CD31/PECAM-1 Is a Ligand for α₄β₃ Integrin Involved in Adhesion of Leukocytes to Endothelium

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Abstract. To protect the body efficiently from infectious organisms, leukocytes circulate as nonadherent cells in the blood and lymph, and migrate as adherent cells into tissues. Circulating leukocytes in the blood have first to adhere to and then to cross the endothelial lining. CD31/PECAM-1 is an adhesion molecule expressed by vascular endothelial cells, platelets, monocytes, neutrophils, and naïve T lymphocytes. It is a transmembrane glycoprotein of the immunoglobulin gene superfamily (IgSF), with six Ig-like homology units mediating leukocyte-endothelial interactions. The adhesive interactions mediated by CD31 are complex and include homophilic (CD31-CD31) or heterophilic (CD31-X) contacts. Soluble, recombinant forms of CD31 allowed us to study the heterophilic interactions in leukocyte adhesion assays. We show that the adhesion molecule α₄β₃ integrin is a ligand for CD31. The leukocytes revealed adhesion mediated by the second Ig-like domain of CD31, and this binding was inhibited by α₄β₃ integrin-specific antibodies. Moreover, α₄β₃ was precipitated by recombinant CD31 from cell lysates. These data establish a third IgSF-integrin pair of adhesion molecules, CD31-α₄β₃ in addition to VCAM-1, MadCAM-1/α₄ integrins, and ICAM/β₂ integrins, which are major components mediating leukocyte-endothelial adhesion. Identification of a further versatile adhesion pair broadens our current understanding of leukocyte-endothelial interactions and may provide the basis for the treatment of inflammatory disorders and metastasis formation.

Integrins are members of a large family of adhesion receptors, composed of two noncovalently associated chains, the α and the β chain. They play a central role in cell adhesion and migration. Integrins function in both, cell-cell and cell-substratum adhesion. Different combinations of α and β subunits give rise to receptors with different ligand specificities. The α chain carries three to four divalent cation-binding sites making the functioning of integrins crucially dependent on these cations (23, 33, 34, 44, 75). The integrin α₄β₃ is expressed by several cell types including endothelial cells (14), dendritic epidermal T cells (38, 46), activated T cells (49, 58, 71), B lymphoblastoid cell lines (61), mast cells (7, 83), NK cells (57), and lymphokine-activated killer (LAK) cells (56, 72). It is a cellular receptor for the extracellular matrix molecules (ECM) vitronectin, fibronectin, laminin, thrombospondin, osteopontin, bone sialoprotein, and denatured collagen type I and also recognizes products of the coagulation-cascade like fibrinogen and von Willebrand-factor. The recognition epitope on these molecules is the amino acid sequence Arg-Gly-Asp (i.e., RGD) (13, 25, 37, 66, 67). So far there is no ligand described for α₄β₃ which mediates cell-cell adhesion.

CD31/PECAM-1 is a single chain molecule containing six Ig-like domains of the C2 subclass, a transmembrane stretch and a cytoplasmic tail (52, 81). It is expressed on cells of the vascular compartment and is a cell-adhesion molecule (CAM) implicated in many physiological events. These include leukocyte-endothelial interactions, transendothelial migration, inter-endothelial cell adhesion and angiogenesis (for review see 20, 21). Cell-cell adhesion mediated by CD31 can occur in both homophilic and heterophilic ways. It is thought that during heterophilic interactions, one CD31 molecule interdigitates with another on the adjacent cells making antiparallel contacts requiring all six domains. Active binding sites for homophilic adhesion were proposed to reside within domains 2-3 and 5-6 (24). Loss of any one domain of CD31 completely disables homophilic binding due to incorrect spacing. Heterophilic
Animals and Cell Lines

For this study, all cell lines and reagents are of murine origin or directed against antigens of murine origin, except where otherwise stated. 10-20-wk-old, male or female C57BL/6, DBA-2, Balb-c, and Lewis mice were used. 10-wk-old C.B-17 severe combined immunodeficient (SCID) mice were from Iffa-Credo, L’Arbresle, France. The generation of polyclonal antibodies, a female 3-month-old New Zealand white rabbit was used. Animal handling and euthanasia was performed according to local regulations.

