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Abstract

Chemotherapy alteration of the bone marrow microenvironment has the potential to influence hematopoietic recovery following transplantation. To discern the effect of specific drugs on components of the complex marrow microenvironment, *in vitro* models have significant utility. In the current study we sought to determine whether dermal (HMEC-1) and marrow derived endothelial cells (BMEC-1) respond differently to identical chemotherapy exposure. BMEC-1 cells were consistently more sensitive to etoposide exposure than HMEC-1 cells, measured as reduced viability. BMEC-1 also had reduced focal adhesion kinase (FAK) and VCAM-1 protein expression following chemotherapy, in contrast to dermal derived endothelial cells in which neither protein was influenced dramatically by etoposide. The two endothelial cell lines had markedly different levels of baseline VE-Cadherin protein, which was modestly altered by treatment. These data indicate that marrow derived endothelial cells have disruption of specific proteins following chemotherapy that may influence their ability to facilitate hematopoietic cell entry or egress from the marrow. In addition, these observations suggest that while BMEC-1 and HMEC-1 share a variety of characteristics, they differ significantly in their response to stress and should be incorporated into specific models with this consideration.

Keywords: chemotherapy - endothelial cells - chemotaxis

Introduction

Efficient hematopoietic recovery following transplant requires a bone marrow microenvironment capable of supporting diverse processes. Transplanted stem and progenitor cells must home to the marrow, adhere to endothelial cells, and subsequently migrate to supportive hematopoietic niches. Bone marrow microvascular endothelial cells line the luminal surfaces of the marrow microenvironment in a position to serve as gatekeepers to the marrow microenvironment. They have a key role in the regulation of hematopoietic cell chemotaxis into and out of the marrow space. This function is regulated by the complex interplay of cellular adhesion molecules including vascular endothelial cell adhesion molecule-1 (VCAM-1),

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endothelial cell associated selectin (E-Selectin), vascular endothelial cell cadherin (VE-Cadherin), and many others.

Interactions between hematopoietic cells within the marrow space and endothelial cells have also been implicated in the control of hematopoietic differentiation and management of the hematopoietic progenitor compartment. Iatrogenic damage incurred by this intricate system, in the setting of dose intense chemotherapy or bone marrow transplant conditioning regimens, could potentially lead to dysregulated chemotaxis as well as hematopoiesis. In previous reports, radiation and chemotherapy induced damage of endothelial cells in the lungs and liver has been implicated in the pathophysiology of transplant complications [1-7].

Human umbilical vein endothelial cells (HUVEC) have served as the major model for investigating endothelial pathophysiology. These cells are relatively easy to derive and maintain for finite periods of time and as such, have broad utility. However, there is evidence to suggest that HUVEC may not be the most representative model to investigate all aspects of endothelial cell function.

Marrow derived endothelial cells have been shown to better support adhesion and migration of hematopoietic cells than HUVEC or lung endothelial cells [8,9]. Sulfation patterns of proteoglycans, important in the presentation of chemokines such as stromal cell derived factor-1 (SDF-1), have also been found to differ between the two cell sources [10,11]. HUVEC express high levels of Fas ligand (CD95) which is not expressed by endothelial cells from adult tissue sources [12]. Fas ligand induces activation of several proinflammatory cytokines which could influence cell adhesion, chemotaxis, and response to stress as well as inducing apoptosis in susceptible cells [13].

HUVEC also express higher levels of Steel factor and its receptor, Kit, compared to primary adult aorta cells either constitutively or following stimulation [14]. HUVEC expression of intracellular adhesion molecule-1 (ICAM-1) and endothelial linked adhesion molecule-1 (ELAM-1/E-Selectin) were found to be reduced in comparison to adult aortic endothelial cells in this study as well. Tan and colleagues compared human saphenous vein endothelial cells (HSVEC) with HUVEC and found that HSVEC could upregulate CD80 via CD40 ligation while HUVEC could not, that HSVEC do not express CD54, and that HUVEC were more responsive to stimulation by incitatory cytokines [15]. Together, these observations emphasize differences between endothelial cells of varied origins.

Much work has focused on irradiation induced endothelial cell damage. Many investigators have shown that irradiation results in alterations to endothelial cells including increased expression of adhesion molecules [16], cytokine production [17], von Willebrand factor [17], and increased apoptosis [18]. Gaugler, *et al*. have shown that irradiation of a human bone marrow endothelial cell line resulted in increased transmigration of CD-34+ cells [19] with increased permeability accompanied by increased ICAM-1, PECAM-1, and numerous myeloid differentiating cytokines. Investigations into the effects of chemotherapy agents on endothelial cells have centered on complications of veno-occlusive disease [1-3], and pulmonary fibrosis [5,20] with less focus on marrow endothelial cells.

