CD31/PECAM-1 Is a Ligand for $\alpha_v\beta_3$ 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 naive 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 $\alpha_v\beta_3$ 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 $\alpha_v\beta_3$ integrin-specific antibodies. Moreover $\alpha_v\beta_3$ was precipitated by recombinant CD31 from cell lysates. These data establish a third IgSF-integrin pair of adhesion molecules, CD31-$\alpha_v\beta_3$ in addition to VCAM-1, MadCAM-1/$\alpha4$ integrins, and ICAM/$\beta2$ 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 $\alpha_v\beta_3$ 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)[1] 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 $\alpha_v\beta_3$ 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 homophilic 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

1. *Abbreviations used in this paper.* CAM, cell-adhesion molecule; CD, cluster of differentiation; D-PBS, Dulbecco's phosphate buffered saline; ECM, extracellular matrix molecules; GAG, sulfated glycosaminoglycans; ICAM, intercellular adhesion molecule; IgSF, immunoglobulin gene superfamily; IL-2, Interleukin-2; LAK, lymphokine-activated killer; PECAM, platelet endothelial cell adhesion molecule; VCAM, vascular cell adhesion.

cell–cell adhesion has been shown to be mediated by the second Ig-like domain which contains a heparin-binding consensus sequence. The heterophilic binding is perturbed by sulfated glycosaminoglycans (GAGs) and is completely dependent on divalent cations (19, 22, 50). However, there is no direct evidence that cells indeed interact with each other via GAG-CD31, and the divalent cation dependency has remained unexplained. Identification of the heterophilic ligand for CD31 would lead to a better understanding of the function of CD31 and its involvement in physiological and pathological events.

Materials and Methods

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. For 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 5×10^{-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 BCMG 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 FTF1.26 (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 FTF1.26 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 endothelioma 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

Polyclonal antibodies were generated by standard methods in rabbits (15). Briefly, purified CD31-6D (see below) was resuspended in complete Freund's adjuvant (Difco Laboratories, Detroit, MI) by sonication. The antigen was injected into multiple subcutaneous sites. Booster immunizations with incomplete Freund's adjuvant (Difco) were started 4 wk after the priming immunization, which was repeated twice after two subsequent weeks. Before the priming and after each immunization the animal was bled and serum prepared from whole blood. The polyclonal Abs were at 2.2 mg/ml IgG (antiCD31) and 6.3 mg/ml IgG (anti-αvβ3) and binding to their respective antigens was saturating at a dilution of 1:3,000 (anti CD31) and 1:300 (anti-αvβ3) as determined by FACS® analysis (not shown). For blocking experiments, only affinity-purified, preservative-free mAbs were used. The following antibodies were used: anti-αv integrin (H9.2B8) (46) and anti-β3 integrin (HmB3) (Pharmingen, San Diego, CA); anti-αL integrin (FD441.8) (63) and anti-α4 integrin (PS/2) (48) (ATCC); anti-β1 integrin (9EG7) (given by Dr. Dietmar Vestweber, Max Planck Institute for Immunobiology, Freiburg, Germany) (43), anti-αv integrin (RMV-7) (obtained from Dr. Hideo Yagita, Juntendo University, Tokyo, Japan) (72); and anti–heparan-sulfate proteoglycan mAb 1948 (17) (Chemicon, Temecula, CA). The RGDS and TKPR peptides (Calbiochem, La Jolla, CA), EDTA (Fluka) and Heparin (Sigma Chem. Co., St. Louis, MO) were of the highest purity available.

