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## Identification of $\alpha_v\beta_3$ as a heterotypic ligand for CD31/PECAM-1

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### SUMMARY

CD31 (PECAM-1) is a member of the immunoglobulin gene superfamily (IgSF) and has an important role in a number of endothelial cell functions including angiogenesis, inflammation, integrin activation and cell-cell adhesion. CD31 has both homotypic and heterotypic adhesive properties and in common with other IgSF members contains multiple functional domains. Using chimaeric fusion proteins of CD31 and a panel of haematopoietic cell lines we show that CD31 can bind cells in a predominantly homotypic or heterotypic manner depending on the cell line used. Heterotypic binding was found to be cation and temperature dependent and enhanced by  $Mn^{2+}$ : all features of integrin mediated

binding. Using a panel of anti-CD31 and anti-integrin antibodies we show that  $\alpha_v\beta_3$  is a ligand for CD31 on the monocytic cell line U937. The specificity of the interaction between  $\alpha_v\beta_3$  and CD31 was further confirmed by solid phase binding assays and the use of  $\alpha_v\beta_3$  transfected cells which bound CD31 specifically. Furthermore, we have mapped the binding site for  $\alpha_v\beta_3$  to domains 1 and 2 of CD31. The interaction of CD31 with  $\alpha_v\beta_3$  may be important in many aspects of endothelial function including leukocyte-endothelial transmigration and angiogenesis.

Key words: Cell adhesion molecule, Integrin, Endothelium, Monocyte

### INTRODUCTION

CD31 (PECAM-1) is a cell adhesion molecule (CAM) the extracellular domain of which consists of six Ig C2-related domains (Simmons et al., 1990; Newman et al., 1990; Stockinger et al., 1990). It is highly expressed on endothelium, platelets, about 50% of lymphocytes and cells of myeloid lineage (De Lisser et al., 1994b). It is also expressed on CD34<sup>+</sup> haematopoietic progenitor cells in human bone marrow (Watt et al., 1993, 1995a,b). In both endothelial and monocytic cells the turnover of CD31 at the cell surface is rapid; the  $t_{1/2}$  being approximately 24 hours (Goldberger et al., 1994).

CD31 has been implicated in a number of biological events, primarily involving inter-endothelial adhesion (Muller et al., 1989; Schimmenti et al., 1992) and leukocyte-endothelial adhesion and transmigration (Muller et al., 1993). A number of animal model studies, using CD31 antibodies, have shown that CD31 is involved in monocyte and neutrophil recruitment during inflammation (Vaporciyan et al., 1993; Bogen et al., 1994). However, CD31 expression on leukocytes is not a prerequisite for transendothelial migration (Bird et al., 1993).

CD31 is also involved in adhesion amplification via  $\beta_1$  and  $\beta_2$  integrins (Tanaka et al., 1992; Piali et al., 1993; Berman and Muller, 1995). The molecular basis of this cross talk between CD31 and integrins is undefined. However, it is not restricted to haematopoietic cells as it can also occur in heterologous

primate cells (Fawcett et al., 1995). Moreover, co-ligation of CD31 with Fc $\gamma$ RII in monocytes leads to the production of pro-adhesive cytokines (Chen et al., 1994). CD31 has also been implicated in T cell and platelet activation with both de novo phosphorylation on serine and threonine residues and cell surface redistribution of CD31 being observed as cells are activated (Newman et al., 1992; Zehnder et al., 1992; Romer et al., 1995). Together these results suggest that CD31 may act as a signalling molecule.

CD31 mediated adhesion is complex involving both homotypic and heterotypic interactions. Homotypic binding involves extensive interdigitation of CD31 on apposing cells and requires all six domains. Key binding sites reside in domains 2-3 and 5-6 (Fawcett et al., 1995). A heterotypic binding site is located within domain 2 which contains a consensus sequence for glycosaminoglycan (GAG) binding (Muller et al., 1992; DeLisser et al., 1993). However, domain 6 also appears to be involved in heterotypic binding as the epitopes for two monoclonal antibodies that block heterotypic but not homotypic binding map to this domain (DeLisser et al., 1994; Yan et al., 1995). CD31 therefore appears to contain multiple functional domains capable of mediating quite different ligand-receptor interactions. How these adhesive options are regulated is not fully understood but a direct role for the cytoplasmic tail has been demonstrated (Baldwin et al., 1994; DeLisser et al., 1994a).

The CD31 gene has a relatively complex structure encompassing more than 65 kb of DNA organized into 16 exons (Kirschbaum et al., 1994). At least three alternatively spliced forms have been identified, one of which encodes a soluble form lacking the transmembrane domain (Goldberger et al., 1994) and in the mouse several splice variants of the cytoplasmic tail have been defined. In view of the recent report that soluble forms of E-selectin and VCAM-1 can act as potent angiogenic stimuli (Koch et al., 1995), soluble and secreted variants of CD31 may have a role in angiogenesis.

Studies on the role of CD31 have mainly concentrated on its function on endothelium and leukocytes. However, the role of CD31 in haematopoietic development is poorly understood. Haematopoietic progenitor cells as well as macrophages in the stroma of long term bone marrow cultures both express CD31 (Watt et al., 1993, 1995a,b). Moreover, ligation of CD31 on CD34<sup>+</sup> haematopoietic cells seems to result in the activation of VLA-4 but not LFA-1 providing further evidence for the role of CD31 as an adhesion amplifier (Leavesley et al., 1994).

In order to explore the repertoire of binding modes of CD31 on haematopoietic cells we screened a panel of cell lines representing various stages of myeloid and lymphoid lineage development for adhesion to recombinant chimeric forms of human CD31. We found that some cell lines (KG1B, MIK ALL) bound to CD31 in a predominantly homotypic manner whereas others (U937) bound in a predominantly heterotypic manner. Recently, another laboratory examining the adhesion of murine lymphokine-activated killer (LAK) cells and pro-T-cells to recombinant forms of truncated murine CD31 reported that the integrin  $\alpha_v\beta_3$  is an additional receptor for CD31 (Piali et al., 1995). In agreement with these findings, we confirm that in the human, CD31 can mediate heterotypic adhesion to  $\alpha_v\beta_3$ . Furthermore, by using a series of truncated soluble recombinant forms of CD31 in combination with blocking monoclonal antibodies we map the integrin heterotypic binding site to domains 1 and 2 of CD31.

## MATERIALS AND METHODS

### Cells and reagents

All the cell lines used in this study except MIK-ALL were provided by the Imperial Cancer Research Fund (ICRF) Cell Production Service and were maintained in RPMI/10% FCS. MIK-ALL was cultured as previously described (Watt et al., 1993). The production of K562 cells stably transfected with  $\alpha_v\beta_3$  (Kavb3) or vector alone (KRc/RSV) has been described (Blystone et al., 1995). These cells were maintained in either RPMI or IMDM/10%FCS containing 1.2 mg/ml G418 (Genticin, Gibco). All reagents used were from Sigma Chemical Co., unless otherwise specified.

