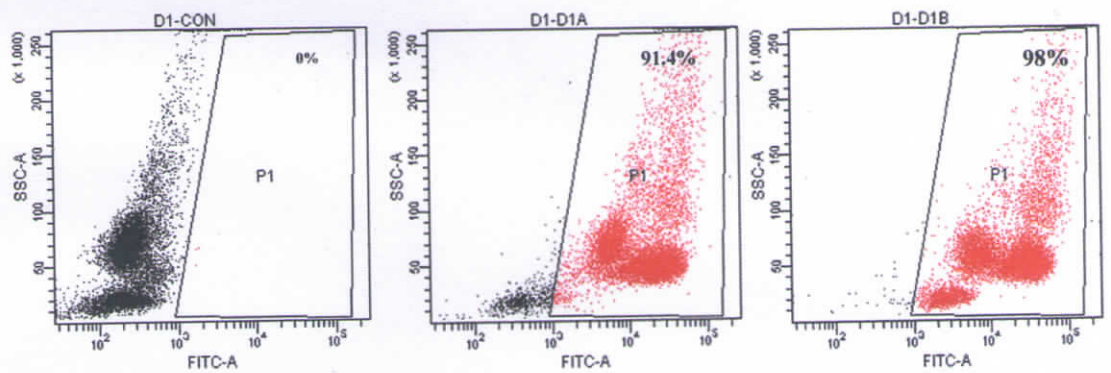

Fig.III.C.2.Dot plot of MAC sorted cells. Representative plot for determining % of cells after MAC sorting. A, represent unstained control, B.MAC sorted cell of donor 1 and C MAC sorted cell of donor 2.The percentage of cell are 91.4% and 98% respectively in two donors.

III.C.3.Culture of CD 14+ cells on 2D culture matrix

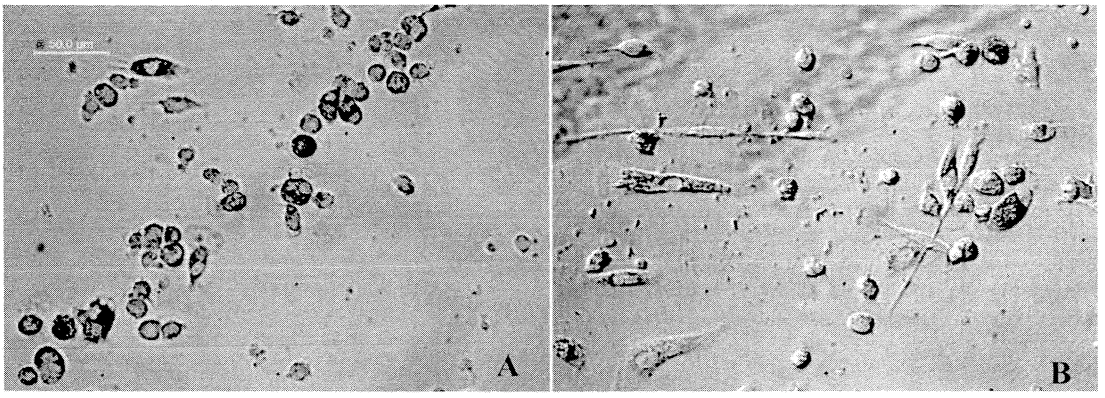

Fig.III.C.3.Photomicrographs of CD14 sorted cells on 2D culture. Representative phase contrast images showing CD14 sorted cells on 8th dayof the starting culture.A,cells on control matrix and B,cells on test matrix with 8 μg PD. Magnification is marked in the image.

MACS sorted cells were cultured in DMEM/F12 media with 10% human serum on fibrin coated control wells and wells coated with platelet debris at a concentration of 8μg as test wells. After 8 days of culture at 37°C with 5% CO_2 , the cells were visualized under phase contrast microscope and found to have resulted a prominant number of macrophages in control wells. III.C.4. Culture of CD 14+ cells on 2D culture, spindle shaped cells were seen in test wells along but foam-like macrophages were much less (Fig.III.C.3).