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. Immunocytochemistry was carried out using a standard APAAP technique (16). All the incubations were done at room temperature. The antibodies used for visualization of mouse cκ 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-dimethyl formamide, 0.4 mM naphtol-AS-BI-phosphate, 1.5 mM Fast Red TR salt and 0.8 mM levamisole (all Sigma Chem. Co., St. Louis, MO) in Michaelis buffer (143 mM sodium acetate and 143 mM 5,5-diethylbarbituric 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 Axiophot microscope (Zeiss, Oberkochen, Germany) and photographs printed 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'-ATTAGAGCTCACCATGC-TCCTGGCTCTGGGACT-3' (5' primer for CD31-3D, CD31-6D and CD31-6DΔ2), EMBL accession number L06039, 5'-TATACTTA CCTTT-CGGCTTGGGAAACAGCTCTGT-3' (3' primer for CD31-3D), 5'-TA-TACTTACCTTTCTTCCATGGG GCAAGGAAGAC-3' (3' primer for CD31-6D and CD31-6DΔ2), 5'-ATTAGAGCTCATGCCTGTGAAGA-TGGTCGCGGTC-3' (5' primer for VCAM-1), 5'-TATACTTACCT-AGTTCTTTGACAGTCTCCCTTTC-3' (3' primer for VCAM-1), EMBL accession number M84487, 5'-ATTAGAGCTCATGGCTTCAACCCGT-GCCAAGCC-3' (5' primer for ICAM-1) and 5'-TATACTTACCTTCG-AAAGT CCGGAGGCTCCTGGC-3' (3' primer for ICAM-1), EMBL accession number X16624. The amplified products were purified and inserted into the pHT4 vector containing the mouse Igκ constant region as a fusion partner (76) and analyzed by nucleotide sequence analysis. A difference to the previously published mouse CD31 sequence (81) was found. Position #381 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 competent E. coli K803 cells and J558L myeloma cells were thereafter transfected by protoplast fusion (12). Supernatants of transfected cell clones were screened by ELISA with goat anti–mouse cκ reagents (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 CD3-cκ 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 (Basel 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 CD31-6DΔ2 molecule. Also the polyclonal Abs stained all three soluble recombinant CD31 isoforms and precipitate CD31 from ^{125}I-surface–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 Microtiter Wells

Approximately 20 μg of soluble recombinant molecule was labeled with ^{125}I by using Iodobeads™ (Pierce, Rockford, IL) as described in the manufacturer's instructions. Free ^{125}I 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 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 mm^2 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 20 mM Hepes, hereafter referred to as RPMI-Hepes medium, were added to the wells. Antibodies and peptides were diluted in RPMI-Hepes medium at triple concentrations. The cells were released from the culture flask, washed three times with RPMI-Hepes medium, saturated with $Na^{51}CrO_4$ (Amersham, UK) (35 µCi/5 × 10^6 cells) for 30 min at 37°C, washed twice with RPMI-Hepes medium and adjusted to 10^6 cells/ml. 100 µl of the cell suspension were added to appropriately pretreated wells and incubated for 30 min on ice to allow antibodies to bind. After this the plates were rapidly warmed to 37°C and incubated for 45 min on a gyratory platform (Kühner, Basel, Switzerland) at 50 rpm. Unbound cells were removed by washing three times with D-PBS containing 1% BSA prewarmed at 37°C. The level of radioactivity retained in each well was released by short treatment of the wells with 1% Triton X-100 in RPMI-Hepes medium and γ-ray emissions of well contents were determined. The addition of 0.5% FCS did not alter the results, since adhesion assays performed without addition of protein to the RPMI-Hepes solution, 0.5% BSA or 0.5% normal mouse serum showed similar blocking effects with anti-α_V antagonists. Also, adhesion assays to CD31-3D was repeated with two further independently cloned CD31-3D molecules to exclude the possibility that somatic mutations were introduced to the CD31cDNA while integrated in the myeloma genome.

The quantification of LAK cell binding to the cultured endothelioma cell line eEnd.2 was performed as described (56). Briefly, 3 × 10^4 endothelioma cells were seeded into 96-well microtiter plates (Costar) and cultured for 24 h. After washing the cells twice with RPMI-Hepes medium, antibodies and/or additional reagents were added. LAK cells were added, incubated for 30 min on ice and then incubated at 15°C for 45 min and analyzed as described above.

Data are expressed as % binding relative to the control group. 10^6 ^{51}Cr-labeled LAK cells represented 3.3–8.5 × 10^5 cpm, 10^6 ^{51}Cr-labeled FTF1.26 cells represented ~1.1 × 10^5 − 1.2 × 10^6 cpm. Coating of wells by 10 µg/ml CD31 generally lead to the binding of 4–20% adhesion according to the batch of LAK cells used. Coating of 120 µg/ml doubled the number of bound cells. In both cases, binding could be inhibited by anti-$\alpha_V\beta_3$ antibodies. When assayed on endothelial cells, 20–30% of all added LAK cells bound.