In this study we compared alterations in survival, adhesion molecule expression, and chemotaxis across two endothelial cell lines following etoposide (VP-16) exposure. The human bone marrow endothelial cell line-1 (BMEC-1), derived from human bone marrow, and the human microvascular endothelial cell line-1 (HMEC-1) derived from human foreskins differ in adhesion molecule expression, survival, and ability to regulate chemotaxis following treatment. In addition, alterations in focal adhesion kinase (FAK) and VE-Cadherin protein in response to Etoposide (VP-16) were shown to be unique to the cell line evaluated. Collectively, these data suggest functional distinctions between the two cells lines that warrant careful consideration when designing models to study bone marrow alterations associated with chemotherapy exposure.

Materials and methods

Cell lines

Growth factor independent transformed microvascular endothelial cell lines were obtained from the CDC. The dermal derived microvascular endothelial cell line, HMEC-1, was initially derived by Dr. Thomas Lawley of Emory University and Dr. Edwin Ades and Mr. Francisco J Candal of the CDC [21,22]. The marrow

derived microvascular endothelial cell line, BMEC-1, was also a kind gift of Mr. Candal and Dr. Rafii [23]. Both cell lines were maintained and grown as previously described [21-23].

JM-1 cells were used to evaluate chemotaxis across endothelial cell layers established on transwell membranes. These factor independent cells were purchased from the ATCC (ATCC#CRL-10423) and were grown according to the recommendation of the depositor. They have a pre-B cell acute lymphocytic leukemia (pre-B ALL) phenotype, and are CXCR-4 and very late antigen-4 (VLA-4) positive.

Chemotherapeutic drugs

VP-16 was stored at -20°C at 20 mg/ml and was diluted in the appropriate media immediately prior to use at concentrations of 50, 75, and 100 μM. These chemotherapeutic drug doses were chosen to approximate doses described in clinical settings of transplantation and high-dose chemotherapy regimens [24].

Evaluation of endothelial cell viability

Confluent layers of BMEC-1 and HMEC-1 were pre-stimulated with 10 ng/ml tumor necrosis factor-α (TNF-α) for 24 hours and then exposed to VP-16 at concentrations of 50, 75, and 100 μM. After an additional 24 hours, cells were collected with Trypsin/ EDTA and analyzed by trypan-blue exclusion counting. The ratio of unstained cells compared to the total number of cells per culture condition was calculated as the percent survival. Adherent and non-adherent cells for each condition were analyzed. Each treatment group was evaluated in triplicate.

Assessment of apoptosis and flow cytometry

To evaluate apoptosis of endothelial cells following chemotherapy exposure, cells were stained with fluorescein isothiocyanate (FITC)-conjugated annexin-V using the TACS apoptosis detection kit (R & D systems, Minneapolis, MN). Adherent cells, as well as any cells that detached during treatment, were collected for evaluation. Following annexin staining, samples were evaluated by flow cytometry and data analyzed using CellQuest (Becton Dickinson, San Jose, CA).

FACS Analysis of endothelial cell adhesion molecule expression

Endothelial cell layers grown to confluency in tissue culture plates were pre-stimulated with TNF-α 10ng/ml 24 hours prior to exposure to VP-16 100 μM for 24 and 48 hrs. Cells receiving pre-stimulation only and unstimulated cells served as controls. Endothelial cells were then detached with Trypsin/EDTA and washed in cold phosphate buffered saline (PBS). Cells were then incubated for 30 minutes with 100ng/sample of the following antibodies: mouse-anti-human VCAM-1 (CD106, Pharmingen, San Diego, CA), mouse- anti-human-PECAM (CD-31), mouse-anti-human ICAM-1 (CD-54), mouse- anti-human-E-Selectin (CD-62E), P-selectin (CD-62P) or L-Selectin, (CD-62L, R&D, Minneapolis MN), diluted in PBS/3%BSA. Samples were subsequently washed with PBS/3%BSA/0.2% Tween 20 before incubating with 100ng/sample goat-anti-mouse IgG – RPE (Southern Biotechnology Associates, Birmingham, Ala) for 30 minutes. After additional washes with PBS/3% BSA/0.2% Tween 20, cells were fixed in 1% paraformaldehyde, collected on a FACScan (Becton Dickinson, San Diego, CA), and analyzed by CellQuest software (Becton Dickinson, San Diego, CA). Matched isotype control samples were stained in all experiments to evaluate nonspecific background fluorescence.