III.C.4.Culture of CD 14+ cells on 3D culture matrix

When MACS separated CD14+ cells were grown in 3-D fibrin, clot dissolution was noticed after 4 days, but it was overcome by adding protease inhibitor aprotinin. Clot was made by the same concentration of fibrinogen, 3.75 IU thrombin and 2IU aprotinin (Fig.III.C.4). Cells entrapped in the clot were grown in DMEM/F12 media at 37°C with 5% CO_2 and were harvested after 8 days for RNA isolation and staining. Cells grown in three dimensional cultures were observed by phase contrast microscopy and in the periphery where clot started degradation cells could be visualized (Fig.III.C.5).

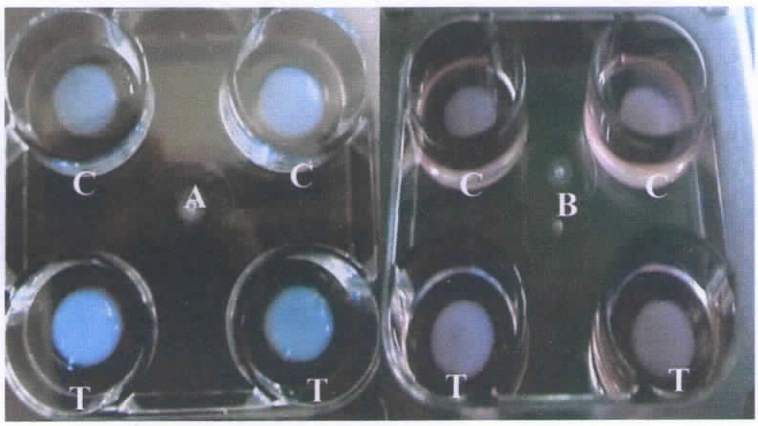

Fig.III.C.4 Three dimensional culture matrix. Photograph showing 3D culture system. A, Fibrin clot incorporated with CD14+ cells, B, fibrin clot suspended in DMEM/F12 media. C-controls & T- tests.

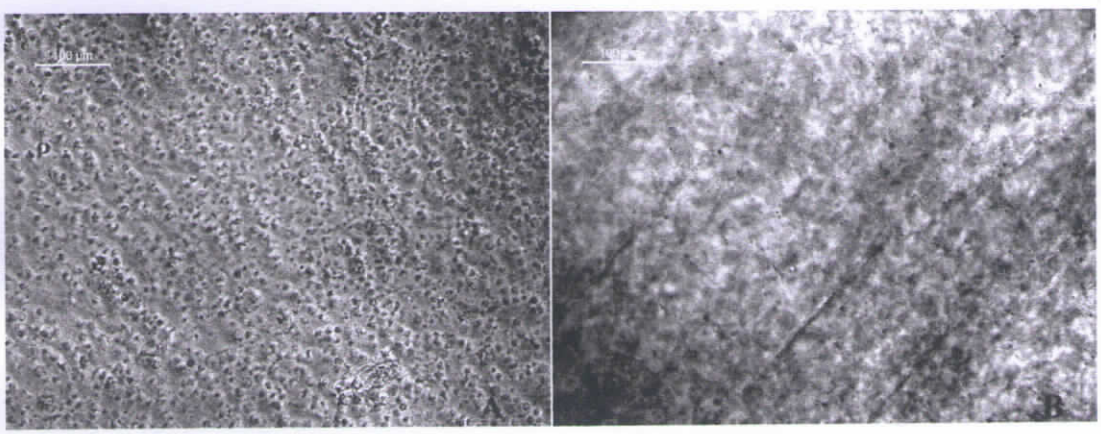

Fig.III.C.5.Photomicrographs of CD14 sorted cells on 3D culture. Representative phase contrast images showing CD14 sorted cells on 8th day of the starting culture. A,cells on control matrix and B, cells on test matrix with 8 µg PD. Magnification is marked in the image. Culture details is described in II.5.1

Since the clots were opaque in nature, it was not possible to analyze if cells are trapped or bound to the matrix even at the termination of the study, i.e. at the end of 8days. It was also not possible to analyze the morphological features of the cells in the clot. Therefore, to analyze if cells are bound to the matrix, the clot was washed and fixed with 10% formaldehyde for 4 to 6h, dehydrated using automated tissue processor, micro sections were made and stained with hemotoxylin-eosin staining solution. Presence of

spherical cells was observed, but no elongation of cells was evident (Fig.III.C.6). This clearly shows that in 3D culture systems the cells are present within the fibrin matrix but its morphology was not changed.

