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Vascular Progenitor Cell and Extracellular Matrix Protein Interactions in Cancer

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Vascular progenitor cell and extracellular matrix protein interactions in cancer

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Abstract

Vascular progenitor cells (VPCs) facilitate angiogenesis and vascular repair in damaged tissues where inflammatory cytokines coordinate healing through VPC mobilization. VPCs also facilitate tumor angiogenesis and induce angiogenic switching in metastatic sites.

VPC binding to extracellular matrix proteins enriched in injured healing tissues and metastatic sites and the participation of integrins in this binding was investigated. VPC binding profiles on fibronectin and laminin of "healthy" controls was different from those of "cancer" patients and patients with acute tissue damage. Specific integrin-mediated binding of cells in VPC clusters were matrix protein-dependent. Patients with oral cancer and lymphoma had elevated plasma vascular endothelial growth factor (VEGF) levels compared to healthy controls. VEGF facilitated VPC clustering on fibronectin and incubating cells from healthy controls with VEGF switched binding profiles from "healthy" to "cancer". This study provided new insights regarding VPC-matrix interactions that help distinguish VPC involvement in vascular repair from vascular-mediated metastasis.

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CAC	circulating angiogenic cell
CFU-EC	endothelial cell colony-forming units
DAPI	4',6-diamidino-2-phenylindole
EC	endothelial cell
ECFC	endothelial colony- forming cell
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
IgG	immunoglobulin G
MMP	matrix metalloproteinase
MNC	mononuclear cell
PE	phycoerythrin
PBS	phosphate-buffered saline
SEM	standard error of the mean
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VPC	vascular progenitor cell

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Chapter 1: Introduction

1.1 Overview

Vascular repair is a critical process for tissue healing. Cells involved in angiogenesis and repair are attracted to sites of wounding or ischemia by a multitude of growth factors and cytokines. Once they come into contact with the extracellular matrix of the tissue to which they are homed, integrins allow them to directly interact with matrix proteins which promote endothelial cell migration and vessel formation. Interestingly, interactions in tumor angiogenesis occur in an analogous way. Often patients with solid tumors undergo tumor-removal surgery. However, as tissue repair occurs at the site of the surgical wound, angiogenic cells also come into close proximity to remaining cancer cells, which frequently results in further metastasis and cancer relapse.

Therefore, understanding the binding that occurs between cells involved in vascular repair and matrix proteins underlies the basis of allowing us to find ways to promote tissue healing while preventing relapse. This thesis provides a basis for understanding the different factors that mediate the binding of vascular progenitor cells (VPCs) to extracellular matrix proteins for which tumor and healthy or remodelling tissues are enriched and identifies important targets for therapies aimed at preventing tumor angiogenesis.

1.2 Vascular progenitor cells (VPCs)

VPCs are endothelial precursor cells that are recruited to sites of tissue injury and facilitate vascular repair and recovery of organ function by both inducing and modulating angiogenesis. VPCs support the re-endothelialization of injured blood vessels through the replacement of dysfunctional endothelial cells (Rabelink *et al.*,2004). They include bone marrow-derived progeny from "angioblast"-like precursors, first described in the developing embryo which form the walls of "blood islands" that later grow into the vasculature (Sirker *et al.,* 2009; Asahara *et al.,* 1999), vessel wall progenitor cells, and monocyte-derived vascular cells (Rehman *et al.,* 2003; Ubrich *et al.,* 2003). Vascular progenitors can be collected from peripheral blood, bone marrow and umbilical cord blood.

VPC levels have been shown to be an important and useful marker for vascular function and cumulative cardiovascular risk (Hill *et al.,* 2003), whereby increased VPC levels are highly correlated with increased endothelial function and inversely correlated with cardiovascular risk (as measured using the Framingham risk score). Hill *et al.,* (2003) suggest that cardiovascular risk factors such as elevated cholesterol, hypertension and diabetes, bring about endothelial injury that in turn leads to impaired endothelial function. Consequently, in the absence of sufficient VPCs, repeated endothelial injury leads to progression of cardiovascular disease (Hill *et al.,* 2003).

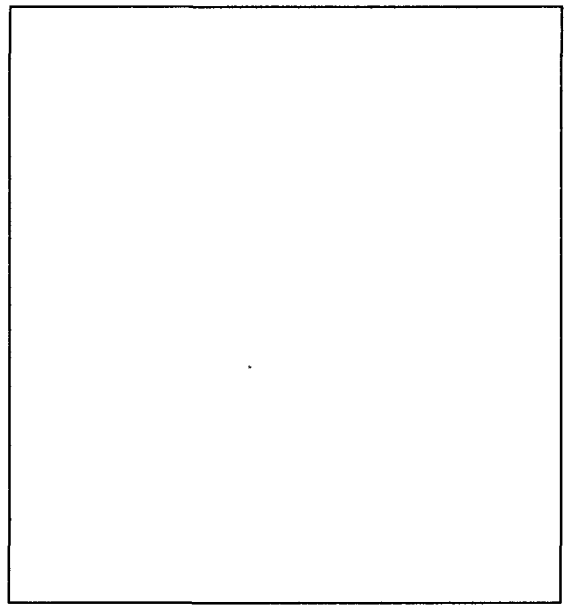


Figure 1 Typical VPC cluster at day 7 plated on fibronectin, cluster shown at 10X objective, demonstrating more than 30 cells with at least 3 attached spindle shaped cells (as described by Hill *et al.,* 2003).

The therapeutic role of VPCs and their ability to incorporate into sites of active neo-vascularization has been shown in animal models of ischemic tissue damage (Asahara *et al.,* 1999; Zhang *et al.,* 2002) and in clinical studies of ischemic injury such as myocardial infarction and stroke where favorable repair responses were associated with increased levels of circulating VPCs (Shintani *et al.,* 2001; Ghani *et al.,* 2005). The administration of VPCs or bone marrow-derived cell populations enriched for VPCs into patients with cardiovascular disease, however, has had limited success with regards to new vessel formation (Yoder *et al.,* 2007). It appears that paracrine effects of infused VPCs are responsible for the modest clinical effects observed in patients, without evidence of long-term VPC engraftment in newly formed vessels in these patients (Hristov and Weber, 2006; Badorff and Dimmeler, 2006; Dimmeler *et al.,* 2005).

The use of VPC-based cell therapies, however, continues to emerge as a promising therapeutic approach in treating many types of acute tissue injury (Rafii and Lyden, 2003; Dimmeler *et al.,* 2008, Krenning *et al.,* 2009). In 2003, Griese *et al.,* found that the isolation, expansion and autologous transplantation of VPCs may play a crucial role in re-establishing endothelial integrity in injured blood vessels by preventing neo-intimal hyperplasia. Many other studies have reported that cell-based therapy is a promising option for the treatment of cardiac ischemic diseases (see Dimmeler *et al.,* 2008). Particularly, transplantation of bone marrow cells enriched for VPCs has experimentally been shown to increase the functional recovery of the heart after ischemia by forming new blood vessels and by supplying pro-angiogenic and anti-apoptotic factors that promote tissue repair in a paracrine manner (Grunewald *et al.,*

2006). In hematopoietic stem cell transplantation, elevated levels of VPCs in stem cell grafts was associated with increased repair of tissue injury and protection against organ dysfunction and toxicity in recipients of autologous hematopoietic stem cell transplantation (Iqbal *et al.,* 2008). In patients with diabetic vascular complications, cell-based therapies using autologous VPC populations represent a novel treatment option for tissue damage requiring revascularization and vascular repair, whereby VPCs accelerate these processes (Jarajapu and Grant, 2010). Together, these findings further underline the important role that VPCs may play in tissue repair.

1.3 Neo-vascularization

Neo-vascularization involves the recruitment of proliferating endothelial cells including VPCs, to the site of wound healing or tumor growth (Schmeisser *et al.,* 2001). New blood vessels are formed by two major processes; angiogenesis and vasculogenesis. Angiogenesis results from the proliferation and sprouting from existing blood vessels and vasculogenesis is believed to arise via the recruitment of circulating cells, mostly derived from the bone-marrow (Ahn and Brown, 2009) and involves the *in situ* differentiation of the primitive endothelial progenitors into endothelial cells that aggregate to form a primary capillary network (Reyes, *et al.,* 2002). Previously, it was thought that vasculogenesis only occurred during embryonic development and that postnatal neovasularization only occurred through angiogenesis (Asahara and Isner, 2002). However Asahara *et al.,* (1997) isolated endothelial progenitor cells from adult human peripheral blood using magnetic bead

selection of CD34+ hematopoietic stem cells. In vitro, these cells differentiated into endothelial cells. In animal models of ischemia, the endothelial progenitor cells incorporated into active sites of new capillary network formation, suggesting that postnatal vasculogenesis can occur. Since then, endothelial progenitors have been recognized as important components of vascular repair by bone marrow-derived cells and much research has focused on VPCs as markers of vascular health (Hill *et al.,* 2003) and as a possible cell-based therapy for tissue repair.

1.4 Other important cell types involved in angiogenesis

1.4.1 Endothelial colony-forming cells (ECFCs)

ECFCs or late-outgrowth cells have gained much attention due to their clinical relevance and therapeutic potential. They can be isolated from human peripheral blood and originate from somatic vascular precursors within the vasculature (Ingram *et al.,* 2004; Ingram *et al.,* 2005). ECFCs are organized in a hierarchy of progenitor states that possess different levels of proliferative potential and can be identified in clonal plating conditions (Ingram *et al.,* 2004). These ex vivo expanded cells have robust proliferation *in vitro* and significant vessel-forming capacity *in vivo* thereby making them attractive candidates for vascular regenerative therapy (Yoder *et al.,* 2007). However, cells giving rise to ECFCs are found at very low frequency of less than one per million nucleated cells in steady-state peripheral blood (Ingram *et al.,* 2004; Reinisch *et al.,* 2009). These cells and their progeny express endothelial but not hematopoietic cell surface markers, and appear to be clonally distinct from

hematopoietic stem cells (Yoder *et al.,* 2007). Interestingly, the co-injection of VPCs and ECFCs has been shown to synergistically improve neo-vascularization in mice following hind limb ischemic injury (Yoon *et al.,* 2005).

1.4.2 Circulating angiogenic cells (CACs)

CACs were previously considered as putative VPCs. CACs arise from peripheral blood cultured on fibronectin-coated plates under "endothelial" differentiation conditions for 4 days using specific tissue culture medium and growth factors (Critser and Yoder, 2010) after which non-adherent cells are removed and adherent cells, or CACs remain. CACs have been reported to have important angiogenic potential (Kalka *et al.*, 2000) and were found to express endothelial cell surface antigens such as CD144 and von Willebrand Factor, however they have also been shown to be enriched for monocyte/macrophages (Rehman *et al.,*2003). Furthermore, evidence suggests that this method of CAC isolation includes platelets that co-fractionate with the mononuclear cells (MNCs) (Prokopi *et al.,* 2009) and become attached to any adherent MNCs in the culture. As a result, platelet membrane proteins are transferred to the adherent cells, many of which express endothelial-like antigens and confer angiogenic properties to these adherent cells (Critser and Yoder, 2010). Although some studies have reported that CACs can increase blood vessel perfusion and capillary density in athymic nude mouse with hind limb ischemia (Kalka *et al.,* 2000), they are likely cells of hematopoietic origin that are unable to form de-novo blood vessels *in vivo*, but may still contribute to

vascular repair and the regulation of normal and abnormal blood vessel formation (Asosingh *et al.,* 2008).

1.4.3 Vascular progenitor cells (VPCs)

VPCs have largely been identified and enumerated using flow cytometry for cells expressing CD34, CD133 and the vascular endothelial growth factor receptor 2 (VEGFR-2) (Asahara *et al.,* 1997; Peichev *et al.,* 2000). However these markers are also expressed by other hematopoietic stem/progenitor cell populations (Verfaillie, 2002; Shizuru *et al.,* 2005; Bryder *et al.,* 2006) and as a result, the presence of hematopoietic contamination of VPCs is expected (Yoder *et al.,* 2007). VPCs have also been quantified by using cell culture-based progenitor assays that identify "endothelial cell colony-forming units" (CFU-ECs). CFU-ECs are grown by replating non-adherent cells following an adherence depletion-step in culture to remove mature endothelial cells, in comparison to ECFCs which emerge from the culture of adherent cells. Initial spindle-shaped cells attach to matrix coated plates and attract other cells to cluster, including monocytes and lymphocytes under specific cytokine stimulation. This may reflect the role of VPCs *in vivo* where paracrine effects are thought to play a role in vascular repair.

There is much debate regarding the cells that give rise to VPCs, however it appears that CD133(+) cells are enriched for spindle-shaped attaching cells that give rise to the VPC clustering phenomenon (unpublished results). Since these "colonies" or clusters are composed of a heterogeneous cell population and arise from

hematopoietic origin, current evidence suggests that they represent hematopoietic cells that stimulate and regulate angiogenesis which do not become long-term integrated endothelial cells *in vivo* (Yoder *et al.,* 2007). VPC-like clusters, or CFU-ECs from peripheral blood have emerged as a predictive biomarker of vascular disease (Hill *et al.,* 2003), whereas ECFCs are a source of expanded cells for angiogenic therapy (Yoder *et al.,* 2007). However the variability in defining the cells that give rise to CFU-ECs has contributed to the controversy in understanding the role of VPCs in vascular repair and angiogenesis (Yoder *et al.,* 2007).

1.5 VPCs and cancer

Recent studies have reported higher levels of VPCs in the peripheral blood and within tumors of patients with invasive cancer (Naik *et al.,* 2008) or recurrent malignancies (Igreja *et al.,* 2007), suggesting possible involvement of VPCs in tumor progression.

VPCs may contribute to the development of pre-metastatic niches which allow dispersed malignant primary tumor cells to colonize secondary organs. Dormant micrometastases can develop and may undergo (Townsown and Chambers, 2006) an "angiogenic switch" associated with the progression to a macrometastases and widespread cancer (Gao *et al.,* 2008). Using a mouse lung cancer model, Gao *et al.,* (2008) showed that VPCs play a direct role in angiogenesis mediated progression of metastatic lesions, however they do not modulate metastatic initiation which appears to be dependent on other VEGFR-1 expressing cells of hematopoietic origin. By

impairing VPC mobilization, a significant decrease in blood vessel density in metastatic lesions was observed, suggesting that targeting VPCs for the inhibition of the angiogenic switch may be feasible (Gao *et al.*, 2008). Furthermore, recent preclinical studies show that VPCs may determine the sensitivity of tumors to chemotherapeutic (Shaked *et al.*, 2008) or vascular disrupting agents (Shaked *et al.*, 2006) and that vascular progenitor cells contribute to neoangiogenesis at sites of tumorigenesis (Reyes *et al.*, 2002).

1.6 Tumor angiogenesis

Tumor angiogenesis is a dysregulated process that involves VPCs and the activation of endothelial cells on pre-existing venules leading to new vessel branching. The mobilization of VPCs from the bone marrow to the tumor occurs in response to tumor hypoxia and the action of tumor-derived pro-angiogenic factors (Lin *et al.*, 2008).