Chemotaxis Assays

HMEC-1 and BMEC-1 were grown to confluency in 100 mm dishes then transferred to Transwell inserts (5 μm pore size, Costar, Wiesbaden, Germany). The endothelial layers were pre-stimulated with TNF-α in the lower as well as upper chambers of the transwell for 24 hours to insure treatment of both sides of the endothelial layers. The membranes were then washed and moved to new chambers where they were exposed to 100 μM VP-16 for 24 hrs. Identically treated membranes were stained with Accustain (Wright stain, modified, Sigma, St. Louis, MO**)** to visually confirm maintenance of the endothelial layers on the transwell filter. After an additional gentle PBS rinse, the transwells were placed in chambers containing 350 μl media with or without 100 ng/ml SDF-1 in the lower chamber, and 150,000 JM-1 cells in 150 μl media in the upper chamber. After 4 hours, the media in the lower chamber was collected and the number of JM-1 cells that migrated

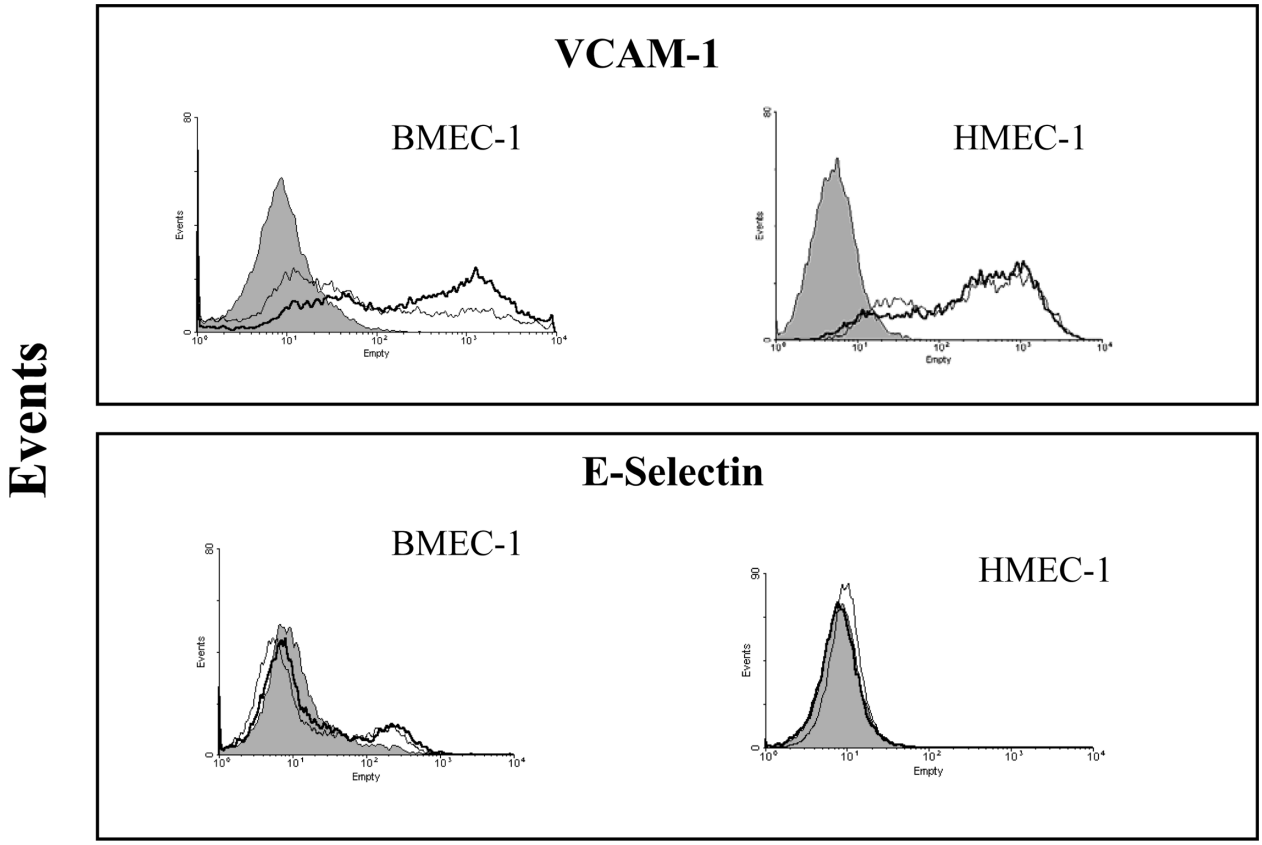

Fig. 1 VCAM-1 alterations following VP-16 exposure differ between BMEC-1 and HMEC-1. BMEC-1 VCAM-1 expression was decreased following VP-16 exposure while HMEC-1 VCAM-1 expression was unchanged. BMEC-1 expressed E-Selectin following TNF-α exposure, while HMEC-1 did not.

was enumerated. Unstimulated endothelial cell layers and stimulated, untreated cell layers served as controls. In addition, chemotaxis of JM-1 cells across transwells without endothelial cell layers was evaluated, with or without SDF-1 in the lower chamber, to measure spontaneous migration as well as chemotaxis toward SDF-1 without any barrier. All treatment groups were evaluated in triplicate.