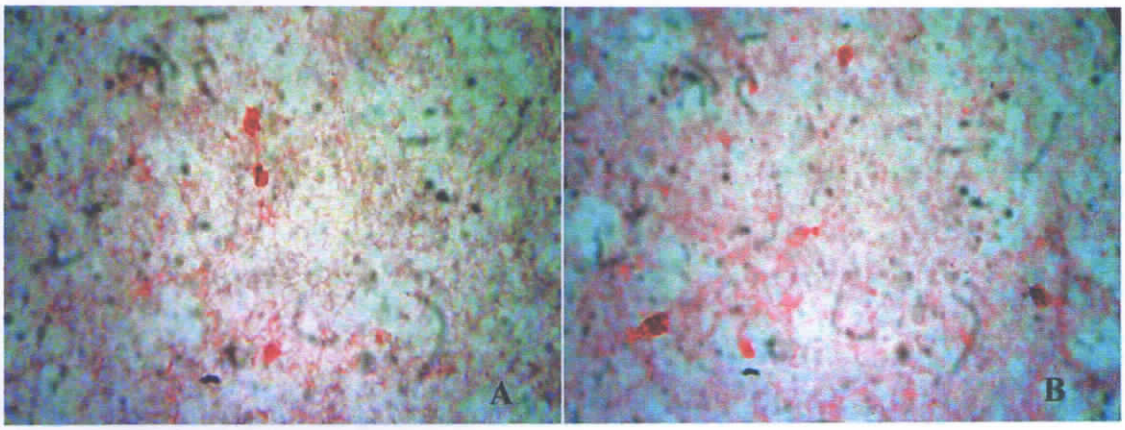

Fig.III.C.6.Histochemistry of cells in 3D culture. Hematoxylin and eosin stained sections of cells in 3D culture under 100X oil immersion objective of compound light microscope. A ,cells on control matrix and B, cells on test matrix. Culture details is described in II.5.1

On analysis of the H&E stained micro sections of the clot after 8 days in culture, blue stained cells are clearly visualized (Fig.III.C.6). Pink stained fibrin net work is also clearly visible in the sections.

The density of fibrin strands formed on clotting fibrinogen with thrombin depends on the fibrinogen concentration and thrombin activity. The more the fibrinogen concentration fiber density will be high and the clot may be less porous. It is also known that lesser the thrombin activity used for clot formation, the fiber thickness will be higher. In this study no experiment was done with different concentration of fibrinogen or thrombin. For cell to cell contact the density of the fibers and the fiber thickness may have advantage. For penetration of the medium and nutrient to the inner part of the clot, it will be better if the fiber density is less and if the clot is more porous. Since in this study, both parameters were not changed and only a single composition was used, it may not be concluded which is the optimum clot density for better angiogenic activity. However, since cells are clearly located in the clot the cellular activity could be estimated in terms of angiogenic marker expression.

III.D. Expression analysis of angiogenic marker gene in PBMNC culture

III.D.1. RNA isolation and cDNA synthesis

RNA was isolated from cells grown on 2D and 3D substrates, after 8 days of culture. Initially the cell count was adjusted to 2×10^6 cells in 1.9 cm^2 wells in 2D culture but the RNA obtained was less so cells were grown in larger surface area (9.6 cm^2 wells).The concentration of PD and cell number were proportionately increased. Cells in culture after 8 days were harvested in TRIzol and RNA was isolated by TRIzol method of RNA isolation. The quantity and quality of RNA was checked using Qubit fluorometer. The concentration of RNA ranged from 0.046-0.170 µg/µl. Oligo dT primers and superscript III reverse transcriptase were used to synthesize cDNA from 1µg of RNA.

