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Distinct profile of vascular progenitor attachment to extracellular matrix proteins in cancer patients

Abstract

Background: Vascular progenitor cells (VPCs) facilitate angiogenesis and initiate vascular repair by homing in on sites of damage and adhering to extracellular matrix (ECM) proteins. VPCs also contribute to tumor angiogenesis and induce angiogenic switching in sites of metastatic cancer. In this study, the binding of attaching cells in VPC clusters that form *in vitro* on specific ECM proteins was investigated.

Methods: VPC cluster assays were performed *in vitro* on ECM proteins enriched in cancer cells and in remodelling tissue. Profiles of VPC clusters from patients with cancer were compared to healthy controls. The role of VEGF and integrin-specific binding of angiogenic attaching cells was addressed.

Results: VPC clusters from cancer patients were markedly increased on fibronectin relative to other ECM proteins tested, in contrast to VPC clusters from control subjects, which formed preferentially on laminin. Specific integrin-mediated binding of attaching cells in VPC clusters was matrix protein-dependent. Furthermore, cancer patients had elevated plasma VEGF levels compared to healthy controls and VEGF facilitated preferential VPC cluster formation on fibronectin. Incubating cells from healthy controls with VEGF induced a switch from the 'healthy' VPC binding profile to the profile observed in cancer patients with a marked increase in VPC cluster formation on fibronectin.

Conclusion: The ECM proteins laminin and fibronectin support VPC cluster formation via specific integrins on attaching cells and can facilitate patterns of VPC cluster formation that are distinct in cancer patients. Larger studies, however, are needed to gain insight on how tumor angiogenesis may differ from normal repair processes.

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Endothelial-like vascular progenitor cells (VPCs) can be isolated or expanded from hematopoietic cell sources including bone marrow, umbilical cord blood (UCB) and peripheral blood (PB). Enumerating vascular clusters according to the method of Hill *et al.* [1] from these blood-derived cell sources correlates with important health outcomes in multiple settings, including patients at risk for cardiovascular events [2,3], critically ill patients with severe organ dysfunction [4-6] and in cancer patients undergoing hematopoietic stem cell transplantation [7,8] or radiation treatment [9]. Changes in VPC cluster number and character may be used to characterize cell sources for vascular repair capacity. In addition, interventions aimed at augmenting regenerative capacity such as the administration of cytokines, growth factors or medications to mobilize vascular progenitors may be monitored and evaluated using the VPC assay.

VPC clusters form by the aggregation of monocytes and lymphocytes on spindle-shaped attaching cells with monocytoïd and endothelial features [10-12]. Moreover, during *ex vivo* expansion of endothelial colony-forming cells (ECFCs) used in therapeutic vascular repair, endothelial-like cells first attach to extracellular matrix proteins and form spindle-shaped cells [13]. Cell populations enriched for angiogenic precursors such as CD133-selected or CD14-selected cells also form attached spindle-shaped cells on ECM proteins *in vitro*, suggesting that interactions between vascular precursors and the ECM is an important initiating step.

VEGF-mediated binding to fibronectin may play an important role in the homing of angiogenic precursors to sites of tissue injury or metastatic cancer *in vivo* [14]. Interestingly, VEGF is often increased in cancer patients [15-17] and contributes to increased tumour angiogenesis. Cell attachment to fibronectin and to other extracellular matrix proteins is facilitated by specific integrins, which are cell surface heterodimers that initiate intracellular signaling cascades after binding to the ECM. The extracellular matrix in sites of tumor involvement is extensively remodeled and highly enriched for fibronectin [18-20] and collagen I [21-23] when compared to that from the matrix environment in healthy or healing tissue which is typically enriched for laminin [24] and collagen IV [25]. The role of ECM proteins, therefore, may influence the homing of vascular precursors in response to angiogenic signals such as VEGF and may preferentially direct angiogenic precursors to sites of tumor involvement. The extent to which angiogenic precursors preferentially contribute to tumor growth at metastatic sites may depend on their capacity to attach to tumour-specific microenvironments, including cancer-specific ECM proteins. Strategies to guide angiogenic precursors to sites of

healing tissue, damaged in cancer patients after surgery or caused by chemotherapy or radiation, are needed to ensure that tissue regeneration does not lead to angiogenic switching and growth of the underlying cancer.

