

Homophilic PECAM-1(CD31) interactions prevent endothelial cell apoptosis but do not support cell spreading or migration

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SUMMARY

PECAM-1 (CD31) is a highly abundant cell surface glycoprotein expressed on haemopoietic and endothelial cells. As well as mediating homophilic (PECAM-1/PECAM-1) adhesion, PECAM-1 can also bind the integrin $\alpha v \beta 3$. Both PECAM-1 and $\alpha v \beta 3$ have been shown to have roles in regulating angiogenesis, endothelial tube formation and in the case of $\alpha v \beta 3$, endothelial cell apoptosis. In this study we show that despite being expressed at equivalent levels, endothelial $\alpha v \beta 3$ is not a ligand for PECAM-1. Rather, PECAM-1 supports homophilic binding on HUVEC with similar characteristics to those we have previously reported for leukocytes and becomes tyrosine phosphorylated after homophilic PECAM-1 and integrin/fibronectin engagement.

Immunoprecipitation studies show that in addition to SHP-2, tyrosine phosphorylated PECAM-1 can interact with at least four other phosphoproteins in pervanadate stimulated HUVEC. While PECAM-1/PECAM-1 interactions support robust endothelial cell adhesion, they do not support cell spreading or migration. In addition PECAM-1 homophilic adhesion rescues HUVEC from serum deprivation-induced apoptosis. Taken together our results indicate that PECAM-1 homophilic interactions play an important role in interendothelial cell adhesion, survival and signalling.

Key words. Endothelium, Adhesion, Apoptosis, CD31, PECAM-1, Signalling

INTRODUCTION

The ability of endothelial cells to proliferate and undergo apoptosis in a coordinated manner is essential for the formation of a continuous vessel lumen. While the initiation of endothelial cell tube formation is thought to critically depend on soluble angiogenic stimuli, cell adhesion molecules and proteolytic remodeling of the extracellular matrix have both been shown to play important roles in the angiogenic process (for recent reviews see Folkman, 1995; Stromblad and Cheresch, 1996; Risau, 1997; Bussolino et al., 1997; Sage, 1997; Shattil and Ginsberg, 1997). This is particularly the case for members of the αv integrin family, in particular the integrin $\alpha v \beta 3$, which in addition to mediating endothelial cell-matrix adhesion, also deliver signals that regulate endothelial cell survival (Brooks et al., 1994a).

Since initial observations that $\alpha v \beta 3$ could be detected on growing but not quiescent blood vessels, a number of studies have directly implicated this integrin in angiogenesis and embryonic neovascularization (Brooks et al., 1994a,b; Frieland et al., 1996; Stromblad et al., 1996; Scatena et al., 1998; Drake et al., 1995). Recent studies have shown that cell attachment via $\alpha v \beta 3$ is required for the maintenance of an

angiogenic program such that prevention of cell contact mediated by this integrin, leads to endothelial cell apoptosis (Brooks et al., 1994b; Stromblad et al., 1996). Intriguingly cyclic RGD containing peptide antagonists and blocking antibodies to $\alpha v \beta 3$ both induce endothelial cell apoptosis in a range of in vivo angiogenic models (Brooks et al., 1994a,b; Stromblad et al., 1996; Hammes et al., 1996). However, the same reagents when immobilized on tissue culture plastic prevent endothelial cell apoptosis (Stromblad et al., 1996). Thus the signals mediated by ligand/ $\alpha v \beta 3$ engagement can have potent effects on endothelial cell function and different ligands may have dramatically different consequences depending on the context in which they are engaged (Bischoff, 1997).

In addition to integrins, recent studies have identified a number of other cell adhesion molecules that are involved in endothelial cell tube formation. One of these is PECAM-1 (CD31), a 130 kDa member of the immunoglobulin superfamily, which is abundantly expressed on endothelium (Newman et al., 1990; Simmons et al., 1990). The molecular basis for its role in angiogenesis remains unexplained, but PECAM-1 may act as a docking molecule, promoting cell-cell adhesion thereby allowing other proteins, such as integrins, to

further stabilize the assembly of vascular structures. This relationship between PECAM-1 and integrins is particularly intriguing since engagement of PECAM-1 has been shown to activate integrins on a number of cells (Tanaka et al., 1992; Piali et al., 1993; Fawcett et al., 1995; Jackson et al., 1997a). However, the ability of PECAM-1 to activate integrins on endothelial cells and the role of PECAM-1 in inter-endothelial adhesion and apoptosis has remained largely unexplored. In addition, the molecular basis by which anti-PECAM-1 reagents mediate their functional effects on vascular cells has had to be re-examined, given recent findings that in addition to its adhesive properties, PECAM-1 can function as a signal regulatory protein, transducing intracellular signals in a variety of cells (reviewed by Newman, 1997, 1999).

PECAM-1 can mediate cell adhesion by a number of pathways. As well as binding to itself via homophilic interactions between domains 1-2 of PECAM-1 on apposed cells (Newton et al., 1997; J. Sun et al., 1996; Q. H. Sun et al., 1996), we and others have demonstrated that $\alpha v \beta 3$ is a heterophilic ligand for PECAM-1 (Piali et al., 1995; Buckley et al., 1996). Other reported heterophilic ligands include glycosaminoglycans (Muller et al., 1992; DeLisser et al., 1993; Prager et al., 1996) and CD38 (Horenstein et al., 1998). We and others have shown that anti-PECAM-1 antibodies can disrupt endothelial tube formation and that soluble recombinant PECAM-Fc fusion proteins prevent the growth of human endothelial cells (Fawcett et al., 1995; Saunders et al., 1995; Sheibani et al., 1997). The receptor-ligand pairings involved in these processes have remained unclear but have been assumed to be homophilic PECAM-1 interactions.