### Antibodies

The following monoclonal antibodies (mAbs) were used. Anti-CD31 mAbs were: 9G11 (Simmons et al., 1990); L133.1 (Becton Dickinson, Oxford, UK); hec 7.2 (Endogen, TCS Biologicals Ltd, Bucks, UK); 5.6E (Immunotech, Marseille, France); 10B8 (British Bio-technology, Oxford, UK); CLB/CD31 (Monosan, Bucks, UK); HC1/6 (Serotec, Kidlington, UK); JC70A (provided by Dr J.Cordell, John Radcliffe Hospital, Oxford, UK); CAL31.1 IgG1 (which maps to domain 6 of CD31) was generated in mice after immunisation with a CD31(D1-D6)-Fc chimeric fusion protein. Anti-integrin antibodies were: anti- $\alpha_2$  integrin clone HAS-6 (ICRF Biotherapeutics and Hybridoma Development Unit, South Mimms, UK); anti- $\alpha_3$  integrin clone PIB5

(Becton Dickinson, Oxford, UK); anti- $\alpha_4$  integrin clone HP2/1 (Immunotech, Marseille, France); anti- $\alpha_5$  integrin clone IIA1 (PharMingen, Cambridge Bioscience, Cambridge, UK); anti- $\alpha_6$  integrin clone GoH3 (Serotec, Kidlington, UK) anti- $\alpha_v$  integrin clone L230 (provided by Dr J. Marshall, Richard Dimpleby Department of Cancer Research, ICRF Laboratory, Guy's and St Thomas's Hospital, London UK); anti- $\beta_1$  integrin clone 13 (Becton Dickinson, Oxford, UK); anti- $\beta_2$  integrin MHM23 (Dako, Bucks, UK); anti- $\beta_3$  integrin clone RUU-PL 7F12 (Becton Dickinson, Oxford, UK); anti- $\alpha_v\beta_3$  integrin clone LM609 (Chemicon International, Harrow, UK) and clone 23C6 (ICRF Biotherapeutics and Hybridoma Development Unit, South Mimms, UK). Control antibody was anti-VCAM clone BBIG-V1 (R&D systems, Abingdon, Oxford). Polyclonal goat anti-human-Fc Ig was obtained from Sigma Chemical Co.

### Chimeric fusion proteins

The CD31 chimeric fusion proteins used in this study consisted of the appropriate NH<sub>2</sub> terminal IgC2 domains of CD31 fused to the Fc region of human IgG1, and have been previously described (Simmons, 1993; Fawcett et al., 1995). A negative control chimeric IgSF-Fc fusion protein (MUC18-Fc) was generated as described previously (Fawcett et al., 1992). All Fc chimeric proteins were purified from COS-1 cell supernatants as previously described (Simmons, 1993), and checked by SDS-PAGE.

### Cell adhesion assays

Cell adhesion to soluble chimeric fusion proteins was carried out as previously described (Fawcett et al., 1995) with the following modifications. 96-well adhesion assay plates (Immulon-3; Dynatech Research Laboratories, Chantilly, Virginia, USA) were precoated with 1  $\mu$ g/well goat anti-human IgGFc and nonspecific sites blocked with 0.4% BSA (Fraction V). Recombinant Fc proteins in PBS were added at 0.5  $\mu$ g/well. This concentration of Fc protein was shown to be saturating by both ELISA and adhesion assays. Cells were labelled by incubation with the fluorescent dye BCECF-AM (Molecular Probes, Oregon, USA) for 30 minutes and washed twice in assay buffer (RPMI-20 mM Hepes, 0.2% BSA). The labelled cells were added at  $1 \times 10^5$  cells/well and incubated for 30-35 minutes at 37°C. Plates were washed with prewarmed assay buffer until the cells in the control wells were sufficiently removed as monitored by visual inspection. Typically this was 2-3 times. The attached cells were quantitated using a fluorescence plate reader Cytofluor II (Millipore, Watford, UK) and the percentage of input cells bound calculated after measuring the total fluorescence prior to washing. For some assays, cells were labelled for 24-48 hours with [<sup>3</sup>H]thymidine (Amersham, Bucks, UK) adherent cells lysed in 1% SDS and incorporated radioactivity counted using a Beckman LS 5000 CE counter.

In experiments where mAbs were added, cells were preincubated with antibodies at a final concentration of 10  $\mu$ g/ml for 10 minutes at room temperature prior to plating and included throughout the assay. In experiments to analyse the effects of Mn<sup>2+</sup> on cell binding, MnCl<sub>2</sub> was diluted in assay buffer at a final concentration of 0.5 mM and added to the plated cells in each well.

All adhesion assays were performed on at least two independent occasions. Each data point represents the mean of three replicates and the data are expressed as the % of input cells bound  $\pm$  1 s.d.

### Purification of $\alpha_v\beta_3$ and solid-phase binding assay

$\alpha_v\beta_3$  was purified from the human melanoma cell line DX3. Cells were lifted with 2 mM EDTA in PBS, washed in PBS and lysed in 50 ml of 150 mM NaCl, 2 mM CaCl<sub>2</sub>, 0.02% NaN<sub>3</sub>, 1% NP40, 20 mM Tris-HCl, pH 7.5, 1 mM PMSF, 5  $\mu$ g/ml leupeptin, 5  $\mu$ g/ml aprotinin, on ice for 1 hour. The cell lysates were centrifuged at 26,000 rpm and then passed over a 23C6 affinity column (prepared by coupling 23C6 to CNBr-activated Sepharose (Pharmacia) according to the manufacturer's instructions). The column was washed in 150 mM NaCl, 2 mM CaCl<sub>2</sub>, 0.02% NaN<sub>3</sub>, 0.1% NP40,

20 mM Tris-HCl, pH 7.5, and bound material eluted with 150 mM NaCl 1% octyl-glucopyranoside, 10 mM sodium acetate, pH 3.1, and 0.5 ml fractions collected and neutralised with 0.1 ml 1 M Tris-HCl, pH 8.0. Pooled fractions were concentrated using a centricon concentrator (Amicon), and aliquots stored at  $-70^\circ\text{C}$ . The integrin preparation was pure as judged SDS-PAGE (Fig. 6A) and ELISA using anti- $\alpha_v\beta_3$  mAbs.

