The L1 Adhesion Molecule Is a Cellular Ligand for VLA-5

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Abstract. The L1 adhesion molecule is a member of the immunoglobulin superfamily shared by neural and immune cells. In the nervous system L1 can mediate cell binding by a homophilic mechanism. To analyze its function on leukocytes we studied whether L1 could interact with integrins. Here we demonstrate that VLA-5, an RGD-specific fibronectin receptor on a wide variety of cell types, can bind to murine L1. Mouse ESb-MP cells expressing VLA-5 and L1 could be induced to aggregate in the presence of specific mAbs to CD24 (heat-stable antigen), a highly and heterogeneously glycosylated glycophosphatidylinositol-linked differentiation antigen of hematopoietic and neural cells. The aggregation was blocked by both mAbs to L1 and VLA-5, respectively. Aggregation was blocked also by a synthetic RGD-containing peptide derived from the Ig-domain VI of the L1 protein. ESb-MP subclones with low L1 expression could not aggregate. In heterotypic binding assays mouse bone marrow cells could adhere in an L1-dependent fashion to platelets that expressed VLA-5. Also purified L1 coated to polystyrene beads could bind to platelets. The binding of L1-beads was again inhibited by mAbs to L1 and VLA-5, by soluble L1 and the L1-RGD peptide in a dose-dependent manner. Thymocytes or human Nalm-6 tumor cells expressing VLA-5 could adhere to affinity-purified L1 and to the L1-derived RGD-containing peptide coated to glass slides. The adhesion was strongly enhanced in the presence of Mn^{2+}-ions and blocked by mAbs to VLA-5. We also demonstrate a direct L1–VLA-5 protein interaction. Our results suggest a novel binding pathway, in which the VLA-5 integrin binds to L1 on adjacent cells. Given its rapid downregulation on lymphocytes after induction of cell proliferation, L1 may be important in integrin-mediated and activation-regulated cell–cell interactions.

Leukocyte function is crucially dependent on cell-adhesion which provides the necessary mechanical stability for cell–cell contact but may also deliver signals from the micro-environment. Integrins have been shown to be important activation-dependent adhesion molecules that possess also signaling potential (for review see reference 37). On leukocytes, integrins are involved in cell–cell and cell–extracellular matrix interactions and play a role in physiological processes such as the regulation of lymphocyte responses to antigenic stimulation, the recirculation into lymphoid tissues or the extravasation at sites of inflammation (for reviews see reference 17, 78). The β2-integrins LFA-1 (αLβ2) and Mac-1 (αMβ2) and the α4-integrins participate in distinct mechanisms for leukocyte–endothelial and leukocyte–leukocyte binding (for review see reference 20). Several members of the Ig superfamily have been identified as cell surface ligands for these integrins. LFA-1, can bind to intercellular cell adhesion molecule (ICAM)-1[1] or ICAM-2 on the surface of stimulated or unstimulated endothelial cells or to ICAM-3 on lymphocytes. ICAM-1 and ICAM-2 are not restricted to endothelial cells but are also expressed by leukocytes and are involved in many leukocyte functions including the recognition of target cells by cytotoxic T lymphocytes and the collaboration of T- and B-lymphocytes. The α4-integrins VLA-4 (α4β1) and LPAM-1 (α4β7) mediate leukocyte adhesion to the vascular cell adhesion molecule VCAM-1 (65), and the addressin MAdCAM-1 (9). These interactions are important for the migration and recirculation of lymphocytes from the blood stream into lymphoid organs. VLA-4 can also support the binding of leukocytes to fibronectin involving an LDV peptide motif in the HepII/IIICS region of fibronectin (CS-1 peptide) (30, 48).

Other integrins of the VLA family promote the binding of leukocytes to extracellular matrix components like laminin, collagen or fibronectin (for review see 34, 36). The VLA-5 integrin is the classical fibronectin receptor and it binds to fibronectin via an RGD-sequence in the

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[1] *Abbreviations used in this paper*: 2-ME, 2-mercaptoethanol; BOG, β-octylglucoside; GPI, glycosylphosphatidylinositol; ICAM, intercellular adhesion molecule; PE, phycoerythrin.

cell binding domain in the center of the molecule which is close to the COOH terminus of the tenth type III repeat of fibronectin (59, 67). VLA-5 is expressed on many cell types including fibroblasts, epithelial and endothelial cells, platelets, thymocytes and T lymphocytes and is involved in the regulation of cell adhesion and matrix assembly (3, 38), in cell migration beneath stromal cells (63), in the adhesion and migration of neural crest cells (7, 13) and has been implicated to influence tumor growth (29, 71, 77). An alternative cellular ligand for VLA-5 has not been identified.