Western Blot Analysis of FAK and VE-Cadherin

Confluent layers of endothelial cells were prestimulated with TNF-α for 24 hours then treated with 100 μM VP-16. Unstimulated cell layers and TNF-α prestimulated cells that were not exposed to chemotherapy served as controls. BMEC-1 or HMEC-1 cell pellets were lysed in CCLB buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% TritonX-100, 0.25% Na-deoxycholate, 0.5% SDS, 1 mM EDTA, 1 mM NaF, 1mM DTT, 1 mM PMSF, 1 mM activated Na_3VO_4, aprotinin, leupeptin, pepstatin) on ice for 15 minutes. Following centrifugation at 14,000 rpm for 15 minutes, supernatants were harvested and stored at -20 °C until use. Protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL). Protein samples were resolved on SDS-PAGE gels and electrotransferred to nitrocellulose membranes. Membranes were blocked in TBS/5% non-fat dry milk/0.05% Tween-20 at room temperature for 1 hour and probed with antibodies specific for human VE-Cadherin (Santa Cruz Biotechnology, Santa Cruz, CA) , or human FAK (BD Pharmingen, San Diego, CA). Isotype matched control antibodies were used to evaluate nonspecific binding. Following washes in TBS/0.1% Tween-20/3% milk, membranes were incubated with horseradish peroxidase labeled goat-anti-mouse IgG, and signal detected with Luminol. Signal for each protein of interest was normalized to GAPDH probed on the same blot.