Given the functional effects of both $\alpha v \beta 3$ and PECAM-1 reagents on endothelial cells, we set out in this study to characterize the nature of PECAM-1 binding on endothelial cells, observe the consequences and examine potential intracellular signalling pathways involved in PECAM-1-mediated signalling. Despite expressing high levels of both PECAM-1 and $\alpha v \beta 3$, we have found that endothelial cells do not use the integrin $\alpha v \beta 3$ as a ligand for PECAM-1. However PECAM-1 can support robust homophilic endothelial cell adhesion but not cell spreading or migration. In addition, PECAM-1 homophilic engagement rescues HUVEC from apoptosis induced by serum withdrawal. Homophilic PECAM-1 interactions on endothelial cells lead to tyrosine phosphorylation. In addition to the protein tyrosine phosphatase SHP-2, endothelial cell PECAM-1 associates with at least four other phosphoproteins. These results suggest that PECAM-1 is a multifunctional signal regulatory protein involved in endothelial cell adhesion, signalling and survival but not cell spreading or migration.

MATERIALS AND METHODS

Monoclonal antibodies, recombinant fusion proteins and reagents

Unless otherwise stated, all materials were purchased from Sigma Chemical Co. (Poole, UK). The anti-PECAM-1/CD31 and anti- $\alpha v \beta 3$ monoclonal antibodies used in this study have all been previously described (Fawcett et al., 1995; Buckley et al., 1996). Antibodies to SHP-2 (clone 42) and anti-phosphotyrosine PY20 antibody were obtained from Affiniti Research Products Ltd, Mamhead, Exeter UK. A sheep anti-mouse horseradish peroxidase-conjugated secondary

antibody was purchased from Amersham (Bucks, UK). All antibodies were used according to suppliers' instructions. PECAM-1 domain deletion series and NCAM(D1-D5)Fc and VCAM(D1-D7)Fc soluble recombinant proteins were produced and purified as previously described (Fawcett et al., 1995; Buckley et al., 1996). The purity of all these proteins was greater than 90% by SDS-PAGE and all proteins were checked for conformational integrity using ELISA.

Cell culture and PECAM-1 tyrosine phosphorylation in HUVEC

HUVEC were isolated and maintained as described previously (Bird et al., 1993). Cells used in this study were between passages 2-4. For most studies cells were detached from gelatin coated plastic using 1 or 2 mM EDTA in PBS or 0.1% trypsin/EDTA. For PECAM-1 phosphorylation studies, HUVEC were allowed to adhere to 10 cm dishes (Life Technologies, Paisley, Scotland) coated with PECAM-1(D1-D6)Fc at 10 $\mu\text{g/ml}$, fibronectin at 5 $\mu\text{g/ml}$, or both PECAM-1 (10 $\mu\text{g/ml}$) in combination with fibronectin at either 0.1 or 0.5 $\mu\text{g/ml}$ for 15 minutes prior to lysis and immunoprecipitation. The amount of PECAM-1 immobilized was unaffected by the presence of fibronectin at either concentration as judged by ELISA from three separate experiments ($99.9 \pm 0.1\%$ for PECAM-1 and 0.1 mg/ml fibronectin and $100 \pm 0.2\%$ for PECAM-1 and 0.5 mg/ml fibronectin compared to PECAM-1 alone). In some experiments HUVEC grown in 15 cm dishes (Life Technologies) were stimulated with 100 μM pervanadate (1:1 mix sodium vanadate with hydrogen peroxide) for 15 minutes at 37°C, prior to lysis and immunoprecipitation.

Immunofluorescence flow cytometry and analysis

Cells were washed in 5 ml PBS and incubated with 50 μl of relevant antibody or control at appropriate dilution for 30 minutes on ice. After washing in PBS, cells were incubated for a further 30 minutes with a secondary antibody, goat anti-mouse phycoerythrin (Southern Biotechnology Associates, Birmingham, AL USA) in PBS. After washing cells were analyzed using a Coulter EPICS XL flow cytometer. The percentage positive was calculated with an arbitrary 1% cut off channel position of the negative control.

HUVEC binding assays

96-well adhesion assay plates (Immulon-3; Dynatech Research Laboratories, Chantilly, VA, USA) were precoated with 1 $\mu\text{g/well}$ goat anti-human IgGFc and non-specific sites blocked with 0.4% BSA (Fraction V, Sigma). Recombinant Fc proteins in PBS were added at 0.5 $\mu\text{g/well}$. BCECF-AM labeled HUVEC ($2 \times 10^4/\text{well}$) were added in adhesion buffer (RPMI 1640 containing 20 mM Hepes and 0.2% BSA) and incubated for 1 hour at 37°C. Cell binding was assessed by measuring fluorescence before and after 2-3 washes. Antibodies were used at 10 $\mu\text{g/ml}$ and preincubated with cells for 10 minutes before use. In experiments using manganese (Mn^{2+}) treatment of HUVEC, MnCl_2 was diluted in assay buffer to 0.5 mM final concentration. All adhesion assays were performed on at least three different occasions. Each data point represents the mean of three replicates and the data are expressed at the % of input cells bound ± 1 s.d.

HUVEC migration assays

Cell migration assays were performed as previously described (Leavesley et al., 1993) with minor modifications. Briefly, the base of 8 μm pore size Transwell inserts (Costar UK Ltd, High Wycombe, Bucks) were coated at 37°C for 30 minutes with 10 $\mu\text{g/ml}$ of extracellular matrix or Fc protein, diluted in HBS (20 mM Hepes, pH 7.45, 150 mM NaCl, 1.8 mM CaCl_2 , 1.8 mM MgCl_2 , 5 mM KCl, 5 mM glucose). 10^5 endothelial cells in HBS containing 1% BSA were added to the insert and left for 4 hours at 37°C to migrate. Non-migrated cells were removed from the upper face of the insert with a cotton swab. Migrated cells (those attached to the lower face) were fixed in 2.5% paraformaldehyde in PBS for 10 minutes and then stained with 0.1% aqueous Crystal Violet for 20 minutes. After

extraction with 10% acetic acid the sample absorbance at 550 nm was determined. Previous studies (Leavesley et al., 1993) have shown that the reading is proportional to the number of cells migrated. Background migration was determined using NCAM-Fc as an irrelevant Fc protein or medium alone.