The L1 cell adhesion molecule is a 200-kD transmembrane glycoprotein belonging to the immunoglobulin superfamily (64, 69). Structurally related antigens that probably represent homologous molecules have been identified in other species including rat (NILE) (56, 61), chick (Ng-CAM) (16), drosophila (neuroglian) (10), and human (46, 86). L1 was first described in the nervous system and appears to mediate binding by distinct mechanisms: (*a*) homotypic binding involving L1–L1 interactions (40, 41, 75); (*b*) assisted homophilic binding between L1 and L1–NCAM complexes at the surface of adjacent cells (40, 41); and (*c*) heterotypic binding of which the interaction with the axon-associated CAM axonin-1 (53) and the chondroitin sulfate proteoglycan phosphacan (62) are well characterized. Further ligands for L1 have been postulated but have not been identified. The L1 molecule plays an important role in cell migration and axon outgrowth in the nervous system. L1 expression was also found on normal and transformed cells of hematopoietic origin (50, 51). Bone marrow cells, mature thymocytes and the majority of peripheral B and T lymphocytes express the antigen. Whereas the function of L1 in the nervous system is well established its role in the adhesion of leukocytes is not known.

Here we report that the L1 adhesion molecule as well as an RGD-containing peptide derived from the L1 sequence can serve as a ligand for VLA-5. In the presence of mAbs to CD24, a highly glycosylated glycophosphatidylinositol-linked differentiation antigen, mouse ESb-MP cells could aggregate in an L1 and VLA-5–dependent manner. Our results suggest that VLA-5 and L1 constitute a novel binding pathway involved in homotypic and heterotypic cell adhesion. Given its rapid downregulation on lymphocytes and neutrophils after cell activation (35), L1 may be important in integrin-mediated and activation-regulated interactions of leukocytes during the immune response. The VLA-5–L1 binding pathway may also be important in other cell–cell interactions.

Materials and Methods

Cell Culture

The murine bend3 endothelioma cell line was kindly provided by Dr. W. Risau (Max-Planck Institut Bad Nauheim, Germany) and was maintained in DME with high Glucose (Life Technologies, Eggenstein, Germany) containing 10% FBS (low endotoxin; Life Technologies). The monocytic tumor cell line ESb-MP was cultivated in RPMI 1640 supplemented with 10% FBS, 2-mercaptoethanol (2-ME), Hepes, and Glutamine as previously described (76). The isolation and characterization of ESb-MP subclones with $L1^{hi}$ or $L1^{lo}$ expression has been described previously (50, 51). All cells were kept at 37°C, 5% CO_2, and 100% humidity. Thymocytes were collected from 6–8-wk-old DBA/2 or Balb/c mice. Platelets were isolated from mouse blood as described (2). Residual erythrocytes were lysed by brief incubation in 155 mM NH_4Cl, 0.1 mM EDTA, 10 mM $KHCO_3$ solution followed by washing of the cells. Purified platelets expressed vonWillebrand factor and P-selectin as described (2). The human Nalm-6 pre–B-cell line was obtained from Dr. R. Schwarz-Albiez (DKFZ, Heidelberg, Germany).

Antibodies

mAbs 79 and M1.69 against mouse CD24 have been described before (42); mAbs 30G12 against mouse CD45; MK2.7 against VCAM-1, YN.1/1.7 against ICAM-1, 324 and 555 against the L1 adhesion molecule, 12-15 against mouse CD2, mAbs PS/2, and 5/3 against α4 integrins were also described before (4, 51). mAbs FD441.8 (TIB 213) and FD 18.5 recognizing the α-chain of LFA-1 and M1/70.15.11.5 (TIB 128) against the α-chain of Mac-1 were obtained from American Type Culture Collection (Rockville, MD) and were described before (2, 51). mAb 5H10-27 (MFR5) against CD49e (mouse α5) (mAb α5(1)) was obtained from Pharmingen (Dianova, Hamburg, Germany). HMα5-1 and HMα5-2 (mAb α5(2)) are blocking hamster mAbs against mouse α5 integrin (88). MAb RMV-7 is a blocking antibody to mouse vitronectin receptor on lymphocytes (79). MAb RB40.34 recognizing mouse P-selectin (12) was a gift of D. Vestweber (Max-Planck Institute für Immunbiologie, Freiburg, Germany). mAb EA-1 (39) against mouse α6-integrin was a gift of B. Imhof (Basel Institute for Immunology, Basel, Switzerland). The mAb to human VLA-5 (Sam-1) was obtained from Dianova. The anti-human CD24 mAb ML5 was obtained from Dr. S. Funderud (Raaium Hospital, Oslo, Norway). mAbs were used in a purified form or as hybridoma supernatants.