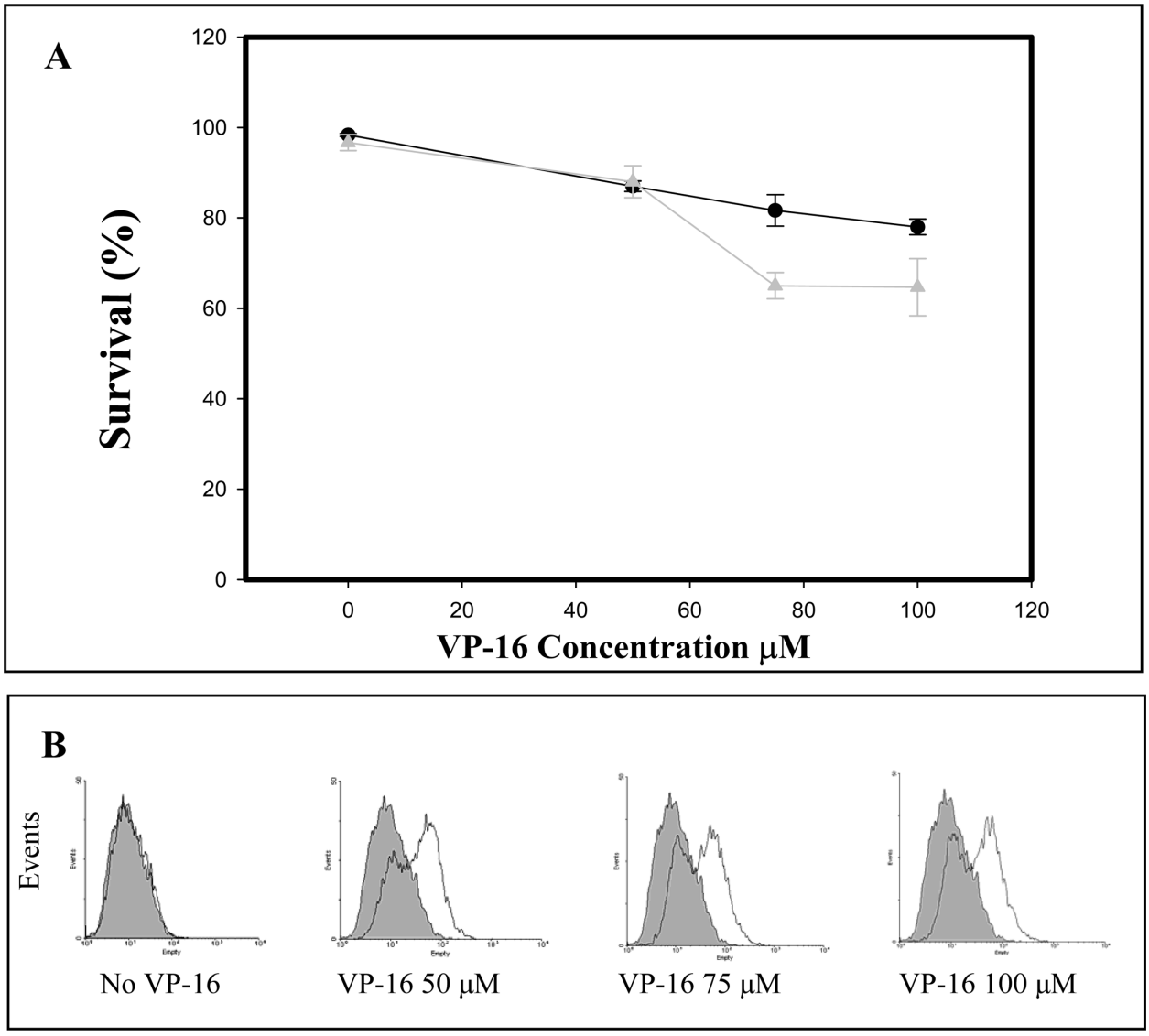

Fig. 2 BMEC-1 viability is reduced following exposure to VP-16. (a) Trypan blue exclusion of BMEC-1 (grey triangle) and HMEC-1 (black circle) following exposure to VP-16 at 50, 75, and 100 μM. (b) Annexin-V-FITC staining of BMEC-1 following exposure to VP-16.

Results

VCAM-1 alterations following VP-16 exposure differ between BMEC-1 and HMEC-1

To investigate changes in adhesion molecule expression by BMEC-1 and HMEC-1 following VP-16 exposure, cell layers were treated with VP-16 and analyzed by FACS as described in the materials and methods. Both cell lines expressed VCAM-1 following TNF-α stimulation as had been reported in the initial descriptions of these cell lines [22,23]. Alterations in VCAM-1 surface expression were detected following treatment with 100 μM VP-16 in BMEC-1 only (Fig. 1). VCAM-1 expression on HMEC-1 cells was not diminished under identical conditions (Fig. 1)

As previously reported [21,23], both endothelial cell lines constitutively expressed PECAM and ICAM (data not shown). P-Selectin and L-Selectin were not detected by FACS analysis (data not shown). BMEC-1 cells expressed E-Selectin following TNF-α exposure, while

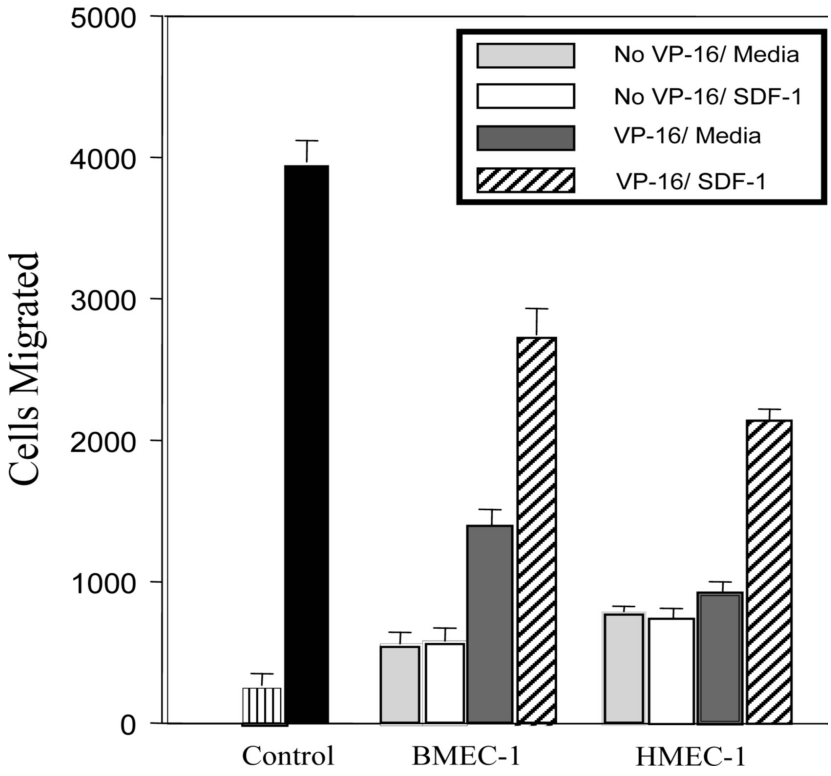

Fig. 3 JM-1 cell migration is increased following VP-16 treatment. The control group represents JM-1 migration across transwell membranes toward media alone (vertical bars) or 100 g/ml SDF-1(black bar). BMEC-1 or HMEC-1 represent JM-1 migration across the specific endothelial cell type indicated. Pretreatment of endothelial cells with VP-16 and chemotactic stimulus in the bottom well is indicated in the figure key.