Affinity Isolation of CD31 Ligand

E.End.2 or LAK cells were surface labeled for 45 min with ^{125}I (Amersham) followed by thorough washes with PBS. The following steps were all performed at 4°C. The pellet was lysed in 30 mM Octyl-β-D-glucopyranoside (Fluka), 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $MnCl_2$, 1 mg/ml Ovalbumin, 1 mM PMSF, 10 µg/ml leupeptin and 2.3 TIU/ml of Aprotinin (all Sigma Chem. Corp., St. Louis, MO) for 10 min. The lysate was then precleared from nonspecifically binding material with ~50 µl of packed Sepharose (Pharmacia). The precleared lysate was then aliquoted and incubated with ~30 µl preformed affinity matrices. The affinity matrices were prepared by incubating 4 mg of rat anti–mouse cκ mAb 187.1 (ATCC) per ml CNBr-activated Sepharose beads overnight in Borate-buffered saline, pH 8.5. After removal of unbound antibodies, the beads were incubated with 20 µg of either CD31-3D or CD4-anti CD3 Janusin, washed and resuspended in the appropriate buffer. After incubating the labeled cell lysates with the affinity matrices for 4 h, the beads were washed five times with Tris-buffered saline (TBS) pH 8.5, 0.05% Triton X-100, 1 mM $MnCl_2$ and twice with a washing buffer lacking $MnCl_2$. Elution was done twice with 40 µl 3 mM EDTA in 50 mM sodium acetate pH