Photomicrography

HUVEC were allowed to adhere to wells on a 24-well plate (Life Technologies) precoated with 5 $\mu\text{g/ml}$ of PECAM(D1-6)Fc protein, vitronectin or fibronectin for 1 hour at 37°C. After three washes in adhesion buffer cells were fixed in 2.5% paraformaldehyde, and viewed using phase contrast optics on a Zeiss Axiovert microscope. Fields of cell were photographed using a Nikon UFX-DX camera loaded with Kodak Ektachrome 100ASA film.

Serum deprivation-induced apoptosis assay

Sterile glass coverslips in 6-well culture plates (Life Technologies) were coated with various proteins; gelatin, fibronectin at 5 $\mu\text{g/ml}$, PECAM(D1-D6)Fc, VCAM-1(D1-D7)Fc all at 10 $\mu\text{g/ml}$ or PBS alone for 18 hours at 4°C, washed in PBS and blocked with 1% BSA (tissue culture grade) in PBS for 1 hour at 37°C. After washing in PBS, HUVEC in chemically defined medium containing 4% BSA but lacking serum (Re et al., 1994) were added at 3.5×10^5 cells per well in 1.5 ml medium. After 24 hour incubation at 37°C coverslips were washed gently to remove unattached cells before nuclear fragmentation was detected by Acridine Orange staining (5 $\mu\text{g/ml}$ in PBS for 1-2 minutes) exactly as described (Meredith et al., 1993). Samples were coded and cells viewed using the $\times 16$ and $\times 40$ dry objective of a Zeiss fluorescent microscope. Five separate fields (100 cells per field) were scored and the percentage of cells with fragmented nuclei calculated.

Immunoprecipitation and western blotting

Cells (typically 10^7) were lysed in 1 ml lysis buffer (20 mM Tris-HCl, pH 8, 1% Triton X-100, 150 mM NaCl, 1 mM Na_2VO_4 , 1 mM AEBSF, 1 $\mu\text{g/ml}$ leupeptin). After centrifugation, the lysate was precleared using Protein G-Sepharose for 30 minutes at 4°C. Precleared lysate was then incubated with specific antibody for one hour before addition of Protein G-Sepharose and incubated overnight at 4°C. Alternatively, immunoprecipitation was carried out by adding relevant monoclonal coupled to Protein G-Sepharose and incubated overnight at 4°C. Sepharose was then washed three times with lysis buffer, boiled in SDS-PAGE sample buffer and eluted proteins resolved using 10% SDS-PAGE. After transfer to PVDF membrane samples were probed with monoclonal antibodies and developed with ECL (Amersham) according to the manufacturer's instructions. All samples were controlled for protein loading by measuring protein concentration using a BCA protein assay (Pierce and Warriner, Chester, UK)

RESULTS

HUVEC express high levels of both PECAM-1 and $\alpha\text{v}\beta 3$

Previous reports have shown that vascular cells express a variety of integrins as well as other hematopoietic cell differentiation molecules (Lampugnani et al., 1991; Mutin et al., 1997). However, $\alpha\text{v}\beta 3$ expression has been reported to be restricted to growing but not quiescent blood vessels (Brooks et al., 1994a). In contrast, PECAM-1 is expressed constitutively on almost all endothelia. In order to examine the relative amounts of PECAM-1 and $\alpha\text{v}\beta 3$ present on HUVEC used in our experiments, cells were stained with appropriate antibodies and examined by fluorocytometry. Fig. 1

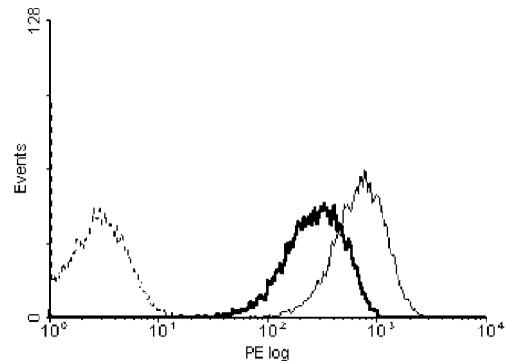


Fig. 1. HUVEC express high levels of $\alpha\text{v}\beta 3$ and PECAM-1. HUVEC were stained for FACS analysis as detailed in Materials and Methods. Anti- $\alpha\text{v}\beta 3$ (clone LM609) and anti-PECAM-1 (clone 9G11) staining are represented by a continuous line and bold line, respectively. Matched isotype control is represented by a broken line.

demonstrates that the HUVEC used in our studies expressed high levels of both PECAM-1 and $\alpha\text{v}\beta 3$.

HUVEC support homophilic PECAM-1/PECAM-1 but not heterophilic PECAM-1/ $\alpha\text{v}\beta 3$ mediated adhesion

In order to examine whether PECAM-1 could act as a ligand for $\alpha\text{v}\beta 3$ expressed on endothelial cells we performed cell adhesion assays in which endothelial cells were allowed to bind to recombinant purified PECAM-1. For comparison, we also examined adhesion of HUVEC to extracellular matrix proteins. Fig. 2A shows that PECAM-1 supported specific HUVEC attachment. However this binding was not as avid as that to collagen I, vitronectin or fibronectin. Further proof that binding of HUVEC to PECAM-1 was specific is indicated by the fact that function-blocking antibodies, clones L133.1, and 5.6E (Fawcett et al., 1995), but not a non-functional anti-PECAM antibody, clone 9G11, inhibited binding to almost background levels. Background binding in these assays was assessed by binding to the control Fc protein, NCAM-Fc, or BSA and was less than 5%.

To further characterize the nature of PECAM-1 mediated binding to HUVEC the ability of function blocking antibodies to $\alpha\text{v}\beta 3$, clone LM609, and αv , clone L230, both known to inhibit the binding of PECAM-1 to $\alpha\text{v}\beta 3$ (Buckley et al., 1996) was examined. As shown in Fig. 2B, neither LM609 nor L230, or a control non-functional anti- $\alpha\text{v}\beta 3$ antibody, 23C6, were able to reduce the binding of HUVEC to purified PECAM-1. As further supporting evidence for a homophilic rather than heterophilic pattern of binding, the ability of a nested series of PECAM-1 truncation mutants to support HUVEC attachment was examined. Only PECAM(D1-D6)Fc was able to support significant HUVEC binding (Fig. 2C). Previous work has shown that the pattern of homophilic PECAM-1/PECAM-1 binding to these nested truncation mutants is characteristic and distinct from heterophilic PECAM-1/ $\alpha\text{v}\beta 3$ interactions (Buckley et al., 1996). These results therefore suggest that despite the high levels of expression of $\alpha\text{v}\beta 3$ on HUVEC, these cells bind PECAM-1 predominantly via a homophilic mechanism rather than via endothelial $\alpha\text{v}\beta 3$.