Tumor angiogenesis influences a variety of angiogenic molecules, including VEGF, which can be expressed by virtually every cell type (Leung *et al.*, 1989). VEGF promotes angiogenesis by interacting with one of its two tyrosine kinase receptors VEGFR-1 and VEGFR-2. Tumor-derived VEGF can co-mobilize VPCs via VEGFR-2 and hematopoietic cells via VEGFR-1 to the tumor tissue where they contribute to neo-vessel formation (Rafii *et al.*, 2002). VPCs and hematopoietic cells collaborate to facilitate the differentiation and integration of cells into the rapidly growing tumor

vasculature and the inhibition of either VPC or hematopoietic cell mobilization slows the growth of tumors (Rafii *et al.,* 2002).

Over-expression of VEGF by tumor cells promotes rapid angiogenesis and the formation of atypical vessels, which are often dilated (Folkman *et al.,* 1989; Carmeliet and Jain, 2000), leaky (Dvorak *et al.,* 1988), and consist of a disorganized array of peri-endothelial pericytes and smooth muscle cells (Morikawa *et al.,* 2002) when compared to normal vasculature. The resulting vascular fragility can lead to micro-hemorrhages and vessel collapse (Bergers *et al.,* 1999), and sluggish circulation through tumor capillaries contributing to tumor hypoxia and a tumor microenvironment with low pH, which tends to mobilize further VPCs for angiogenesis (Rafii *et al.,* 2002).

The recruitment of bone-marrow-derived precursors in tumor angiogenesis appears to be regulated by additional factors such as the constitution of the tumor extracellular matrix, and the chemokine and cytokine signal repertoire of the tumor (Rafii *et al.,* 2002). Matrix metalloproteinase (MMP)-9, a factor produced by tumor-infiltrating neutrophils, mast cells and macrophages, can promote tumor angiogenesis (Coussens *et al.,* 2000) and transplantation of MMP-9-expressing bone-marrow cells enhanced tumor growth in *Mmp9 -/-* mice with impaired angiogenesis. Tumor-cell production of MMPs promotes the release of extracellular matrix-bound cytokines and growth factors, including VEGF, which can mobilize VPCs (Vu and Werb, 2000). Similarly tumor cells can release signals that will

activate MMPs, such as VEGF-mediated MMP-9 activation, which promotes the release of the stem cell cytokine-soluble KIT ligand and facilitates hematopoietic-cell proliferation and motility (Heissig *et al.,* 2002).

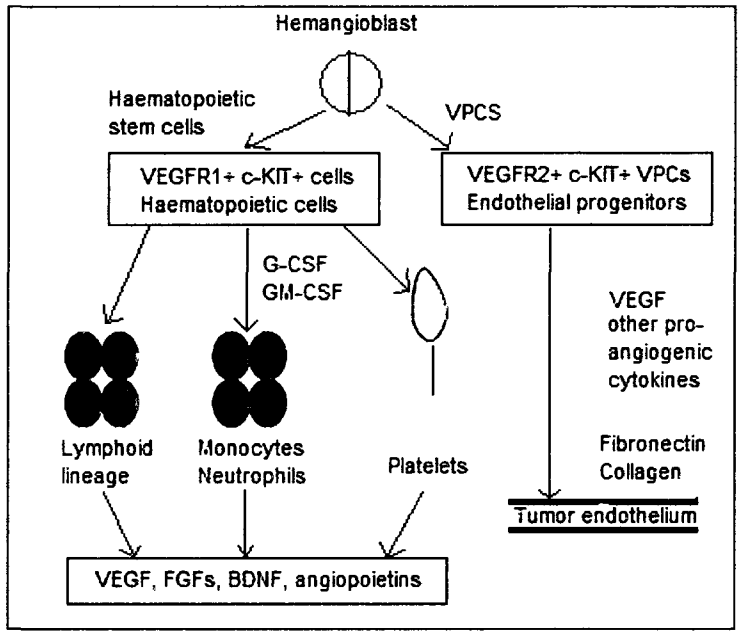


Figure 2 Haematopoietic stem cells and circulating endothelial precursors (which include VPCs) are believed to originate from a common precursor cell, known as the haemangioblast. Much debate remains regarding whether the haemangioblast exits in the adult bone marrow. Lineage-specific differentiation of haematopoietic stem cells depends on the availability of cytokines that support selective differentiation of these cells into myeloid, megakaryocytic and lymphoid cells. Angiogenic factors, along with the extracellular matrix proteins fibronectin and collagen, promote differentiation of CEPs, which express the VEGFR-2, into functional tumour endothelium (modified from Rafii *et al.,* 2002).

1.7 Integrins

Angiogenesis is a highly complex process which involves the migration, proliferation and apoptosis of several cell types. Many of these cellular functions are modulated by an intricate series of intracellular signaling events (Ramjaun and Hodivala-Dilke, 2009).

Interactions between cell surface receptors and the extracellular matrix serve to physically anchor vascular cells to the substratum and to each other, but also activate intracellular signaling pathways that regulate various aspects of vascular cell function (Hodivala-Dilke *et al.,* 2003; Liebner *et al.,* 2006). One of the most important functions of cell adhesion signaling is to maintain cell viability (Ramjaun and Hodivala-Dilke, 2009), a process facilitated by cell adhesion-mediated stimulation of pro-survival pathways as well as the inhibition of apoptotic pathways (Chavakis and Dimmeler, 2002).

The integrin receptors, heterodimeric molecules made up of a α and a β subunit, are expressed on the cell surface and interact with extracellular matrix proteins. During angiogenesis, integrins are responsible for initiating many of the signaling pathways that result when cells bind to and interact with the extracellular matrix. For example, focal adhesion kinases are activated through direct binding of the cytoplasmic tail of the β1 integrin subunit (Schaller *et al.,*1995) and regulate signaling molecules as well as cytoskeletal remodelling (Ramjaun and Hodivala-Dilke, 2009). Similarly, the activation of extracellular-signal regulated kinases following integrin ligation has

been shown to regulate endothelial cell survival, migration and proliferation as well as controlling cell cycle progression (Wary *et al.,* 1996) possibly via the direct regulation of cell cycle proteins (Roovers and Assoian, 2000; Roovers *et al.,* 1999). The fibronectin receptor α5β1 is a key regulator in the activation of cell adhesion pathways in endothelial cells (ECs). Its expression is up-regulated during angiogenesis (Kim *et al.,* 2002, 2000) and activates protective pathways for ECs as well as enhances their migration and proliferation when bound to fibronectin. The α2β1 integrin, which binds to collagens and laminins, protects ECs from apoptosis and enhances their proliferation by supporting the activation of growth factor induced extracellular-signal regulated kinase signaling (Perruzzi *et al.,* 2003; Senger *et al.,* 2002).

In tumors, integrins bind to the basement membrane and the extracellular matrix where they are involved in mediating tumor cell migration and invasion. Interestingly VEGF can interact directly with certain integrins such as α5β1, during tumor angiogenesis through a direct physical association between the VEGFR-2 and α5β1 found on endothelial precursors which may amplify the effects of these cells when they bind to fibronectin at the tumor site (Wijelath *et al.* 2002).

A common aspect of integrin signaling involves a close association with cytokine-induced pathways (Ramjaun and Hodivala-Dilke, 2009). Consequently integrins are emerging as significant mediators of tumor metastatic behavior (Guo and Giancotti, 2004).

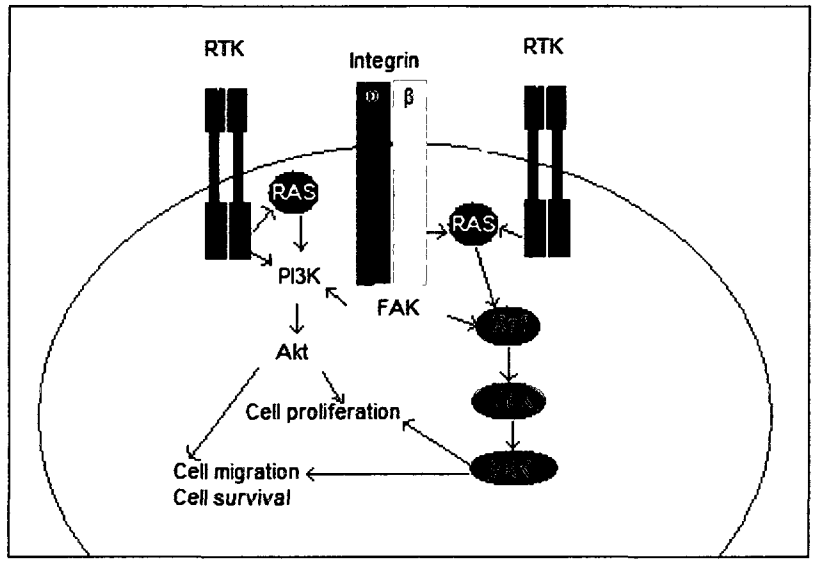


Figure 3 Integrin signaling pathways in angiogenesis. Cells involved in angiogenesis express integrins that activate a number of common signaling pathways following contact with the extracellular matrix. Many of these signaling molecules are common to cytokine signaling pathways, particularly downstream of receptor tyrosine kinases (RTK). As a result, signals from integrins and cytokine-receptors integrate at various levels of the signaling pathway to control cellular response (modified from Ramjaun and Hodivala-Dilke, 2009).

1.8 Extracellular-matrix

Complex interactions between the vasculature and normal and malignant cells have been described, including the role of growth factors and the extracellular matrix. The components that make up the extracellular matrix are crucial for the differentiation of many cell types and these exchanges exist as a homeostatic system that may become distorted during cancer progression (Brooks *et al.,* 2010).

During cancer progression, the extracellular matrix of the tumor bearing tissue is extensively remodeled due to the combined effects of degradation of pre-existing extracellular matrix proteins and by the synthesis of new extracellular matrix components which, generally, are not present in the extracellular matrix of normal tissues (Kaspar *et al.,* 2005). The extracellular matrix proteins contribute to creating a tumor microenvironment conducive to metastatic cancer cell behavior. Particularly, the production of fibronectin by tissues has been implicated in promoting the transition from dormant cancer cells to metastatic growth (Barkan *et al.,* 2008). For example in a metastatic mammary cell line, a transition from a quiescent state to proliferation was brought about upon the production of fibronectin and signaling through β1 integrins (Barkan *et al.,* 2008). In a dormant metastatic cell line, addition of fibronectin was able to induce transient proliferation (Barkan *et al.,* 2008). Interestingly, fibronectin has also been shown to contribute to the pre-metastatic niche through its recruitment and clustering of VPCs and hematopoietic cells (Barkan *et al.,* 2010; Kaplan *et al.,* 2006). Increased fibronectin expression in cells residing at the pre-metastatic site, have been reported to be vital for the adhesion of

VPCs (Barkan *et al.,* 2010). Similarly type I collagen expressed by fibroblasts has been identified as a prognostic marker for poor health outcomes, metastases, and tumor recurrence (Ramaswamy *et al.,* 2003; Qiu *et al.,* 2004; Feng *et al.,* 2007). Collagen I deposition induces extracellular matrix stiffening and promotes malignant transformation (Paszek *et al.,* 2005). High levels of pro-collagen type I, a marker for collagen I synthesis have been reported in the serum of patients with recurrent breast cancer (Jensen *et al.,* 2002), and increased breast density associated with increased stromal collagen I has been shown to promote tumor initiation, progression, and increased risk of metastases, and local recurrence after mastectomy and radiotherapy (Park *et al.,* 2009). These clinical phenomena suggest that collagen-I enrichment at the metastatic site may be an important determinant of cytoskeletal reorganization in dormant tumor cells leading to the transition from cancer cell dormancy to metastatic growth (Barkan *et al.,* 2010).

Interestingly other matrices have been shown to inhibit angiogenesis such as type IV collagen (Maeshima *et al.,* 2000). The basement membrane of blood vessels is an important structural component which undergoes many alterations during angiogenesis. It also influences cell behavior such as differentiation and proliferation. The major matrix proteins found in the basement membrane include type IV collagen and laminin and some fibronectin. Type IV collagen plays an important role in promoting cell adhesion, migration, differentiation and growth, however, it can also inhibit the proliferation of capillary endothelial cells and blood vessel formation in *in vivo* and *in vitro* models of angiogenesis and tumor growth, as well as being able to induce endothelial cell-specific apoptosis (Maeshima *et al.,*

2000). Laminin, much like collagen IV, promotes cell adhesion, migration and differentiation, but importantly also cell survival. It is an integral part of the structural scaffolding in almost every tissue found in the body and is critical for their maintenance and survival, and defects can cause many conditions such as muscular dystrophy and defective kidney filtration etc. (Yurchenco and Patton, 2009).

1.9 Statement of the problem

Interactions between VPCs and components of the extracellular matrix may be critical in regulating new blood vessel formation in cancer and in the healing process following tissue injury although insight regarding these progenitor-matrix interactions is currently lacking. Characterizing how extracellular matrix proteins regulate VPC binding may provide a foundation for better understanding the angiogenic function of VPCs.

1.10 Objectives

In this study we addressed the following specific objectives: (i) how different extracellular matrix proteins influence attaching cells in VPC cluster formation; (ii) compare the matrix binding profile of attaching cells in VPC clusters from cells isolated from patients with cancer, tissue injury, and from umbilical cord blood; iii) to identify specific integrins expressed on the surface of attaching cells in VPC clusters

that mediate binding to matrix proteins; and (iv) investigate VEGF as a signaling molecule in VPC cluster formation on different matrix proteins.

1.11 Hypothesis

We hypothesize that VPCs bind to different extracellular matrix proteins in a distinct manner depending on the matrix protein and whether the cells are isolated from cord blood, peripheral blood from healthy individuals, patients with cancer or with acute tissue injury and that this binding occurs through specific cell surface integrins and signaling molecules including VEGF.

Chapter 2: Materials and Methods

2.1 Sample collection

All samples used in this study were collected after having received informed consent and in accordance with protocols approved by the Ottawa Hospital Research Ethics Board.

Mononuclear cells were isolated from several blood sources including: umbilical cord blood, peripheral blood from healthy adult donors, pre-operative peripheral blood from adult patients with oral cancer, peripheral blood from adult patients with non-Hodgkin's lymphoma, and peripheral blood from adult patients with acute kidney injury.

Cord blood samples were collected from the birthing unit at the Ottawa Hospital following informed consent from mothers undergoing uncomplicated vaginal delivery or elective Caesarean section. Samples were obtained prior to delivery of the placenta using sterile collection kits (Macopharma INC., France).

Peripheral blood from healthy adult donors, patients with oral cancer, lymphoma or acute kidney injury were collected in 7 mL vacutanor tubes containing ethylenediaminetetraacetic acid (EDTA) to prevent coagulation.