Solid-phase binding was performed using a modification of the method of Charo et al. (1990). Purified  $\alpha_v\beta_3$  (0.2 mg/ml) was diluted 1:50 in TBS 0.02%  $\text{NaN}_3$  containing 1 mM  $\text{CaCl}_2$  (Buffer A) and 100  $\mu\text{l}$  aliquots added to a 96-well Nunc-Maxisorp ELISA plate (Gibco, Life Technologies). Plates were incubated overnight at  $4^\circ\text{C}$  and blocked with 200  $\mu\text{l}$  5% BSA in buffer A for 1-3 hours at room temperature. Wells were then washed 3 times in TBS, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MnCl}_2$ , 1 mg/ml BSA (Buffer B). Aliquots (100  $\mu\text{l}$ ) of the Fc protein ligands (at 5  $\mu\text{g}/\text{ml}$ ) were added with or without mAbs and the plate incubated at  $30^\circ\text{C}$  for 3 hours. Wells were washed 3 times in buffer B and bound Fc ligand detected by the addition of peroxidase-conjugated anti-human Fc antibody (1:2,000) in buffer B for 30 minutes at room temperature. Wells were then washed 4 times in buffer B and colour developed using OPD substrate (Sigma). The level of non specific binding was determined by the level of ligand binding to wells coated with BSA alone; these values were subtracted from all samples.

### Immunofluorescence

Cells were incubated in PBS 0.2% BSA, 5 mM azide at  $4^\circ\text{C}$  for 20 minutes with the appropriate antibody at 10  $\mu\text{g}/\text{ml}$  followed by FITC labelled goat anti-mouse Ig (or in some cases anti-rat Ig). The median fluorescence intensity was measured on a FACScan (Becton Dickinson, Oxford, UK) and at least 2,000 cells per sample were analysed.

### Synthetic peptides

Synthetic peptides LKREKN, NKERKL and REKNLK, used in this study, were synthesised on a Zinsser SMPS 350 multiple peptide synthesiser using standard Fmoc solid phase chemistry and were purchased from Zinsser Analytic, Maidenhead, Berks, UK. These peptides have been used in a previous study (DeLisser et al., 1993) and correspond to residues 150-155 of CD31 (LKREKN), the reverse sequence (NKERKL) and a peptide with the same residue in a scrambled order (REKNLK). Both the 150-155 peptide and its reverse sequence have been shown to block glycosaminoglycan (GAG) mediated heterotypic binding (DeLisser et al., 1993). The peptides were purified by high performance liquid chromatography (HPLC) prior to use and sequence confirmed by mass spectrometry. All three peptides were water soluble and stored in PBS at  $-20^\circ\text{C}$  prior to use.

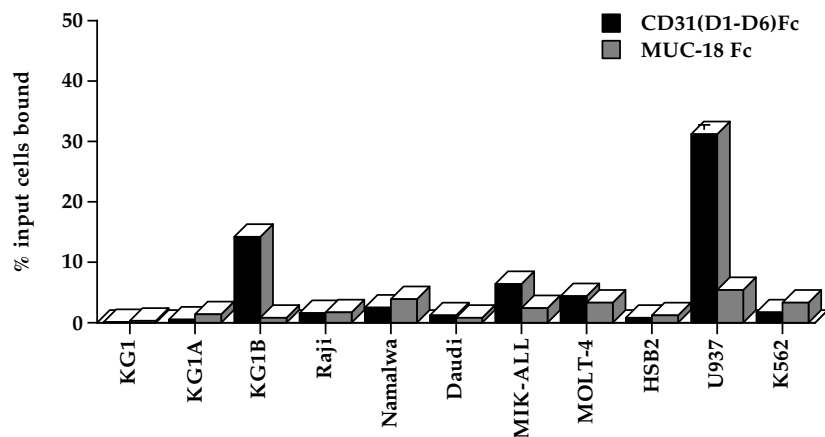
## RESULTS

### Cell-chimeric protein adhesion screen for CD31 binding modes

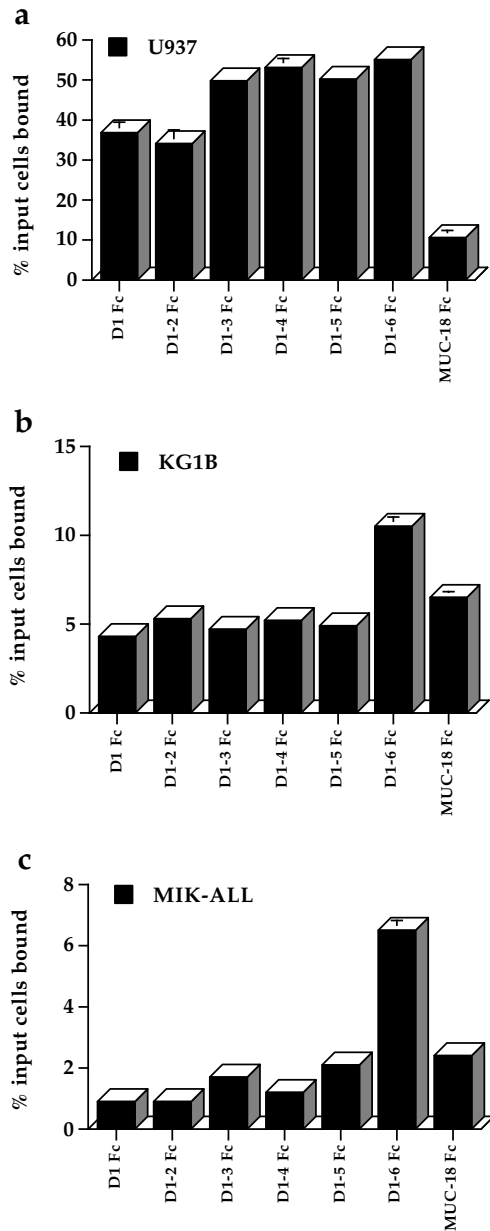
In order to further explore the binding modes of CD31, we screened a panel of 11 haematopoietic cell lines representing different lineages and stages of development: KG1, KG1A and KG1B (myeloblastic progenitors); Raji, Namalwa and Daudi (B cell), MIKALL (pre-B cell); Molt 4 and HSB2 (T cell); U937 (pro-monocyte), and K562 (erythroleukaemic), for their ability to bind to soluble recombinant CD31(D1-D6)Fc chimeric protein immobilized on plastic via anti-Fc polyclonal antibodies (Fig. 1). The Fc fusion proteins were used at 0.5  $\mu\text{g}/\text{well}$ . This system eliminates the possibility of accessory cell-cell interactions which may contribute to background binding in a two-cell system, and is quantitative and reproducible. Moreover, it allows for normalisation of the amount of chimeric protein presented to input cells. We have previously used this approach to define and dissect the homotypic binding mode of CD31 (Fawcett et al., 1995). Of the 11 cell lines screened, three (KG1B, MIK-ALL and U937) bound constitutively and specifically to CD31(D1-D6)Fc and not to a control IgSF-Fc fusion protein MUC-18Fc. (Fig. 1). All three of these cell lines express CD31 on their surface. However, there was no correlation between the level of CD31 expressed by these cells and their ability to bind to CD31(D1-D6)Fc. The binding of the pro-monocytic cell line U937 to CD31(D1-D6)Fc was much stronger than any homotypic adhesion previously measured on haematopoietic cells e.g. MIK-ALL (Fawcett et al., 1995) and suggested that additional mechanisms of CD31 adhesion were operating in this cell line. This was confirmed by the following analysis.