5.2, 0.05% Triton X-100. Eluted proteins were separated by SDS-PAGE under nonreducing and reducing conditions and detected by fluorography using Kodak X-OMAT X-ray films or a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using the ImageQuant™ software (Microsoft Corp. Redmond, WA). For blocking, the precipitations were performed in the presence of the anti-$\alpha_V\beta_3$ antibody, (dilution: 1:100), anti-β_3 antibody (30 µg/ml), and anti-β_1 antibody (30 µg/ml).
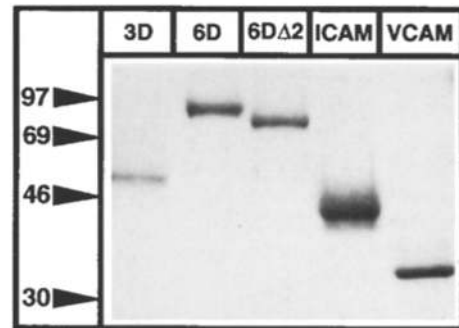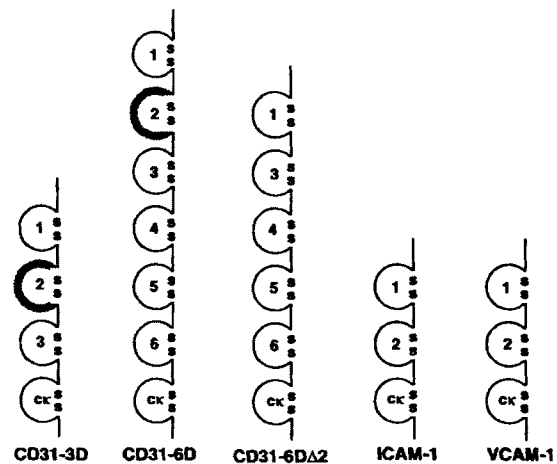
Figure 1. Production of soluble recombinant adhesion molecules. (*A*) The molecules were produced in myeloma cells, purified from supernatants by affinity columns, and analyzed on SDS-PAGE under nonreducing conditions after staining with Coomassie blue. CD31 Ig-domains 1–3 linked to the constant region of the κ light-chain, CD31-3D (*3D*); CD31 Ig-domains 1–6 linked to cκ, CD31-6D (*6D*); CD31 Ig-domains 1–6 lacking domain 2 linked to cκ, CD31-6DΔ2 (*6DΔ2*); ICAM-1 Ig-domains 1–2-cκ, ICAM-1 (ICAM); and VCAM-1 Ig-domains 1–2-cκ, 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 disulphur 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 cκ. 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 (cκ) of the mouse Ig light chain (CD31-3D, -6D, and -6DΔ2, respectively). The RNA for CD31-6DΔ2 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-cκ 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 spleno-cytes (29), were studied. These cells adhered considerably to the 3D-, moderately to the 6D- and not to the 6DΔ2-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 CD31-6DΔ2 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 CD31-6DΔ2, 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-cκ antibody, the CD31-3D and CD31-6D, but not the CD31-6DΔ2, constructs were able to stain heparin-containing mast cells on frozen sections of
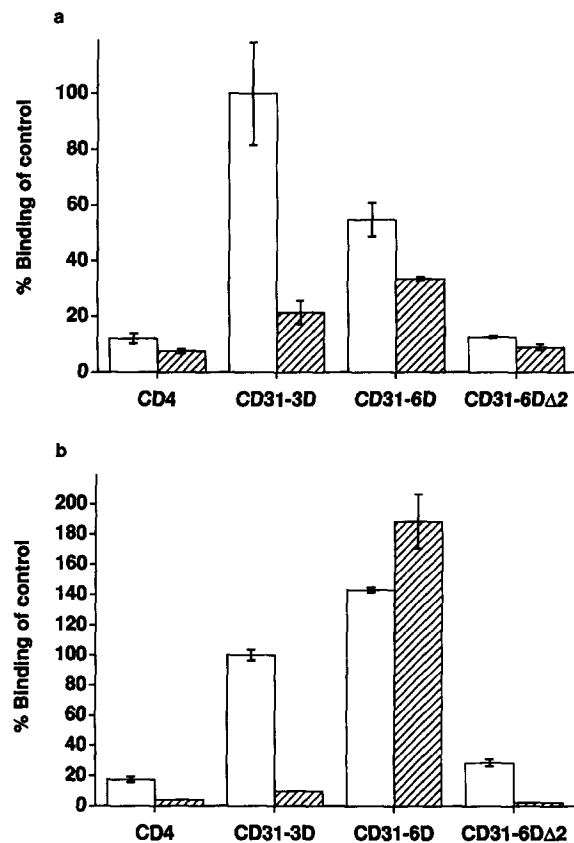
Figure 3. Leukocyte binding: cation-dependent and cation-independent CD31 interactions. Cells were allowed to bind to plates coated with recombinant molecules. (a) LAK cell binding to CD4, CD31-3D, CD31-6D, and CD31-6DΔ2 soluble molecules in the presence of 1 mM Ca^{++} and 1 mM Mg^{++} (*open bars*) or in the presence of 5 mM EDTA (*hatched bars*). (b) FTF1.26 cell binding to CD4, CD31-3D, CD31-6D, and CD31-6DΔ2 molecules in the presence of 1 mM Ca^{++} and 1 mM Mg^{++} (*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.
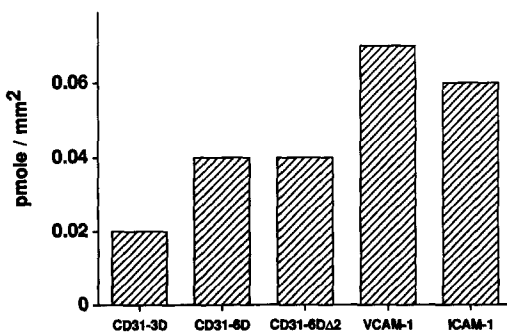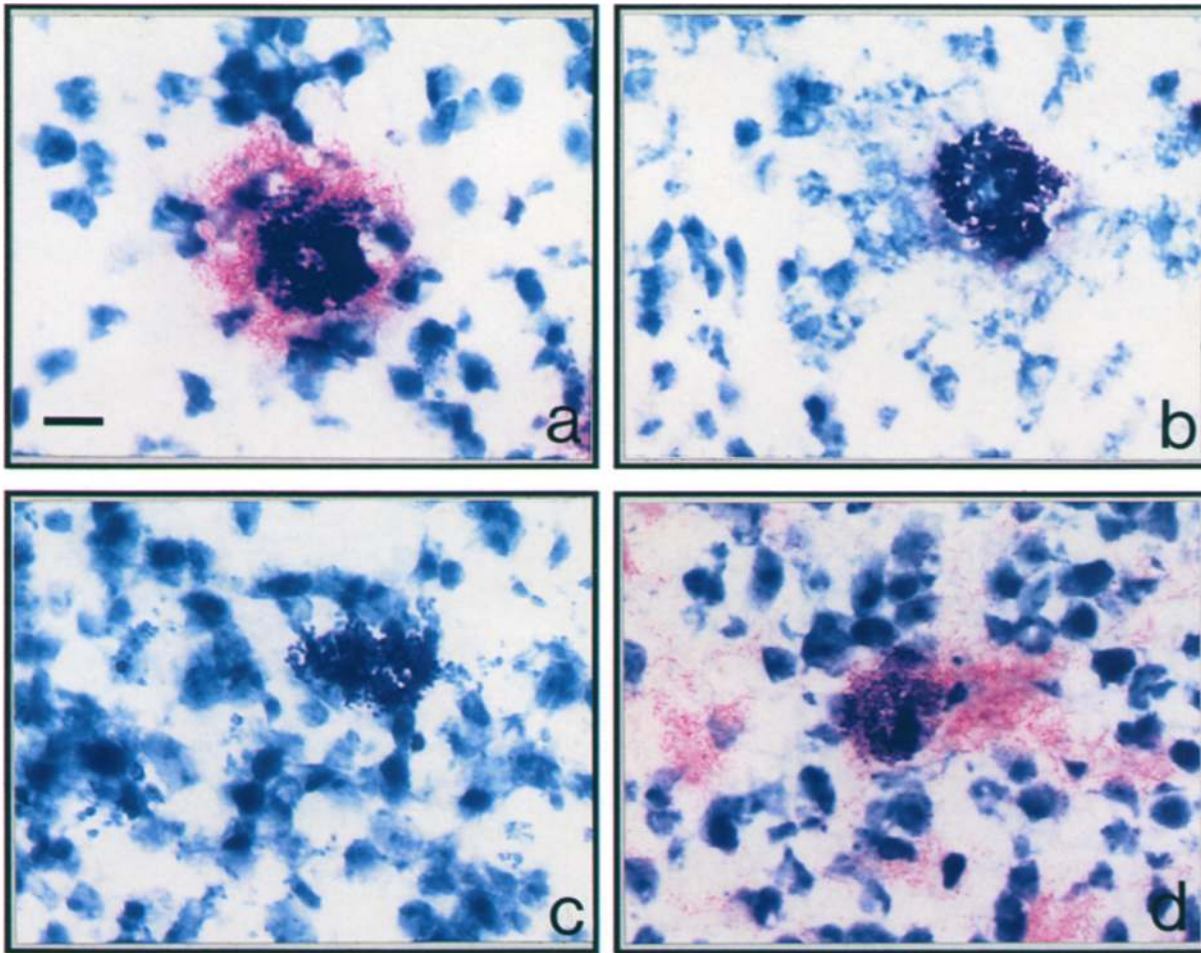
Figure 2. Binding of soluble recombinant adhesion molecules to microtiter well surface. The number of molecules bound on the surface of the microtiter well was determined as described in the Materials and Methods section. The amount of CD31-3D, CD31-6D, CD31-6DΔ2, VCAM-1, and ICAM-1 bound to the plates is expressed as pmol per mm².