2.2 Mononuclear cell preparation

MNCs were isolated using Ficoll density centrifugation. Blood samples were diluted 1:1 with sterile 1X phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA) with 2% fetal calf serum (FCS) (Invitrogen Canada Inc., Burlington, ON, Canada) and overlaid onto Ficoll-Paque Premium (GE Healthcare, Uppsala, Sweden) and centrifuged at 1500 rpm at 20°C for 30 minutes. MNCs were subsequently collected and washed three times with 1XPBS with 2% FCS (see figure 4 for schematic of subsequent performed experiments).

2.3 VPC assay and enumeration

VPC assays were performed based on the methods described by Hill *et al.*, (2003). VPCs were cultured using the EndoCult Liquid Medium Kit (StemCell Technologies, Vancoucer, BC, Canada) as stated in the manufacturer's protocol. MNCs were resuspended in complete EndoCult medium and seeded at $5x10^6$ cells/well on 6-well matrix pre-coated tissue culture plates (coated with either fibronectin, collagen I, collagen IV or laminin) (BD Biosciences, Mississauga, ON, Canada). Plates were then placed in a 37°C humidified incubator with 5% CO_2 in air. After 48 hours, wells were gently rinsed with media and non-adherent cells were collected and re-plated at 10^6 cells/well on 24-well matrix-coated tissue culture plates (coated with either fibronectin, collagen I, collagen IV or laminin) (BD Biosciences, Mississauga, ON, Canada), and incubated for an additional 3-5 days. At days 5-7, VPC cluster (see fig. 1 for example of cluster) formation was quantified

by enumerating the number of clusters formed on each matrix using an inverted microscope.

2.4 Immunohistochemistry of surface integrins

VPC clusters were analyzed by immunohistochemistry by removing the media in the wells of the matrix-coated tissue culture plates and the remaining attached VPC clusters were gently washed three times with 1XPBS (Sigma-Aldrich, St. Louis, MO, USA) and fixed using 4% paraformaldehyde (diluted in 1XPBS both from Sigma-Aldrich, St. Louis, MO, USA) for 10 minutes at 4°C and then washed three times with 1XPBS. Cells were then incubated with a blocking solution of 10% FCS (Invitrogen Canada Inc., Burlington, ON, Canada) (diluted in 1XPBS) for 20 minutes at room temperature. Cells were then incubated for 1 hour at room temperature with primary antibody diluted in incubation buffer (2%FCS diluted in 1X PBS). The following primary antibodies were used to stain the cells: mouse anti-human α5 conjugated to Phycoerythrin (PE) antibody and β1 conjugated to Fluorescein isothiocyanate (FITC) antibody (diluted 1:100), α2β1 conjugated to FITC antibody (1:100), α3β1 antibody (1:100) (all antibodies from Life Span Biosciences Inc, Seattle, WA, USA). Controls were performed in parallel by incubating with no primary antibody (to confirm that secondary antibodies did not auto-fluoresce) or with an isotype control of mouse immunoglobulin G (IgG) antibody (1:100) (IgG 1k, BD Biosciences, Mississauga, ON, Canada). Control cells were then washed three times with 1XPBS and incubated for 1 hour at room temperature with secondary antibody diluted in incubation buffer. Secondary antibodies against mouse were

conjugated to FITC or Texas Red (1:100) (Invitrogen, Eugene, OR, USA). Cells were washed three times with 1XPBS, and then incubated with 1mL of 4', 6-diamidino-2-phenylindole (DAPI) (Pierce Biotechnology Inc., USA) for 1 minute at room temperature. Cells were then mounted with 1-2 drops of DAKO mounting media (Dako North America Inc., Carpinteria, CA, USA). Cells were visualized using the Zeiss Observer.Z1 inverted microscope and images captured with the AxioCam HRm camera by Zeiss.

2.5 Inhibiting integrin function

MNCs were isolated from 3 samples of peripheral blood from healthy volunteers. Each sample was divided into 4 treatment types and cells received media containing either of the following: neutralizing antibody against α5β1 integrin (clone JBS5, Millipore, Billerica, Massachusetts, USA) (Qin, *et. al.*, (2003); Furumatsu, *et.al.*, (2002); Ria *et. al.*, (2002); Gu and Park (2001); Akimov and Belkin (2001)), neutralizing antibody against α2β1 integrin (clone BHA2.1, Millipore, Billerica, Massachusetts, USA) (Wang and Frazier (1998)), mouse anti human IgG (IgG 1k, BD Biosciences, Mississauga, ON, Canada) (control) or no treatment (control). Antibodies were diluted to a concentration of 10ug/mL as per manufacturer's protocol. Cells were then plated on fibronectin or laminin according to the VPC assay described in section 2.3 and grown in the media containing the antibodies throughout the entire VPC culture period. At days 5-7, VPC cluster formation was quantified by enumerating the number of clusters formed on each matrix using an inverted microscope.

2.6 VEGF ELISA

An enzyme-linked immunosorbent assay (ELISA) for VEGF (R&D Systems, Minneapolis, MN, USA) was performed using the plasma collected from the peripheral blood samples of healthy controls and patients with oral cancer, lymphoma and acute kidney injury in accordance with the manufacturer's protocol. All samples were tested in duplicate. A standard curve of VEGF concentrations was generated using 8 standard VEGF solutions provided by the manufacturer ranging from 0-2000 pg/mL against which our plasma samples of unknown VEGF levels were tested and from which VEGF concentrations were interpolated. To compare and analyze plasma VEGF concentrations, a Student's *t*-test was used.

2.7 Anti-VEGF treatment and VEGF treatment

2.7.1 Anti-VEGF treatment

VPC assays were performed using MNCs from 2 cord blood samples and 2 pre-operative peripheral blood samples from patients with oral cancer and plated on fibronectin or laminin coated 6-well tissue culture plates. Half of each sample was treated with Bevacizumab (Genentech Inc, San Francisco, CA, USA), a diffusible VEGF inhibitor introduced to block the effects of VEGF in VPC-matrix interactions. Cells were incubated for 24 hours with 1ug/mL of Bevacizumab prior to being plated on fibronectin or laminin. Following incubation, Bevacizumab remained in the media throughout the entire VPC culture period. At days 5-7, VPC cluster formation was

quantified by enumerating the number of clusters formed on each matrix using an inverted microscope.

2.7.2 VEGF treatment

VPC assays were performed using cells from 5 healthy adult peripheral blood samples that were plated on fibronectin or laminin coated 6 well tissue culture plates. Each sample was divided into two, and mononuclear cells were incubated with either 0 or 1 ug/mL of human recombinant $VEGF_{165}$ (BD Biosciences, Mississauga, ON, Canada) in sterile 1XPBS (Sigma-Aldrich, St. Louis, MO, USA) for 24 hours prior to being plated on fibronectin or laminin. Following incubation, the cells receiving VEGF were grown in the media containing the 1 ug/mL of VEGF throughout the entire VPC culture period. At days 5-7, VPC cluster formation was quantified by enumerating the number of clusters formed on each matrix using an inverted microscope.

2.8 Flow cytometry to measure integrin expression

MNCs were isolated from 3 samples of peripheral blood from healthy volunteers. Samples were divided into 2 and MNCs were given either no VEGF or 1 ug/mL of human recombinant VEGF(BD Biosciences, Mississauga, ON, Canada) in sterile 1X PBS (Sigma-Aldrich, St. Louis, MO, USA). Flow cytometry was performed on MNCs immediately after isolation (baseline) and after 24 hours on both groups of cells. Aliquots of cells with or without VEGF were analyzed by flow cytometry using

no antibody (control), or 1 uL/100 uL of primary staining mouse anti-human α5 integrin conjugated to PE primary antibody or mouse anti-human α2 integrin conjugated to FITC primary antibody, or both (all antibodies from Life Span Biosciences Inc, Seattle, WA, USA) in 1X PBS. Cells were incubated for 15-30 minutes at room temperature prior to FACS analysis.

Flow cytometry was performed using the Beckman Coulter Cytomics-FC 500 MPL flow cytometer and MxP Cytometer Software (both from Beckman Coulter, Mississauga, ON, Canada). Data were analyzed using Summit 4.3 Software (Beckman Coulter, Mississauga, ON, Canada). All gating was maintained for analysis.

2.9 Statistical analyses

Data are expressed as mean ± standard error of the mean (SEM). Statistical significance was determined using an unpaired, two-tailed Student's *t*-test with a significant value of $p < 0.05$, except for the VEGF treatment data and flow data which was analyzed using a paired, two-tailed Student's *t*-test. GraphPad Prism version 5.00 (Graphpad Software, San Diego, California) for Windows was used for statistical analysis.

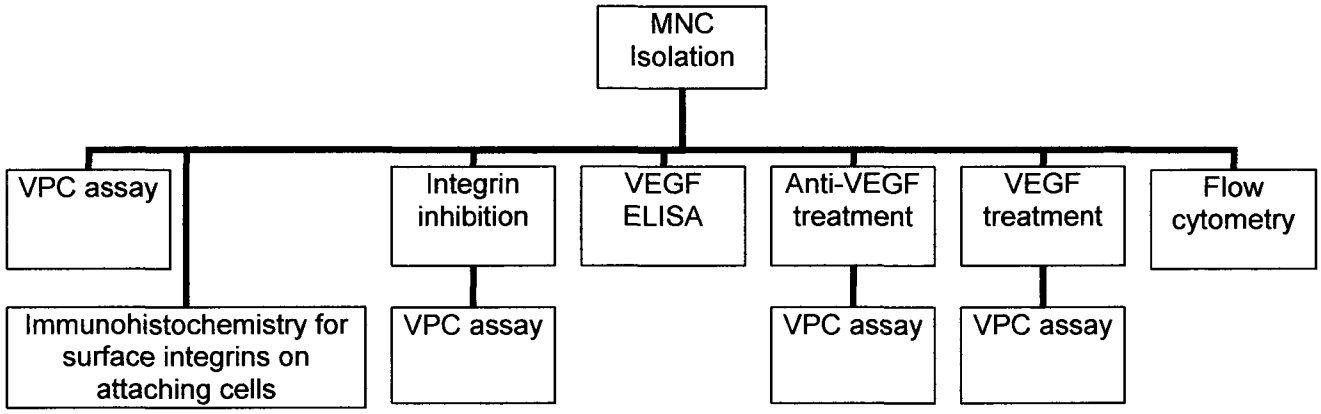


Figure 4 Schematic showing experimental methods

Chapter 3: Results

3.1 Patient and control characteristics

In our study we used a total of 5 cord blood samples, 10 peripheral blood samples from healthy controls, 4 peripheral blood samples from patients with oral cancer, 5 peripheral blood samples from patients with non-Hodgkin lymphoma and 4 peripheral blood samples from patients with acute kidney injury. The mean age of the healthy control was 35 years, whereas the mean age of patients with oral cancer was 66, patients with lymphoma 60 and patients with acute kidney injury 57. There was a relatively equal distribution of samples from females and males. The disease status of cancer patients and patients with acute kidney injury, as well as their prior chemotherapy or radiation treatment is listed in table 1.

Patient and healthy control characteristics

	Healthy Controls	Oral Cancer	Non-Hodgkin Lymphoma	Acute Kidney Injury
n	10	4	5	4
Age, mean yrs (range)	35 (23-45)	66 (52 – 75)	60 (51 – 67)	57 (43-73)
Gender, F / M (%)	5/5 (50/50)	2/2 (50/50)	2/3 (40/60)	2 / 2 (50/50)
Disease status	N/A	*T1N0M0: 3 T2N0M0: 1	Remission: 3 Relapse: 2	**Risk: 1 Injury: 0 Failure: 3
Prior Chemotherapy or radiation, n (%)	0 (0)	1 (25)	5 (100)	0 (0)

Table 1 Patient cohort characteristics, * TNM staging refers to tumor size (T), presence of nodes (N) and metastases (M) as per (Denoix PF, 1946). ** Risk, Injury and Failure refer to specific categories of renal injury as defined by the RIFLE criteria (Bellomo *et al.*, 2004)

3.2 VPC cluster numbers and the role of different matrix proteins

Fibronectin and collagen I are matrix proteins highly enriched in tumor microenvironments and laminin and collagen IV are matrix proteins enriched in healthy or remodeling tissues. We compared the mean number (±SEM) of VPC clusters that formed on each matrix type using the VPC assay to better understand how angiogenic precursors may contribute to tumor angiogenesis coupled with vascular repair in healing or remodeling tissues.

We first enumerated VPCs derived from MNCs isolated from healthy peripheral blood samples. A significantly greater number of VPC clusters formed when cells were plated on laminin (31.6±6.5 clusters/mL of blood) than when plated on any of the other three matrices (fibronectin 8.5±0.7 clusters/mL of blood $p=0.02$, collagen I 6.9±1.3 clusters/mL of blood $p=0.03$, collagen IV 6.1± 2.0 clusters/mL of blood $p=0.04$) (see fig. 5a).

We enumerated VPCs derived from MNCs isolated from cord blood. A statistically significantly greater number of VPC clusters were observed when cells were plated on fibronectin (25.8±6.7 clusters/mL of blood) and laminin (36.1±2.5 clusters/mL of blood) than when plated on collagen I (4.77±1.4 clusters/mL of blood) (fibronectin vs. collagen I $p=0.03$, laminin vs. collagen I $p=0.004$) or collagen IV (7.1±2.4 clusters/mL of blood) (fibronectin vs. collagen IV $p=0.04$, laminin vs. collagen IV $p=0.009$). VPC cluster numbers on fibronectin and laminin were not different (see fig. 5b).

VPCs derived from MNCs isolated from peripheral blood samples from patients admitted to the intensive care unit with acute kidney injury were then enumerated. Greater numbers of clusters were observed when cells were plated on fibronectin (14.1±4.8 clusters/mL of blood) and laminin (11.9±5.5 clusters/mL of blood) compared with collagen I (6.3±2.5 clusters/mL of blood) ($p=0.04$ for fibronectin vs. collagen I, $p=0.03$ for laminin vs. collagen I) or collagen IV (1.9±0.8 clusters/mL of blood) ($p=0.04$ for fibronectin vs. collagen IV, $p=0.03$ for laminin vs. collagen IV) (see fig. 5c).

Finally, we enumerated VPCs derived from MNCs isolated from pre-operative peripheral blood samples from patients with oral cancer. Greater numbers of VPC clusters were observed when cells were plated on fibronectin (42.1±8.1 clusters/mL of blood) compared with the other three matrices (laminin 7.5±8.2 clusters/mL of blood $p=0.01$, collagen I 3.7±1.3 clusters/mL of blood $p=0.03$, collagen IV 7.8± 4.0 clusters/mL of blood $p=0.02$). Similarly, VPCs derived from MNCs isolated from peripheral blood of patients with lymphoma produced statistically significantly greater number of clusters when plated on fibronectin (86.5±13.8 clusters/mL of blood) than when plated on the other three matrices (laminin 27.9±10.1 clusters/mL of blood $p=0.003$, collagen I 39.6±7.5 clusters/mL of blood $p=0.01$, collagen IV 11.9±1.9 clusters/mL of blood $p=0.004$). A significantly greater number of VPC clusters were also observed on collagen I in these patients (39.6±7.5 clusters/mL of blood) when compared to VPC cluster formed on collagen IV (11.9±1.9 clusters/mL of blood, $p=0.02$). The overall number of VPC clusters that formed from MNCs

isolated from patients with lymphoma was higher than from oral cancer samples on all matrices (see fig. 5d).