HMEC-1 did not demonstrate TNF-α induced expression of this protein (Fig. 1). Surface expression of PECAM, ICAM, or E-Selectin, did not change in response to VP-16 treatment (data not shown).

BMEC-1 viability is reduced by VP-16 exposure more than HMEC-1

BMEC-1 were more sensitive to equal doses of VP-16 when compared to HMEC-1 by trypan blue staining (Fig. 2, A). Following exposure to 100 μM VP-16, the highest dose evaluated, mean survival of BMEC-1 cells was 65% (standard error (SE) 6.3%) as compared to mean HMEC-1 viability which remained at 78% (SE 1.7%). This difference was also observed following exposure to 75 μM VP-16 with a mean survival of BMEC-1 cells of 65% (SE 2.9%) and mean survival of HMEC-1 cells of 82% (SE 3.5%). Annexin-V-FITC staining of VP-16 treated BMEC-1 cells indicated that VP-16 induced cell death was apoptotic in nature, and did not differ significantly across the dosages tested (Fig. 2, B).

Hematopoietic cell chemotaxis is increased by VP-16 treatment of endothelial cell layers

To elucidate potential alterations in endothelial cell function following exposure to VP-16, chemotaxis assays were completed as described in the materials and methods section. Both marrow and dermal derived cell layers diminished chemotaxis across transwell membrane when compared to control wells without endothelial cell layers (Fig. 3). Exposure of endothelial cell layers to VP-16 resulted in increased spontaneous JM-1 cell migration across the endothelial layers, as well as JM-1 chemotaxis toward recombinant SDF-1 in the bottom chamber. Migration of JM-1 cells across VP-16 treated BMEC-1 was increased to a greater extent than across HMEC-1 treated identically.

Alterations in FAK and VE-Cadherin

To further investigate potential mechanisms for the alterations in chemotaxis patterns across BMEC-1 and HMEC-1 described above, Western blot analy-

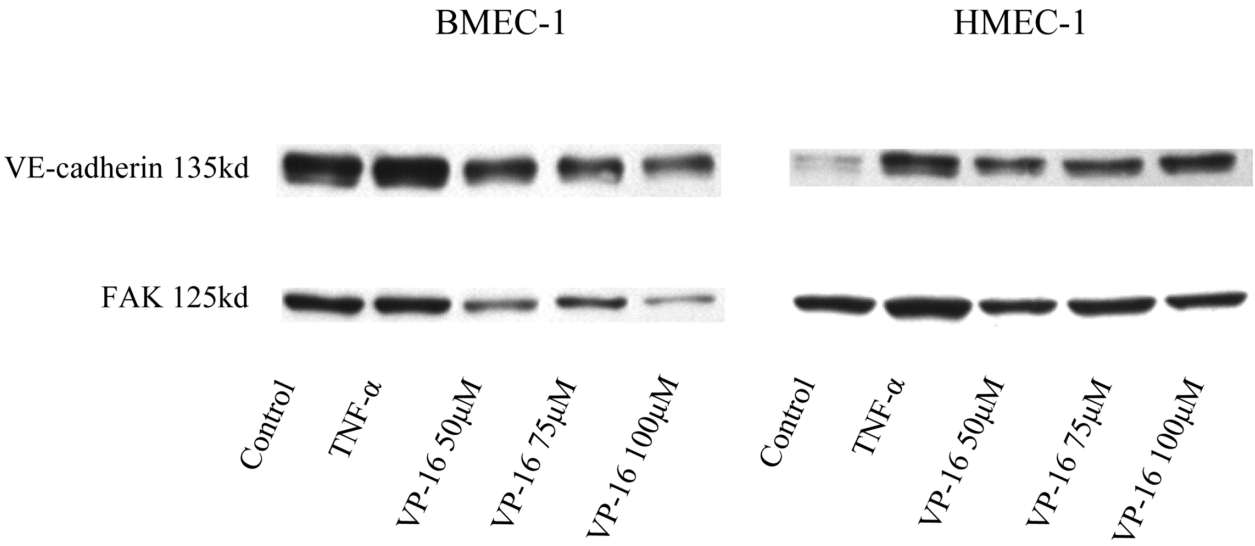

Fig. 4 Alterations in VE-cadherin and FAK following VP-16 exposure. Western analysis for VE-cadherin or FAK was carried out on BMEC-1 or HMEC-1 following prestimulation with TNF-α as indicated below the columns. Unstimulated cells were used as controls.