Lymphokine-activated killer (LAK) cells were prepared as described elsewhere (29). Briefly, splenocytes were resuspended in 50 ml RPMI 1640 medium (Gibco, Paisley, Scotland) supplemented with 10% FCS (Boehringer Mannheim, Germany), nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin (all Gibco), and 5 x 10⁻⁵ M 2-ME (Fluka, Buchs, Switzerland) (hereafter referred to as complete medium) and 1,000 IU/ml of interleukin-2 (IL-2) derived from supernatants of IL-2-producing X63/0 BCGM Neo cells (36). After 3 d of incubation, the adherent cells were washed twice with prewarmed (37°C) medium and cultured for an additional 2 d. They were harvested with 0.02% EDTA in PBS, washed, and resuspended with the appropriate buffer before use. The pro-T cell line FTI.1.26 (a generous gift from Dr. R. Palacios, MD Anderson Cancer Center, Houston, TX) was grown as described (55). Briefly, the cells were cultured in DME medium supplemented with 10% FCS (Sera-lab, Sussex, UK), 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 IU/ml of IL-2. The cells were harvested and used as LAK cells at day three after passage. Both LAK and FTI.1.26 cells were transfected with pCMV-βgal-LACZ at day three after passage. Both LAK and FTI.1.26 cells express CD31 on the surface (56 and data not shown). The myeloma cell-line J558L was from American Type Culture Collection (ATCC) (Rockville, MD) and was maintained in DME medium supplemented with 10% FCS (Sera-lab). The endothelium cell line eEnd.2 was a generous gift of Dr. W. Risau, Max-Planck Institute for Physiological and Clinical Research (Bad Nauheim, Germany).

Reagents and Antibodies

Polycional antibodies were generated by standard methods in rabbits (15). Briefly, purified CD31-6D (see below) was resuspended in complete Freund’s adjuvant (Difco) and injected i.p. into New Zealand white rabbits. The rabbits were bled 4 wk after the last immunization. The antisera were purified by affinity chromatography with goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA). The purified IgG was stored at -20°C until further use. The antibodies were directed against two epitopes on murine CD31: a) a D-terminus specific antibody (1:1000) to a peptide corresponding to a synthetic peptide mapped to the C-terminus of human CD31 (19). The epitope consists of residues 218-222 of human CD31, and therefore is not conserved in murine CD31. This antibody was used to detect murine CD31 protein by immunoblot analysis. b) A C-terminus specific antibody (1:1000) was made using the peptide corresponding to the murine CD31 C-terminus (residues 231-246) as immunogen. This antibody was also used to detect murine CD31 protein by immunoblot analysis, but was not used for immunohistochemical studies because it had no effect on the expression of CD31 in murine endothelial cells.

Materials and Methods

Immunohistochemistry

For immunohistochemical analysis of CD31 ligands, organs of SCID mice were removed, embedded, and frozen in Tissue-Tek, O.C.T. compound (Miles Inc., Elkart, IN). 6-μm-thick frozen sections were prepared. Immunohistochemistry was carried out using a standard APAAP technique (16). All the incubations were done at room temperature. The antibodies used for localization of mouse ccl were rabbit anti-mouse Ig (Dako, Glostrup, Denmark) followed by mouse anti-alkaline phosphatase complexed with alkaline phosphatase (Dako). For the detection of rat mAb binding, rabbit anti-rat Ig (Dako) followed by rat anti-alkaline phosphatase complexed with alkaline phosphatase (Dako) were used. The developing reagent was 137 mM N,N-dimethylformamide, 0.4 μM naphtol-AS-BT-phosphate, 1.5 mM Fast Red TR salt and 0.8 mM levamisole (all Sigma Chem. Co., St. Louis, MO), washed, and 143 buffer (143 mM sodium acetate and 143 mM diethybarbituric acid). The solution was mixed for 15 min and filtered before use. After a reaction time of 1 h, the sections were counterstained with Mayer’s Hematoxylin (Merck, Darmstadt, Germany), embedded in Mowiol 4-88 (HOECHST, Frankfurt, Germany), examined under an Axioshot microscope (Zeiss, Oberkochen, Germany) and photographed with a color videoprinter UP-500P (Sony, Tokyo, Japan).