### Pattern of cell binding to CD31(D1-D6)Fc deletion series

To further define the nature of CD31 mediated adhesion to U937, KG1B and MIK-ALL, we examined the ability of a nested series of chimeric truncation mutants of CD31-Fc to support binding of these three cell lines. This series of truncated forms of CD31 consists of  $\text{NH}_2$  terminal 1, 1-2, 1-3, 1-4, 1-5 and 1-6 IgC2 domains of CD31 fused to the Fc region of human IgG1 which are designated CD31(D1)Fc, CD31(D1-D2)Fc, CD31(D1-D3)Fc, CD31(D1-D4)Fc, CD31(D1-D5)Fc and CD31(D1-D6)Fc. Fig. 2B,C, shows that for both KG1B

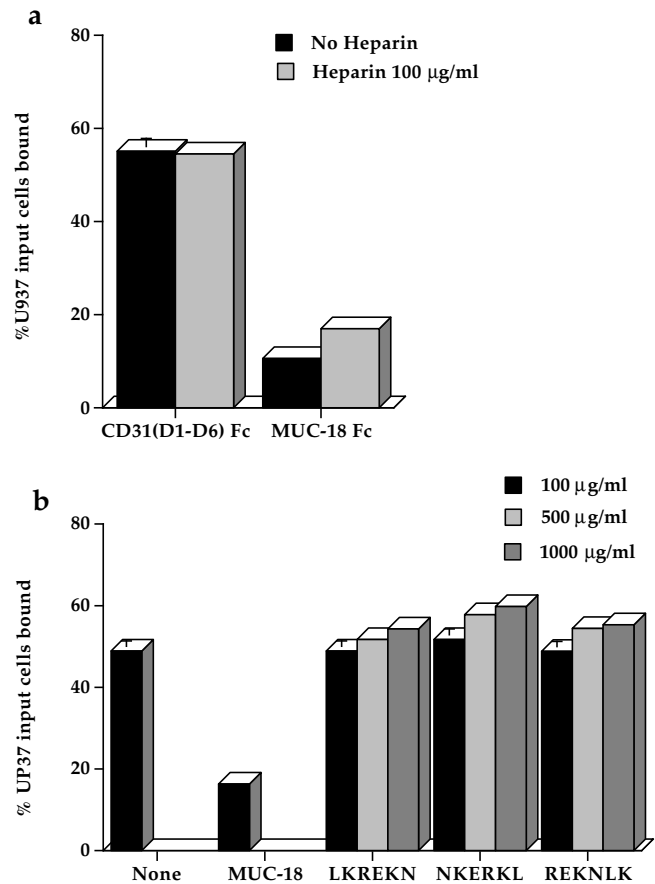


**Fig. 1.** Cell-chimeric protein adhesion screen of haematopoietic cell lines. Adhesion of 11 haematopoietic cell lines (KG1, KG1A, KG1B, Raji, Namalwa, Daudi, MIK-ALL, MOLT-4, HSB2, U937 and K562) to CD31(D1-D6)Fc or a control IgSF-Fc fusion protein. MUC-18Fc is included as a negative control for each cell line and represents the background level of adhesion in the assay. Results are expressed as the percentage of total input cells bound  $\pm$  1 s.d. ( $n=3$ ).



**Fig. 2.** Pattern of cell binding to CD31Fc chimeric deletion series. Adhesion of (a) U937, (b) KG1B, (c) MIK-ALL to CD31 deletion chimeras. The series used was CD31(D1)Fc, CD31(D1-D2)Fc, CD31(D1-D3)Fc, CD31(D1-D4)Fc, CD31(D1-D5)Fc and CD31(D1-D6)Fc. Background adhesion is to a control protein, MUC18-Fc. Results are expressed as percentage of total input cells bound  $\pm$  1 s.d. ( $n=3$ ).

and MIK-ALL the presence of domain 6 of CD31 was necessary to support significant binding. However, for U937, all the truncation mutants, including the minimal unit CD31(D1)Fc, were able to support significant binding, although for maximal binding the minimal unit was CD31(D1-3)Fc. Domains 4, 5 and 6 did not contribute further to the binding of U937 (Fig. 2A). This suggested that U937 interacted with CD31 in a completely different manner to KG1B and MIK-ALL, and not at all like the previously established homotypic mode. Since homotypic binding has been shown to



**Fig. 3.** Lack of involvement of GAGs in adhesion of U937 to CD31(D1-D6)Fc. (a) Effect of heparin. U937 binding to CD31(D1-D6)Fc is not inhibitable by heparin. Cells were allowed to bind in the presence or absence of 100  $\mu$ g/ml heparin. Background binding is represented by binding to MUC18-Fc. (b) Effect of peptide mimetics. U937 binding to CD31(D1-D6)Fc is not inhibited in the presence of peptide mimetics of the putative GAG recognition sequence in CD31. Cells were incubated in the absence or presence of three concentrations (100  $\mu$ g/ml, 500  $\mu$ g/ml and 1,000  $\mu$ g/ml) of the peptide LKREKN which exactly mimics the sequence of CD31 (residues 150-155); the reverse sequence peptide NKERKL, and a scrambled sequence peptide REKNLK. Background binding is represented by binding to MUC18-Fc. Results are expressed as percentage of total input cells bound  $\pm$  1 s.d. ( $n=3$ ).

require the correct alignment of all six domains of CD31 (Fawcett et al., 1995), this suggested that U937 was binding CD31 in a predominantly heterotypic rather than homotypic manner, and that a novel CD31 counter receptor was expressed by U937.