In this report, differential ECM-binding profiles of angiogenic precursors from patients with cancer compared to normal controls are described. Furthermore, the pattern of cell surface integrin expression from these patients is specific for particular ECM proteins and may be informative regarding the readiness of vascular repair mechanisms and the risk of vascular switching in cancer patients. Moreover, enhanced binding of angiogenic precursors to fibronectin in cancer patients was confirmed to be mediated by VEGF through specific interactions with integrin $\alpha 5\beta 1$. Our observations provide a foundation for developing regenerative therapies in cancer patients with the goal of promoting healing of normal tissues without stimulating new vessel growth at sites of micrometastases.

Methods

Sample collection

Blood samples were obtained following informed consent and in accordance with protocols approved by the institutional research ethics board at The Ottawa Hospital. Mononuclear cells (MNCs) were isolated from blood samples from controls and from pre-operative samples from adult patients with oral cancer and peripheral blood from adult patients with non-Hodgkin's lymphoma. Peripheral blood samples were collected in 7 mL sterile vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA).

Cell preparation and isolation

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 and centrifuged for 30 min.

Vascular progenitor cell cluster assay (CFU-Hill)

VPC assays were performed in accordance with the methods described by Hill *et al.* [1]. VPCs were cultured using the EndoCult Liquid Medium Kit (StemCell Technologies, Vancouver, BC, Canada) as stated in the manufacturer's protocol.

MNCs were resuspended in complete EndoCult medium and seeded at 5×10^6 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% CO₂ 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), and incubated for an additional 3-5 days. VPC cluster formation was quantified by enumerating the number of clusters formed on each matrix using an inverted microscope. All values are reported as the mean number of VPC clusters observed/ mL of blood collected prior to Ficoll gradient centrifugation.

Immunohistochemistry

VPC clusters were analyzed by immunohistochemistry for cell surface integrin expression. Media in the wells of the matrix-coated tissue culture plates was removed and the remaining attached VPC clusters were gently washed and fixed for 10 minutes at 4°C and washed again. Cells were incubated with a blocking solution for 20 minutes at room temperature and 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: mouse anti-human $\alpha 5$ conjugated to Phycoerythrin (PE) antibody and $\beta 1$ conjugated to Fluorescein isothiocyanate (FITC) antibody (diluted 1:100), $\alpha 2\beta 1$ conjugated to FITC antibody (1:100), $\alpha 3\beta 1$ antibody (1:100) (all antibodies from Life Span Biosciences Inc, Seattle, WA, USA). Controls were performed in parallel by incubating with no primary antibody or with an isotype control of mouse immunoglobulin G (IgG) antibody (1:100) (IgG 1k, BD Biosciences). Controls were also washed 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 and 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) and visualized using the Zeiss Observer.Z1 inverted microscope where images were captured with the AxioCam HRm camera by Zeiss.

Inhibiting integrin function

MNCs were isolated from three samples of peripheral blood from healthy volunteers. Each sample was divided into four treatment types and cells received media containing either of the following: neutralizing antibody against $\alpha 5\beta 1$ integrin (clone JBS5, Millipore, Billerica, Massachusetts, USA), neutralizing antibody against $\alpha 2\beta 1$ integrin (clone BHA2.1, Millipore), mouse anti-human IgG (IgG 1k, BD Biosciences) (control) or no treatment (control). Antibodies were diluted to a concentration of 10 μ g/mL as per manufacturer's protocol. Cells were then plated on fibronectin or laminin in the presence of the same antibody conditions throughout the VPC culture period.

VEGF studies

VEGF ELISA: An enzyme-linked immunosorbent assay (ELISA) for VEGF (R&D Systems, Minneapolis, MN, USA) was performed using plasma from healthy controls, patients with oral cancer, patients with lymphoma and patients with 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 eight 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.

Anti-VEGF treatment: VPC assays were performed using MNCs from two cord blood samples and two 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 1 μ g/mL of bevacizumab prior to being plated on fibronectin or laminin. Bevacizumab remained in the media throughout the entire VPC culture period.