Peptides

Peptides were synthesized using Fmoc strategy and purified by preparative HPLC. They were characterized further by analytical HPLC and mass spectroscopy. The L1-RGD peptide was CW**RGD**GRDLQE**RGD**SDK. For control the peptide VAIYDDMESLPLTGT was used. The cyclic peptide GA*CRRETAWAC*GA has been reported to inhibit specifically human $\alpha5\beta1$-mediated cell binding to fibronectin (47). The **RGDS** peptide was obtained from Sigma (Taufkirchen, Germany). Peptide-carrier protein conjugates for cell adhesion were produced by crosslinking *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) activated rabbit IgG with peptides via the NH_2-terminal Cystein residue (4). Unconjugated IgG was used for control purposes in the binding assays. Peptide-carrier conjugates were coated to glass slides as described below. The cyclic RGD peptide 66203 (cycloRGDfV) that inhibits preferentially αv (Brooks et al., 1994) and 69601 (cycloRADfV) (control peptide) were obtained from Dr. A. Jonczyk (E. Merck, Darmstadt, Germany).

Cytofluorography

The staining of cells with saturating amounts of mAbs, either hybridoma supernatants or purified antibodies, and phycoerythrin (PE)-conjugated goat antibodies to rat immunoglobulins (SERVA, Heidelberg, Germany), has been described elsewhere (50). Stained cells were analyzed with a FACScan fluorescence activated cell analyzer (Becton Dickinson, Heidelberg, Germany).

Affinity Purification of Cell Surface Antigens

L1 was purified by affinity chromatography on a mAb 324 column from lysates of ESb-MP cells or N2A neuroblastoma cells essentially as described elsewhere (35). CD2 was isolated from mouse thymocyte lysate on a mAb 12–15-sepharose column. The elution buffer contained 100 mM diethylamine/HCl, pH 11.5, 150 mM NaCl with 50 mM β-octylglucoside (BOG). Soluble L1 from mouse brain was a gift of Dr. G. Kadmon (DKFZ, Heidelberg, Germany). VLA-5 was isolated from thymocyte lysate on a HMα5-1-sepharose column. The antigen was eluted with 0.2 M acetic acid, 50 mM BOG, 500 mM NaCl containing 2 mM $CaCl_2$ and $MgCl_2$. Eluted fractions were neutralized and analyzed by ELISA with respective antibodies and by SDS-PAGE. Purified antigens were stored at −20°C. For coating to polysterene beads L1 antigen ($\sim$150 μg/ml) in BOG was incubated with the beads (SERVA) and the detergent was removed by dialysis as described (35). Remaining binding sites were blocked by incubation with 1% BSA in PBS for 1 h. The density of coated L1 was determined by FACS analysis using biotinylated mAb 324 followed by Streptavidin-Phycoerythrin (Dianova). The soluble mouse CD2

Cκ fusion protein (the extracellular domains of CD2 fused to the constant part of the mouse κ light chain) was a kind gift of R. Rutschmann (Basel Institute for Immunology, Basel, Switzerland).

Homotypic Cell Aggregation Assay

For homotypic aggregation ESb-MP cells at 2 × 10^6/ml were incubated in the presence of 10–50 μg/ml of purified mAb 79 to mouse CD24 in complete RPMI 1640 medium for 30 min at room temperature in 1.5-ml Eppendorf tubes. Tubes were rotated headwise to keep cells in suspension. At the end of incubation time the cells in medium were transferred to 24-well tissue culture plates, were allowed to sediment for 5 min and were then scored for aggregation.

Heterotypic Cell Binding Assay

For binding assays the bend3 endothelioma cells were grown to confluency in LABTEK glass chamber slides (Nunc, Wiesbaden, Germany). For platelet binding experiments the platelets were stimulated with PMA (10 ng/ml) for 10 min at room temperature, fixed with 2% formaldehyde, and adsorbed to LABTEK-slides for overnight at 4°C (2). Before the binding assay the slides were incubated in tissue culture medium containing 10% FBS to block residual binding sides. Cells or coated polysterene beads (2 × 10^6 /ml or 4 × 10^6 /ml, respectively) in HBSS containing 10 mM Hepes, 2 mM Ca^{2+} and Mg^{2+} (binding buffer) were incubated for 20 min at room temperature on the cell monolayer under constant shaking on a rotary platform (70–80 rpm). Slides were then dipped in PBS to remove unbound beads or cells, fixed with 2% glutaraldehyde and counted.