Generation of Soluble Recombinant Adhesion Molecules

Total RNA was isolated from the murine endothelial cell line eEnd.2 (80) and reverse transcribed into cDNA by standard methods (62). The primers used for the amplification of coding regions for the extracellular domains of adhesion molecules were: 5'-ATTAGAGCTCACCAGTGC TCTCTGGTGGGGATAGCATCGATGAGCTGACGACCTCG-3' (5' primer for CD31-3D, CD31-6D and CD31-6DA2), 5'-ATTAGAGCTCACCAGTGC TCTCTGGGAAACAGCTCTGT-3' (3' primer for CD31-3D), 5'-TAC TACTCTCTCTCTCTCCTCGGTTTGCAAGGAAAGACG-3' (5' primer for CD31-6D and CD31-6DA2), 5'-ATTAGAGCTCACCAGTGC TCTCTGGGAAACAGCTCTGT-3' (5' primer for VCAM-1), 5'-TATAC TACTCTCTCTCTCTCCTCGGTTTGCAAGGAAAGACG-3' (5' primer for ICAM-1), and 5'-GAATACCTCTCTCTCTCTCCTCGGTTTGCAAGGAAAGACG-3' (5' primer for ICAM-3). The amplified products were purified and inserted into the pET15b vector containing the mouse Igκ constant region as a fusion partner (76) and analyzed by nucleotide sequence analysis. A difference of 1 bp was found to be a G instead of an A, changing the amino acid sequence from Arg 95 to Gly 95. To exclude a PCR artefact, two independently isolated CD31 cDNAs were resequenced and the mutation confirmed. All other residues were as published (81). The vectors were cloned into E. coli K03 cells and 55SL myeloma cells were transfected with the fusion partners. Supernatants were purified by affinity chromatography with goat anti-mouse cκ Abs (Southern Biotechnologies Asc. [SBA], Birmingham, AL). Supernatants were purified by affinity chromatography with goat anti-mouse cκ Abs (SBA). The purified molecules were equilibrated with PBS and aliquots frozen at ~70°C until further use. The CD4-anti-cκ 3AKs Janusin molecule is composed of the two first Ig-like domains of human CD4 linked to an Fv fragment specific for the human CD3 molecule and coupled to mouse cκ. This molecule was a kind gift of A. Traunecker (Basil Institute for Immunology, Switzerland) (74). To assess whether the soluble recombinant CD31 molecules were folded in a conformation comparable to native CD31, ELISA assays were performed. The rat-anti-mouse CD31 mAb EA-3 (56) was able to recognize the CD31-3D and CD31-6D but not the CD31-6DA2 molecule. Also the polyclonal Abs stained all three soluble recombinant CD31 isoforms and precipitate CD31 from ~25I-labeled endothelial cells (not shown). This indicated that all three CD31 isoforms were folded in a manner analogous to native CD31.

Determination of Soluble Recombinant Adhesion Molecules Bound to Microtubell Winters

Approximately 20 μg of soluble recombinant molecule was labeled with 125I using IodoGen™ (Pierce, Rockford, IL) as described in the manufacturer’s instructions. Free 125I was separated from the labeled proteins by size fractionation using a Sephadex G-25 column (Pharmacia, Uppsala, Sweden). For the assessment of the number of plate-bound molecules 0.2 μg/ml of radioactively labeled soluble recombinant molecule was mixed with...
with graded concentrations of unlabeled material in a total volume of 50 μl Dulbecco’s phosphate buffered saline (D-PBS). The range of concentrations varied between 0.2 μg/ml (radioactive molecule alone) to 100 μg/ml. Buffers and the type of microtiter plates used were identical with those used for adhesion assays. The plates were left for 1 h at room temperature, whereupon supernatants were discarded and the plates washed twice with D-PBS. Finally, bound protein was released with 0.2 M NaOH and 1% Triton X-100 (Fluka) in D-PBS and the radioactivity determined in a γ-counter. By comparing the input radioactivity with bound radioactivity, the percentage of bound soluble recombinant molecules was calculated for each protein concentration and the quantity of bound soluble recombinant molecules per mm² was determined.

**Cell Adhesion Assays**

Purified adhesion molecules were directly coated onto microtiter wells (Costar, Cambridge, MA) at 10 μg/ml in 50 μl D-PBS for 2 h at room temperature. The wells were blocked with 20% BSA for 1 h at room temperature and washed five times with D-PBS. 50 μl of RPMI 1640 medium supplemented with 0.5% FCS and 2 mM Hepes were added to the wells. Antibodies and peptides were diluted in RPMI-Hepes medium at triple concentrations. The cells were incubated with 3 × 10⁴ endo-thelioma cells in a total volume of 50 μl Dulbecco’s phosphate buffered saline (D-PBS). The range of concentrations varied between 0.2 μg/ml (radioactive molecule alone) to 100 μg/ml. Buffers and the type of microtiter plates used were identical with those used for adhesion assays. The plates were left for 1 h at room temperature, whereupon supernatants were discarded and the plates washed twice with D-PBS. The percentage of bound soluble recombinant molecules was calculated for each protein concentration and the quantity of bound soluble recombinant molecules per mm² was determined.