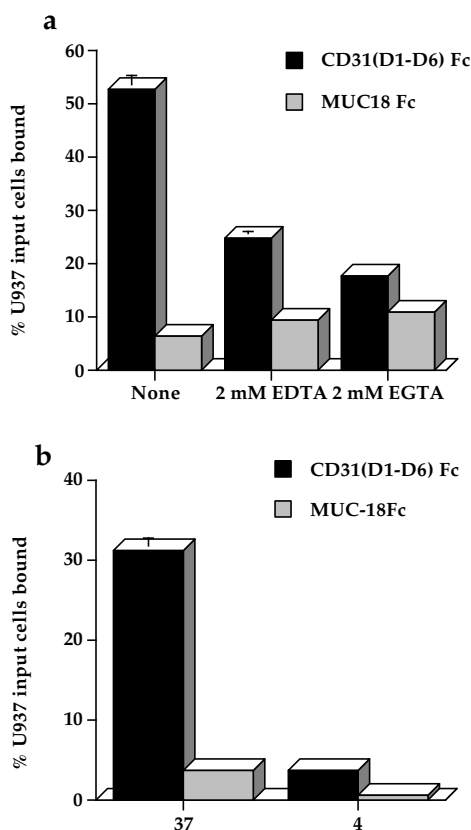
### Heterotypic U937-CD31(D1-D6)Fc interaction does not occur via glycosaminoglycans

A CD31 heterotypic adhesion mode has been reported that involves a site within the second Ig domain of CD31 binding to as yet undefined cell associated glycosaminoglycans (GAGs) (Muller et al., 1992; DeLisser et al., 1993). This binding can be completely blocked by heparin at 100  $\mu$ g/ml. In addition, short linear peptide sequence recognition motifs have been identified within domain 2 of CD31 (position 150-155 LKREKN, and the reverse NKERKL) that are believed to

engage the GAG structures (DeLisser et al., 1993). We performed adhesion assays in the presence of these reagents. However, none of these reagents blocked the binding of U937 to CD31(D1-D6)Fc (Fig. 3A and B). These data, together with the fact that CD31(D1)Fc, could support significant binding suggested that a novel, non-GAG dependent counter-receptor existed on U937.

#### Cation, temperature dependence and $Mn^{2+}$ regulation of CD31 mediated adhesion to U937

To further investigate the nature of this new counter-receptor, we examined whether binding to CD31 was dependent on cations and temperature. As shown in Fig. 4A,B the binding of U937 to CD31(D1-D6)Fc was sensitive to both divalent cations and temperature. The lack of complete inhibition by EDTA and EGTA suggests that a small component of CD31 homotypic, cation independent binding was occurring when U937 bound CD31(D1-D6)Fc. This cation and temperature dependence suggested that the heterotypic counter-receptor on U937 might be an integrin. This was further enforced by the



**Fig. 4.** Cation and temperature dependence of U937 adhesion to CD31(D1-D6)Fc. (a) Cation dependence. Adhesion of U937 cells to plastic coated with CD31(D1-D6)Fc. Cells were incubated in assay buffer alone (0.42 mM  $Ca^{2+}$ , 0.4 mM  $Mg^{2+}$ ) or washed three times in HBSS containing 2 mM EDTA and then assayed in HBSS in the presence of 2 mM EDTA or 2 mM EGTA. (b) Temperature dependence. Adhesion of U937 cells to CD31(D1-D6)Fc either incubated at 37°C or 4°C. For both a and b, the background level of binding is indicated by adhesion to MUC18-Fc. Results are expressed as percentage of total input cells bound  $\pm$  1 s.d.  $n=6$  for a;  $n=3$  for b.

fact that the addition of 0.5 mM  $Mn^{2+}$  doubled the binding of U937 to CD31(D1-D6)-Fc. However, the same concentration of  $Mn^{2+}$  did not increase the binding of KG1B or MIK-ALL (data not shown).  $Mn^{2+}$  regulation is a characteristic feature of integrin mediated binding (Karecla et al., 1995). This strongly suggested that the novel counter-receptor on U937 cells was an integrin.

#### Adhesion of U937 to CD31(D1-D6)Fc is inhibited by antibodies to $\alpha_v\beta_3$

To identify the integrin that binds CD31, we established the surface integrin profiles of the three cell lines, U937, KG1B and MIK-ALL, known to bind CD31(D1-D6)Fc. U937 binds CD31 predominantly by the novel integrin heterotypic counter-receptor, compared with KG1B and MIK-ALL, which bind CD31 solely by the homotypic mode. The aim of establishing surface integrin profiles was to determine if there were any integrins that were unique to and/or expressed on U937, that were not on KG1B and MIK-ALL. By using a panel of anti-integrin  $\alpha$  and  $\beta$  chain antibodies and flow cytometry we found that the integrin  $\alpha_v\beta_3$  was expressed on U937, but not on KG1B or MIK-ALL (Table 1).

We then screened a further panel of anti-integrin adhesion blocking antibodies for their ability to inhibit the binding of U937 to CD31(D1-D6)Fc. Only antibodies to  $\alpha_v$  (L230) and  $\alpha_v\beta_3$  (LM609) were able to completely inhibit binding to background levels (Fig. 5). In contrast, a non-blocking antibody to  $\alpha_v\beta_3$  (mAb 23C6) did not affect binding suggesting that blockade by LM609 was not due to non specific steric effects. Blocking mAbs to other integrins expressed on U937 ( $\alpha_3$ ,  $\alpha_5$ , and  $\beta_2$ ) did not affect binding. Although some inhibition did occur with antibodies to  $\alpha_4$ ,  $\beta_1$  and  $\beta_3$  this inhibition was within the limits of plate to plate variation of control cell binding. These results demonstrated that  $\alpha_v\beta_3$  was the integrin ligand mediating U937 binding to CD31(D1-D6)Fc.

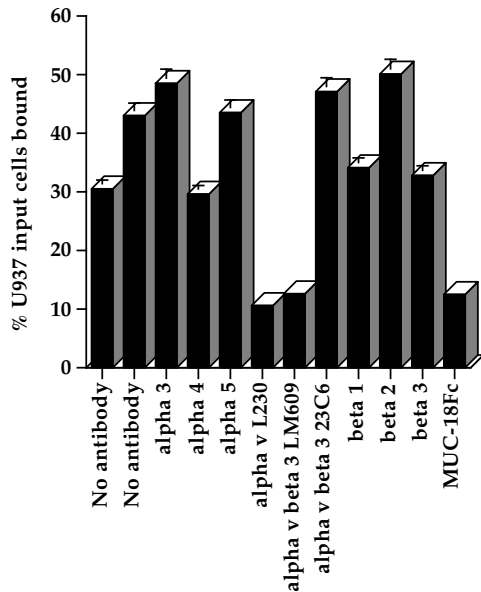
#### Specificity of the $\alpha_v\beta_3$ CD31 interaction

To provide additional evidence for a specific interaction between  $\alpha_v\beta_3$  and CD31 we examined the binding of CD31(D1-D6)Fc to purified  $\alpha_v\beta_3$  using a solid phase assay (Fig. 6A,B). This showed that the interaction between CD31 and  $\alpha_v\beta_3$  was specific as a control protein MUC-18Fc did not bind  $\alpha_v\beta_3$ . Antibodies to both CD31 and  $\alpha_v\beta_3$  inhibited