For binding of cells to purified L1 the antigen in BOG was diluted 1:10 to 1:30 with 10 mM Tris-HCl, pH 8.0, 150 mM NaCl (TBS), and were then coated to LABTEK slides for 16 h at 4°C. Wells were blocked with 1% BSA in PBS or 1% ovalbumin in TBS for 2 h at room temperature, washed with binding buffer and used for the assay. For binding, cells (5–10 × 10^6/ml) were suspended in the same buffer and 0.2-ml aliquots were added to the coated slides. The binding assay was performed for 30 min at room temperature without shaking and the slides were washed and fixed as described above. For antibody or peptide-blocking studies, cells were preincubated with purified antibody or peptides at the indicated concentration for 10 min at room temperature and then transferred to the chamber slides. For Mn^{2+}-activation, the Ca^{2+} and Mg^{2+} ions in the buffer were substituted with 0.5 mM Mn^{2+}. Cell binding was measured by counting six independent 10× fields by video microscopy using IMAGE 1.47 software.

Biochemical Analysis

SDS-PAGE and Western blotting procedures have been described previously (40, 42, 54). Biotinylation of soluble L1 or the CD2-Cκ fusion protein was performed at ~100 μg/ml using a similar procedure as previously described for mAbs (35). Procedures for ELISA were also described before (41). For the binding of biotinylated antigens to VLA-5 the affinity-