Figure 1. Production of soluble recombinant adhesion molecules. (A) The molecules were produced in myeloma cells, purified from supernatants by affinity columns, and analyzed on SDS-PAGE under nonreducing conditions after staining with Coomassie blue. CD31 Ig-domains 1–3 linked to the constant region of the α light-chain, CD31-3D (3D); CD31 Ig-domains 1–6 linked to βα, CD31-6D (6D); CD31 Ig-domains 1–6 lacking domain 2 linked to κc, CD31-6DΔ2 (6DΔ2); ICAM-1 Ig-domains 1–2–κα, ICAM-1 (ICAM); and VCAM-1 Ig-domains 1–2–κε, VCAM-1 (VCAM). Molecular mass markers are indicated (in kD). (B) Schematic representation of the truncated recombinant adhesion molecules. Open circles represent immunoglobulin-like domains. Putatively conserved cysteines forming intradomain disulphide bridges are represented by a double s. The enumeration of domains starts at the NH2 terminus, the constant domain of the Igκ light chain is represented by κc. The bold circles highlight the second Ig domain of CD31 as putative heterophilic-binding site.
Results

Recombinant, Soluble Adhesion Molecules

We generated soluble, recombinant forms of truncated mouse CD31, ICAM-1, and VCAM-1. To obtain various CD31 isoforms, the first three Ig-domains, all six domains, or an isoform lacking domain 2 were fused to the c Kappa-domain (ck) of the mouse Ig light chain (CD31-3D, -6D, and -6DA2, respectively). The RNA for CD31-6DA2 was identified as a naturally occurring splice product found in many different endothelial and hematopoietic cell lines of mouse origin. The splice product gives rise to an in-frame RNA species and protein (unpublished observation). The ICAM-1 and VCAM-1 fusion proteins contained the first two Ig-domains which included the integrin-binding sites (54, 69). All molecules were constitutively secreted by transfected J558L myeloma cells and were affinity purified with anti-cK columns (Fig. 1). The observed differences in apparent molecular weight between ICAM-1 and VCAM-1 are not readily explicable but could represent glycosylation differences. Indeed, the two first Ig-domains of VCAM-1 contain no glycosylation sites whereas ICAM-1 bears three potential N-glycosylation sites (5, 31).

Cation-dependent and Cation-independent Adhesion Mediated by CD31

The truncated forms of CD31 were directly coated onto microtiter wells and used for cell-adhesion experiments. A solution of 10 μg/ml of recombinant soluble CAMs were incubated in microtiter wells and the amount bound to the plastic surface was determined (Fig. 2). The adhesive properties of lymphokine-activated killer (LAK) cells, a heterogeneous cell line derived from IL-2-activated spleenocytes (29), were studied. These cells adhered considerably to the 3D-, moderately to the 6D- and not to the 6DA2-form of CD31 although double the amount of CD31-6D than CD31-3D molecules were ligated to the microtiter well (Figs. 2 and 3 a). The binding to the 3D form was exquisitely dependent on divalent cations since adhesion was inhibited with 5 mM EDTA. The cation dependence and the lack of adhesion to CD31-6DA2 suggested that LAK cells might interact with CD31-3D in a heterophilic way. In contrast, the pro-T-cell line FTF1.26 moderately adhered to the 3D form but strongly bound to the full-length CD31 molecule. In the presence of EDTA, residual binding of the cells to the 3D form was lost but binding to the 6D molecule persisted (Fig. 3 b). These results pointed out that FTF1.26 cells may preferentially adhere to CD31 in a homophilic way. Similar to LAK cells, FTF1.26 cells did not bind to CD31-6DA2, confirming that cation-independent interaction between two CD31 molecules requires six Ig domains (24). Taken together, these data show that truncated, recombinant CD31 molecules can function as cation-independent or cation-dependent ligands, depending on the cell line and the type of recombinant CD31 molecule used.