**Table 1.** Expression of integrin subunits by cell lines

Antibody	Integrin	Median fluorescence intensity		
		U937	KG1B	MIK-ALL
IgG1	Control	5.8	7.7	5.2
HAS-6	$\alpha_2$	33.9	8.0	n/t
P1B5	$\alpha_3$	16.5	4.8	6.0
HP2/1	$\alpha_4$	67.3	45.3	69.7
IIA1	$\alpha_5$	54.3	31.6	77.7
GoH3	$\alpha_6$	12.8	10.7	40.6
13	$\beta_1$	107.5	69.8	119.7
MHM23	$\beta_2$	58.3	74.9	11.9
LM609	$\alpha_v\beta_3$	22.9	8.6	5.8
9G11	CD31	67.3	56.3	46.9

Fluorocytometry was used to determine integrin expression on the three cells lines as described in the materials and methods. Results are expressed as the median fluorescence intensity. Background fluorescence was determined by incubation with a mouse IgG1 control mAb (BBIG-V1). n/t, not tested.



**Fig. 5.** Antibodies to  $\alpha_v\beta_3$  block adhesion of U937 to CD31(D1-D6)Fc. Adhesion of U937 to CD31(D1-6)Fc in the absence or presence of a panel of blocking mAbs to integrins expressed on the surface of U937. Cells were incubated with mAbs at 10  $\mu\text{g/ml}$  for 10 minutes at room temperature before the assay and then the cells were plated in 96-well plates coated with CD31(D1-D6)Fc as described in Materials and Methods. mAbs were present throughout the assay. The mAbs were: anti- $\alpha_3$  integrin P1B5, anti- $\alpha_4$  integrin HP2/1, anti- $\alpha_5$  integrin IIA1, anti- $\alpha_v$  integrin L230, anti- $\alpha_v\beta_3$  integrin LM609, anti- $\alpha_v\beta_3$  integrin 23C6, anti- $\beta_1$  integrin 13, anti- $\beta_2$  integrin MHM23, anti- $\beta_3$  integrin RUU-PL 7F12. Background binding is represented by binding to MUC18-Fc. Two 'no antibody' values from the two separate plates used in this experiment are shown to indicate plate to plate variation of control binding. Results are expressed as percentage of total input cells bound  $\pm$  1 s.d. ( $n=3$ ).

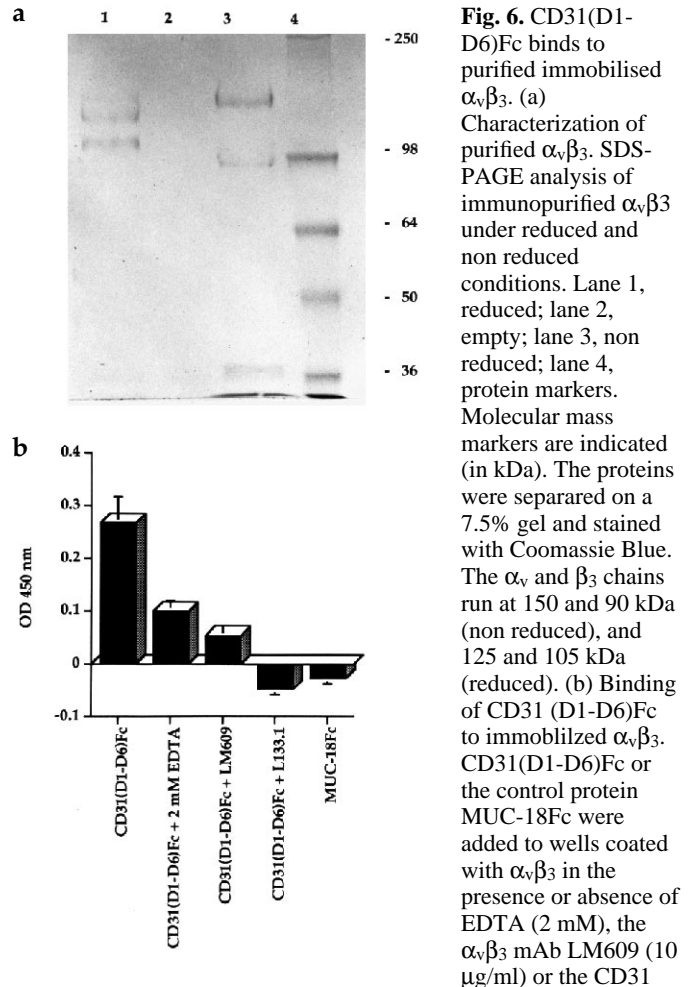
binding as did 2 mM EDTA confirming the specificity of the CD31/ $\alpha_v\beta_3$  interaction.

As further evidence for the specificity of the CD31/ $\alpha_v\beta_3$  interaction we examined the binding of K562 cells (which do not express endogenous  $\alpha_v\beta_3$ ) transfected with  $\alpha_v\beta_3$  to CD31(D1-D6) Fc. Fig. 7 shows that only cells transfected with  $\alpha_v\beta_3$  bound CD31(D1-D6)Fc. Mock transfected cells did not bind CD31(D1-D6)Fc. Furthermore neither transfected nor mock transfected cells bound MUC-18Fc. These K562 transfected cells express  $\alpha_v\beta_3$  but no CD31 and therefore can only engage in heterotypic binding.

### Mapping the $\alpha_v\beta_3$ binding site on CD31 by adhesion blockade

From the pattern of U937 binding to the CD31-Fc deletion series it was apparent that the binding site for  $\alpha_v\beta_3$  on CD31 resided at least in part in domain 1 (Fig. 2A).

To determine whether any other domains of CD31 had additional contribution to CD31- $\alpha_v\beta_3$  mediated adhesion, a panel of 9 anti-CD31 mAbs were screened for their ability to block CD31- $\alpha_v\beta_3$  binding (Fig. 8). This also allowed us to directly compare the blocking abilities of mAbs known to inhibit homotypic binding (Fawcett et al., 1995) with their ability to inhibit  $\alpha_v\beta_3$ -CD31 adhesion. The anti-CD31 domain 2 mAbs