Together, this analysis supports our hypothesis that attaching cells in VPC clusters have extracellular matrix protein binding profiles that allows one to distinguish the normal or “repair” profile from a cancerous or "malignant" one.

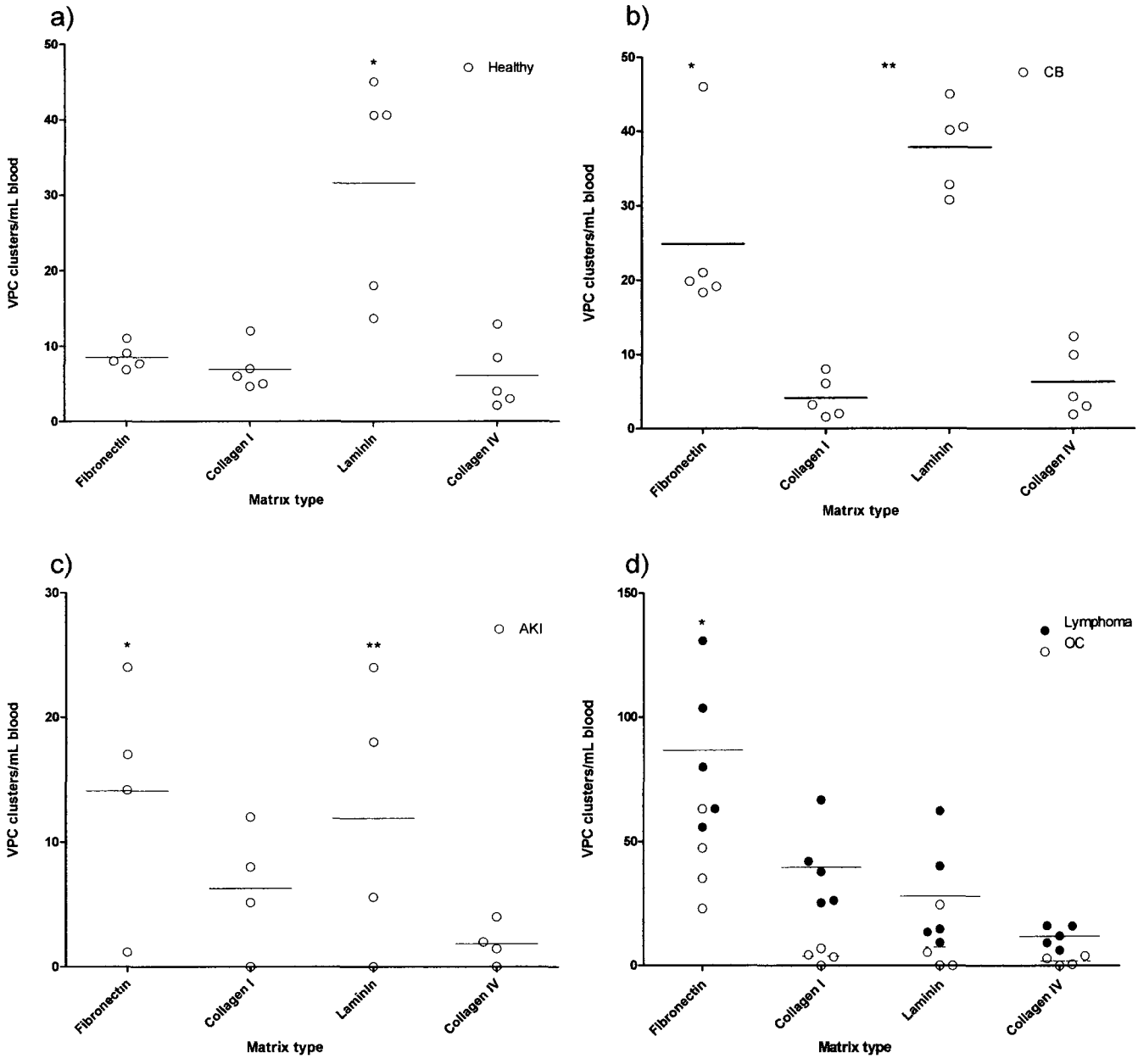


Figure 5 (a) Number of VPC clusters/mL of blood for 5 peripheral blood samples from healthy adult volunteers, * denotes a significantly greater number of VPC cluster on laminin than on fibronectin (p=0.02), collagen I (p=0.03) and collagen IV (p=0.04), (b) number of VPC clusters/mL of blood for 5 cord blood (CB) samples, * denotes a significantly greater number of VPC clusters on fibronectin than on collagen I (p=0.03) and collagen IV (p=0.04) and ** on laminin than on collagen I (p=0.004) and collagen IV (0.009), (c) number of number of VPC clusters/mL of blood for 4 peripheral blood samples from acute kidney injury (AKI) patients, * denotes a significantly greater number of VPC clusters on fibronectin than on

collagen I (p=0.04) and collagen IV (p=0.04) and ** on laminin than on collagen I (p=0.03) and collagen IV (0.03), (d) comparison between number of VPC clusters/mL of peripheral blood between lymphoma and oral cancer (OC) samples, * denotes a significantly greater number of VPC clusters on fibronectin from OC and lymphoma samples respectively than on laminin (p=0.01, p=0.003) collagen I (p=0.03, p=0.01) and collagen IV(p=0.02, p=0.004), ** denotes a significantly greater number of VPC clusters from peripheral blood lymphoma samples on collagen I than on collagen IV (p=0.02).

Based on the results we obtained in these VPC assay experiments, it appears that in most cases, VPCs form greater cluster numbers on fibronectin and laminin than on the collagen matrix proteins and the different binding profile between VPCs from healthy and cancer samples can be distinguished using fibronectin and laminin. Consequently, subsequent studies focused on the binding of VPCs with fibronectin and with laminin.

3.3 Attaching cells in VPC clusters bind fibronectin and laminin via specific integrins

To determine which factors may be involved in the different binding profiles we observed on different matrix proteins, we investigated the role of integrin receptors on attaching cells in VPC clusters.

Integrins α5β1 (Pankov and Yamada, 2002; Kim *et al.,* 2000; Wijelath *et al.*, 2004), α2β1(Languino *et al.*, 1989; Mizejewski, 1999) and α3β1 (Mizejewski, 1999; Saito *et al.,* 2010) were selected for further study. Integrin α5β1 binds to fibronectin and not laminin, and is involved in angiogenesis and tumor angiogenesis, whereas the α2β1 integrin binds to laminin and not fibronectin and has also been reported to be involved in angiogenesis. The α3β1 integrin binds to both laminin and fibronectin.

Immunohistochemistry was performed to assess the expression of the α5β1 integrin. Cells in VPC clusters derived from MNCs isolated from peripheral blood samples

from healthy volunteers plated on fibronectin were positive for α5β1 while cells were negative for α2β1 integrin expression.

In contrast, the expression of the α2β1 integrin was observed on the cell surface of attaching cells in VPC clusters plated on laminin derived from the same peripheral blood samples while clusters were negative for α5 integrin expression.

The expression of α3β1 was observed on the cell surface of attaching cells in VPC clusters plated on both laminin and fibronectin. This observation is in keeping with the known binding affinity of α3β1 integrin for these two matrix proteins.

Isotype controls and negative controls lacking any primary antibody confirmed that there was no auto-fluorescence during integrin staining and that observed fluorescence was the result of antibody-integrin interactions (see fig.6).

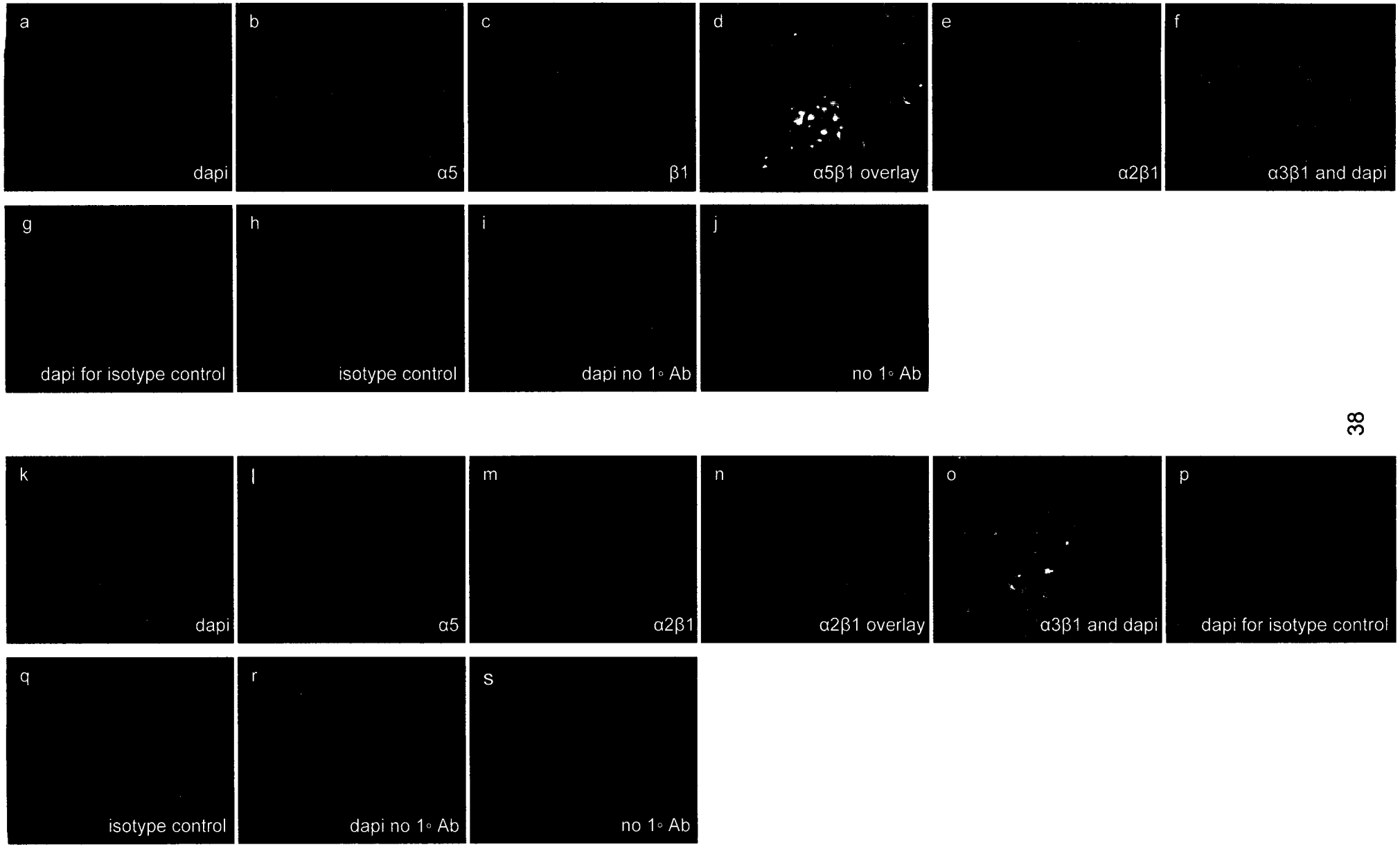

a
dapi
b
α5
c
β1
d
α5β1 overlay
e
α2β1
f
α3β1 and dapi
g
dapi for isotype control
h
isotype control
i
dapi no 1◦ Ab
j
no 1◦ Ab
k
dapi
l
α5
m
α2β1
n
α2β1 overlay
o
α3β1 and dapi
p
dapi for isotype control
q
isotype control
r
dapi no 1◦ Ab
s
no 1◦ Ab

Figure 6 Representative 6 day old VPC clusters formed from MNCs isolated from a peripheral blood sample from a healthy volunteer. Cells were plated on fibronectin (a-j) and expressed α5 integrin (b), β1 integrin (c) (a dimerized α5β1 integrin primary fluorescent antibody was not commercially available). Clusters on fibronectin were negative for α2β1integrin (e) and positive for α3β1 integrin (f). Isotype control and control with no primary antibody (Ab) shown in (g,h) and (i,j). Cells were plated on laminin (k-s) and were negative for α5 integrin (l) however expressed α2β1 integrin (m, n). Clusters were also positive for α3β1 (o). Isotype control and control with no primary Ab shown in (p,q) and (r,s).

To assess whether integrin mediated binding is required for VPC function, we performed VPC assays in the presence of neutralizing antibodies against α5β1 and α2β1 integrins. IgG was used as a negative control, and cells that did not receive any neutralizing antibodies were used as positive controls.

MNCs from 3 peripheral blood samples from healthy volunteers were isolated, and VPC assays were performed on fibronectin and laminin in the presence or absence of anti- α5β1, anti- α2β1, IgG or without neutralizing antibodies.

A reduced number of VPC clusters was observed when cells were plated on fibronectin and treated with neutralizing antibody against α5β1 (30.2±6.9 clusters/mL of blood) when compared to any of the other culture conditions (anti-α2β1 133.7±21.9 clusters/mL of blood p=0.03, IgG 133.0±10.5 clusters/mL of blood p=0.004, no treatment 121.0± 13.1 clusters/mL of blood p=0.008) (see fig. 7a). Similarly, a statistically significant lower number of clusters was produced when cells were plated on laminin and treated with neutralizing antibody against α2β1 (3.0±2.1 clusters/mL of blood) than with any of the other culture conditions (anti-α5β1 130.8±8.6 clusters/mL of blood p=0.003, IgG 110.2±12.4 clusters/mL of blood p=0.01, no treatment 105.9± 17.8 clusters/mL of blood p=0.03) (see fig. 7b). Together, these observations support the important role of integrin-specific binding to extracellular matrix proteins during VPC cluster formation.