Studies with other endothelial cell types of lung and dermal microvessel origin gave similar results supporting only homophilic and not $\alpha\text{v}\beta 3$ -mediated heterophilic adhesion (C.

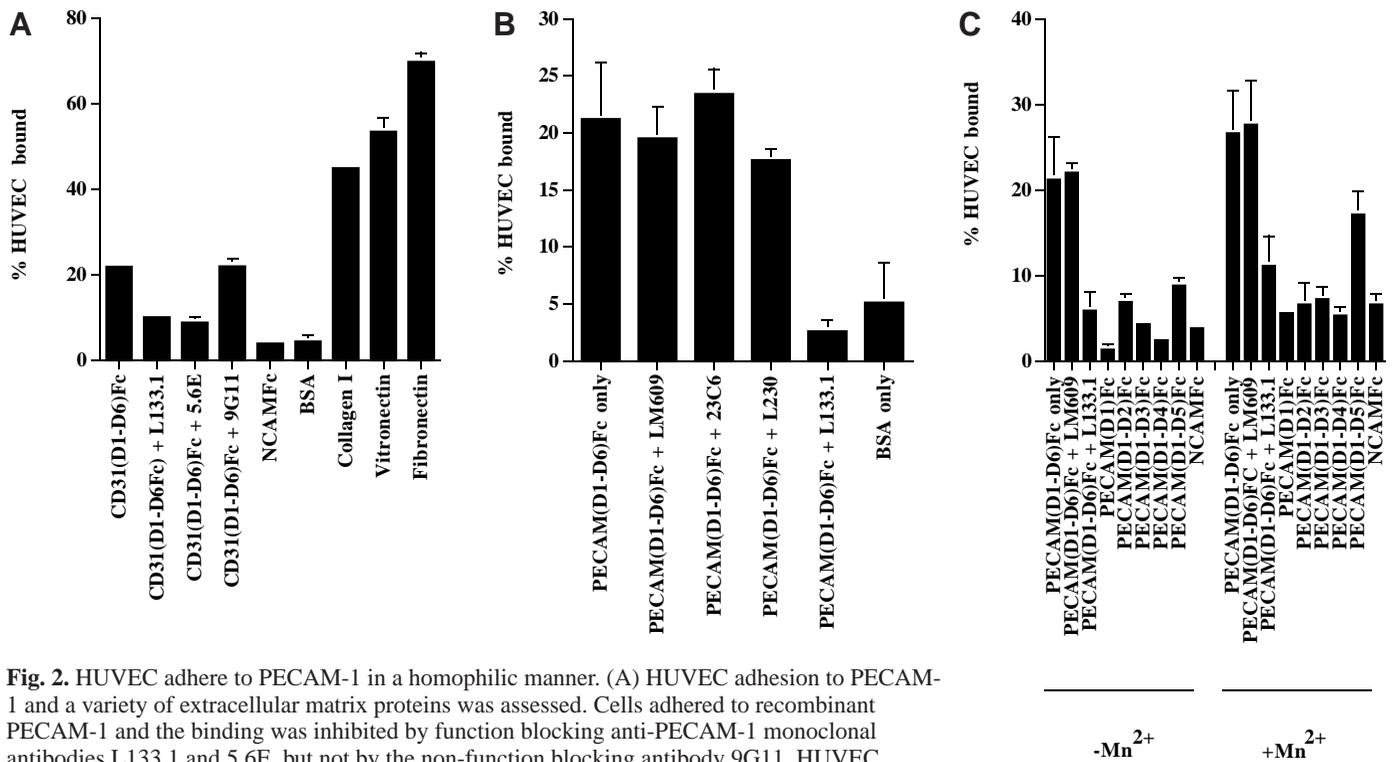


Fig. 2. HUVEC adhere to PECAM-1 in a homophilic manner. (A) HUVEC adhesion to PECAM-1 and a variety of extracellular matrix proteins was assessed. Cells adhered to recombinant PECAM-1 and the binding was inhibited by function blocking anti-PECAM-1 monoclonal antibodies L133.1 and 5.6E, but not by the non-function blocking antibody 9G11. HUVEC binding to PECAM-1 was not as avid as to extracellular matrix proteins collagen I, vitronectin or fibronectin. The background level of binding in this assay is indicated by adhesion of HUVEC to an irrelevant Fc control protein, NCAM-Fc or BSA. (B) HUVEC adhesion to recombinant PECAM-1 was assessed in the presence or absence of function blocking anti- α v β 3 (LM609), anti- α v (L230), anti-PECAM-1 (L133.1) or non functional anti- α v β 3 (23C6) antibodies. Cell adhesion to PECAM-1 was unaltered by anti- α v β 3 antibodies. However, anti-PECAM-1 antibody reduced cell adhesion to background (BSA) levels. (C) HUVEC adhesion to a C-terminal domain deletion series of recombinant PECAM-1 was determined either in the presence or absence of 0.5 mM Mn²⁺. HUVEC only bound PECAM-1 containing all six extracellular domains. Mn²⁺ did not alter HUVEC adhesion profile to the PECAM-1 domain deletion series. The inhibition of HUVEC binding to PECAM-1 by anti-PECAM-1 antibody L133.1 was Mn²⁺ insensitive. Anti- α v β 3 (LM609) did not affect binding either in the presence or absence of Mn²⁺. All the above data are representative of at least three experiments (mean \pm s.d.).