III.D.2. Primer standardization for real time PCR

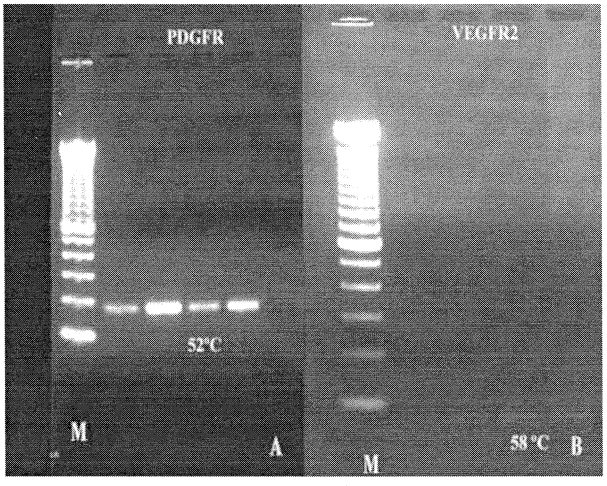

Fig.III.D.1.Primer standardization of PDGFR and VEGFR2 primers. Agarose gel images after electrophoresis of SMC and HUVEC cDNA amplified by PDGFR and VEGFR2 primers at different annealing temperatures. A, represent bands of PDGFR expressed gene and B, represent VEGFR2 expressed gene. The suitable annealing temperature was found to be 52°C for PDGFR and 58 °C for VEGFR2.

cDNA was synthesized using RNA isolated from HUVEC and human SMC to determine the annealing temperature of VEGFR2 and PDGFR primers respectively. The primer was diluted to 10 picomolar concentrations and amplified at different annealing

temperature within the range of primer melting temperature. Amplified products were checked on 1.5% agarose gel. Annealing temperature was selected at which there is no primer dimers and non specific amplification. The annealing temperature for VEGFR2 and PDGFR primer was found to be 58°C and 52 °C respectively (Fig.III.D.1).

III.D.3.Real time PCR analysis

The expression analysis of angiogenic markers in 2D and 3D substrates were analyzed by amplification of cDNA by real time PCR. The amplification of the genes was determined from the amplification plot (Fig.III.D.2) and specificity of the product by melt curve analysis and agarose gel electrophoresis (Fig.III.D.3). In two dimensional cultures the PDGFR and VEGFR2 expression was seen and the Ct values obtained were as shown in table: 1 and table: 2 respectively. This clearly shows that the angiogenic marker PDGFR was expressed in cells grown in presence of platelet debris in 2D culture. PDGFR expression indicates that the cell may be differentiating towards SMC like phenotype.

Table: 1 2D culture PDGFR expression-Ct values obtained in Real Time PCR

Sample	Control	Test
Donor 1	39.21	35.66
Donor 2	39.58	35.46
Donor 3	39.11	37.19
Average	39.4	35.56
SD	0.26	0.82

But no amplification was detected when the RNA from 3D culture was used. This observation indicates that no cells are differentiation to SMC when MACS sorted cells were grown on 3D fibrin clot. It is not clear from this study why SMC differentiation was

not seen, even though the matrix composition was similar. But matrix density was different, so the SMC differentiation was found to be retarded.

Table: 2 2D culture VEGFR2 expression-Ct values obtained in Real Time PCR

Sample	Control	Test
Donor 1	36.6	35.3
Donor 2	35.2	35.81
Donor 3	32.9	33.3
Donor 4	33.4	33.26
Average	34.5	34.4
SD	1.69	1.32

In 2D culture the amplification of VEGFR2 was comparable in both control and test. There was amplification of this gene even when mRNA isolated from PBMNC before culture and the Ct value was comparable slightly more (35.8) than to that after culture on 2D substrate for 8 days. Thus a clear indication is that there is no marked differentiation of monocytes to endothelial cells without or with the influence of PD entrapped in culture substrate.