VEGF supplementation: VPC assays were performed using cells from five 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 μ g/mL of human recombinant VEGF₁₆₅ (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

a) Healthy controls

b) Cancer patients

FIGURE 1. *Profile of VPC cluster formation on different matrix proteins in healthy controls and in cancer patients.* (a) Number of VPC clusters/mL from peripheral blood of healthy adult volunteers (n=5). (*) denotes a significantly greater VPC clusters on laminin than on fibronectin (p=0.02), collagen I (p=0.03) and collagen IV (p=0.04); (b) VPC clusters/mL of peripheral blood from patients with lymphoma (●, n=5) and oral cancer (○, n=4). (*) denotes greater number of VPC clusters on fibronectin from patients with oral cancer and lymphoma, respectively, compared to 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 of patients with lymphoma on collagen I than on collagen IV (p=0.02).

grown in the media containing the VEGF throughout the entire VPC culture period.

Statistical analyses

Data are expressed as mean $\pm$ 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 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.

Results

VPC clusters on different extracellular matrix proteins in cancer patients

To better understand the status of cells that give rise to VPC clusters in patients with cancer, VPC clusters were measured on ECM proteins that are more typical of cancer environments and compared the cluster formation on ECM proteins that are typical of normal remodeling tissue. VPC clusters from peripheral blood (PB) samples of healthy controls (n=10) were more numerous when plated on laminin (31.6 ± 6.5 clusters/mL PB)

compared with any of the other matrices tested (fibronectin: 8.5 ± 0.7 clusters/mL PB, $p=0.02$; collagen I: 6.9 ± 1.3 clusters/mL PB, $p=0.03$; and collagen IV: 6.1 ± 2.0 clusters/mL PB, $p=0.04$) (see figure 1). VPC clusters were also enumerated using a similar panel of matrix proteins and PB samples from patients with cancer. VPC clusters from pre-operative PB samples from patients with oral cancer (n=4) were more abundant when cells were plated on fibronectin (42.1 ± 8.1 clusters/mL) compared with the other three matrices (laminin: 7.5 ± 8.2 clusters/mL, $p=0.01$; collagen I: 3.7 ± 1.3 clusters/mL, $p=0.03$; collagen IV: 7.8 ± 4.0 clusters/mL, $p=0.02$). Similarly, PB samples from patients with lymphoma (n=5) yielded increased VPC clusters on fibronectin (86.5 ± 13.8 clusters/mL) compared with the other matrices (laminin: 27.9 ± 10.1 clusters/mL, $p=0.003$; collagen I: 39.6 ± 7.5 clusters/mL, $p=0.01$; and collagen IV: 11.9 ± 1.9 clusters/mL, $p=0.004$).

Taken together, our observations suggest that VPC cluster formation can occur to varying degrees on different matrix proteins with a profile that is specific for patients with cancer in comparison to healthy controls. In particular, preferential VPC cluster formation on fibronectin with no accompanying increase in VPC cluster formation on laminin was associated with patients with oral cancer and lymphoma. Moreover, can-

TABLE 1. Patient cohort characteristics

	Healthy controls	Oral Cancer	Non-Hodgkin Lymphoma
n	10	4	5
Age, mean years (range)	35 (23-45)	66 (52 – 75)	60 (51 – 67)
Gender, F / M (%)	5/5 (50/50)	2/2 (50/50)	2/3 (40/60)
Disease status	N/A	*T1N0M0: 3 T2N0M0: 1	Remission: 3 Relapse: 2
Prior chemotherapy or radiation, n (%)	0 (0)	1 (25)	5 (100)

* TNM staging refers to tumor size (T), presence of nodes (N) and metastases (M).

cer patients had increased overall levels of VPCs. In contrast, a distinct VPC binding profile was observed in steady state controls with more abundant cluster formation on laminin compared with the other ECM proteins. Although the profile of VPC cluster formation differs between patients with cancer and healthy controls, the absolute levels of VPC clusters on any one matrix did not allow us to distinguish between the groups. The clinical characteristics of the study subjects are summarized in Table 1. While gender was similar for the various categories of subjects enrolled in this study, patients and controls differed significantly with regards to age and prior chemotherapy or radiation exposure.

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

The different binding profiles of VPC clusters from healthy controls and from cancer patients can be distinguished using fibronectin and laminin alone as an abbreviated matrix panel based on our initial results described above. Fibronectin and laminin, therefore, were used for subsequent studies to address factors involved with VPC cluster formation on different matrix proteins. The role of integrins in the initial stage of cell attachment during VPC cluster formation was then investigated.