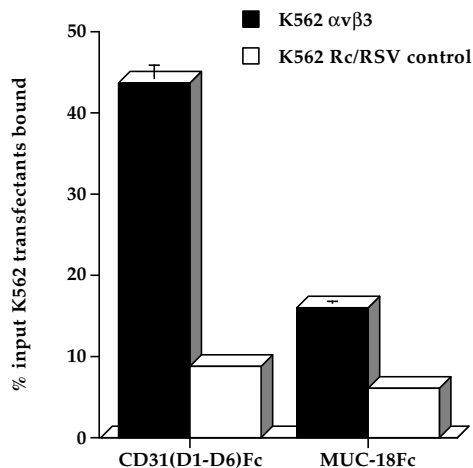
**Fig. 6.** CD31(D1-D6)Fc binds to purified immobilized  $\alpha_v\beta_3$ . (a) Characterization of purified  $\alpha_v\beta_3$ . SDS-PAGE analysis of immunopurified  $\alpha_v\beta_3$  under reduced and non-reduced conditions. Lane 1, reduced; lane 2, empty; lane 3, non-reduced; lane 4, protein markers. Molecular mass markers are indicated (in kDa). The proteins were separated on a 7.5% gel and stained with Coomassie Blue. The  $\alpha_v$  and  $\beta_3$  chains run at 150 and 90 kDa (non-reduced), and 125 and 105 kDa (reduced). (b) Binding of CD31(D1-D6)Fc to immobilized  $\alpha_v\beta_3$ . CD31(D1-D6)Fc or the control protein MUC-18Fc were added to wells coated with  $\alpha_v\beta_3$  in the presence or absence of EDTA (2 mM), the  $\alpha_v\beta_3$  mAb LM609 (10  $\mu\text{g/ml}$ ) or the CD31

mAb L133.1 (10  $\mu\text{g/ml}$ ) and incubated for 3 hours at 30°C. A peroxidase-conjugated anti-human Fc antibody was used to detect bound Fc proteins, and background binding of this antibody to BSA subtracted from all samples. Results are expressed as the mean (OD<sub>450 nm</sub>)  $\pm$  1 s.d. ( $n=6$ ).

5.6E and L133.1 inhibited binding to background levels. Both these mAbs have been shown to inhibit CD31 homotypic binding. (Fawcett et al., 1995). The anti-CD31 mAb hec 7.2 which maps to the D1-D2 boundary (Muller et al., 1993), partially inhibited binding. None of the other anti-CD31 mAbs 9G11, JC70A, CLB/CD31, HC1/6, 10B8 and CAL31.1 affected binding. These results suggest that domain 2 of CD31 may contribute to the binding site for  $\alpha_v\beta_3$ .

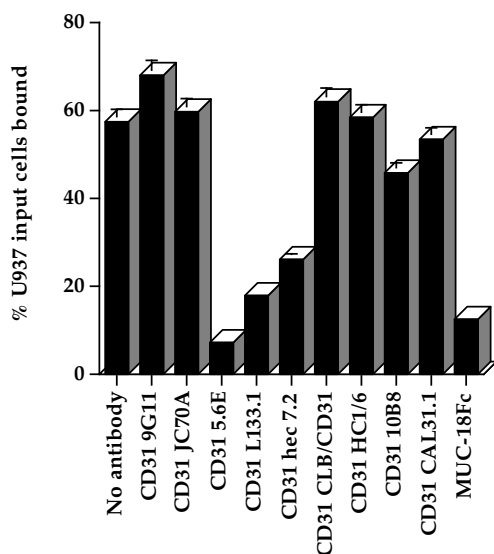
### DISCUSSION

It has already been established that CD31 exhibits both homotypic and heterotypic adhesive properties. The homotypic mode involves anti-parallel inter-digitation of apposing CD31 molecules. However, the identity of the heterotypic counter-receptor(s) has not been defined, but may involve glycosaminoglycans. By using an adhesion assay that relies on the presentation of a nested series of truncated forms of CD31 made as Fc fusion proteins we have been able to define an alternative CD31 binding mode on the pro-monocytic cell line



**Fig. 7.** K562 cells transfected with  $\alpha_v\beta_3$  bind CD31(D1-D6)Fc. Adhesion of K562 cells stably transfected with  $\alpha_v\beta_3$  or vector alone to wells coated with either CD31(D1-D6)Fc or MUC-18Fc. Cells were washed three times in TBS and incubated in TBS with 0.5 mM  $Mn^{2+}$  for 30 minutes at 37°C. Background binding is represented by binding to MUC-18Fc. Results are expressed as percentage of total input cells bound  $\pm$  1 s.d. ( $n=6$ ).

U937. This strategy has allowed us to extend our previous studies on the mapping of the homotypic binding sites in CD31 (Fawcett et al., 1995) to demonstrate that the CD31<sup>+</sup> U937 cell line binds to CD31(D1-D6)Fc in a very different manner to other CD31<sup>+</sup> leukocytic cell lines. Significant, though not maximal, adhesion occurred with the CD31(D1)Fc fusion



**Fig. 8.** Antibodies to D1-D2 of CD31 block binding of U937 to CD31(D1-D6)Fc. Adhesion of U937 to CD31(D1-D6)Fc in the absence or presence of a panel of mAbs to various domains of CD31. Assays were performed as in Fig. 5. The mAbs were: anti-CD31(D1) 9G11 and JC70A; anti-CD31(D2) 5.6E, L133.1 and CLB/CD31; anti-CD31(D1-D2) hec 7.2; anti-CD31(D4-D5) HC1/6; anti-CD31(D5) 10B8 and anti-CD31(D6) CAL31.1. Background binding is represented by binding to MUC18-Fc. Results are expressed as percentage of total input cells bound  $\pm$  1 s.d. ( $n=3$ ).

protein. This pattern of binding was unlike that observed for homotypic binding where the lack of any one of the six domains of CD31 leads to loss of binding (Fawcett et al., 1995) and suggested that a novel heterotypic counter-receptor for CD31 existed on U937 cells.

Using a combination of biochemical and mAb blocking assays we found that the heterotypic ligand on U937 did not involve cell surface GAGs but instead was the integrin  $\alpha_v\beta_3$ . Direct evidence for the specificity of the CD31/ $\alpha_v\beta_3$  interaction was obtained through a combination of solid phase binding assays and the binding of  $\alpha_v\beta_3$  transfectants to CD31. These results confirm for human CD31 a similar observation made by Piali et al. (1995) for murine CD31.

From the work of Piali et al. (1995), and our present study, the concomitant expression of  $\alpha_v\beta_3$  and CD31 by a cell may modulate any potential CD31 homotypic binding, with heterotypic binding dominating over homotypic binding.

We have shown that CD31 can bind cells in a predominantly homotypic or heterotypic manner depending on the cell line used. We found that U937 bound CD31 in a predominantly heterotypic manner but that homotypic adhesion could also occur at a low level, since U937 binding to CD31 was not completely inhibited by EDTA or EGTA (Fig. 4A). This suggests that both homotypic and heterotypic modes of binding can operate in parallel. The preferential usage of one adhesion pathway over another to regulate overall cell adhesion has been demonstrated for other integrin/IgSF interactions. For example, there appears to be a hierarchy of binding pathways for the integrins LFA-1 and VLA-4 which are co-expressed on leukocytes (Van Kooyk et al., 1993). Moreover, recent observations in both humans and the mouse have shown that E-cadherin can mediate both homotypic (between epithelial cells) and also heterotypic interactions through adhesion to the mucosal T cell integrin  $\alpha_E\beta_7$  (Cepek et al., 1994; Karecla et al., 1995).