sis of FAK and VE-Cadherin were completed. Expression of either protein was normalized to GAPDH probed on the same membrane to control for lane loading error. VE-Cadherin protein was detected in both cell lines at baseline; however, BMEC-1 had higher baseline levels of VE-Cadherin than HMEC-1 (densitometry readings normalized to GAPDH of 4.8 and 0.4 respectively). Densitometric analysis indicated only modest changes in VE-Cadherin following exposure of TNF-α prestimulated cell lines to VP-16 (Fig. 4).

FAK was present at baseline in relatively equal amounts in both cell lines. Densitometric analysis indicated a reduction in TNF-α prestimulated BMEC-1 FAK levels following VP-16 exposure (densitometry readings normalized to GAPDH and control of 1.3 reduced to 1.1), while no change was detected in FAK levels of VP-16 treated HMEC-1 (Fig. 4).

Discussion

The success of bone marrow transplantation depends, in part, on the ability of transplanted hematopoietic progenitors to migrate into the host's marrow cavities and establish interactions with supporting cells of the microenvironment. Endothelial cells play a critical role in hematopoietic reconstitution by serving as the barrier between the blood spaces and marrow microenvironment. Transplanted stem or progenitor cells must efficiently interact with bone marrow endothelial cells prior to further migration into marrow niches that support hematopoietic survival and further development. Chemotherapy damage of these microvascular endothelial cells may disrupt the efficiency of this early, and essential, step in recovery.

In the current study we show that survival of BMEC-1 was decreased to a greater extent following VP-16 exposure than HMEC-1 (Fig. 2, A). Annexin-V-FITC staining of HMEC-1 indicated initiation of apoptosis in response to VP-16 exposure (Fig. 2, B). Differences in cell line sensitivity to stressors, such as exposure to chemotherapeutic agents, could potentially bias interpretation of results if not carefully considered. Comparison of several different endothelial cell lines, derived from different anatomic locations, may enable investigators to understand the mechanisms behind these differences and how they may influence pathophysiology in their specific original anatomic locations.

Alterations in VCAM-1 expression following VP-16 exposure were also found in this study (Fig. 1). VLA-4 binding to VCAM-1 is required for pro-

genitor cell attachment to endothelial cells and subsequent binding to stromal cells within the marrow parenchyma [24-27]. Unlike other ligand interactions, VLA-4 activation is required for functional binding to VCAM-1. This has been shown for VLA-4 interaction with VCAM-1 on endothelial cells, an essential initiating event in hematopoietic cell immigration into marrow spaces. Antibodies directed against VCAM-1 or VLA-4 have been previously shown to inhibit stem cell homing [28-30]. Treatment of human umbilical cord endothelial cells with etoposide has been shown to down-regulate VCAM-1 expression [28], indicating that modulation of adhesion molecule expression is one means by which chemotherapy may alter endothelial cell characteristics. Consistent with these reports, both BMEC-1 and HMEC-1 were found to have decreased expression of VCAM-1 following exposure to VP-16. VCAM-1 binding to activated VLA-4 also facilitates the majority of selectin-independent interactions at this site [29]. Consistent with the proposed importance of the role for VCAM-1 in stem cell homing, engraftment of SCID mice following transplantation of hematopoietic progenitor cells has been shown to be dependent on expression of VLA-4 and VLA-5 [33-35]. As such, these molecules are logical targets of investigation in attempts to better understand mechanisms that underlie disrupted hematopoiesis following aggressive chemotherapy treatment. Differential sensitivity and VCAM-1 expression responses exhibited by endothelial cell lines should be considered.

Initial interactions between transplanted hematopoietic cells and the microvascular endothelium involve engagement of selectins. Both E- and P-selectin are expressed constitutively on marrow endothelial cells. E-selectin has been shown to play a major role in initial binding of hematopoietic progenitor cells to marrow endothelium in an *in vitro* human bone marrow endothelial cell line model using the transformed line HBMEC [30]. Attachment of normal mobilized peripheral blood progenitors was blocked by anti-E-selectin antibody in this model. CD34+ cells also express ligands for P-selectin. In a NOD/SCID mouse model, mice lacking both P- and E-selectin expression were found to have a 90 percent reduction in homing of transplanted human CD34+ cells by intravital microscopy [31]. E-Selectin expressed on bone marrow endothelial cells has also been shown to play a potential role in the regulation of hematopoiesis through inhibition of proliferation of CD-34+ cells and induction of apoptosis in a subset of hematopoietic cells [32].