Integrins $\alpha 5\beta 1$ [26-28], $\alpha 2\beta 1$ [29,30] and $\alpha 3\beta 1$ [30,31] were selected to address the role of specific integrin-mediated binding of attaching cells in the VPC clusters. Integrin $\alpha 5\beta 1$ binds to fibronectin and not laminin, while $\alpha 2\beta 1$ integrin binds to laminin and not fibronectin and both integrins have been associated with angiogenesis. The $\alpha 3\beta 1$ integrin binds to both laminin and fibronectin.

Immunohistochemistry was used to assess cell surface expression of integrins in VPC clusters. Attaching cells in VPC clusters derived from samples of healthy volunteers and plated on fibronectin expressed $\alpha 5\beta 1$ integrin but not $\alpha 2\beta 1$ (see figure 2). $\alpha 2\beta 1$ integrin was present on attaching cells in VPC clusters plated on laminin derived from the same samples while $\alpha 5$ integrin expression was negative. The expression of $\alpha 3\beta 1$ was ob-

FIGURE 2. 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) and expressed $\alpha 5$ integrin (b), and $\beta 1$ integrin (b inset). Clusters on fibronectin were negative for $\alpha 2\beta 1$ integrin (c) and positive for $\alpha 3\beta 1$ integrin (d). Isotype controls were negative (insets). Cells were plated on laminin (e-h) and expressed $\alpha 2\beta 1$ integrin (g). Clusters were negative for $\alpha 5$ integrin (f) on laminin but positive for $\alpha 3\beta 1$ (h). Isotype controls were negative (insets).

served on the cell surface of attaching cells in VPC clusters derived from healthy donors when plated on both laminin and fibronectin. This data suggests that populations of cells with the appropriate integrin repertoire and the capacity to form VPC on both fibronectin and laminin are present in healthy donors, but taken together with our previous observations, the relative frequency of fibronectin-binding or laminin-binding integrin profile appears to shift.

To assess whether integrin mediated binding of attaching cells is required for VPC cluster formation, VPC assays were performed in the presence of inhibiting antibodies against $\alpha 5\beta 1$ and $\alpha 2\beta 1$ integrins. VPC assays were performed using MNCs isolated from PB of healthy controls and plated on fibronectin and laminin in the presence or absence of anti- $\alpha 5\beta 1$, anti- $\alpha 2\beta 1$, or polyclonal IgG. VPC cluster levels were reported as a ratio compared with the number of clusters observed in controls lacking antibody. VPC clusters on fibronectin were markedly reduced in the presence of anti- $\alpha 5\beta 1$ antibody ($25 \pm 16\%$ of controls, $p=0.008$) whereas anti- $\alpha 2\beta 1$ and non-specific IgG had no effect on VPC cluster number on fibronectin ($111 \pm 28.9\%$ and $110 \pm 19.5\%$ of controls, respectively, $p=NS$) (Figure 3A). Similarly, reduced number of VPC clusters were observed when cells were incubated with anti- $\alpha 2\beta 1$ antibody and plated on laminin ($2.8 \pm 18.8\%$ of controls, $p=0.03$) whereas anti- $\alpha 5\beta 1$ and non-specific IgG had no effect on VPC cluster number on laminin ($123 \pm 24\%$ and $104 \pm 28.5\%$ of controls, respectively, $p=NS$) (Figure 3B). Together, these observations support the important role of integrin-specific binding to extracellular matrix proteins during VPC cluster formation.

VEGF enhances VPC cluster formation on fibronectin

Prior studies report that cancer patients have increased plasma VEGF levels that correlate with worse prognosis and increased likelihood of metastasis [15-17]. Moreover, as VEGF can modulate $\alpha 5\beta 1$ signalling [27], it may directly contribute to increased levels of $\alpha 5\beta 1$ expressing cells that influence VPC cluster formation. Plasma VEGF levels were measured in our patients and the association of these levels with VPC cluster number on fibronectin were assessed.