Table: 3 Ct values for VEGFR2 gene expression in 3D cultures

Sample	Control	Test
Donor 1	36.92	36.63
Donor 2	36.01	36.37
Donor 3	34.99	37.17
Donor 4	36.01	37.77
Donor 5	34.6	34.12
Average	35.71	36.41
SD	0.92	1.39

The results obtained clearly explain that the fabricated 3D substrate may not be suitable for the differentiation of CD14+ cells to EC or SMC those express angiogenic markers. Thus in our study the 2D substrate is better suited for the differentiation of CD14+ cells and to induce angiogenic marker expression. Only once different composition of fibrin clot is tested, it may be concluded if 3 D fibrin clot incorporated with PD has an effect or not on differentiation of monocytes.

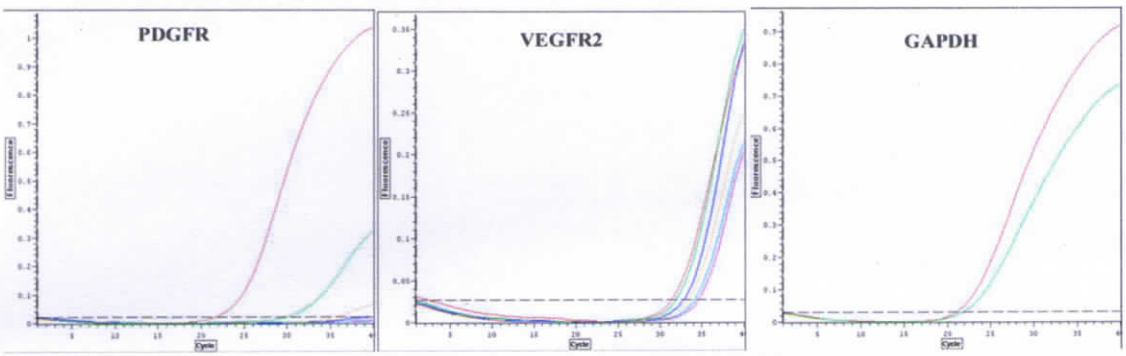

Fig.III.D.2.Amplification plot of real time PCR. Representative amplification plot for GAPDH,VEGFR2 and PDGFR

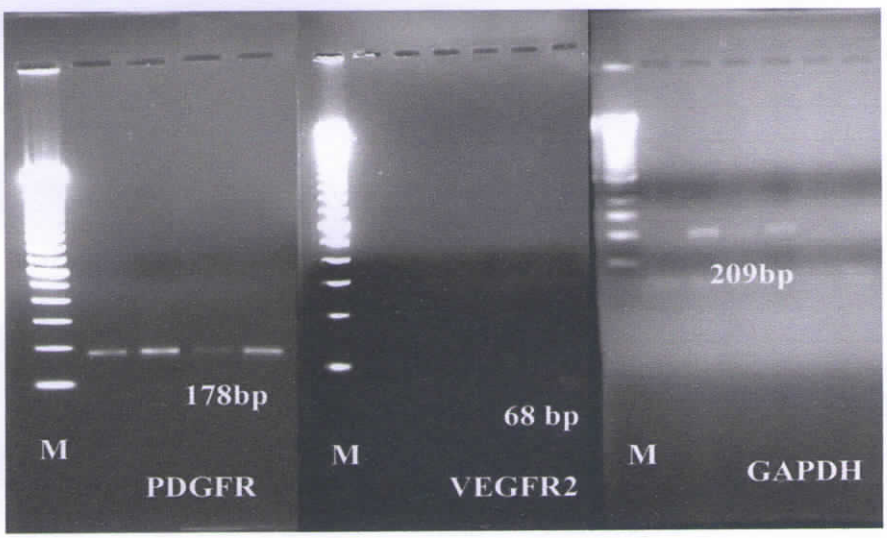

Fig.III.D.3.Agarose gel images of Real time amplified products. Representative gel pictures showing the real time PCR amplified product .A, represent PDGFR2 amplified product of 178bp,B, represent VEGFR2 amplified product of 68bp and C, represent GAPDH amplified product of 209bp.