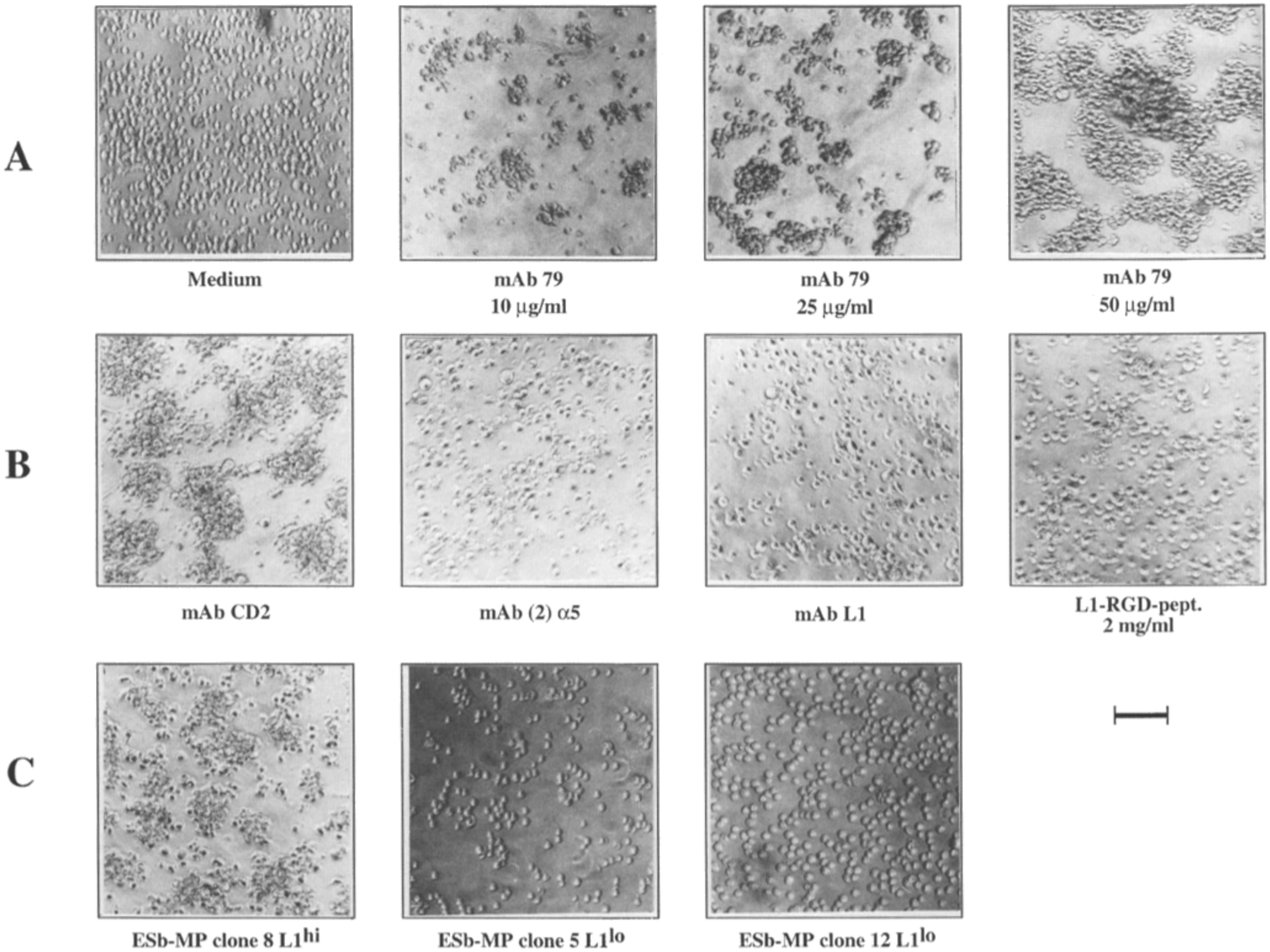

Figure 1. CD24 induced homotypic aggregation of ESb-MP cells. (*A*) ESb-MP cells (2 × 10^6 /ml) were incubated under constant head-over head rotation for 30 min in the presence of increasing concentrations of the inducing CD24 mAb (mAb 79) at room temperature. The cells were then transferred to a microtiter plate and photographed. (*B*) ESb-MP were activated for 30 min by mAb 79 (50 μg/ml) in the presence or absence of the indicated mAbs to L1 (324 and 555), VLA-5 (HMα5-1), L1-RGD peptide or a mAb to CD2 for control. (*C*) ESb-MP subclones with different L1 phenotype ($L1^{hi}$ or $L1^{lo}$) were activated as described above. The phenotype of each subclone as assessed by FACS analysis is indicated. Bar, 83 μm.

isolated VLA-5 antigen (~5 μg/ml) in BOG was coated to ELISA plates by dilution (1:10) with 10 mM Tris-HCl, pH 8.0, 150 mM NaCl containing 1 mM of each Ca^{2+}, Mg^{2+}, and Mn^{2+} ions (TBS plus ions). Affinity-purified L1 or CD2 were coated under similar conditions. All following washes and incubation steps with biotinylated L1 or CD2-Cκ fusion protein (1 μg/ml) were carried out in the same buffer. Bound biotinylated antigens were detected by streptavidin-conjugated peroxidase (Dianova) and 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) as substrate.

Statistical Analysis

Tests for statistical significance between the quantitative effects of different treatments were done using the Wilcoxon's rank sum test. Statements regarding differences are significant with a value of $p \leq 0.0043$.

Results

L1-mediated Homotypic Aggregation of ESb-MP Cells

We have shown in earlier studies that ESb-MP cells form homotypic cell aggregates that can be blocked by L1-specific antibodies (40, 50). This spontaneous aggregation occurred within 3 h at 37°C and was not seen in $L1^{lo}$-expressing subclones of ESb-MP cells that were obtained from the parental subline suggesting an important role for L1 in this process (50). We now show that strong homotypic aggregation in shorter time can be induced in the presence of mAbs against mouse CD24. Fig. 1 *A* indicates that the CD24-specific mAb 79 caused aggregation within 30 min at 24°C in a dose-dependent manner. This effect was not dependent on the Fc-portion of the mAb since an isotype-matched control mAb (30G12) did not induce aggregation. Another mAb against CD24, i.e., M1.69, also caused aggregate formation (data not shown).

To examine whether the CD24-induced aggregation also involved L1, blocking studies were performed. As summarized in Table I and depicted in Fig. 1 *B*, a mixture of L1-specific mAbs (324 and 555) inhibited the aggregation, whereas control mAbs against CD2 (12-15), CD45 (30G12), ICAM-1 (YN 1/1.7), VCAM-1 (MK2.7) showed no effect. Soluble L1 at 2 μg/ml significantly reduced aggregate formation. Fig. 1 *C* indicates, that the $L1^{lo}$-expressing subclones (subclones 5 and 12) in contrast to the $L1^{hi}$ subclone 8 could not undergo CD24-induced homotypic aggregation although the levels of CD24 and VLA-5 (see below) were comparable as judged by FACS analysis (not shown). These results suggested, that CD24 mAbs can drastically enhance the L1-mediated homotypic aggregation of ESb-MP cells.