It has been shown that CD31 binds to GAGs via a consensus heparin-binding motif in the second Ig-like domain (22). In fact, when used as primary reagents and then developed with an anti-cK antibody, the CD31-3D and CD31-6D, but not the CD31-6DA2, constructs were able to stain heparin-containing mast cells on frozen sections of

Figure 2. Binding of soluble recombinant adhesion molecules to microtiter well surface. The number of molecules bound on the surface of the microtiter well was determined as described in the Materials and Methods section. The amount of CD31-3D, CD31-6D, CD31-6DA2, VCAM-1, and ICAM-1 bound to the plates is expressed as pmol per mm².

Figure 3. Leukocyte binding: cation-dependent and cation-independent CD31 interactions. Cells were allowed to bind to plates coated with recombinant molecules. (a) LAK cell binding to CD4, CD31-3D, CD31-6D, and CD31-6DA2 soluble molecules in the presence of 1 mM Ca²⁺ and 1 mM Mg²⁺ (open bars) or in the presence of 5 mM EDTA (hatched bars). (b) FTF1.26 cell binding to CD4, CD31-3D, CD31-6D, and CD31-6DA2 molecules in the presence of 1 mM Ca²⁺ and 1 mM Mg²⁺ (open bars) or in the presence of 5 mM EDTA (hatched bars). Data are expressed as mean percentage of binding ± SE of mean of three replicate wells. 100% is equivalent to 1,400 cpm in a and 11,500 in b, two independent experiments with similar results were done.
spleen and thymus, and this was inhibitable by an excess of heparin (Fig. 4 and data not shown). An mAb directed against heparan sulfate proteoglycan leads to a staining comparable to CD31-3D (Fig. 4 d). However, in the cell adhesion assays cation-dependent binding to CD31 was not inhibitable by the same heparin preparation (Fig. 5). Thus, the CD31-3D and CD31-6D constructs bind GAGs by the second Ig-like domain and an additional, yet unidentified, heparin-independent ligand.

Leukocyte-Endothelial Adhesion: Inhibition of CD31-mediated Leukocyte Binding by Anti-αvβ3 Antagonists

The heparin-independent interaction of LAK cells with CD31-3D was further investigated. Binding of LAK cells was inhibited by saturating concentrations of a polyclonal antiserum raised against CD31 (Fig. 6). Furthermore, a series of antibodies directed against cell surface adhesion molecules were screened for inhibitory activities. Surprisingly, saturating concentrations of a polyclonal rabbit anti-serum raised against native α₄β₃ integrin reduced binding of LAK cells to CD31-3D to background levels (Fig. 6). Two blocking monoclonal antibodies (mAbs) reactive with the cell surface α₄ integrin chain (H9.2B8 [46], and RMV-7 [72]) and a β₃ integrin chain specific mAb were also able to inhibit binding of LAK cells to CD31-3D. In contrast, blocking antibodies against α₄- or β₃ integrin (43, 63) chains did not inhibit CD31-3D-LAK cell binding (Fig. 6).

To exclude artefactual binding of LAK cells, adhesion assays were performed with the recombinant cell–cell adhesion molecules ICAM-1 and VCAM-1. LAK cell binding to ICAM-1 was inhibited by antibodies against αL integrin (FD441.8) but not by the anti-α₄ antibody H9.2B8 (Fig. 7). Binding to VCAM-1 was inhibited by antibodies against α4 (PS/2) (48) integrin, but not by anti-α₃ (Fig. 7). In contrast, binding of LAK cells to CD31-3D was inhibited by both anti-α₄ mAbs H9.2B8 and RMV-7 (Fig. 7 and not shown). This showed that the inhibition exerted by H9.2B8 and RMV-7 to CD31-3D was specific. It is well established that RGD specific integrins can be inhibited by peptides containing the RGD motif. The peptide RGDS inhibited binding to CD31-3D in a dose-dependent manner and reached maximal blocking activity at 5 μg/ml. A
control peptide of the same size and similar charge had no effect at up to 50 μg/ml (Fig. 8). Since the CD31 molecule does not contain RGD sequences, we conclude that the binding epitope on the αvβ3 molecule making contact with CD31 is either influenced by or identical with the RGD-binding site. Since both adhesion molecules, αvβ3 and CD31, are strongly expressed by subsets of leukocytes (30, 46, 58, 71, 72) and endothelial cells (14, 18, 28), we tested whether αvβ3 interaction with CD31 is involved in leukocyte-endothelial adhesion. To this end, LAK cells were allowed to bind to monolayers of endothelial cells. Saturating concentrations of antibodies directed against CD31 inhibited cell binding by ∼30%, those against αv integrin by ∼20% (Fig. 9). The high amount of anti-CD31 antibodies used for blocking did not allow CD31 cross-linking which would result in integrin activation, and thus enhanced binding, as described previously (56, 73). To prove that the interaction of αvβ3 with CD31 was involved, combinations of both Abs were applied and no additive blocking effect was observed. This shows that αvβ3 integrin can recognize CD31 in its native conformation state in the membrane of another cell.