Healthy controls had lower plasma VEGF concentrations (75.6 ± 13.6 pg/mL) than patients with oral cancer (210.7 ± 38.0 pg/mL, $p=0.01$) or lymphoma (261.0 ± 66.7 pg/mL, $p=0.02$). Concerning patients with cancer, plasma VEGF levels were similar between patients with oral cancer and lymphoma ($p=0.5$). Furthermore, VPC assays performed in the presence of bevacizumab, a soluble VEGF-inhibitor, and plated on fibronectin completely abrogated cluster formation in

a. Fibronectin

b. Laminin

FIGURE 3. Number of VPC clusters/mL of peripheral from healthy volunteers relative to controls (n=3). (a) Fewer VPC clusters formed on fibronectin when treated with anti- $\alpha 5\beta 1$ antibody than with anti- $\alpha 2\beta 1$ or IgG. (b) Fewer VPC clusters formed on laminin when treated with anti- $\alpha 2\beta 1$ antibody than with anti- $\alpha 5\beta 1$ or IgG. Means are shown with dashed lines. (*) denotes $p < 0.05$ compared to other mean values.

healthy controls (0 ± 0 clusters/mL *vs.* 27.5 ± 1.5 clusters/mL in controls) and markedly reduced VPC clusters from PB samples of patients with oral cancer (10.5 ± 1.5 clusters/mL *vs.* 150 ± 5 clusters/mL) (Figure 4). Cells from the identical samples incubated with bevacizumab and plated on laminin did not reduce VPC cluster numbers.

Next, MNCs from PB of healthy controls were exposed to elevated levels of VEGF and assessed the profile of VPC formation on fibronectin and laminin to address whether a pattern of increased VPC formation on fibronectin could be induced by VEGF. Increased numbers of VPC clusters were observed using MNCs from healthy control subjects incubated with VEGF (1 μ g/mL) when plated on fibronectin compared to controls without VEGF (167 ± 33 clusters/mL *vs.* 79 ± 29 clusters/mL, $p=0.04$). MNCs isolated from healthy peripheral blood samples did not produce greater numbers of VPC clusters on laminin when incubated with high levels of VEGF compared to controls without VEGF (67 ± 16 clusters/mL *vs.* 69 ± 12 clusters/mL, $p=0.9$).

Discussion

Our results from this small study highlight the role of specific integrin-mediated interactions with proteins of the extracellular matrix during the attachment of spindle-shaped cells in VPC cluster formation. Further, enhanced cluster formation on fibronectin, mediated by integrin $\alpha 5 \beta 1$ binding, was observed in patients with oral cancer and lymphoma whereas greater cluster formation on laminin, mediated by integrin $\alpha 2 \beta 1$ binding, was preferentially observed in control subjects. Our data demonstrate that enhanced VPC cluster formation on fibronectin is facilitated by VEGF and that blocking VEGF abrogates cluster formation on fibronectin but has no effect on cluster formation on laminin. Taken together, our work has identified an apparent pattern of VPC cluster formation on ECM proteins that may be specific to cancer and differs from healthy controls. Although more study is needed in a broader range of tumor types and stage of disease, our results may encourage the development of strategies to selectively block vascular progenitor recruitment to sites of tumor involvement without interfering with normal repair mechanisms by targeting VEGF-mediated changes in angiogenic attaching cells.

Consistent with our observations that VEGF enhances VPC cluster formation on fibronectin, VEGF elaborated by tumors may facilitate vascular precursors and hematopoietic cells with angiogenic potential to tumor microenvironments that are enriched for fibronectin, likely via VEGFR-1 and/or VEGFR-2 mediated cell interactions with specific integrins [32-34]. Once VPCs are in close proximity to fibronectin-rich

a) Cells plated on fibronectin

b) Cells plated on laminin

FIGURE 4. Blocking VEGF reduces VPC cluster formation on fibronectin but not laminin. (a) Blocking VEGF with bevacizumab abrogated VPC cluster formation from umbilical cord blood (•, $n=2$) and from peripheral blood of patients with oral cancer (o, $n=2$) when plated on fibronectin. (b) Incubating cells from cord blood and peripheral blood of patients with oral cancer with bevacizumab had no significant effect on VPC clusters/mL when plated on laminin.