D. Buckley and I. N. Bird, unpublished results). One *in vitro* tool that has been used to activate integrins generically is Mn²⁺ ions. Even when Mn²⁺ ions were added to the assay buffer HUVEC were still unable to adhere to PECAM-1 in a heterophilic manner as measured by the pattern of binding to PECAM-1 truncation mutants (Fig. 2C) and by the inability of the blocking α v β 3 antibody LM609 to block binding. Interestingly Mn²⁺ increased endothelial cell binding to PECAM-1(D1-D5) but not to any of the other domain constructs. Pretreatment of HUVEC with TNF α did not change the cell adhesion profile to PECAM-1 from that seen with non-activated HUVEC (data not shown). In summary, these results suggest that PECAM-1 is unlikely to be a physiologically important ligand for endothelial cell α v β 3.

HUVEC adhere to, but do not spread or migrate on PECAM-1

In contrast to the attachment and spreading of HUVEC on collagen, fibronectin and vitronectin, HUVEC rapidly attached but did not spread on PECAM-1, even after 60 minutes (Fig. 3). This observation prompted us to examine the ability of PECAM-1 to support endothelial cell migration. Previous studies have shown that HUVEC migrate in the absence of serum, growth factors or exogenous chemoattractants, through a microporous membrane, towards extracellular matrix, in a

time and concentration dependent manner (Leavesley et al., 1993). In such assays, PECAM-1 was unable to support HUVEC migration through the Transwell polycarbonate filters, even when coated at high density (Fig. 4). As previously reported other extracellular adhesive substrates such as collagen, fibronectin and vitronectin, were all able to induce HUVEC migration (Fig. 4). When PECAM-1 was admixed with these matrix proteins, endothelial cell migration was unaffected (data not shown). These results imply that PECAM-1/PECAM-1 interactions can neither initiate, nor modulate established endothelial cell migration.

PECAM-1 engagement rescues HUVEC from serum deprivation-induced apoptosis

The adhesion of endothelial cells to extracellular matrix and the attainment of an appropriate cell shape have been shown to be crucial for endothelial cell survival and proliferation (Meredith and Schwartz, 1997; Re et al., 1994). When endothelial cells are cultured under conditions that prevent adhesion and spreading, they stop growing and die by apoptosis. In order to examine whether PECAM-1 mediated adhesion of HUVEC could modify cell survival, HUVEC were plated onto different adhesive substrates and apoptosis monitored after 24 hours in chemically defined medium lacking serum but containing growth factors (Fig. 5).

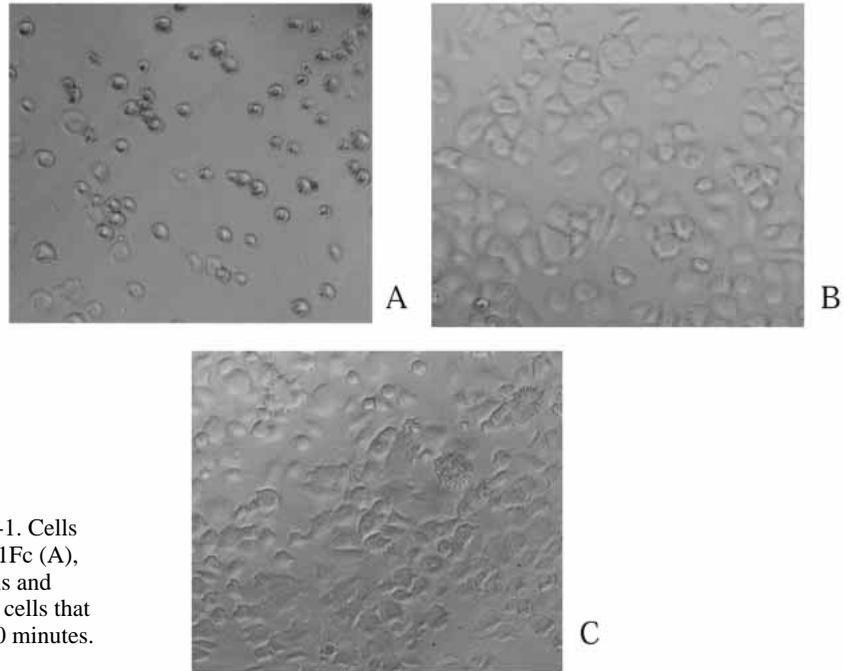


Fig. 3. HUVEC adhere to but do not spread on PECAM-1. Cells were allowed to settle on surfaces coated with PECAM-1Fc (A), fibronectin (B) or vitronectin (C), as detailed in Materials and Methods. HUVEC adhered to all three surfaces but only cells that settled onto fibronectin or vitronectin had spread after 60 minutes. (original magnification $\times 400$).

Apoptosis was assessed by staining cells with Acridine Orange and scoring for nuclear fragmentation. Attachment of HUVEC to PECAM-1(D1-D6)Fc but not a control Fc fusion protein VCAM-1(D1-D7)Fc rescued cells from apoptosis. As expected

there was minimal apoptosis of cells adherent to gelatin but substantial nuclear fragmentation in cells adherent to BSA coated glass slides. These results suggest that, PECAM-1 homophilic adhesion prevents serum deprivation-induced apoptosis in those HUVEC that adhere to PECAM-1.

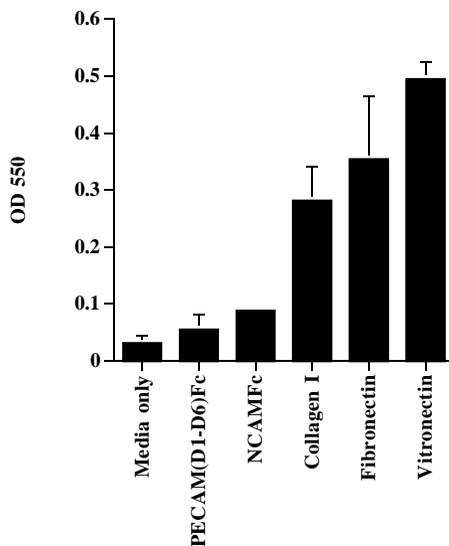


Fig. 4. PECAM-1 does not support HUVEC haptotactic migration. To determine whether PECAM-1 could support HUVEC migration, 8 μ m pore diameter Transwell filters were coated with either PECAM-1Fc, NCAMFc, Collagen I, fibronectin or vitronectin. HUVEC were added into Transwells in chemically defined media and transmigration measured after 4 hours as described in Materials and Methods. Migration was only observed through Transwells coated with the extracellular matrix proteins. In the presence of PECAM-1, HUVEC migration was at levels equivalent to media only and NCAMFc controls. The OD₅₅₀ is directly proportional to the number of transmigrated cells. Data shown is representative of 5 experiments (mean \pm s.d.).