**Precipitation of αvβ3 by CD31**

Direct evidence for binding of CD31 to αvβ3 integrin was obtained by immunoprecipitation experiments using CD31-3D as an affinity matrix. Elution with EDTA revealed two major proteins with apparent molecular masses of 145 kD and 85 kD on SDS-PAGE under nonreducing conditions. The apparent sizes of the precipitated molecules corresponded to the published molecular weights of the αv and β3 integrin chains (49). A control construct, the CD4-anti-

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**Figure 5.** Leukocyte binding to CD31 is not inhibitable by Heparin. LAK cells were allowed to bind to CD31-3D in the presence of graded concentrations of Heparin. Data are expressed as mean percentage of binding ± SE of mean of three replicate wells. 100% binding is equivalent to 1,500 cpm, two independent experiments with similar results were done.

**Figure 6.** Binding of LAK cells to CD31 is dependent on integrin αvβ3. LAK cell binding to CD31-3D without antibody (co) or in the presence of 50 μg/ml blocking antibodies. The monoclonal antibodies were anti-αv integrin H9.2B8 (αv), anti-αL integrin RMV-7 (αL), anti-αL integrin FD441.8 (αL), anti-β3 integrin 9EG7 (β3) and anti-β3 integrin Hmβ3 (β3). The polyclonal rabbit antibodies were used at a dilution of 1:10. Preimmune serum (co), antisera directed against αvβ3 integrin (αvβ3) or antiserum raised against CD31-6D (CD31). Data are expressed as mean percentage of binding ± SE of mean of three replicate wells. 100% binding is equivalent to 1,600 cpm, three independent experiments with similar results were done.

**Figure 7.** Inhibition by anti-αv is specific. LAK cell binding to CD31-3D, VCAM-1, and ICAM-1 without antibody (co) or in the presence of 50 μg/ml blocking antibodies. The monoclonal antibodies were anti-αv integrin H9.2B8 (αv), anti-α4 integrin PS/2 (α4), and αL integrin FD441.8 (αL). Data are expressed as mean percentage of binding ± SE of mean of three replicate wells. 100% binding is equivalent to 1,400 cpm, three independent experiments with similar results were done.

**Figure 8.** Binding of LAK cells to CD31 is inhibited by RGD peptides. LAK cell binding to CD31-3D in the presence of graded concentrations of peptides. The peptides were TKPR (open bars) and RGDS (hatched bars). Data are expressed as mean percentage of binding ± SE of mean of three replicate wells. 100% binding is equivalent to 1,500 cpm, four independent experiments with similar results were done.
CD3-cK Janusin chimaeric molecule (74), was not able to precipitate this protein. Identity of the molecules detected, was documented by precipitations done in the presence of anti-αβ3 polyclonal antibodies, anti-β3, and anti-β1 monoclonal antibodies (Fig. 10 A). Alternatively, the material was precipitated by the CD31-3D affinity matrix, eluted by EDTA treatment and reprecipitated by an antibody directed against the αβ3 integrin (Fig. 10 B). Upon reduction, the α, molecule precipitated by CD31-3D migrated at 125 kD. The apparent molecular weight of the β1 chain increased upon reduction to 100 kD (34) (Fig. 10 B). Moreover, an additional protein with diffuse appearance was precipitated with CD31-3D from endothelial cell lysates (Fig. 10 A). Recognition of this molecule by CD31-3D was not perturbed by antibodies to αβ3 and could represent an additional cation-dependent, heterophilic ligand for CD31.

**Discussion**

Taken collectively, we conclude that CD31 can directly bind to the αβ3 integrin, and that the αβ3 integrin and CD31 constitute a heterophilic receptor ligand pair. This adds a third Ig-SF-integrin pair to the previously described ICAM/β2 integrins and VCAM-1, MadCAM-1/α4 integrin pairs.