tissues, cells expressing $\alpha 5\beta 1$ integrin may bind to fibronectin and produce signals that initiate angiogenesis [28]. Fibronectin and VEGF are key regulators of blood vessel growth, an observation that is supported by gene deletion studies that confirm fibronectin and VEGF, as well as their receptors $\alpha 5\beta 1$ integrin and VEGFR-2, are critical for vascular development [14]. Endothelial cells (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 [35]. 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 EC responses to growth factors [35,36]. Indeed, activation of $\alpha 5\beta 1$ integrin by VEGFR-2, in the presence of VEGF, appears to facilitate binding to fibronectin [37] and the continued activation of focal adhesion kinase and extracellular-regulated kinase, which can promote enhanced survival and proliferation of erythroid progenitors [38]. The activation of $\alpha 5\beta 1$ integrin by VEGFR-2 may also increase vascular precursor migration as a result of prolonged extracellular-regulated kinase activation [39]. It is possible that when the $\alpha 5\beta 1$ integrin and VEGF bind fibronectin in close proximity, fibronectin acts as a "chaperone" to support the sustained binding of VEGF to VEGFR-2 while simultaneously engaging $\alpha 5\beta 1$ [28]. As such, when fibronectin and VEGF are bound to their cognate receptors, cellular responses to VEGF are enhanced [35].

Based on the idea that there is an important interaction between the $\alpha 5\beta 1$ integrin and VEGF in a fibronectin-rich environment, $\alpha 5\beta 1$ integrin may be an important target for therapies aimed at preventing or slowing metastatic tumor growth. Indeed, preclinical models have suggested that selective antagonists of $\alpha 5\beta 1$ integrin can reverse tumor growth [40,41] and these inhibitors have been studied in preliminary clinical trials [42]. 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 depriving ECs from essential signals for growth and survival [40,41].

Although VEGF may have an important role in the setting of cancer, we recognize that it is elevated in other inflammatory conditions and is implicated in other biological processes. For instance, VEGF expression can be up-regulated in vascular cells at sites of inflammation in response to basic fibroblast growth factor, which is released by the action of proteases and heparinases found on the extracellular matrix [43]. In addition, uterine activation during labor is associated with increased production of pro-inflammatory cytokines which increase the synthesis of VEGF [44]. Additional studies in patients with acute

tissue injury may provide insight regarding the specificity of the pattern of VPC cluster formation on fibronectin that we observed in cancer patients in our study.

Defining cell populations most enriched for vascular precursor activity has been challenging. It appears VPC clusters include a heterogeneous cell population and specific cell surface molecules that allow for the prospective isolation of pure VPC populations in humans or other vertebrate species is lacking [45]. Moreover, the precise origin, proliferative potential and differentiation capacity of cells contributing to clusters observed using the VPC assay, also referred to as the CFU-Hill assay, has been recently summarized [46,47]. It appears that several cell types from different cell lineages likely contribute to neovascularisation during normal conditions and during disease. Chief among these angiogenic cell types are blood-derived monocytes that can form spindle-shaped attaching cells in the VPC assay [10-12]. Although there is a well described correlation between various health outcomes and the number of VPC clusters measured using the VPC assay, the relevance and impact of our observations requires confirmation using *in vivo* models where the extracellular matrix can be manipulated. The VPC assay, also referred as the CFU-Hill method, does not assess vessel-forming capacity which may limit its physiological relevance. Importantly, VPC assays typically use fibronectin alone and may not reflect the full scope of vascular progenitor function. In addition, other cell types associated with vascular repair may need to be examined to understand the full spectrum of integrin-mediated binding of vascular precursors to the extracellular matrix during health and in cancer patients. Additionally, the relatively small number of patient samples used in our study limits our ability to consider other variables which could influence VPC cluster formation. More study is required in a broader range of tumor types, in patients with various stages of cancer, and at different time points during treatment to confirm our observations and to appreciate the full scope of our findings. Moreover, advanced age [48], diabetes [49], smoking [50] and certain medications such as HMG-co reductase inhibitors [51] are known to influence the number of VPC clusters in the peripheral blood. Larger cohorts of patients will be needed to perform multivariable analysis of clinical factors that may influence the pattern of VPC cluster formation. Our results using the VPC cluster assay, however, provide a foundation for better understanding interactions between VPCs and extracellular matrix proteins.

In summary, our work suggests distinct profiles of VPC cluster formation seem to occur in cancer patients and our observations highlight the importance of integrin-specific binding of attaching cells to extracellular matrix proteins. VEGF

appears to be a key determinant in facilitating integrin-mediated binding to fibronectin through the $\alpha 5 \beta 1$ integrin. The downstream signaling effects of specific matrix proteins, such as laminin and fibronectin, which bind specific integrins on cells of the vascular repair system, require further investigation to better understand how mechanisms associated with tumor angiogenesis are distinct from normal repair processes.

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