Adhesion of HUVEC to PECAM-1 and fibronectin leads to PECAM-1 tyrosine phosphorylation

Previous studies have shown that PECAM-1 expressed on platelets and leukocytes becomes phosphorylated upon integrin mediated cell-cell and cell-matrix interactions (Jackson et al.,

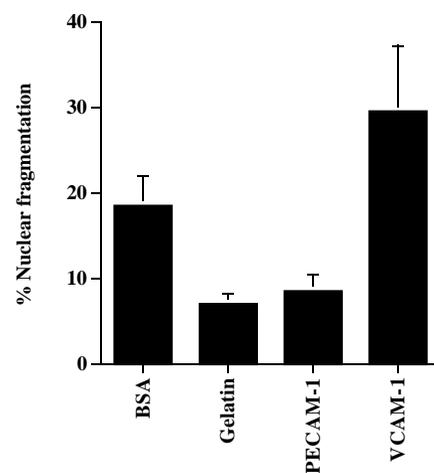


Fig. 5. HUVEC adhesion to PECAM-1 prevents serum deprivation-induced apoptosis. HUVEC were added to glass coverslips coated with BSA, gelatin, PECAM-1Fc and VCAM-1Fc in media lacking serum. After 24 hours adherent cells were stained with Acridine Orange and nuclear fragmentation assessed as described in Materials and Methods. VCAM-1 is included as control Fc fusion protein. Bars represent the average of 5 separate fields (100 cells per field) (\pm s.d.).

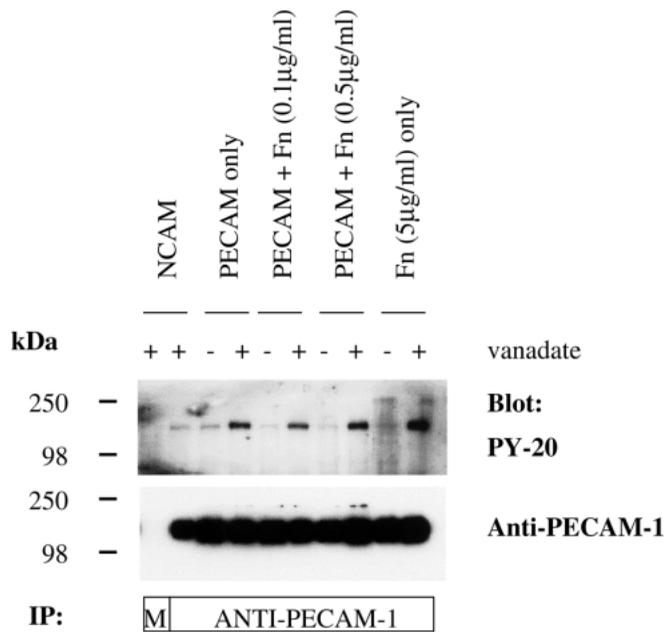


Fig. 6. Homophilic PECAM-1/PECAM-1 interactions and HUVEC adhesion to fibronectin promotes PECAM-1 tyrosine phosphorylation. HUVEC were allowed to adhere to wells coated with either PECAM-1Fc (10 μg/ml) and fibronectin (5 μg/ml) alone or PECAM-1Fc (at 10 μg/ml) with varying concentrations of fibronectin. Adhesion to NCAMFc was used as an Fc fusion protein containing control. After 15 minutes, cells were lysed in lysis buffer with (+) or without (-) vanadate. After immunoprecipitation (IP) with control mouse Ig (M) or anti PECAM-1 (9G11) blots were probed with anti-phosphotyrosine (PY20) antibody or anti PECAM-1 (9G11) as a gel loading control. Molecular mass markers are shown on the left.

1997a,b; Sagawa et al., 1997a). A further report using endothelial cells has suggested that PECAM-1 becomes dephosphorylated upon integrin mediated adhesion to extracellular matrix proteins (Lu et al., 1996). In order to examine the effects of HUVEC attachment to PECAM-1 and extracellular matrix proteins on the phosphorylation of the cytoplasmic region of PECAM-1, we examined cells allowed to adhere to PECAM-1, fibronectin or combinations of the two adhesive substrates, in the presence and absence of vanadate, in the lysis buffer in order to preserve any transient phosphorylation on PECAM-1. Fig. 6 shows that when PECAM-1 immunoprecipitates were examined for tyrosine phosphorylation using the phosphotyrosine specific antibody PY20, both PECAM-1 and fibronectin were able to induce tyrosine phosphorylation on PECAM-1. Mixtures of the two adhesive substrates did not significantly modify the level of tyrosine phosphorylation relative to either substrate alone. Since the PECAM-1 protein used in these assays was an Fc chimeric protein we used NCAM(D1-D5)Fc as a control Fc fusion protein to eliminate any potential effects that the Fc portion might have on PECAM-1 phosphorylation via Fc receptor signalling (Sagawa et al., 1997b). Both VEGF and crosslinking HUVEC with the functional anti-PECAM-1 antibody PECAM-1.2 (Varon et al., 1998) lead to tyrosine phosphorylation of PECAM-1 (data not shown). These data therefore suggest that adhesion of HUVEC to both PECAM-1