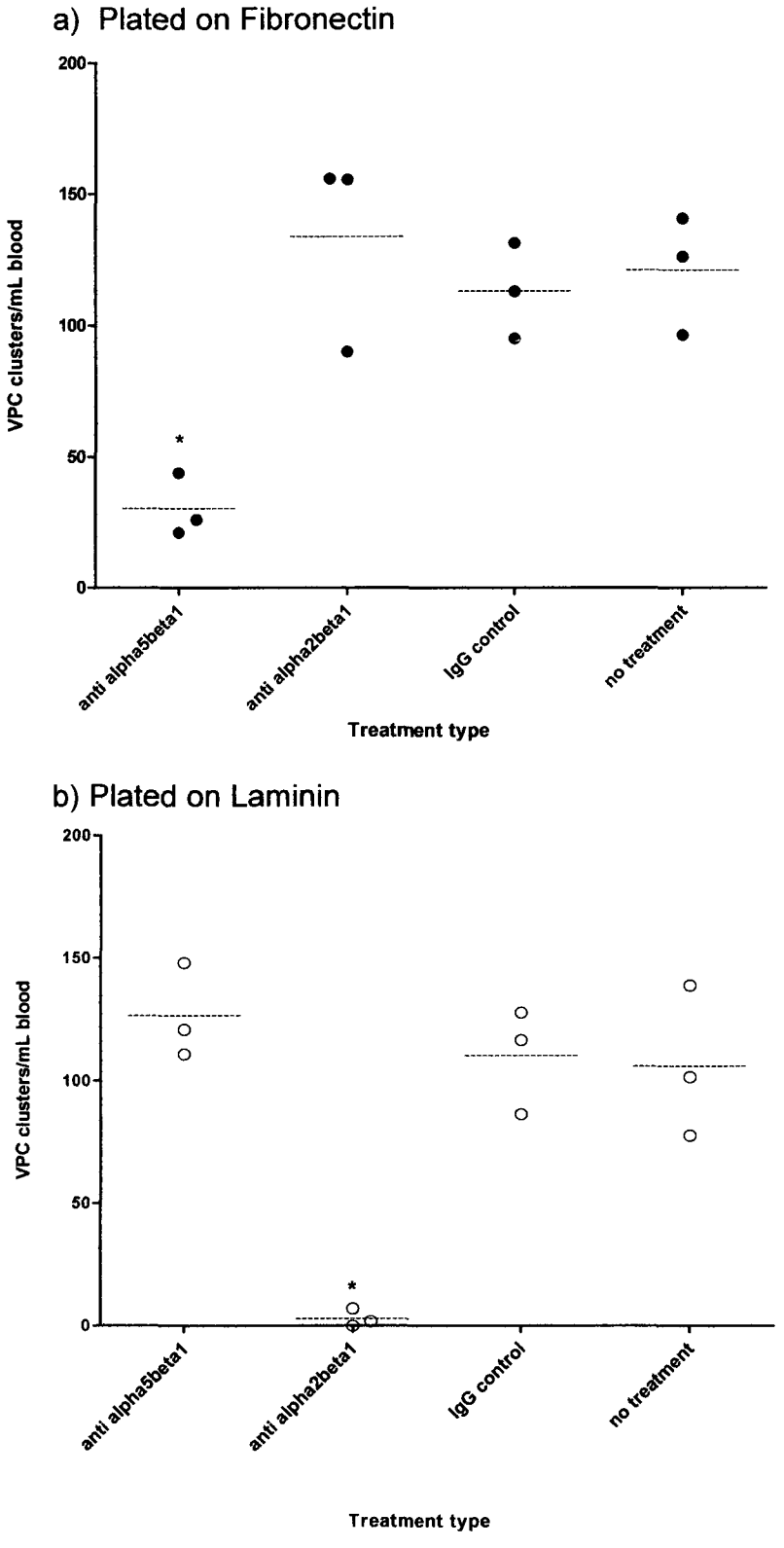


Figure 7 Number of VPC clusters/mL of blood for 3 peripheral blood samples from healthy volunteers, a lower number of VPC clusters formed on fibronectin when treated with anti- α5β1 antibody than with anti- α2β1 ($p=0.03$), IgG ($p=0.004$) and

with no treatment ($p=0.008$) (a), a lower number of VPC clusters formed on laminin when treated with anti- $\alpha 2\beta 1$ antibody than with anti- $\alpha 5\beta 1$ ($p=0.003$), IgG ($p=0.01$) and with no treatment ($p=0.03$), * denotes a significantly lower number of VPC clusters.

3.4 VEGF enhances VPC cluster formation on fibronectin

Many prior studies report that cancer patients have higher plasma or serum VEGF levels that correlate with poor prognosis and metastasis (Mitsuhashi *et al.*, 2005; Belgore *et al.,* 2001; Tamura M and Ohta Y, 2003) and that VEGF can modulate α5β1 signaling (Wijelath *et al.,* 2004). We measured plasma VEGF levels (mean ± SEM) in our patient cohort and in healthy controls to determine whether VEGF could influence the binding of VPCs to either of our extracellular matrix proteins of interest. Healthy volunteers had lower plasma VEGF concentrations (75.6±13.6 pg/mL) than that of oral cancer (308.9±101.8 pg/mL, $p=0.04$) and lymphoma (261.0±66.7 pg/mL, $p=0.02$) patients and also patients with acute kidney injury (355.5±22.4 pg/mL, $p<0.0001$) samples. Oral cancer and lymphoma sample VEGF concentrations were not significantly different from each other ($p=0.7$) and plasma VEGF levels from lymphoma and oral cancer samples were not significantly different from levels in samples from patients with acute kidney injury ($p=0.3$, $p=0.8$ respectively) (see fig. 8). These results suggest that in our patient cohort, cancer patients and patients with acute injury had higher mean VEGF plasma concentrations than those of healthy plasma.,

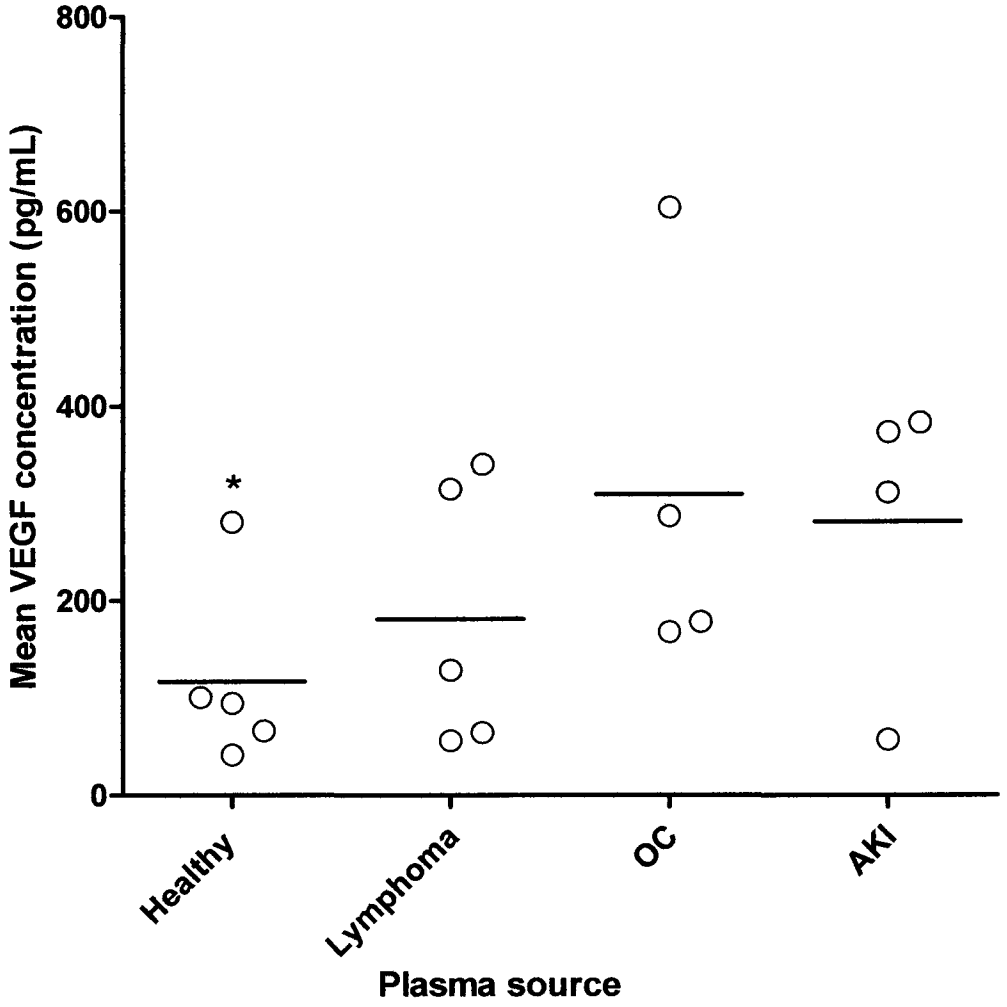
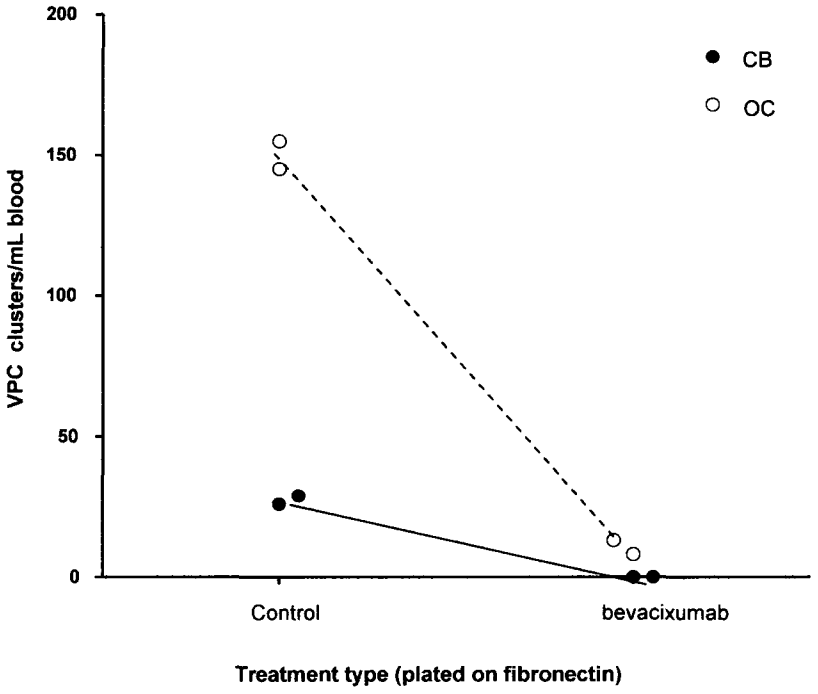


Figure 8 VEGF concentrations (pg/mL) in peripheral blood plasma samples collected from healthy controls, patients with lymphoma, oral cancer (OC) and acute kidney injury (AKI). * denotes a significantly lower mean VEGF concentration in the plasma from healthy volunteers than from patients with lymphoma ($p=0.02$), OC ($p=0.04$) and AKI ($p<0.0001$). Lines denote mean values for each group.

To determine whether VEGF was involved in the binding of VPCs to fibronectin, MNCs isolated from cord blood and peripheral blood samples from patients with oral cancer were incubated with Bevacizumab (VEGF inhibitor) and plated on fibronectin in the VPC assay. The presence of Bevacizumab reduced the mean number of VPC clusters for both cord blood (0±0 clusters/mL blood) and oral cancer (10.5±1.5 clusters/mL blood) samples when compared to cells that did not receive Bevacizumab (cord blood 27.5±1.5 clusters/mL blood, oral cancer 150 ± 5 clusters/mL blood)(see fig. 9a). MNCs from the same cord blood and peripheral blood samples from patients with oral cancer were incubated with Bevacizumab and plated on laminin, however Bevacizumab did not reduce VPC cluster numbers for either cord blood (31.5±0.5 clusters/mL blood without Bevacizumab and 23.5± 1.5 clusters/mL blood with Bevacizumab) or oral cancer samples (39±1 cluster/mL of blood without Bevacizumab and 32.5±1.5 clusters/mL blood with Bevacizumab) (see fig. 9b). No statistics could be performed due to the limited number of samples (n=2).

These results suggest that VEGF is important in the binding of cells to fibronectin in the VPC assay, but not required in VPC cluster formation on laminin.

a)



b)

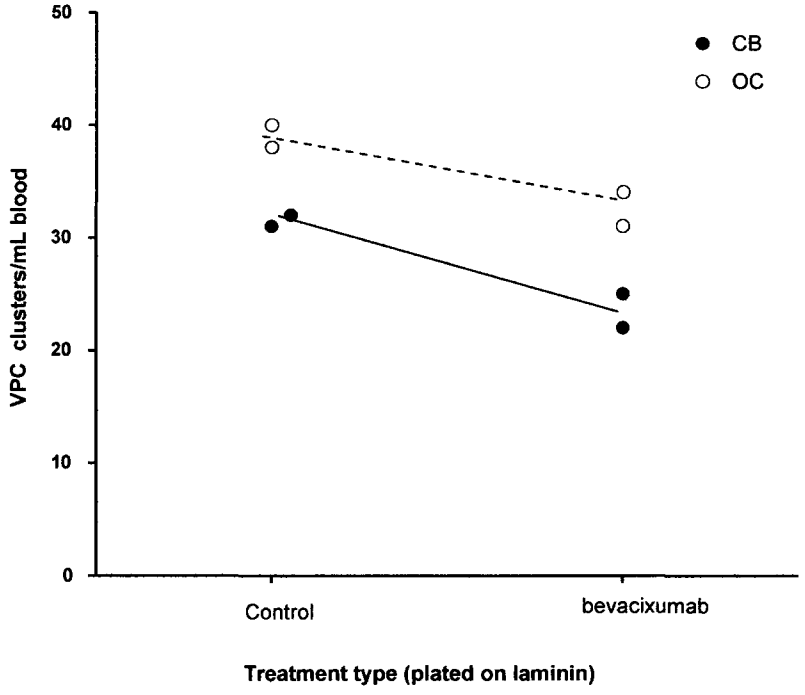
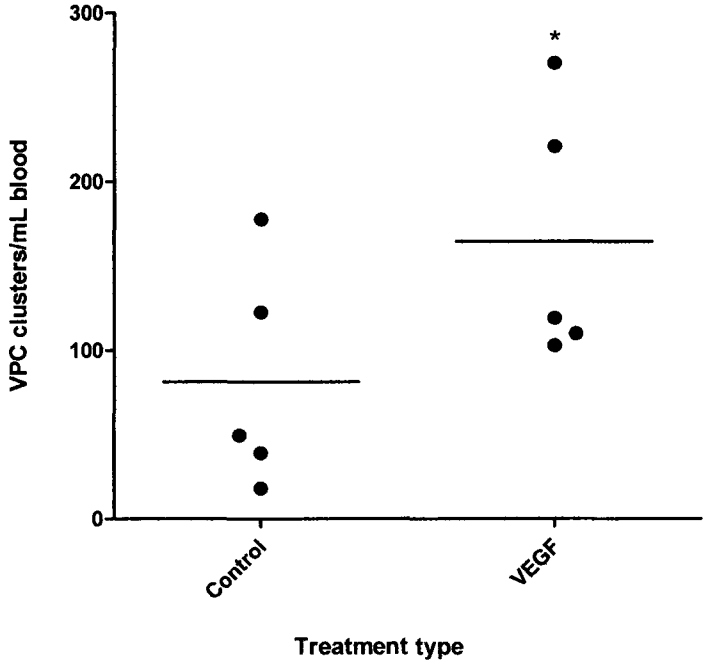


Figure 9 (a) Number of VPC clusters/mL of blood for 2 cord blood (CB) and 2 oral cancer (OC) samples without Bevacizumab and incubated with Bevacizumab plated on fibronectin, (b) number of VPC clusters/ mL of blood for 2 CB and 2 OC samples without Bevacizumab and incubated with Bevacizumab plated on laminin.