In the present study, and consistent with previous reports, BMEC-1 were found to express E-selectin following TNF-α stimulation by FACS analysis, while HMEC-1 did not express E-selectin (Fig. 1). BMEC-1 E-selectin expression was not altered following exposure to VP-16. This difference may contribute to the increased migration of JM-1 cells across BMEC-1 compared to HMEC-1 observed in this study. It also highlights the potential utility of using these two cell lines to further expand our understanding of the roles of E-selectin in hematopoiesis.

To further investigate potential factors that may contribute to the differences observed between BMEC-1 and HMEC-1 as regulators of JM-1 cell chemotaxis, western analysis of VE-Cadherin and FAK was completed (Fig. 4). VE-Cadherin is a transmembrane protein that is crucial to cell-cell adhesion between endothelial cells. Earlier reports indicate that antibody blocking of VE-Cadherin led to increased neutrophil extravasation in a mouse peritonitis model suggesting a role for this protein in regulating permeability of vascular endothelial cell layers [33]. In the current study, the VE-Cadherin level was modestly decreased in BMEC-1 following VP-16 exposure (Fig. 4), coincident with increased JM-1 cell chemotaxis toward SDF-1, as well as random migration across BMEC-1 cell layers (Fig. 3). Chemotaxis of JM-1 cells across VP-16 treated HMEC-1 was also increased compared to untreated control HMEC-1 layers but not to the same degree as that observed with BMEC-1. These observations suggest that altered VE-Cadherin may be one factor that contributes to increased permeability of bone marrow derived endothelial cells following VP-16 exposure. Theoretically, chemotherapy induced alterations of endothelial cell junction integrity may influence both the “ease” with which transplanted progenitor cells enter the marrow spaces, and may also affect the efficiency with which specific chemotherapeutic agents mobilize hematopoietic stem and progenitor cells.

Recent work has linked VCAM-1 engagement with decreased expression of VE-Cadherin on the surface of a human marrow derived endothelial cell

line independent of the cell line used in this report [34]. This process was found to be mediated by production of reactive oxygen species and led to accompanying reorganization of the cytoskeleton, widening of endothelial cell-cell gaps, and increased migration of CXCR4+ cells across endothelial layers towards an SDF-1 gradient. However, in the current study, BMEC-1 demonstrated a greater decrease in VCAM-1 expression following exposure to VP-16, while also demonstrating a greater promotion of chemotaxis. This suggests either a decreased role of VLA-4: VCAM-1 engagement in promoting intracellular gaps or alternative means of activation of this pathway in our model.

Focal adhesion kinase (FAK) is a major tyrosine kinase involved in signaling pathways downstream of focal adhesions [35]. Focal adhesions consist of integrins and the associated intracellular proteins linking them to the cytoskeleton. Interactions with cytoskeletal proteins in turn lead to changes in cell shape, mobility, and permeability of cell layers. Activation of FAK along with related adhesion focal tyrosine kinase (RAFTK/Pyk2) by vascular endothelial cell growth factor (VEGF) has been shown to result in accelerated construction of focal adhesions in neural microvascular endothelial cells [36,37]. Blocking of this pathway led to loss of focal adhesions, decreased spreading of the endothelial cells, and decreased endothelial cell migration [36,37]. In this study we report that exposure to VP-16 lead to modestly decreased levels of FAK in BMEC-1 alone. This difference may influence increased permeability of BMEC-1 layers, measured as increased JM-1 spontaneous migration or SDF-1 driven chemotaxis, compared to HMEC-1 layers following exposure to VP-16.

Taken together, differences between HMEC-1 and BMEC-1 following VP-16 exposure evaluated as viability, regulation of JM-1 cell chemotaxis, and VCAM-1, VE-Cadherin, and FAK protein expression demonstrate the need to consider the source of endothelial cells used in establishing *in vitro* models of the bone marrow microenvironment. Investigation of the mechanisms underlying the differences observed in this study may increase our understanding of the regulation of chemotaxis in the specific context of the bone marrow. Ultimately, a better understanding of chemotherapy induced changes in marrow endothelial cells may lead to improvements in bone marrow transplantation procedures including optimized conditioning regimens, supportive care to prevent endothelial cell related complications, and improvements in engraftment and patient outcomes.

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