ESb-MP Cell Aggregation Is Blocked by mAbs to VLA-5

L1 has been shown to participate in homophilic and heterophilic interactions (40, 53, 62). Since the homotypic aggregation of ESb-MP cells did not occur at 4°C (data not shown) we wondered whether the putative counter-receptor for L1 might be an integrin. We therefore analyzed ESb-MP cells for integrin expression. As shown in Fig. 2 *A*, ESb-MP cells expressed LFA-1, Mac-1, α4-, α5-, α6-integrins, and the vitronectin receptor αVβ3 as revealed by FACS staining with the respective mAbs. When these mAbs were tested for blocking capacity in the homotypic aggregation assay, only the two mAbs to VLA-5 showed significant inhibition (Table I and Fig. 1 *B*). All other mAbs to integrins or their respective cellular ligands as listed in Table I had no effect.

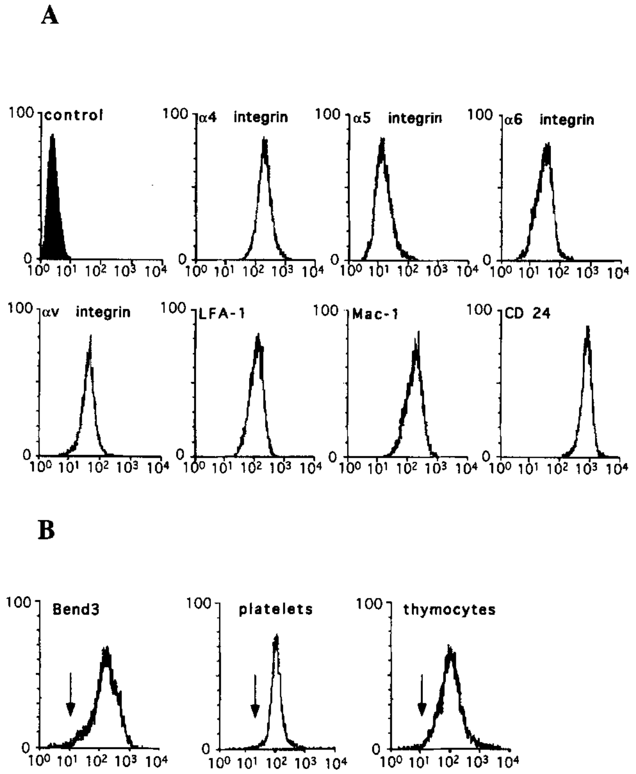

Figure 2. Integrin expression pattern of ESb-MP cells. (*A*) ESb-MP cells were stained with saturating amounts of mAbs to the indicated antigens followed by PE-conjugated second antibodies and then analyzed for fluorescence. (*B*) Analysis of VLA-5 expression on mouse platelets, bend3 endothelioma cells, and thymocytes. Arrows indicate the right border of background fluorescence (second antibody only).

An L1-derived RGD-containing Peptide Inhibits Homotypic Aggregation

VLA-5 (α5β1) is a fibronectin receptor and binds to an RGD site in fibronectin. We reasoned that if VLA-5 would be a counter-receptor for L1 it might bind to the two RGD sites known to be present in close proximity in the Ig-domain VI of the L1 molecule (64). We therefore examined the effects of RGD-containing peptides on the CD24 induced aggregation of ESb-MP cells. As shown in Table I the peptide **RGD**S at 2 mg/ml effected the homotypic aggregation only weakly. In contrast, the RGD peptide CW**RGD**GRDLQER**GD**SDK derived from the 6th domain of L1 showed a good blocking effect that was dose dependent. At a concentration of 5 mg/ml peptide there was virtually no aggregation observed even after longer incubation times at 37°C although the cells remained viable as judged by tryphan blue exclusion. A control peptide VAIYDDMESLPLTGT in the same buffer as the L1-RGD peptide and tested in the same range of concentration (1–5 mg/ml) had no effect on the aggregation. The cyclic peptide GA*CRRETAWAC*GA which has been reported to inhibit specifically human α5β1-mediated cell binding to fibronectin (47) also showed an inhibitory ef-

Table I. Inhibition of the CD24-induced Homotypic Aggregation of ESb-MP Cells by mAbs and Peptides

Reagent	Antigen	Homotypic aggregation
Antibodies		
Medium	–	++++
mAb 30G12	CD45	++++
mAb 12-15	CD2	++++
mAbs 324+555	L1	+
mAb MFR5	α5	+
mAb MHα5-2	α5	+
mAb EA-1	α6	++++
mAb RMV-7	αV	++++
mAb FD18-5	LFA-1	++++
mAb TIB213	LFA-1	++++
mAb TIB128	Mac-1	++++
mAb 5/3	α4	++++
mAb P/S2	α4	++++
mAb YN 1/1.7	ICAM-1	++++
mAb MIC2/4	ICAM-2	++++
mAb MK2/7	VCAM-1	++++
Peptides		
RGDS 2 mg/ml		+++
L1-RGD 1 mg/ml		++
L1-RGD 2 mg/ml		+
L1-RGD 5 mg/ml		–
control peptide 2 mg/ml		++++
GA*RRETAWAC*GA 1 mg/ml		+
cycloRGDfV(66203) 2 mg/ml		++++
cycloRADfV(69601) 2 mg/ml		++++