Since VEGF can influence the number of VPC clusters that form on fibronectin, and VEGF levels are elevated in the plasma of cancer patients, we wanted to determine if exposing MNCs isolated from peripheral blood of healthy controls to VEGF could change the binding profile of VPCs to fibronectin or laminin and yield a profile similar to the cancer patients.

MNCs from peripheral blood of healthy controls incubated with VEGF (1ug/mL) produced a greater number of clusters when plated on fibronectin (167.4±33.0 clusters/mL blood $p=0.008$) compared to controls without VEGF (78.6±28.8 clusters/mL blood) (see fig. 10a). However, MNCs isolated from healthy peripheral blood samples did not produce a greater ($p=0.9$) number of VPC clusters when incubated with VEGF and plated on laminin (68.8±12.1 clusters/mL blood) compared to controls without VEGF (66.9±16.3 clusters/mL blood) (see fig. 10b).

a) Cells plated on fibronectin



b) Cells plated on laminin

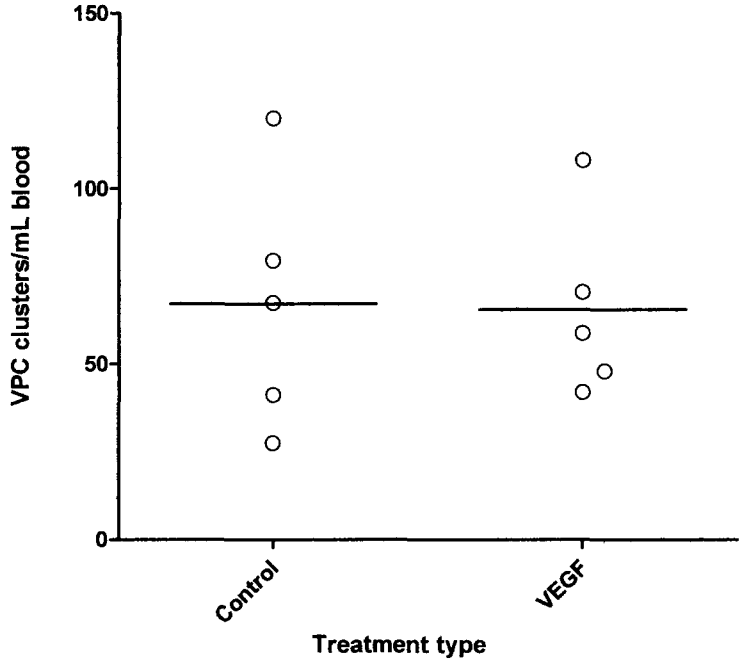


Figure 10 (a) the number of VPC clusters/mL of blood for 5 peripheral blood samples from healthy controls incubated with 0 or 1 ug/mL of VEGF and plated on fibronectin, * denotes a significantly greater number of VPC clusters when cells are treated with VEGF (p=0.04) (b) number of VPC clusters/mL of blood for 5 peripheral blood samples from healthy controls incubated with 0 or 1 ug/mL of VEGF and plated on laminin.

These data suggest that elevated VEGF levels can induce greater VPC cluster formation from MNCs derived from peripheral blood of healthy controls when plated on fibronectin, in a manner similar to samples from peripheral blood of cancer patients.

Together, our analysis suggests that VEGF enhances fibronectin-associated VPC cluster formation and that this can be inhibited by a soluble VEGF inhibitor.

3.5 VEGF and surface expression of α5 and α2 integrins on MNCs

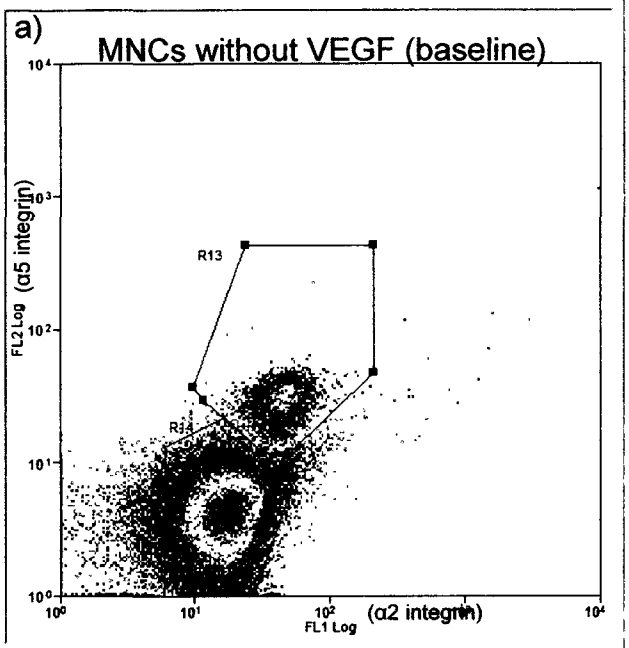
In order to determine the impact of soluble VEGF on the surface expression of α5 or α2 integrins, α5 and α2 integrin expression was assessed by flow cytometry on MNCs isolated from 3 peripheral blood samples from healthy volunteers at two time points: baseline (immediately after isolation with or without 1ug/mL of VEGF) and at 24 hours (after baseline with or without 1ug/mL of VEGF). All data shown represent the mean ±SEM percentage of cells expressing integrins.

Our results (see fig.11 and Appendix A figs. 1S and 2S) suggest that MNCs from healthy patients prior to 24 hours of VEGF exposure are divided into three populations including: one expressing neither integrins (α2-/α5-),one expressing α2 but not α5 integrin (α2+/α5-), and one positive for both integrins (α2+/ α5+). There does not appear to be a cell population that expresses the α5 integrin but not α2 integrin. At baseline the presence of VEGF did not change the proportion of cells

expressing either phenotype where on average 7.1±2.1% of cells without VEGF were α2-/α5-, 10.7±0.8% of cells were α2+/ α5+ and 76.1±2.8% of cells were α2+/ α5-. Cells exposed to 1ug/mL of VEGF included on average 10±1.6% of α2-/α5- cells, 10.8±0.6% of α2+/ α5+ cells and 72.8±3.4% of α2+/ α5- cells.

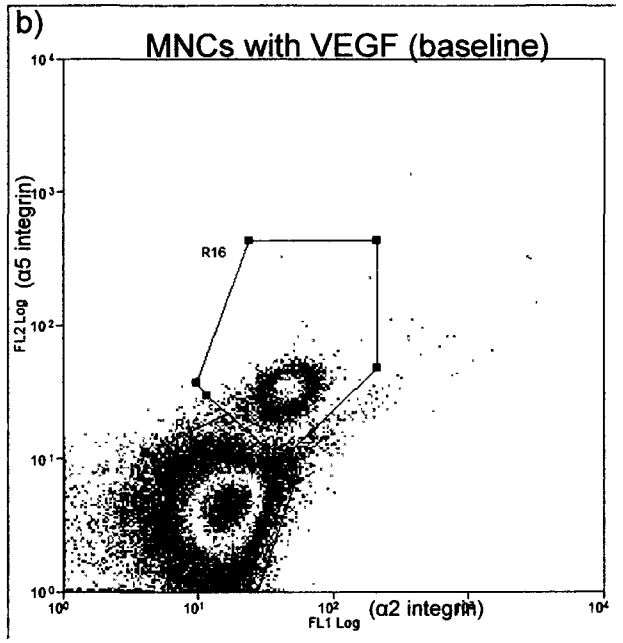
After 24 hours, controls without VEGF had a slight increase in the mean number of α2-/α5- cells (8.4±1.3%, p=0.5), a decrease in the mean number of α2+/ α5+ expressing cells (7.2±1.5%, p=0.2) and a slight increase in α2+/ α5- cells (78.1±1.1%, p=0.3). Whereas cells incubated with VEGF for 24 hours, had a slight increase in the mean number of α2-/α5- cells (16.1±2%, p=0.1) no change in the mean number of α2+/ α5+ cells (10.3±2.8%, p=0.9) and no significant change in the mean number of α2+/ α5- cells (68.7±5%, p=0.5) (see fig.11 for representative data and supplementary data in Appendix A figs. 1S (for sample 1) and 2S (for sample 2)). None of these relationships were statistically significant as shown by the p values which are all greater than 0.5.

a)



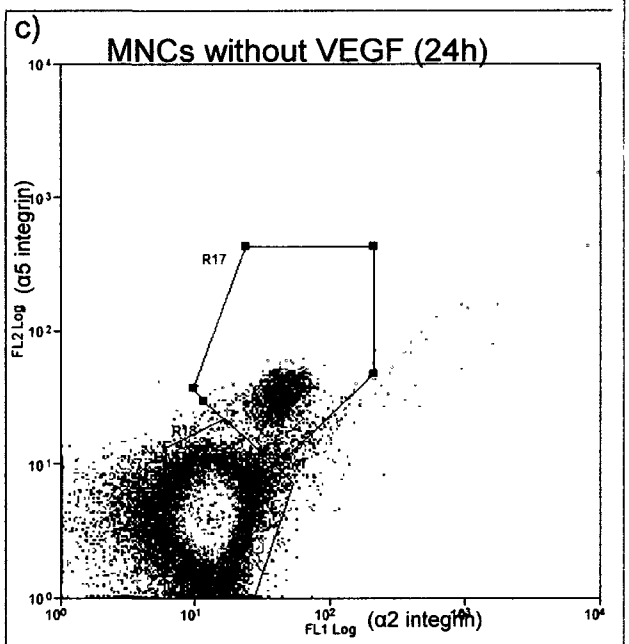
Region	Count	% Hist	% All	Mean	Median	Std Dev.
Total	46965	100.00	100.00	20.77, 6.61	16.73, 4.09	57.94, 10.56
R13	4272	9.10	9.10	45.64, 29.26	44.37, 28.76	13.47, 8.23
R14	37867	80.63	80.63	17.17, 4.35	16.14, 3.95	7.13, 2.40

b)



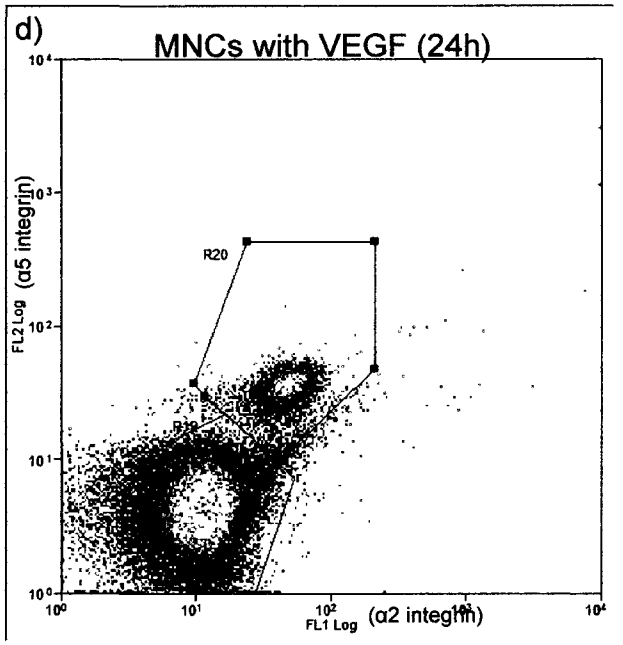
Region	Count	% Hist	% All	Mean	Median	Std Dev.
Total	46583	100.00	100.00	19.72, 7.22	15.57, 4.24	40.34, 11.43
R15	36939	79.30	79.30	16.20, 4.48	15.01, 4.09	6.95, 2.45
R16	4512	9.69	9.69	47.35, 32.06	46.00, 32.05	14.05, 9.58

c)



Region	Count	% Hist	% All	Mean	Median	Std Dev.
Total	30415	100.00	110.04	17.02, 6.61	12.53, 4.09	97.58, 54.66
R17	1949	6.41	7.05	44.88, 31.67	42.79, 32.05	15.52, 9.30
R18	24323	79.97	88.00	13.70, 4.46	12.53, 3.95	5.71, 2.59

d)



Region	Count	% Hist	% All	Mean	Median	Std Dev.
Total	35613	100.00	100.00	18.65, 8.76	11.66, 4.73	95.47, 20.31
R19	24629	69.16	69.16	13.07, 4.78	11.66, 4.24	5.94, 2.68
R20	4657	13.08	13.08	47.99, 33.46	46.00, 33.23	15.57, 9.41

Figure 11 Scatter plots showing α5 and α2 integrin expression on MNCs from 1 peripheral blood sample from a healthy volunteer (sample 3) at baseline without VEGF (a) or with VEGF (b) and at 24 hours without VEGF (c) and with VEGF (d).The top gates in each plot show the cell population positive for both α5 and α2 integrin expression, and the bottom gates delineate the cell population negative for α5 expression but positive for α2 integrin expression. The value of these gates is seen in the "% All" section under the plot. The set gates were maintained for all cells from the same samples when data was analyzed. The gates showing the α5-/α2- are not included.

Chapter 4: Discussion

In this study we wanted to determine how different extracellular matrix proteins influence attaching cells in VPC cluster formation and compare the matrix binding profile of VPC clusters derived from MNCs that were isolated from cord blood, peripheral blood from healthy individuals and patients with cancer or acute tissue injury. We wanted to identify the surface integrins on attaching cells in VPC clusters that are involved in binding to matrix proteins and to determine the role of VEGF in mediating VPC cluster formation.

We found (i) that attaching cells in VPC clusters bind to extracellular matrix proteins differently and therefore allows one to distinguish a normal profile from an "activated" or stressed profile and also a profile associated specifically with cancer. We observed increased VPC cluster formation on fibronectin and laminin from peripheral blood samples of patients with acute kidney injury, or from cord blood units which represent "activated" samples. Increased VPC cluster formation on fibronectin alone was observed using peripheral blood from patients with oral cancer and patients with lymphoma, thereby establishing a profile specific to cancer, and increased VPC cluster formation on laminin was observed from peripheral blood of healthy or "steady state" volunteers. We also demonstrated (ii) that this binding involves specific integrins. In particular, enhanced fibronectin-associated VPC cluster formation predominantly involves the α5β1 integrin and not the α2β1 integrin and that the use of a α5β1 integrin blocking antibody significantly reduced the number of clusters that formed on fibronectin. Furthermore, (iii) VEGF appears to be

an important mediator of binding to fibronectin, as cluster formation can be inhibited with the use of a soluble anti-VEGF antibody. The role of VEGF in modulating VPCs and their interactions with fibronectin was further supported by our data showing that when VPCs from healthy volunteers were treated with VEGF, their matrix binding profile changed from preferentially clustering on laminin, to mimicking that of VPCs from cancer sources that preferentially cluster on fibronectin.

Laminin-associated VPC cluster formation involves the α2β1 integrin and not the α5β1 integrin, as support our immunohistochemistry data and the use of a α2β1 integrin blocking antibody which significantly reduced cluster formation on laminin. In contrast to the case with fibronectin, VEGF does not appear to play a significant role in the binding of VPCs to laminin since the use of a soluble anti-VEGF antibody had no significant effect on the number of clusters that formed on laminin when compared to untreated cells. Furthermore, the addition of VEGF to VPCs from healthy volunteers plated on laminin did not significantly increase cluster formation.