The PDGFR expression in two dimensional cultures indicates that the cells could be stimulated by protein moieties on PD and PDGFR is expressed on the transformed cells. PDGFR signaling is involved in diseases like glomerulonephritis, liver cirrhosis, fibrosis and atherosclerosis [Alvarez et al.2006]. In normal condition, the PDGFR expression is low in SMCs but during inflammatory response its expression increases. Because the PD is devoid of soluble proteins from platelets, the result from this experiment suggest that the integrin induced signaling has resulted in expression of PDGFR in some of the monocyte during culture in contact with PD. In pathogenesis of atherosclerosis the monocytes are attracted towards the arterial wall and there occurs the accumulation and proliferation of macrophages and SMC and leads to the formation of connective tissue. [Raines and Ross, 1995].

Receptors of VEGF play a major role in angiogenesis and cell migration. The amplification of VEGFR2 in cells after 2D and 3D culture was similar to that in PBMNC on the day of sample collection. No up regulation of the gene after 8 days of culture on test or control substrate was indicated. VEGFR2 is involved in angiogenesis by binding to VEGF, these receptors are expressed on vascular ECs and lymphatic ECs and it regulate vascular endothelial function. During this study, no angiogenic differentiation was found to be stimulated by PD on cells from PBMNCs.

Integrin play a vital role in biological processes, including cell survival, growth, differentiation, migration, inflammatory responses, platelet aggregation, tissue repair and tumor invasion. They are integral membrane protein that serves as a bridge between the extra cellular matrix and the cytoskeleton of the cell [Thomas S. Kickler, 2005]. The interaction of monocytes and platelets are mediated by the integrin molecules (GP IIb/IIIa). The signals from these adhesion receptors are integrated with those originating from growth factor receptors in order to organize the cytoskeleton, stimulate cell proliferation and rescue cells from matrix detachment and induced programmed cell death [Chandra Kumar,1998] .These functions are critical in the regulation of gene

expression, tissue development, inflammation, angiogenesis, tumor cell growth and metastasis.

Though endothelial cell differentiation was absent, there is indication for SMC differentiation in 2D cultures. The culture substrate was composed as model of matrix that may be formed on the vessel wall during minor injuries. The debris of platelets that are activated during the injury may be entrapped into the fibrin matrix. An example of such an occurrence is during angioplasty, when stent is deployed in the vessel. Inflation of the balloon and simultaneous expansion of metallic stent can cause minor injuries to the blood vessel. Activation of coagulation, thrombin generation, fibrin formation, platelet activation etc. can take place during balloon angioplasty. Platelet debris may get lodged in to fibrin clot and influence anchorage of circulating monocytes to the site of injury which may get differentiated to smooth muscle cells. The excessive growth of SMC and deposition of connective tissue may lead to restenosis.

This study was initiated to find out if platelet debris can influence angiogenesis after vessel injury. But there is no evidence in this study which suggested initiation of angiogenesis. So the hypothesis was disproved. On the other hand, it was observed that differentiation of monocytes to SMC may be influenced by the PD embedded in fibrin clot. The finding is significant because it might explain how restenosis may occur after balloon angioplasty. The same process may be involved in the progression of atherosclerosis, where excessive growth of SMC and connective tissue formation may lead to vessel thickening and calcification. Various risk factors such as diabetes, hypertension and hypercholesterolemia may cause endothelial dysfunction and damage and as a result, the activated platelets may bind to the damaged site which in turn may attract monocytes. So there is a chance for SMC differentiation at such sites of minor injury, which may lead to vessel thickening and atherosclerosis progression. Since SMC differentiation in 3D culture was not observed, it is suggested that dense fibrin clots may not be suitable for SMC differentiation. This is a preliminary finding that PD influences SMC differentiation from PBMNC. More detailed studies are required to prove that such

PD induced SMC formation can take place. Therefore, involvement of PD in restenosis and atherosclerosis is a problem for future study.

CHAPTER IV SUMMARY AND CONCLUSION

IV.1 Summary

Platelets are non nuclear cellular fragment discovered by Giulio Bizzozero in 19th century. It plays a major role in hemostasis that leads to the arrest of bleeding. Plasma proteins, blood vessels and endothelial cells are also involved in this process. Under normal conditions platelets do not adhere to endothelial cells. The non-thrombogenic nature of endothelial cells is due to a negative charge on its surface and many active processes. Platelets have specialized secretory elements that release their intracellular granules upon activation. Released granule contents are also involved in angiogenesis, inflammation and tissue repair. The integrin molecules exposed upon platelet activation have a role in binding to adjacent cells as well as recruitment of progenitor cells. The goal of this study was to identify if such integrins, present on the activated platelet debris, induces angiogenic differentiation of the CD14+ monocytic population.