Cells at 2 × 10^6/ml were induced to aggregate in the presence of 50 μg/ml mAb 79. Blocking antibodies were used at the concentration of 10 μg/ml. Peptides were used at the indicated concentrations. Note that all mAbs shown in this table can bind to ESb-MP cells as revealed by fluorescent staining except for the mAb to VCAM-1 for which the cells are negative. Data are compiled from >10 experiments in which each antibody was tested at least twice for inhibition. Aggregation scores are: – all cells free; + 10–20% cells aggregated; ++ 20–50% cells aggregated; +++ 50–75% cells aggregated; ++++ nearly all cells in aggregates.

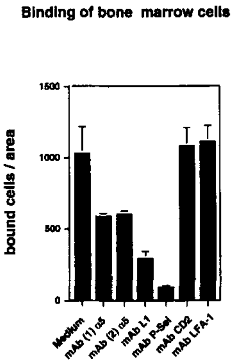

Figure 3. Binding of bone marrow cells to platelets. Platelets were immobilized to LABTEK chamber glass slides after treatment with PMA and formaldehyde fixation. Bone marrow cells at 2 × 10^6/ml were allowed to bind to the platelet monolayer in the presence or absence of the indicated antibodies. Under these conditions ~25% of input cells bind to the monolayer. After cell binding the slides were washed, fixed in glutaraldehyde, and bound cells were counted. Data are expressed as mean values ± SE. Three independent experiments were done with similar results. Area = 0.4 mm^2.

fect in the aggregation assay (see Table I). In contrast, a cyclic RGD peptide that was shown to be specific for αv-integrins (15) and a cyclic control peptide had no effect.

Heterotypic Binding of Bone Marrow Cells to Platelets Involves VLA-5

The homotypic aggregation studies had suggested a possible interaction of VLA-5 with L1 on adjacent cells as a novel mechanism of cell–cell binding. We examined whether this mechanism might also be effective in heterotypic cell adhesion. First we studied the binding of bone marrow cells (expressing L1; reference 50) to platelets that expressed VLA-5 (see Fig. 2 *B*) but no L1 (data not shown) and that had been immobilized to glass slides. Fig. 3 shows that the bone marrow cells could bind to the platelet monolayer and this binding was completely blocked by a mAb to P-selectin in agreement with previous results (2). Both mAbs to L1 and VLA-5, respectively, were also able to block the binding by ~40–70% whereas control antibodies could not (Fig. 3).

We also analyzed the binding of ESb-MP cells ($L1^{hi}$) and $L1^{lo}$ subclones to the platelet monolayer. ESb-MP cells bound well whereas the binding of $L1^{lo}$ subclones 5 and 12 was strongly reduced (65 and 50%, respectively). Both mAbs to L1 and VLA-5, respectively, were able to block the binding of ESb-MP cells to platelets by ~25% whereas control antibodies could not (data not shown). As expected, the cell binding was also blocked in the presence of the mAb to P-selectin (2). Collectively, the data from both types of binding experiment suggested that in the heterotypic binding of bone marrow cells or ESb-MP cells to platelets the VLA-5/L1 binding pathway was active in addition to the P-selectin–ligand interaction.

L1-Beads Bind to Platelets via VLA-5

We next investigated whether affinity-isolated L1 could mediate binding to platelets via VLA-5. L1 was purified by affinity chromatography from ESb-MP or N2A neuroblastoma cell lysate, respectively. As shown in Fig. 4 *A*, the purified antigen showed the expected bands of 200 and 150 kD characteristic for L1 and its subfragment. The L1 antigen was adsorbed to polysterene beads and as shown in Fig. 4 *B* could be detected by FACS analysis using L1-specific mAbs. We examined the binding of the L1-coated beads to platelets expressing VLA-5. Fig. 5 *A* shows that the L1 beads bound well to the immobilized platelets whereas control beads that had been coated with BSA did not bind. The binding was completely blocked by mAbs to L1 and to a lesser extent by mAbs to VLA-5. Control antibodies to CD2 or LFA-1 did not have an effect. The binding of L1 beads to platelets was significantly reduced in the presence of soluble L1 antigen. It was also blocked in the presence of the L1-RDG peptide in a dose-dependent manner whereas a control peptide had no effect.