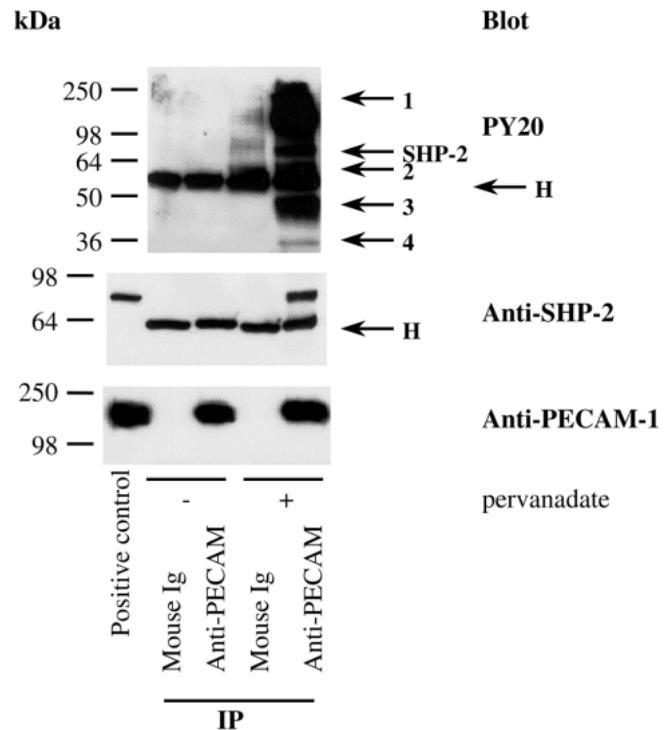


Fig. 7. PECAM-1 associates with SHP-2 and at least 4 other proteins in pervanadate stimulated HUVEC. HUVEC treated with or without pervanadate (+, -) lysed, immunoprecipitated (IP) either with control mouse Ig or anti-PECAM-1 (9G11) and then blotted with anti-phosphotyrosine (PY20 top panel), anti SHP-2 (clone 42 middle panel) or PECAM-1 (9G11 bottom panel) antibodies as indicated. Jurkat cell lysate was run as a positive control for the respective blots. Molecular mass standards are shown to the left. Arrows (numbered 1-4) denote additional phosphotyrosine proteins associated with PECAM-1. SHP-2 (72 kDa) is also shown. The background band between 50 and 64 kDa is the IgG heavy chain (H).

and fibronectin is able to directly induce PECAM-1 tyrosine phosphorylation and supports previous data showing cross-talk between integrins and PECAM-1 in platelets (Varon et al., 1998; Jackson et al., 1997b) and endothelial cells (Lu et al., 1996).

Phosphorylated PECAM-1 binds SHP-2 and associates with at least four other phospho-tyrosine containing proteins in HUVEC

Recent observations in platelets and leukocytes have shown that the activation of immune receptors and integrin mediated aggregation leads to tyrosine phosphorylation of PECAM-1 which then associates with the protein tyrosine phosphatase SHP-2 (Varon et al., 1998; Sagawa et al., 1997a,b). Others have shown that PECAM-1 is a major SHP-2 binding protein in bovine endothelial cells (Masuda et al., 1997). In order to examine whether phosphorylated PECAM-1 could associate with other phosphotyrosine containing proteins in HUVEC, we treated cells with pervanadate to maximally stimulate tyrosine phosphorylation on PECAM-1. Cell lysates were then immunoprecipitated with anti-PECAM-1 antibody and immunoprecipitates analysed by immunoblotting with anti-phosphotyrosine antibodies (Fig. 7). In addition to PECAM-1

(molecular mass 120-130 kDa) and SHP-2 (molecular mass 72 kDa), tyrosine phosphorylated PECAM-1 was able to associate with other phosphoproteins with molecular masses of 36 kDa, 45 kDa and 65 kDa. There also appeared to be an additional major phosphotyrosine containing protein which co-immunoprecipitated with PECAM-1 and which migrated above the PECAM-1 band. In order to estimate the relative stoichiometry of the PECAM-1 associated proteins, a coomassie stain of the immunoprecipitated proteins was performed which showed that the most abundant co-precipitating protein was SHP-2 (data not shown).

DISCUSSION

We and others have previously demonstrated that PECAM-1 can mediate both homophilic and heterophilic adhesion to the integrin $\alpha v \beta 3$ (Piali et al., 1993; Fawcett et al., 1995; Buckley et al., 1996). In view of the similarities in the distribution of $\alpha v \beta 3$ and PECAM-1 and the fact that PECAM-1 like $\alpha v \beta 3$ has been implicated in many aspects of endothelial cell biology including tube formation and angiogenesis, we set out in this study to examine whether PECAM-1 was a functional ligand for $\alpha v \beta 3$ expressed on endothelial cells. In addition given the emerging signalling properties of PECAM-1 (Newman, 1999), we examined the functional consequences and downstream signalling events associated with PECAM-1 engagement in these cells.

Despite high levels of expression of both $\alpha v \beta 3$ and PECAM-1 on HUVEC, PECAM-1 is not a ligand for $\alpha v \beta 3$ when expressed on endothelial cells, even when stimulated with Mn^{2+} . Others have suggested that homophilic PECAM-1 binding is favoured in areas of high PECAM-1 density (J. Sun et al., 1996). We observed no change in homophilic PECAM-1 binding after cytokine stimulation, where PECAM-1 is more diffusely spread over the cell (Ioffreda et al., 1993; Romer et al., 1995). Various sized mRNAs and protein isoforms of PECAM-1 have been reported in HUVEC and other cells, some of which engage only in heterophilic and not homophilic adhesion (Zehnder et al., 1992; Kirschbaum, and Newman, 1993; Goldberger et al., 1994; Kirschbaum et al., 1994; Famiglietti et al., 1997; Almendro et al., 1996). However, it remains unclear which PECAM-1 isoform(s) are expressed by endothelial cells and what function they perform. While it is clear that PECAM-1 can bind $\alpha v \beta 3$, in cell free systems, with a calculated affinity of 4 nM (A. Sharma and C. D. Buckley, unpublished), our data suggest that it is unlikely that $\alpha v \beta 3$ /PECAM-1 engagement is of any physiological significance in endothelial cells. However, heterophilic PECAM-1/ $\alpha v \beta 3$ binding is likely to play a role in endothelial-leukocyte interactions particularly when endothelial expressed PECAM-1 is presented to $\alpha v \beta 3$ expressed on leukocyte or tumor cells (Brown, 1997; Imhof et al., 1998; Rainger et al., 1999).