Activation of platelet-monocyte mixture with different concentrations of thrombin identified that 0.6 to 1 IU thrombin is the concentration required to expose the integrins and to get stable platelet-monocyte complex. The interaction of thrombin activated platelets with monocytes was studied by flow cytometry which suggest that at low thrombin concentration the interaction is weak and is less stable with respect to increase in time. But at higher thrombin concentration there is an increase in platelet monocyte complex which exposes more CD14 monocytes upon 60 min incubation. Increase in CD14 exposure is significant inflammatory processes as well.

For the preparation of PD, platelets were activated with 1 IU thrombin. Protein content in the PD samples was estimated and the proteins were separated by SDS-PAGE. The presence of integrin molecules in the PD was evident from PAGE based on their molecular sizes which are reported in the literature for major integrins. The PD obtained from 1 unit of platelet rich plasma was sufficient for carrying out the analysis and for carrying out all the culture studies. As the integrins play roles in adhesion of PD to fibrin

matrix, adhesion to monocytes and probably in lineage commitment, their presence in the debris was to be identified. The presence of specific integrin molecule CD61 Integrin β, CD62 P and CD41 integrin α- 2b were confirmed by Western blotting, in which specific bands were developed.

The PBMNCs were cultured in a 2D and 3D culture system that resembles the *in vivo* fibrin matrix, which served as control. To the control fibrin matrix, PD was incorporated and was considered as the test substrate. In the preliminary experiments with PBMNC, three different composition of PD was included to check if the change on PBMNC morphology was concentration dependant. Cells were cultured in DMEM/F12 media with 10% human serum derived from fresh frozen plasma, so that no growth factors from platelets would be present in the serum. No additional growth factors were supplemented. It was evident that in control, majority of cells were in foam cell like appearance, where as in test more spindle shaped cell existed after 8 days of culture. The result indicated that on fibrin substrate most of the PBMNCs were converted to macrophages. On the other hand with addition of PD to the substrate, macrophagic differentiation was prevented. No marked difference was observed when the concentration of PD was varied. So for all further studies 8 μg/1.9cm^2 PD was incorporated to the substrate. In 2D culture, the matrix was fabricated by smearing the fibrinogen on to thrombin treated polystyrene surface where as for fabricating 3D substrate, fibrinogen into which cells and PD were incorporated was clotted by mixing with thrombin. The PD was added to obtain 8 μg/ml of clot. In order to prevent clot lysis and disintegration, protease inhibitor aprotinin was added.

To know if a homogenous cell population is cultured on the matrix, the phenotypic change is similar to that of PBMNC culture, CD14+ cells were separated by MACS using CD14 specific microbeads and magnetic column. The percentage recovery of CD14+ cells were analyzed by flow cytometry and showed that MACS resulted in >90% positive cells. The MACS separated cells were seeded in both 2D and 3D substrates. The cells were observed under phase contrast microscope in the case of 2 D culture. For analyzing

if cells are present in 3D substrate, clot was sectioned; H&E stained using histopathological technique and was analyzed under light microscope. In 2D culture, elongated cells were observed in PD incorporated substrate and macrophages were abundant in substrate having fibrin matrix alone. So, in 2D culture crude PBMNC and MACS separated CD14+ cells showed same behaviour. In the case of 3 D culture, cells were seen frequently in H&E stained sections also. No morphological features could be identified from these sections. So the analysis for differentiation was restricted to the RT PCR using RNA that was isolated from cultures by TRIzol method. Appropriate controls were included for standardization of RT PCR and internal controls for real time PCR. The receptor, PDGFR and VEGFR2 expression in these cells were analyzed by real time PCR. The analysis of Ct (cycling threshold) showed that PDGFR expression is influenced by platelet debris in 2D culture but not in 3D culture.VEGFR2 expression was comparable after the cells were cultured on fibrin substrate to that on PBMNC isolated; in the presence and absence of PD, and irrespective of the use of 2D or 3D substrate.