Cells can undergo cation-dependent, heterophilic or cation-independent, homophilic adhesion mediated by CD31 (19, 22, 50). We showed that FTF1.26 cells preferentially undergo cation-independent CD31 interactions whereas LAK cells bind via heterophilic CD31-αβ3 contacts. These binding differences are not merely exclusive since LAK cell binding in the presence of EDTA to the CD31-3D molecule is not completely abrogated (Fig. 3 a) and FTF1.26 cell binding to the CD31-3D molecule is blocked by EDTA (Fig. 3 b). Thus, both mechanisms can operate in parallel. However, cation-independent, homophilic, and cation-dependent, heterophilic interactions may offer the possibility for regulation of the adhesion type. It has been shown that a regulatory domain of CD31 is located in the cytoplasmic tail. Experimental truncation of this domain leads to CD31 that can only interact in a homophilic manner (19). Indeed, such regulations of CD31 function may

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**Figure 9.** Binding of LAK cells to endothelioma cells is partially mediated by αβ3-CD31 interactions. LAK cell binding to monolayers of the endothelioma cell line eEnd.2 in the presence of antibodies directed against αβ3 (α/33), CD31 (CD31), combinations of both (CD31 + αβ3) or control preimmune serum (co 2) at dilutions of 1:30. Data are expressed as mean percentage of binding ± SE of mean of three replicate wells. 100% binding is equivalent to 23,000 cpm, three independent experiments with similar results were done.

**Figure 10.** CD31-3D specifically precipitates αβ3 integrin. (A) Immunoprecipitations with 125I surface-labeled endothelial cell lysates. The affinity matrices were prepared with CD4-anti-CD3-cK Janusin as control (CD4) and CD31-3D (CD31) used as such or in the presence of anti-αβ3 antibodies (αβ3), anti-β3 antibodies (β3) or anti-β1 antibodies (β1) and separated under nonreducing (NR) and reducing conditions (R). (B) Immunoprecipitations with 125I surface-labeled LAK cell lysates. The affinity matrices were prepared with CD31-3D (CD31) and the control CD4-anti-CD3-cK Janusin (CD4). LAK cell lysate was immunoprecipitated with anti-αβ3 antibody. The material eluted from the CD31 and CD4 affinity matrices was further immunoprecipitated with anti-αβ3 antibody and analyzed under reducing conditions (R). The proteins were separated on 8% polyacrylamide gels. Molecular mass markers are indicated (in kD).
naturally occur in cells. The genomic organization of the cytoplasmic region comprises seven exons and several alternatively spliced mRNAs have been reported, leading to CD31 variants showing homophilic or heterophilic binding (6, 39). Our finding of an integrin being a heterophilic ligand for CD31 now offers a possibility for rapid changes of CD31-mediated adhesion. Integrins can switch from low to high affinity states by cell activation, addition of Mn^{++} leads to a fully activated high affinity state (34). Indeed, addition of 0.5 mM Mn^{++} to the medium increases the binding of LAK cells to CD31-3D approximately six-fold (data not shown). Since CD31 interacts with αβ_{3} integrin, regulation of the adhesive capacity of CD31 depends on the regulation of the integrin.

It has been recognized that in order for leukocytes to arrest and leave the bloodstream during normal recirculation and inflammation, it would require a multitude of adhesive interactions with the endothelium. These include rolling, mediated by selectins, tight adhesion to the endothelium (Fig. 10, A and B). Furthermore, the adhesion of leukocytes to endothelium monolayers was inhibitable with antibodies directed to native CD31 (Fig. 9). Since the blocking effect of the antibodies was not additive, we conclude that indeed αβ_{3} and CD31 form an adhesion molecule pair involved in the adhesion of leukocytes to endothelium. Formerly, integrin αβ_{3} has been shown to be a major receptor mediating migration of cells on ECM substrates (41, 42). CD31 expressed by endothelial cells might represent a surrogate substrate for lymphocyte migration on the endothelial cell surface and intercellular junctions, where immobilized ECM components are mostly absent.

The transendothelial migration of monocytes has been shown to be crucially dependent on CD31 (51). It was however not possible to discriminate whether the CD31 molecules mediated this process by homophilic interactions or in a heterophilic manner. Two circumstances point to heterophilic interactions, first, the transendothelial migration of monocytes was inhibited by an mAb directed against domain 2 and second, the soluble recombinant CD31 molecule used for blocking, lacked half of domain 6, a domain essential for homophilic binding (24, 51). However, transendothelial migration was blocked irrespective of whether the monocytes or the endothelial cells were preincubated alone with anti-CD31 antibodies. This could be due to CD31 and αβ_{3} integrin expression by monocytes (64) as well as endothelial cells (14). Thus both endothelial and monocyte αβ_{3} integrin may use CD31 as a ligand. In contrast to monocytes, lymphocytes which migrated through endothelial cell layers did not express CD31. This would allow only heterophilic interactions if CD31 was involved in the migration of these cells (8).