PECAM-1 mediated HUVEC binding observed in our studies has the same characteristics as PECAM-1 homophilic binding previously reported for leukocytes (Fawcett et al., 1995; Buckley et al., 1996). Maximal stable binding requires all six extracellular Ig domains of the molecule (but not the cytoplasmic tail), is Mn^{2+} insensitive and is divalent cation independent. Our results are in agreement with those of others (Q. H. Sun et al., 1996) who observed the binding of

proteoliposomes containing full length PECAM-1 or an IgG fusion protein containing the 6 extracellular domains of PECAM-1, to HUVEC. The fact that endothelial microvessel cells, like HUVEC, bound PECAM-1 only in a homophilic manner, suggests that PECAM-1-PECAM-1 homophilic interactions are the common mode of binding on endothelial cells.

Since anti-PECAM-1 antibodies have been shown to inhibit endothelial tube formation and angiogenesis, and cell adhesive events are known to be important in regulating angiogenesis (reviewed by Bischoff, 1997) we examined whether purified recombinant PECAM-1 protein could support HUVEC cell migration. We were unable to demonstrate any effect of these proteins in either initiating cell migration, or in preventing migration mediated by matrix proteins such as fibronectin, collagen or vitronectin (Fig. 4). Others have suggested that PECAM-1 expression in bovine aortic endothelial cells and the immortalized endothelial cell line ECV304 transfected with PECAM-1 modulates endothelial sheet migration on type I collagen (Kim et al., 1998). However, sheet migration differs from cell motility; our migration assays were much shorter (4 hours versus 5-7 days), examined individual cell migration, not endothelial sheets and studied migration stimulated by PECAM-1/PECAM-1 interactions rather than overexpression of PECAM-1. Our results are consistent with the findings of Lampugnani et al. (1995) who found that PECAM-1 does not partition with the Triton-insoluble fraction of endothelial cell lysates, suggesting that PECAM-1 does not associate with the cytoskeleton in endothelial cells.

In this study we show that both PECAM-1 homophilic engagement and adhesion to fibronectin result in tyrosine phosphorylation of PECAM-1 in HUVEC. This supports previous data showing cross-talk between integrins and PECAM-1 and extends the number of pathways that lead to PECAM-1 phosphorylation to include homophilic engagement. While our observations in HUVEC, and those from studies in platelets (Varon et al., 1998), rat basophilic leukaemic RBL-2H3 cells and Jurkat T cells (Sagawa et al., 1997a) show that cell adhesion to matrix proteins such as fibronectin leads to PECAM-1 tyrosine phosphorylation, others (Lu et al., 1996) have shown that integrin-mediated adhesion in endothelial cells leads to dephosphorylation of PECAM-1 and changes in endothelial cell motility. The reasons for these differences remain unclear but may be related to the time at which tyrosine phosphorylation was measured.

Of the five tyrosine residues in the cytoplasmic tail of PECAM-1 only one (Y686) has been reported as being a substrate for Src and Csk family protein-tyrosine kinases (Masuda et al., 1997; Lu et al., 1997; Cao et al., 1998). However, both Y663 and Y686 form consensus binding motifs for SH2 domains of SHP-2 and are required for the association with SHP-2 in human platelets, rat basophilic leukaemic RBL-2H3 cells and endothelial cells (Jackson et al., 1997a,b; Sagawa et al., 1997a,b; Lu et al., 1996; Masuda et al., 1997) and SHP-1 in COS-1 cells transfected with PECAM-1, SHP-1 and Lck (Cao et al., 1998). In order to determine whether additional phosphotyrosine signalling molecules might interact with PECAM-1 in endothelial cells, we examined PECAM-1 immunoprecipitates from endothelial cells that had been stimulated with pervanadate. As has been reported by others in human embryonic kidney 293 cells (Jackson et al., 1997b) we

identified a number of potential PECAM-1-associated phosphoproteins in HUVEC. Experiments to identify these potential signalling molecules are currently underway. It is likely that SHP-2 accounts for the majority of the associated proteins since only this protein could be detected in coomassie stains of the co-precipitated PECAM-1 proteins.

Homophilic engagement of PECAM-1 on HUVEC protects cells from serum deprivation-induced apoptosis. Other groups have shown that β integrin occupancy is not enough to rescue endothelial cells from apoptosis; integrin occupancy and cell shape change are both required (Re et al., 1994). Cells adhering to PECAM-1 alone did not undergo shape change but remained round (Fig. 3). This suggests that PECAM-1 mediated interactions on HUVEC might regulate apoptosis in different manner to integrins. We have previously shown that recombinant PECAM-1Fc fusion proteins inhibit endothelial cell growth in subconfluent HUVEC cells (Fawcett, 1995). It is therefore tempting to speculate that PECAM-1 homophilic interactions, through the association with cytoplasmic signalling proteins such as SHP-2, might prevent endothelial cell apoptosis, perhaps by localizing these associated proteins to the sites of endothelial cell-cell contact (Yang et al., 1996). Thus, in addition to the known requirement for adhesion to extracellular matrix in order for endothelial cells to survive (Re, 1994), it is possible that PECAM-1-PECAM-1 interactions at endothelial cell borders may provide an additional survival but non-proliferative signal for these cells (Korff and Augustin, 1998).

In summary, these studies have further characterized the role of PECAM-1 on endothelial cells. Our observations suggest that the main role of PECAM-1 on endothelium is not to support strong adhesive interactions but to act as an adhesion-dependent signalling receptor. Such a model for PECAM-1 has been suggested from studies in platelets (Newman, 1997) basophilic leukaemic cells (Sagawa et al., 1997), monocytes (Chen et al., 1994), natural killer cells (Poggi et al., 1996) and is fully consistent with the findings that PECAM-1 is an important component of the mechanosensing machinery and junctional signalling complex in endothelial cells (Osawa et al., 1997; Litwin et al., 1997). Like a growing number of cell surface receptors, PECAM-1 appears to act as a multifunctional signal regulatory protein capable of interacting with both protein tyrosine kinases and phosphatases and other as yet unidentified cytoplasmic signalling molecules (Newman 1999). How these different cytoplasmic signalling molecules interact with the cytoplasmic domain of PECAM-1 and the functional consequences of these interactions in different blood and vascular cells represents an important area for future research.

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