Figure 4. Histochemical staining of C.B-17 SCID mouse thymus sections. Frozen sections of the thymus (6 μm) were incubated with soluble recombinant molecules and developed with alkaline phosphatase–coupled secondary antibodies. (*a*) Labeling of SCID thymus with 10 μg/ml CD31-3D. (*b*) Labeling of SCID thymus with 10 μg/ml CD31-3D in the presence of 100 μg/ml Heparin. (*c*) Control labeling of SCID thymus with CD4-anti CD3 Janusin. (*d*) Labeling with rat-anti–mouse heparan sulfate proteoglycan mAb 1948. Original magnification: 1,000; Bar, 8.5 μm.

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 antiserum raised against native $\alpha_v\beta_3$ integrin reduced binding

of LAK cells to CD31-3D to background levels (Fig. 6). Two blocking monoclonal antibodies (mAbs) reactive with the cell surface α_v integrin chain (H9.2B8 [46], and RMV-7 [72]) and a β_3 integrin chain specific mAb were also able to inhibit binding of LAK cells to CD31-3D. In contrast, blocking antibodies against α_L- or β_1 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-α_v antibody H9.2B8 (Fig. 7). Binding to VCAM-1 was inhibited by antibodies against α4 (PS/2) (48) integrin, but not by anti-α_v (Fig. 7). In contrast, binding of LAK cells to CD31-3D was inhibited by both anti-α_v 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
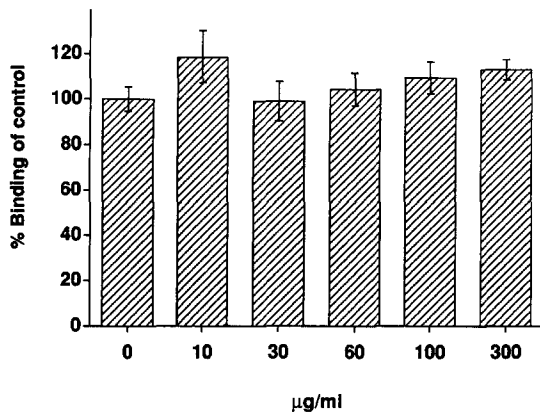
Figure 5. Leukocyte binding to CD31 is not inhibitable by Heparin. LAK cells were allowed to bind to CD31-3D in the presence of graded concentrations of Heparin. Data are expressed as mean percentage of binding ± SE of mean of three replicate wells. 100% binding is equivalent to 1,500 cpm, two independent experiments with similar results were done.