Based on our flow cytometry analysis, surface expression of α5β1 is not significantly changed following brief exposure to VEGF for 24 hours. It is possible that further changes in the cell surface density of specific integrins are induced once the cells are plated on the matrix proteins.

Together, our observations provide key insights that contribute to a foundation for the development of strategies to block the mobilization and integration of VPCs into

sites of tumour development without compromising the healing capacity of normal tissues by VPCs.

The differences in VPC cluster formation on different matrix proteins suggests that different signaling pathways may be implicated in the interactions between vascular precursors and extracellular matrix proteins in cancer microenvironments as compared to healthy or other activated settings such as acute kidney injury and post-delivery cord blood.

In a cancer setting VPCs appear to mostly interact with fibronectin. In conjunction with our observations of enhanced VPC cluster formation on fibronectin when VPCs were isolated from cancer patients, others have reported that tumor cells release elevated soluble VEGF into circulation leading to higher plasma VEGF levels compared to non-cancer controls (Mitsuhashi *et al.*, 2005; Belgore *et al.,* 2001; Tamura M and Ohta Y, 2003). VEGF can bind and attract VEGFR-2 expressing vascular precursors and hematopoietic cells expressing VEGFR-1 to tumor microenvironments and also into areas of acute tissue injury (Manabe *et al.,* 1999; Redington *et al.,* 2001; Kikuchi *et al.,* 2005). Once VPCs are in close proximity to tumor sites, α5β1 integrin on VPCs can facilitate binding to fibronectin and initiate angiogenesis (Wijelath *et al.,* 2004).

Fibronectin and VEGF are key regulators of blood vessel growth. Gene deletion studies have shown that both fibronectin and VEGF, as well as their receptors α5β1 integrin and VEGFR-2 are critical for vascular development (Francis *et al.,* 2002).

ECs respond to growth factors like VEGF and are modulated by the signals conveyed from integrins which often reflect the conditions of the extracellular milieu (Wijelath *et al.,* 2006). Consequently, the local combination of extracellular matrix proteins and the type and density of integrins expressed by cells attracted to a specific tissue site can modulate responses of ECs to growth factors (Byzova *et al.,* 2000; Wijelath *et al.,* 2006). Binding of VEGF to VEGFR-2 can activate certain integrins such as αvβ3 and α5β1. Indeed, activation of α5β1 by VEGF appears to facilitate binding to fibronectin (Van der Loo *et al.,* 1998). Furthermore α5β1 can interact with c-kit and appears to promote the continued activation of focal adhesion kinase and extracellular-regulated kinase which can enhance erythroid progenitor survival and proliferation (Kapur *et al.,* 2001). When VEGF is in the presence of VPCs that bind to fibronectin, there is a collaboration between α5β1 and VEGFR-2 which results in a prolonged extracellular-regulated kinase activation and consequently increased VPC migration (Wijelath *et al.,* 2002). It is possible that when the α5β1 and VEGF binding domains are physically joined, fibronectin can act as a "chaperone" and support the sustained binding of VEGF to VEGFR-2 while simultaneously engaging α5β1 (Wijelath *et al.,* 2004). As such, when fibronectin and VEGF are bound to their cognate receptors, cellular responses to VEGF are enhanced, which may be the most important step in modulating the signalling pathways that lead from receptor ligation to cellular responses (Wijelath *et al.,* 2006).

Flow cytometry analysis revealed that the surface expression of α5β1 integrin is not significantly changed during 24 hours of exposure to VEGF. Consequently, this

exposure does not explain the altered extracellular matrix binding profiles we observed when MNCs from healthy patients were incubated with VEGF in the VPC assay which caused a shift from the healthy VPC-matrix binding profile to the cancer profile. Based on the notions of the unique interactions that occur between VEGF, α5β1 and fibronectin described above, it is possible that the majority of surface α5β1 integrins are induced once MNCs are plated on fibronectin in the presence of VEGF and these factors can interact. Future work should further examine this concept.

Based on the idea that there is an important interaction between the α5β1 integrin and VEGF in a fibronectin rich environment, α5β1 integrin may be an important target for therapies aimed at preventing or slowing metastatic tumor growth. Interestingly preclinical models have shown that the use of selective antagonists of α5β1 can reverse tumor growth (Kim *et al.,* 2002; Stoeltzing *et al.,* 2003) and these inhibitors are under study in some clinical trials (Ricart *et al.,* 2004). These inhibitors are thought to lead to endothelial cell apoptosis via anoikis, a form of cell death induced by anchorage-dependent cells detaching from the surrounding extracellular matrix, thereby losing essential signals for growth and survival (Kim *et al.,* 2002; Stoeltzing *et al.,* 2003).

Although VEGF appears to play an important role in a cancer setting, we recognize that it may be elevated in other inflammatory conditions and that it is involved in a multitude of other biological processes.

It is of interest to note that the VPC- extracellular matrix protein binding profile we observed of patients with acute kidney injury and cord blood is similar. This suggests that in "stressful" settings, such as acute tissue injury, VPCs preferentially interact with fibronectin and laminin. This observation, might be explained by the fact that in acute injury such as acute kidney injury, inflammation leads to the increased release of VEGF (Manabe *et al.,* 1999; Redington *et al.,* 2001; Kikuchi *et al.,* 2005) up-regulation of the α5β1 integrin (van Vliet *et al.,* 2002) and that although, fibronectin only comprises a small fraction of the extracellular matrix in the artery wall of a normal healthy adult, fibronectin can be significantly up-regulated in the diseased wall (Glukhova *et al.,* 1989; van Vliet *et al.,* 2002). Glomerulosclerosis, a severe complication associated with many kidney diseases that eventually results in the loss of renal function, has been shown to up-regulate fibronectin expression which is accompanied by up-regulation of α5β1 integrin expression (van Vliet *et al.,* 2002). VEGF expression can be up-regulated at sites of inflammation by basic fibroblast growth factor which is released by the action of proteases and heparinases found on the extracellular matrix (Bikfalvi *et al.,* 1997). Therefore, the increased presence of fibronectin, α5β1 and VEGF in the patients with acute kidney injury, might explain the enhanced binding on fibronectin when compared to healthy adults with predominant VPC cluster formation on laminin.

Just prior to birth, all uterine tissues progress through staged changes that lead to relative uterine quiescence and maintenance of pregnancy to the activation of the uterus that prepares it for labour. These changes are activated by pro-inflammatory cytokines which increase the synthesis of MMPs and VEGF (Christiaens *et al.,*

2008). Although, plasma fibronectin levels are usually down-regulated in healthy infants and in the plasma of cord blood from healthy labours, whenever a delivery occurs pre-term (Zygmunt *et al.,* 1997) or the mother is suffering of preeclampsia (hypertension) (Kupferminc *et al.,*1995) plasma fibronectin levels are significantly up-regulated. Cord blood was collected for 5 deliveries in our study but cells may still have been "activated" due to the inflammatory milieu of labour and stress related to delivery. The idea of "activated" cells in cord blood units should be pursued in future work.

Although there is a well described correlation between various health outcomes and the number of VPC clusters measured using the VPC assay, the relevance of our model remains to be tested *in vivo* and perhaps even using therapeutically relevant ECFCs. The VPC cluster assay provides a foundation for better understanding interactions between VPCs and extracellular matrix proteins, however as the clusters produced are chiefly diagnostic as opposed to the expression of therapeutic cells, our results may not reflect the interactions occurring between VPCs (or ECFCs) and extracellular matrix proteins *in vivo*. This limitation is also related to the controversial definition of a VPC, which as previously mentioned includes various cell types such as ECFCs and CACs. Unlike ECFCs, CFU-ECs, represent a heterogeneous cell population, and no one has yet been able to identify specific and unique cell surface molecules that allow for the prospective isolation of a VPC in humans or other vertebrate species (Hirschi *et al.,* 2008). Significant progress has been made, however, regarding our understanding that several cell types and different cell lineages contribute to neovascularisation during normal conditions and

during disease. Despite the lack of a unifying VPC phenotype, research efforts are focusing on how all these cells and lineages participate in angiogenesis and new strategies for enhancing or inhibiting angiogenesis are emerging (Hirschi *et al.,* 2008).

In our model we plated our cells on isolated matrix proteins, which does not replicate the true *in vivo* extracellular matrix milieu which, in both healthy and cancer settings, is made up of many extracellular matrix proteins. Having performed our experiments *in vitro* also limits the applications of our findings, since *in vivo,* these matrix proteins and tumor and healthy tissues are exposed to a host of signalling molecules, cytokines, and growth factors which are absent *in vitro.* They are also subject to different stresses which cannot be replicated *in vitro*, as a result our results must be validated using *in vivo* models.

Our work showed that distinct "healthy", "activated" and "malignant" VPC-extracellular matrix protein binding profiles exist and that VEGF appears to be a factor involved predominantly in the binding of VPCs to fibronectin which occurs through the α5β1 integrin and less so in the binding of VPCs to laminin which occurs through the α2β1 integrin. The effects of specific matrix proteins such as laminin and fibronectin on integrin expression should be further investigated using flow cytometry at different time points to see if these proteins can regulate integrin expression. Likewise, the effects of matrix proteins on growth factor and other signaling molecules in tissues should be addressed. Similarly, the role of other important cytokines and/or growth factors on inducing integrin expression should be examined.

Here we identified a role for VEGF, however other signaling molecules that have been identified as important factors involved in angiogenesis have not been assessed. For example, basic fibroblast growth factor, interleukin-8 and tumor necrosis factor-α have been identified as signaling molecules associated with up-regulating the expression of α5β1 on blood vessels (Parsons-Wingerter *et al.,* 2005) and would be interesting candidates to investigate in further studies in order to determine their involvement in VPC- extracellular matrix protein binding. The effects of different matrix proteins on VPCs or ECs at the level of gene expression should be explored in future work using gene profiling. The expression of certain angiogenic genes should be compared when the cells are plated on different matrix proteins, as well as on a combination of proteins (in a manner more representative of the true extracellular matrix milieu). Similarly, the effects of VEGF and other signaling molecules on gene expression should be tested using gene profiling as it would help determine important genes involved in VPC-matrix binding, which may allow new strategies aimed at preventing cancer relapse following surgery to be investigated.

In this study we were able to obtain samples from two different cancer types, oral cancer and lymphoma, and we concluded that VPCs isolated from patients with either of these cancers have the same VPC- extracellular matrix protein binding profiles for the 4 proteins examined in this study. Future work should examine the VPC-binding profile of VPCs isolated from patients with many different cancers to see if the binding profile we observed depends on the type of cancer. Furthermore, we looked at the binding-profile of VPCs isolated from patients with acute kidney injury, and the binding profile of VPCs isolated from patients with other types of acute injury should be further investigated, in order to better understand the role of

VPCs in these patients and the involvement of inflammatory cytokines. A limitation to this work is that our results may have been influenced by patient-specific factors other than the presence of cancer or acute injury such as age, smoking history, diabetes and vascular disease could affect outcomes and should be controlled in future work. It is likely that the differences in mean age between our populations does not account for the different binding profiles we observed since cord blood, a blood product of young "age" had a different binding profile than cells from both our younger "healthy" subjects and older sick patients. We acknowledge that we have a small sample size for our study and larger studies may clarify this issue in the future. Although the feasibility of blocking the α5β1 integrin *in vivo* as an anti-angiogenesis treatment in an animal model of cancer is under investigation and may hold promise for the future, work in this domain should continue, and addressing the impact of blocking the the α5β1 integrin in VPCs or ECFCs could be insightful.

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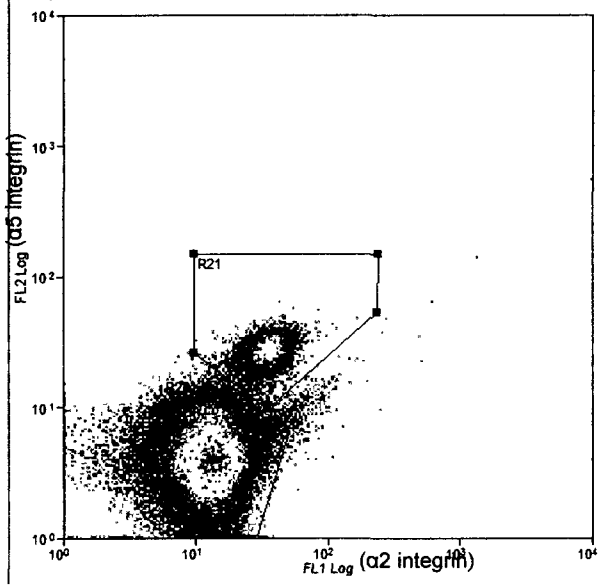
Appendices

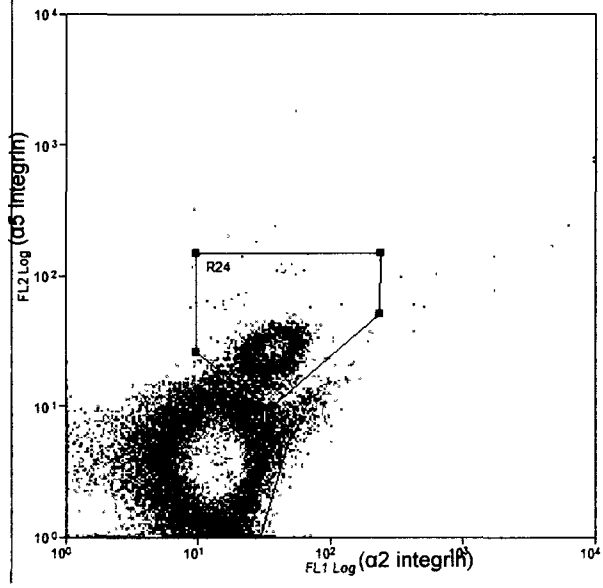
Appendix A- Supplementary flow cytometry data

a) MNCs without VEGF (baseline)



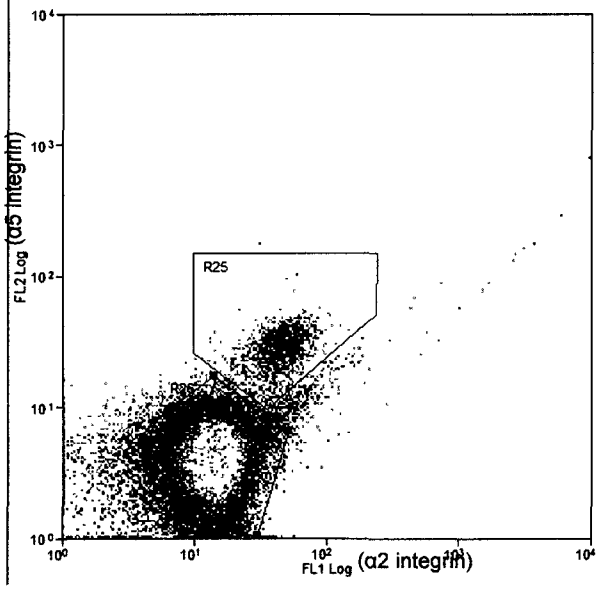
Region	Count	% Hist	% All	Mean	Median	Std Dev.
Total	40002	100.00	100.00	17.01, 7.14	13.47, 4.24	52.56, 8.58
R21	4780	11.95	11.95	35.63, 26.07	34.46, 25.81	10.71, 6.57
R22	28448	71.12	71.12	14.23, 4.60	12.99, 3.95	6.05, 2.52

b) MNCs with VEGF (baseline)