VLA-4 (αβ_{4}) is another integrin involved in leukocyte transendothelial migration. It also recognizes both an IgSF molecule, VCAM-1, and molecules of the ECM, the IIICS domain of fibronectin (2, 32, 53). It appears, that the IgSF members VCAM-1 and CD31 are the migration-substrates for leukocyte integrins αβ_{1} and αβ_{3}, respectively, and that migration within the ECM continues to depend on these integrins. This minimizes complicated regulatory switches from cell-dependent to ECM-dependent migration. In addition, the two integrins have further functional aspects in common. Cross-linking of both αβ_{1} and αβ_{3} by their ligands induces the expression of metalloproteinases (59, 65). These enzymes are crucial for the invasive process, i.e., for the digestion of the basement membrane, a prerequisite for the entry of cells into tissue. It remains to be investigated whether the interaction of αβ_{3} with CD31 may induce the release of proteases.

Heterophilic ligands for CD31 have been identified as cell surface glycosaminoglycans (GAGs) (22). Although our CD31 constructs recognized heparan sulfate proteoglycan-binding epitopes, the cell lines did not show GAG-dependent adhesion to CD31 (Figs. 4 and 5). Here we studied the adhesion of activated leukocytes whereas in other studies L-cell aggregation assays were used (22, 50). It is conceivable that these leukocytes do not express the GAGs binding to CD31. The additional molecule detected by precipitation of endothelial cell lysates with the CD31-3D affinity matrix, might represent the second αβ_{3} independent ligand (Fig. 10 A). This molecule was not detected in precipitations done with LAK cell lysates (Fig. 10 B). Thus it seems that CD31 has two heterophilic cellular ligands, GAGs and αβ_{3} integrin. Since αβ_{3} dependent binding of LAK cells to CD31 occurs in the presence of heparin, it is likely that CD31 uses different binding epitopes, both residing within the second Ig-like domain (Fig. 3 a).

The α integrins have been described as cell adhesion molecules for the ECM (82). The RGD sequence is widely distributed within the molecules of the ECM and is readily used as attachment site for many different integrins (for reviews see 60, 82). Yet CD31 is not a member of the ECM and does not contain an RGD sequence (52, 81). However, α integrins can bind to non-RGD sequences as has been shown for HIV Tat Protein, and fragments of osteopontin (77, 78). Although this osteopontin fragment did not contain an RGD sequence, its interaction with αβ_{3} was inhibitable by RGD peptides. This effect is similar to our finding with αβ_{3} binding to CD31. This confirms that alternative recognition sequences for α integrins other than RGD do exist as reported for αβ_{3} integrin (40). These may be specific for subclasses of RGD-binding integrins, i.e., be specific for αβ_{3}.

Similarly to leukocytes, metastasizing malignant cells are transported by the blood to distant tissues (70). On melanoma cells, increased expression of αβ_{3} is positively correlated with increased malignancy (3, 26, 27, 45). It has been shown that metastatic cells have to adhere to the vascular endothelium before they can reach the subendothelial matrix, containing the ECM ligands for αβ_{3} (79). It is conceivable that CD31 plays a role in the tumor cell adhesion to endothelium, an important step in the metastatic process.

Angiogenesis has been shown to depend on the adhesive interactions of vascular endothelial cells (for reviews see 9, 47). Angiogenic vascular endothelium showed an increase in expression of αβ_{3} integrin (10). Blocking of αβ_{3} by antagonists disrupted the new formation of blood vessels and rendered the endothelial cells apoptotic (11). Fur-
thermore, endothelial cells cultured in the presence of anti-CD31 antibodies failed to form normal cell–cell contacts (4, 21). Although CD31 can mediate cell–cell contact in a homophilic manner and αβ3 may confer endothelial ECM contacts, the novel αβ3-CD31 ligand pair might be involved in angiogenesis.

In view of the possible implications, blocking of αβ3-CD31 dependent adhesion may offer important targets for therapeutic interventions in the treatment of inflammation, angiogenesis, and metastasis formation.

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