IV.2. Conclusions

- ✓ Higher concentration of thrombin increases CD14+ monocytes in circulation which may lead to inflammatory complications.
- ✓ Presence of CD41 and CD61, might have conjugated PD with fibrin matrix and CD62 might have promoted monocyte binding to the PD that is immobilized on fibrin matrix.
- ✓ Absence of VEGFR2 expression suggests that PD has no influence on angiogenesis; thus hypothesis was disproved.
- ✓ PDGFR expression in 2D culture indicates cell differentiation to SMC-like cells.
- ✓ Finding is significant because SMC proliferation in the absence of EC growth is likely to cause restenosis, atherosclerosis progression and vessel occlusion.

- ✓ Such cellular activity may be a consequence of vessel injury and platelet activation.
- ✓ This study suggests that PD could be the culprit in restenosis after angioplasty or in vessel thickening during atherosclerosis progression.
- ✓ This study also suggests that PD could provoke a negative effect on inflammatory response because macrophage –like cells were found in the absence of PD.

IV.3. Future prospects

- 3D- culture model did not show any improvement in the expression of VEGFR2 as compared to 2D culture; whereas PDGFR expression was absent. So the 3D culture matrix has to be modified.
- To conclusively demonstrate SMC differentiation from monocyte in the presence of PD, using specific and confirmative markers.
- Study the detailed mechanism by which PD regulates macrophage.
- Prove that differentiation of monocytes to SMC is a major mechanism in pathology of cardiovascular disease.

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APPENDIX

1.Phosphate Buffered Saline (1X PBS)

NaCl	8.166g
Na2HPO4	1.419g
KH2PO4	0.204g

pH -7.4,Made up to 1L using deionized water

Filtered and stored at room temperature

2.Hank's Balanced Salt Solution(HBSS)

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

pH -7.4,Made upto 1 L using deionised water.

Filtered ,autoclaved and stored at 4°C.

3.Acid Citrate Dextrose

Trisodium citrate	2.20g
Citric acid	0.80g
Dextrose	2.50g

Made upto 1L using deionized water and filtered

Stored at 4-8°C

4.Tyrode's Buffer

Dextrose	1g
MgCl2	0.199g
KCl	0.402g
NaCl	8.12g
Tris base	1.756g

pH was adjusted to 7.4, Made upto 1L using deionized water

Filtered and stored at 4-8°C

5. Reagents for Lowry's Estimation of proteins

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

6. Reagents for SDS PAGE

Resolving gel for 15mL:

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

Preparation of Stacking gel for 10mL:

Deionized Water	6.8mL
30% Acrylamide mix	1.7mL
Tris HCl (pH 6.8)	1.25mL
10% SDS	0.1mL
10% APS	0.1mL

TEMED	0.004mL

Gel Loading Buffer (1X) – For 10mL:

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

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

8. Reagents for Western Blotting:

Transfer buffer for 100ml:

Tris	0.58gm
Glycine	0.29gm
SDS	0.1gm
Methanol	20ml
Distilled Water	80ml

Amido Black for 15ml:

0.1 % Amido Black	0.015gm
45% Methanol	6.75ml
10% Acetic Acid	1.5ml
Distilled Water	6.75ml

Destaining Solution for 15ml:

90% Methanol	13.5ml
2% Acetic Acid	0.3ml
Distilled Water	1.5ml

Chloronaphthol :

Dissolve 0.3 gm Chloronaphthol in 10 ml absolute alcohol and store at -20° C.

9.Culture medium

DMEM/F12

10% human serum

1X antibiotics

Preparation of human serum from fresh frozen plasma

Fresh frozen plasma was thawed at 4°C and clotted using 15 IU/ml thrombin.

The serum was collected by centrifugation at 3500 rpm for 20 min at 4°C.

Dialysed against HBSS for 48 hrs and centrifuged at 13000rpm for 15 min at 4°C.

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

Syringe filtered aseptically using 0.22µm filter and store at -40°C.