Figure 7. Inhibition by anti-α_v 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-α_v integrin H9.2B8 ($\alpha_v 1$), anti-$\alpha 4$ integrin PS/2 (α_4), and αL integrin FD441.8 (α_L). 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.

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 $\alpha_v\beta_3$ molecule making contact with CD31 is either influenced by or identical with the RGD-binding site. Since both adhesion molecules, $\alpha_v\beta_3$ and CD31, are strongly expressed by subsets of leukocytes (30, 46, 58, 71, 72) and endothelial cells (14, 18, 28), we tested whether $\alpha_v\beta_3$ 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 $\alpha_v\beta_3$ 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 $\alpha_v\beta_3$ with CD31 was involved, combinations of both Abs were applied and no additive blocking effect was observed. This shows that $\alpha_v\beta_3$ integrin can recognize CD31 in its native conformation state in the membrane of another cell.

Precipitation of $\alpha v\beta 3$ by CD31

Direct evidence for binding of CD31 to $\alpha_v\beta_3$ 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 α_v and β_3 integrin chains (49). A control construct, the CD4-anti–
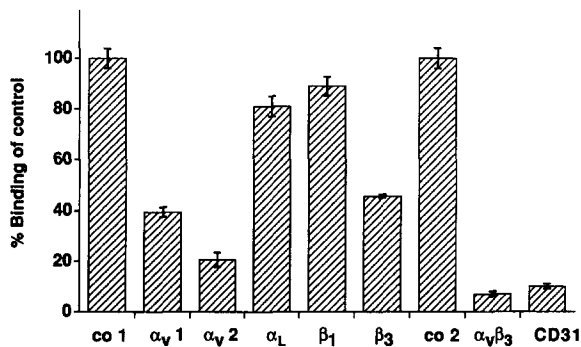

Figure 6. Binding of LAK cells to CD31 is dependent on integrin $\alpha_v\beta_3$. LAK cell binding to CD31-3D without antibody (co 1) or in the presence of 50 µg/ml blocking antibodies. The monoclonal antibodies were anti-α_v integrin H9.2B8 (α_v 1), anti-α_v integrin RMV-7 (α_v 2), anti-α_L integrin FD441.8 (α_L), anti-β_1 integrin 9EG7 (β_1) and anti-β_3 integrin Hmβ3 (β_3). The polyclonal rabbit antibodies were used at a dilution of 1:10. Preimmune serum (co 2), antiserum directed against $\alpha_v\beta_3$ integrin ($\alpha_v\beta_3$) 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.
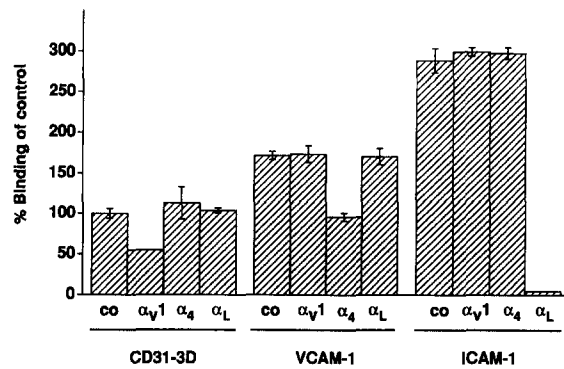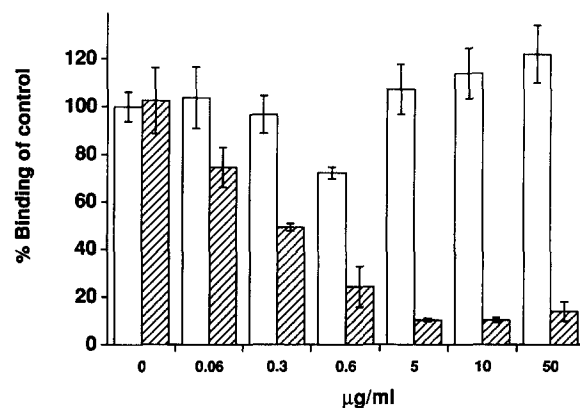