The  $\alpha_v\beta_3$  integrin is expressed on U937 but not on KG1B or MIK-ALL both of which bind CD31 solely in a homotypic manner. U937 cells, however, bind CD31 in a predominantly heterotypic manner. Furthermore, because U937 cells bind to CD31(D1-D6)Fc constitutively, this suggests that  $\alpha_v\beta_3$  on U937 is expressed in an already activated state which can be further activated by  $Mn^{2+}$  ions, a well established external activator of integrin adhesion. The molecular mechanisms that regulate the adhesive phenotype (homotypic versus heterotypic) of CD31 expressing cells remain unknown. Some clues about possible regulatory mechanisms come from the work of DeLisser et al. (1994a) who showed that deletions of the cytoplasmic tail of CD31 can switch CD31 mediated adhesion from predominantly heterotypic (GAG-mediated, not via  $\alpha_v\beta_3$ ) to predominantly homotypic binding. Only the extracellular portions of CD31 are necessary for homotypic binding to occur as measured by direct protein-protein interactions using surface plasmon resonance (our unpublished observations).

The binding site for  $\alpha_v\beta_3$  on CD31 resides at least in part in domain 1. Utilising the CD31-Fc chimeric deletion series, we established that CD31(D1)Fc alone could support nearly maximal binding. In addition, three anti-CD31 blocking mAbs (2 of which by ELISA map to domain 2, and one to the domain 1/domain 2 boundary) block the  $\alpha_v\beta_3$ -CD31 interaction. This implies that domain 2 contributes to the binding site. These mAbs may however exert their blocking activity by sterically



hindering the access of  $\alpha_v\beta_3$  to domain 1 of CD31. Maximal binding of  $\alpha_v\beta_3$  to CD31 occurs with CD31(D1-D3)Fc. This could implicate a contribution from domain 3 but also could be due to optimal accessibility and presentation of CD31 to  $\alpha_v\beta_3$  away from the anti-Fc coated plastic surface used for the adhesion assays. Domains 4, 5 and 6 do not yield any increase in binding. The issue of accessibility of the IgSF chimeric protein to its counter-receptor also seems to be true of the interaction of ICAM-1 with its receptor on *Plasmodium falciparum* infected erythrocytes (Berendt et al., 1992). The ICAM-1-binding site for malaria infected erythrocytes maps to the first 2 domains yet adhesion to a shortened version of ICAM-1 containing these domains is reduced presumably due to steric hindrance. Therefore from our data we conclude that the binding site for  $\alpha_v\beta_3$  on CD31 resides predominantly in domain 1 but that domain 2 may contribute to the binding site.

Both the domain 2 mAbs which are known to block homotypic binding (Fawcett et al., 1995) also blocked heterotypic binding to  $\alpha_v\beta_3$ . Significantly the hec 7.2 mAb which is known to inhibit monocyte transendothelial migration but does not inhibit homotypic or GAG-mediated heterotypic adhesion, partially inhibited  $\alpha_v\beta_3$  mediated heterotypic adhesion. This mAb has been mapped to the D1-D2 boundary (Muller et al., 1993; Yan et al., 1995). Since D2 has been shown to be important on GAG mediated heterotypic binding (De Lisser et al., 1993), these results suggest that D1 and D2 play central roles in mediating homotypic and heterotypic adhesion, and that the binding sites for homotypic and  $\alpha_v\beta_3$  mediated heterotypic adhesion must partially overlap, at least as measured by mAb epitope mapping.

From studies which have attempted to map the binding sites of those IgSF members which bind integrins (LFA-1-ICAM 1, 2, 3; VLA-4-VCAM,  $\alpha_4\beta_7$ -Mad-CAM), a model has emerged which emphasises the dominance of domains 1 and 2 (Holness and Simmons, 1994). Moreover, mutagenesis studies coupled with the crystal structure of vascular cell adhesion molecule-1 (Jones et al., 1995) have suggested that a common structural motif for integrin binding to IgSF members is the presence of an aspartate or glutamate residue in the C-D loop of the Ig like domain to which binding occurs (Bergelson and Hemler, 1995; Loftus et al., 1994). Sequence alignments of CD31 domains 1 and 2 against VCAM-1 domain 1, show that both D1 and D2 of CD31 have aspartate or glutamate residues at exposed regions in their C-D loops (data not shown and Watt et al., 1995a). These residues are key targets for mutagenesis to further define the  $\alpha_v\beta_3$  binding site.

There is much direct evidence implicating CD31 in the transendothelial migration of monocytes (Muller et al., 1993). Our results, and those for murine CD31 (Piali et al., 1995), suggest that CD31 dependent monocyte interaction with endothelium occurs via  $\alpha_v\beta_3$ , since the hec 7.2 mAb, which is known to inhibit transmigration, partially blocks  $\alpha_v\beta_3$ -mediated heterotypic adhesion to CD31(D1-D6)1Fc. Interestingly, the anti- $\beta_3$  mAb RUU-PL 7F12, which is known to block some  $\beta_3$  mediated cell matrix functions, did not fully block binding to CD31(D1-D6)Fc, suggesting that the binding site for CD31 on  $\alpha_v\beta_3$  may be distinct from that for matrix molecules. There are many such examples of selective blocking of ligand binding by mAbs to integrins (Ruiz et al., 1993; Landis et al., 1994; Neugebauer and Reichardt, 1991). The fact that the ligand repertoire of  $\alpha_v\beta_3$  includes both CD31

and ECM molecules such as vitronectin, fibronectin and laminin suggests that as transmigration occurs  $\alpha_v\beta_3$  may become increasingly activated and able to switch from cell-cell to cell-ECM engagement. In fact it appears to be a general trend for integrins to display a broader range of ligand specificity as they become more activated (Chan and Hemler, 1993).

Finally, the CD31- $\alpha_v\beta_3$  receptor-ligand pair has important implications for angiogenesis and endothelial biology since endothelial cells express both  $\alpha_v\beta_3$  and CD31. Blocking  $\alpha_v\beta_3$  either by mAbs or peptide antagonists has been shown to inhibit angiogenesis and increase apoptosis (Brooks et al., 1994). Furthermore, we have previously shown that CD31(D1-D6) Fc inhibits the growth of endothelial cells and mAbs to CD31 disrupt endothelial monolayer formation (Fawcett et al., 1995). Thus, CD31-CD31 homotypic adhesion and CD31- $\alpha_v\beta_3$  heterotypic adhesion may provide key signals to regulate endothelial proliferation and differentiation.

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