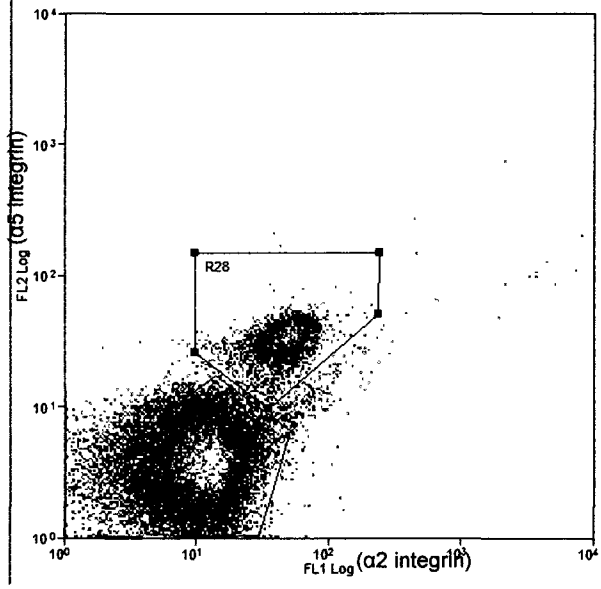
Region	Count	% Hist	% All	Mean	Median	Std Dev.
Total	36333	100.00	100.00	18.46, 6.78	13.97, 3.81	91.05, 14.19
R23	24576	67.64	67.64	14.96, 4.37	13.97, 3.81	6.37, 2.51
R24	4084	11.24	11.24	37.64, 26.25	37.04, 25.81	12.19, 8.69

c) MNCs without VEGF (24 h)



Region	Count	% Hist	% All	Mean	Median	Std Dev.
Total	30763	100.00	100.00	17.89, 5.59	13.47, 3.81	80.48, 22.28
R25	1570	5.10	5.10	45.57, 27.98	44.37, 27.74	15.34, 8.93
R26	23440	76.20	76.20	14.88, 4.32	13.97, 3.95	6.19, 2.23

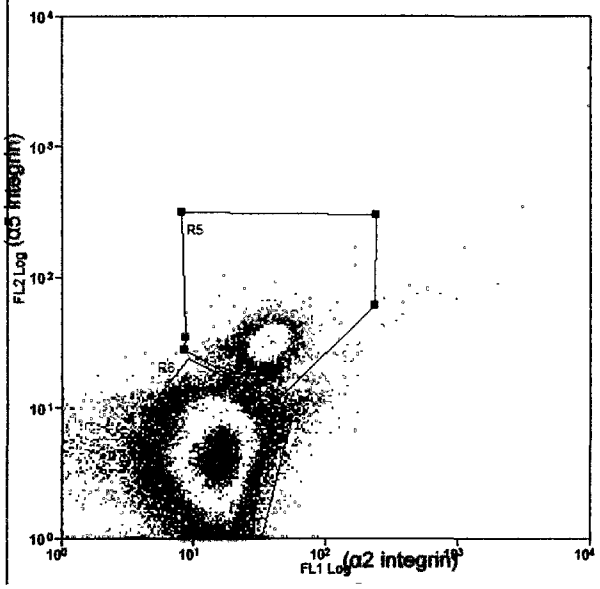
d) MNCs with VEGF (24 h)



Region	Count	% Hist	% All	Mean	Median	Std Dev.
Total	27641	100.00	100.00	19.30, 7.93	11.66, 3.95	100.31, 11.65
R27	16527	59.79	59.79	13.24, 4.40	12.09, 3.81	5.90, 2.41
R28	3571	12.92	12.92	47.57, 32.20	46.00, 32.05	17.09, 8.76

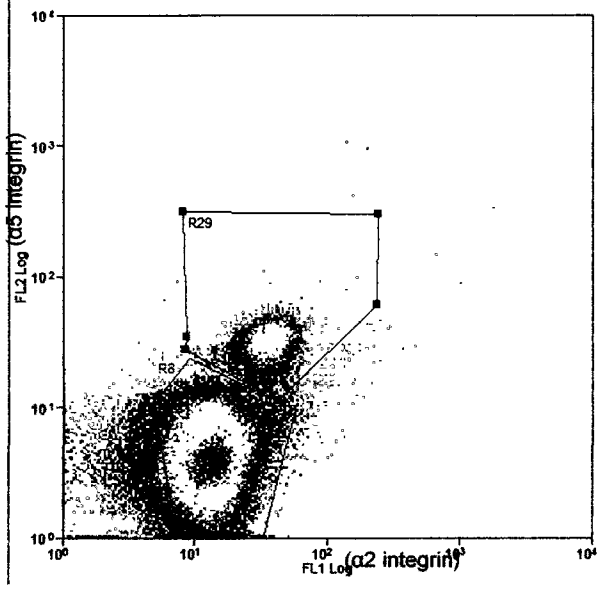
Figure 1S Scatter plots showing α5β1 and α2β1 integrin expression on MNCs from a peripheral blood sample from a 2nd healthy volunteer (sample 1) at baseline without VEGF (a) or with VEGF (b) and at 24 hours without VEGF (c) and with VEGF (d).The top gates in each plot show the cell population positive for both α5β1 and α2β1 integrin expression, and the bottom gates delineate the cell population negative for α5β1 expression but positive for α2β1 integrin expression. The value of these gates is seen in the % All section under the plot. The set gates were maintained for all cells from the same samples when data was analyzed. The gates showing the α5-/α2- are not included but values include 11.3(a), 11.5 (b), 10.3 (c) and 19.7 (d).

a) MNCs without VEGF (baseline)



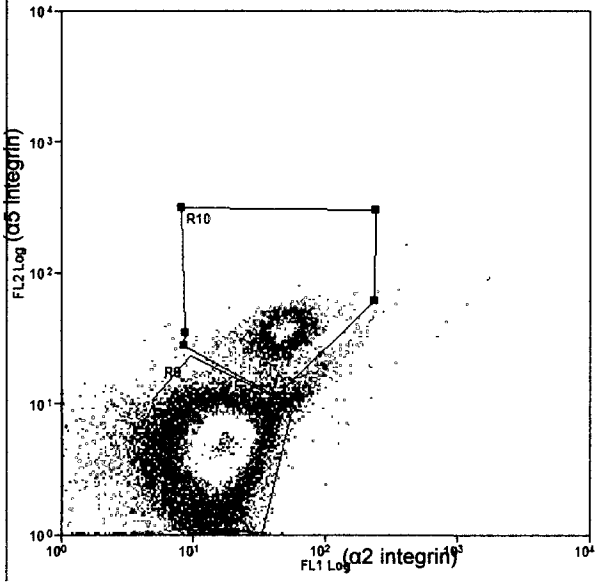
Region	Count	% Hist	% All	Mean	Median	Std Dev.
Total	60662	100.00	100.00	18.76, 7.69	15.57, 4.40	61.73, 20.53
R5	6784	11.18	11.18	37.21, 30.24	35.72, 29.82	11.24, 8.00
R6	46455	76.58	76.58	15.91, 4.96	15.01, 4.24	6.68, 2.81

b) MNCs with VEGF (baseline)



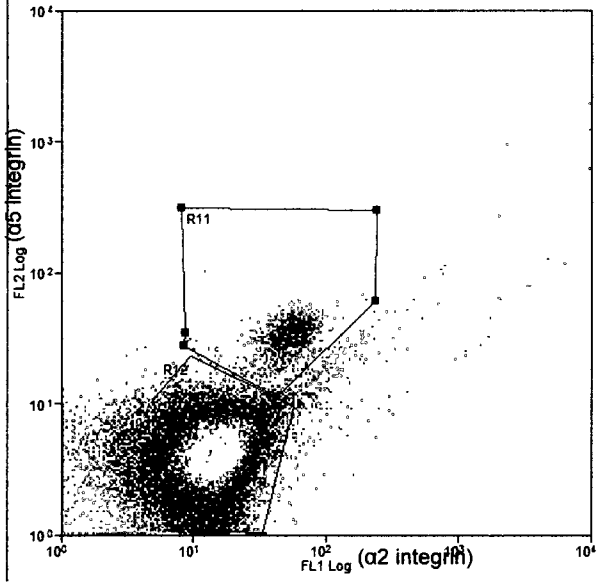
Region	Count	% Hist	% All	Mean	Median	Std Dev.
Total	58013	100.00	100.00	16.52, 7.53	13.47, 4.09	17.98, 11.27
R8	41490	71.52	71.52	14.31, 4.80	12.99, 4.09	6.37, 2.82
R29	6723	11.59	11.59	37.01, 30.20	35.72, 29.82	10.94, 8.40

c) MNCs without VEGF (24 h)



Region	Count	% Hist	% All	Mean	Median	Std Dev.
Total	31037	100.00	100.00	20.83, 7.95	16.14, 4.73	30.77, 20.46
R9	24287	78.25	78.25	16.77, 4.89	15.57, 4.56	7.25, 2.41
R10	3140	10.12	10.12	49.68, 34.54	47.69, 34.46	16.45, 9.00

d) MNCs with VEGF (24 h)



Region	Count	% Hist	% All	Mean	Median	Std Dev.
Total	25094	100.00	100.00	20.66, 6.09	13.47, 4.09	138.68, 18.06
R11	1198	4.77	4.77	53.48, 32.52	53.15, 32.05	17.21, 9.62
R12	19383	77.24	77.24	15.65, 4.63	13.97, 4.24	7.38, 2.31

Figure 2S Scatter plots showing α5β1 and α2β1 integrin expression on MNCs from a peripheral blood sample from a 3rd healthy volunteer (sample 2) at baseline without VEGF (a) or with VEGF (b) and at 24 hours without VEGF (c) and with VEGF (d).The top gates in each plot show the cell population positive for both α5β1 and α2β1 integrin expression, and the bottom gates delineate the cell population negative for α5β1 expression but positive for α2β1 integrin expression. The value of these gates is seen in the % All section under the plot. The set gates were maintained for all cells from the same samples when data was analyzed, although it appears that there is a decrease in the proportion of cells expressing both α5β1 and α2β1 integrins for cells incubated for 24 hours with VEGF in (d), this is likely due to the overall smaller cell count used, and the proportionally greater decrease in the number of cells expressing α5β1 and α2β1 counted when compared to the number of cells only positive for α2β1 counted for this set of cells. The gates showing the α5-/α2- are not included but values include 5.7 (a), 11.6 (b), 6 (c) and 13 (d).

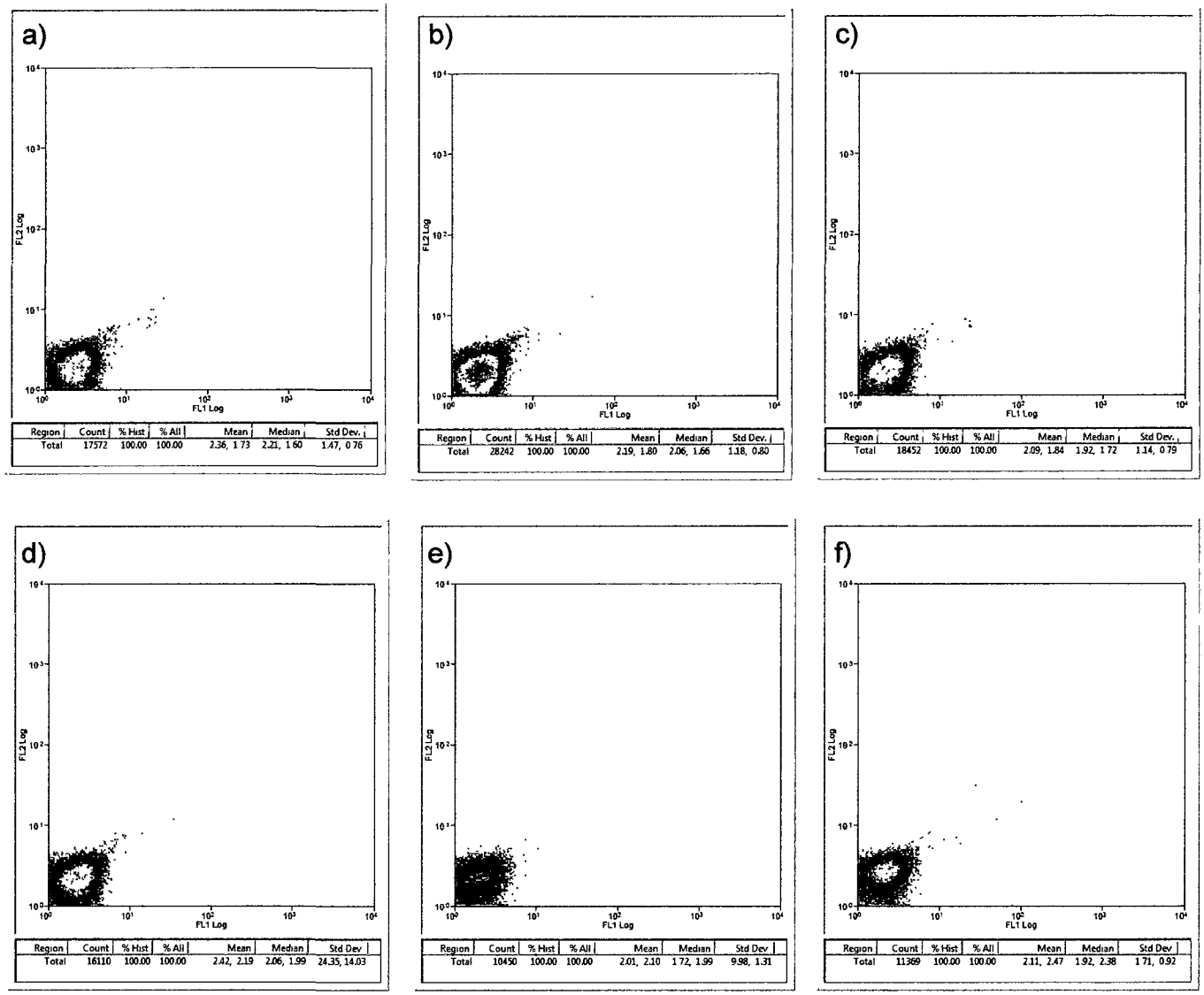


Figure 3S Scatter plots showing the blank samples run containing MNCs from 3 healthy volunteers using flow cytometry for (a) cells from sample 1 at baseline, (b) cells from sample 2 at baseline, (c) cells from sample 3 at baseline, (d) cells from sample 1 at 24 hours, (e) cells from sample 2 at 24 hours, (f) cells from sample 3 at 24 hours.