Figure 8. Binding of LAK cells to CD31 is inhibited by RGD peptides. LAK cell binding to CD31-3D in the presence of graded concentrations of peptides. The peptides were TKPR (open bars) and RGDS (hatched bars). Data are expressed as mean percentage of binding ± SE of mean of three replicate wells. 100% binding is equivalent to 1,500 cpm, four independent experiments with similar results were done.
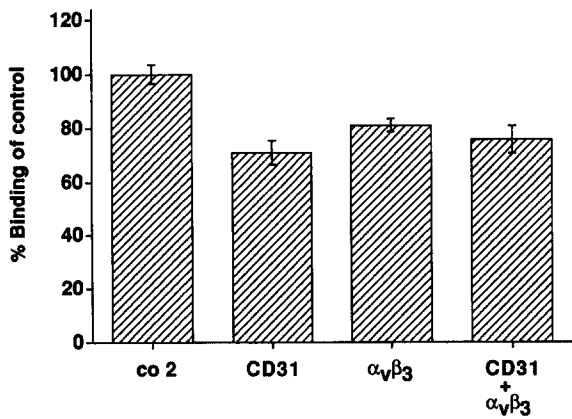
Figure 9. Binding of LAK cells to endothelioma cells is partially mediated by $\alpha_v\beta_3$-CD31 interactions. LAK cell binding to monolayers of the endothelioma cell line eEnd.2 in the presence of antibodies directed against $\alpha_v\beta_3$ ($\alpha_v\beta_3$), CD31 (CD31), combinations of both (CD31 + $\alpha_v\beta_3$) 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.

CD3-cκ 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-$\alpha_v\beta_3$ polyclonal antibodies, anti-β_3, and anti-β_1 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 $\alpha_v\beta_3$ integrin (Fig. 10 B). Upon reduction, the α_v molecule precipitated by CD31-3D migrated at

125 kD. The apparent molecular weight of the β_3 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 $\alpha_v\beta_3$ and could represent an additional cation-dependent, heterophilic ligand for CD31.

Discussion

Taken collectively, we conclude that CD31 can directly bind to the $\alpha_v\beta_3$ integrin, and that the $\alpha_v\beta_3$ integrin and CD31 constitute a heterophilic receptor ligand pair. This adds a third Ig-SF-integrin pair to the previously described ICAM/β_2 integrins and VCAM-1, MadCAM-1/α_4 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-$\alpha_v\beta_3$ contacts. These binding differences are not merely exclusive since LAK cell binding in the presence of EDTA to the CD31-6D 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
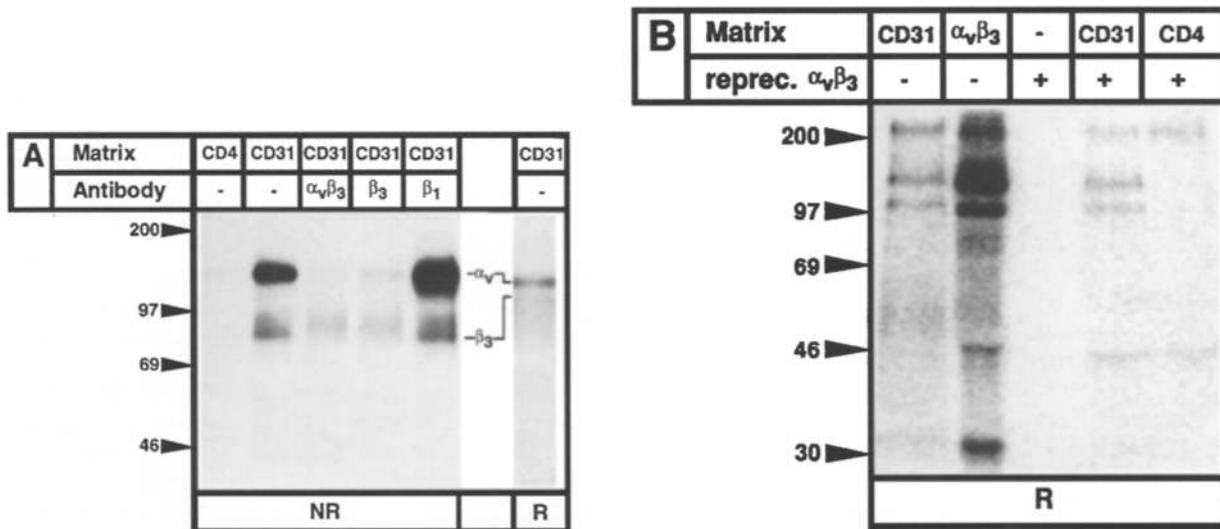

Figure 10. CD31-3D specifically precipitates $\alpha_v\beta_3$ integrin. (A) Immunoprecipitations with ^{125}I surface-labeled endothelial cell lysates. The affinity matrices were prepared with CD4-anti-CD3-cκ Janusin as control (CD4) and CD31-3D (CD31) used as such or in the presence of anti-$\alpha_v\beta_3$ antibodies ($\alpha_v\beta_3$), anti-β_3 antibodies (β_3) or anti-β_1 antibodies (β_1) and separated under nonreducing (NR) and reducing conditions (R). (B) Immunoprecipitations with ^{125}I surface-labeled LAK cell lysates. The affinity matrices were prepared with CD31-3D (CD31) and the control CD4-anti CD3-cκ Janusin (CD4). LAK cell lysate was immunoprecipitated with anti-$\alpha_v\beta_3$ antibody. The material eluted from the CD31 and CD4 affinity matrices was further immunoprecipitated with anti-$\alpha_v\beta_3$ 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 sixfold (data not shown). Since CD31 interacts with $\alpha_v\beta_3$ 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 conferred by integrins and subsequent transendothelial migration, partially due to integrins(for reviews see 1, 35, 68). Our results point to a role of $\alpha_v\beta_3$ in this adhesion cascade. Integrin $\alpha_v\beta_3$ is expressed by LAK cells and endothelial cells (Fig. 10, A and B). Furthermore, the adhesion of LAK cells to endothelial monolayers was inhibitable with antibodies directed to $\alpha_v\beta_3$ integrin and to native CD31 (Fig. 9). Since the blocking effect of the antibodies was not additive, we conclude that indeed $\alpha_v\beta_3$ and CD31 form an adhesion molecule pair involved in the adhesion of leukocytes to endothelium. Formerly, integrin $\alpha_v\beta_3$ 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 $\alpha_v\beta_3$ integrin expression by monocytes (64) as well as endothelial cells (14). Thus both endothelial and monocyte $\alpha_v\beta_3$ 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 ($\alpha_4\beta_1$) 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 $\alpha_4\beta_1$ and $\alpha_v\beta_3$, 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 $\alpha_4\beta_1$ and $\alpha_v\beta_3$ 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 $\alpha_v\beta_3$ 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 $\alpha_v\beta_3$-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 $\alpha_v\beta_3$ integrin. Since $\alpha_v\beta_3$ 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_v 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_v 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 $\alpha_v\beta_3$ was inhibitable by RGD peptides. This effect is similar to our finding with $\alpha_v\beta_3$ binding to CD31. This confirms that alternative recognition sequences for α_v integrins other than RGD do exist as reported for $\alpha_5\beta_1$ integrin (40). These may be specific for subclasses of RGD-binding integrins, i.e., be specific for $\alpha_v\beta_3$.

Similarly to leukocytes, metastasizing malignant cells are transported by the blood to distant tissues (70). On melanoma cells, increased expression of $\alpha_v\beta_3$ 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 $\alpha_v\beta_3$ (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 $\alpha_v\beta_3$ integrin (10). Blocking of $\alpha_v\beta_3$ by antagonists disrupted the new formation of blood vessels and rendered the endothelial cells apoptotic (11). Fur-

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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 $\alpha_v\beta_3$ may confer endothelial ECM contacts, the novel $\alpha_v\beta_3$-CD31 ligand pair might be involved in angiogenesis.

In view of the possible implications, blocking of $\alpha_v\beta_3$-CD31 dependent adhesion may offer important targets for therapeutic interventions in the treatment of inflammation, angiogenesis, and metastasis formation.

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