Binding of L1-Beads to Bend3 Endothelioma Cells

The murine bend3 endothelioma cells expressed VLA-5 as revealed by FACS analysis (see Fig. 2 *B*). Since we observed previously that L1 beads could bind to bend3 endothelioma cells (35) we investigated a possible involvement of the VLA-5/L1 binding pathway in this interaction. As expected, the L1-coated beads could bind to bend3 endothelioma cells in a specific fashion and this binding was inhibited by mAbs to L1 but not by control mAbs (Fig. 5 *B*). In contrast to the platelet binding experiment, both mAbs to VLA-5 and the L1-RGD peptide were unable to block the binding. An inhibitory effect of the VLA-5 mAbs was also not seen after activation of endothelial cells with LPS for 4 h or when the binding assay was performed in the presence of Mn^{2+} ions to activate the VLA-5 integrins (data not shown).

To analyze whether VLA-5 was expressed on the luminal surface of the cells fluorescent staining of the bend3 monolayer with specific mAbs was performed. Significant staining could be detected (data not shown) ruling out the possibility that VLA-5 was not accessible for the binding. Thus, we concluded that VLA-5 on the luminal site was functionally not active in bend3 cells. Importantly, the experiments indicated that bend3 endothelioma cells expressed another ligand for L1 distinct from VLA-5.

Purified L1 and the L1-RGD Peptide Support Cell Binding

We next studied whether purified L1 antigen or the L1-RGD peptide could mediate the adhesion of cells. The purified L1 was coated to glass slides and residual binding sites were blocked before the assay. We analyzed the binding ability of thymocytes that expressed VLA-5 (see Fig. 2 *B*). As shown in Fig. 6 *A*, thymocytes showed weak binding to the immobilized L1 that was however enhanced when the cells were activated in the presence of 0.5 mM

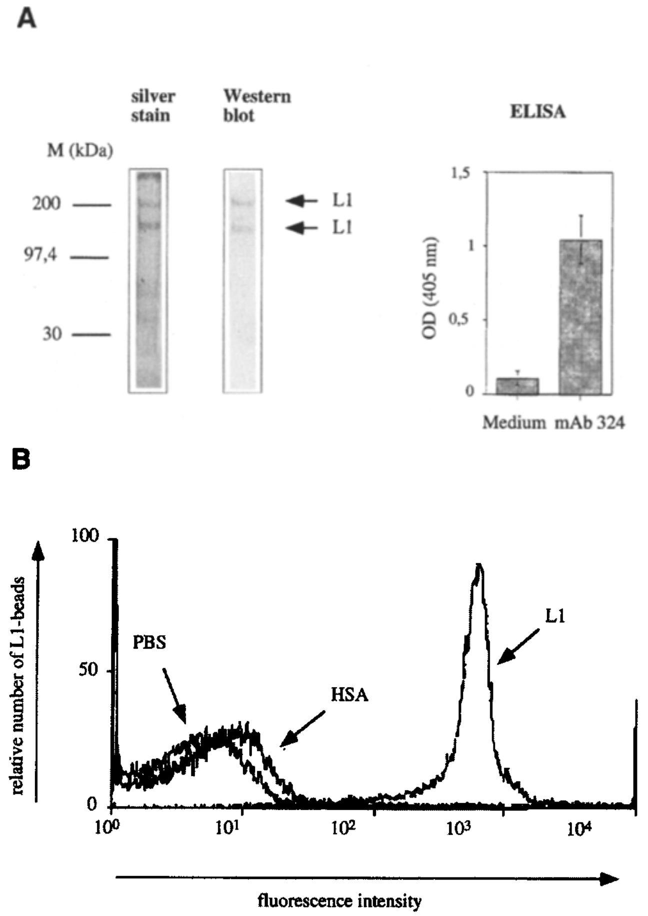

Figure 4. Characterization of purified L1 adhesion molecule. (*A*) Affinity-purified L1 antigen was analyzed by SDS-PAGE followed by silver staining or transferred to nitrocellulose and detected by Western blotting using mAb 324. The same material was also analyzed by ELISA using mAb 324 hybridoma supernatant or tissue culture medium for control. (*B*) Coating of purified L1 to polysterene beads and FACS analysis of the beads using biotinylated mAb 324 specific for L1 and biotinylated mAb 79 to CD24 (for control) followed by Streptavidin-PE. Stained beads were analyzed by FACscan analysis.

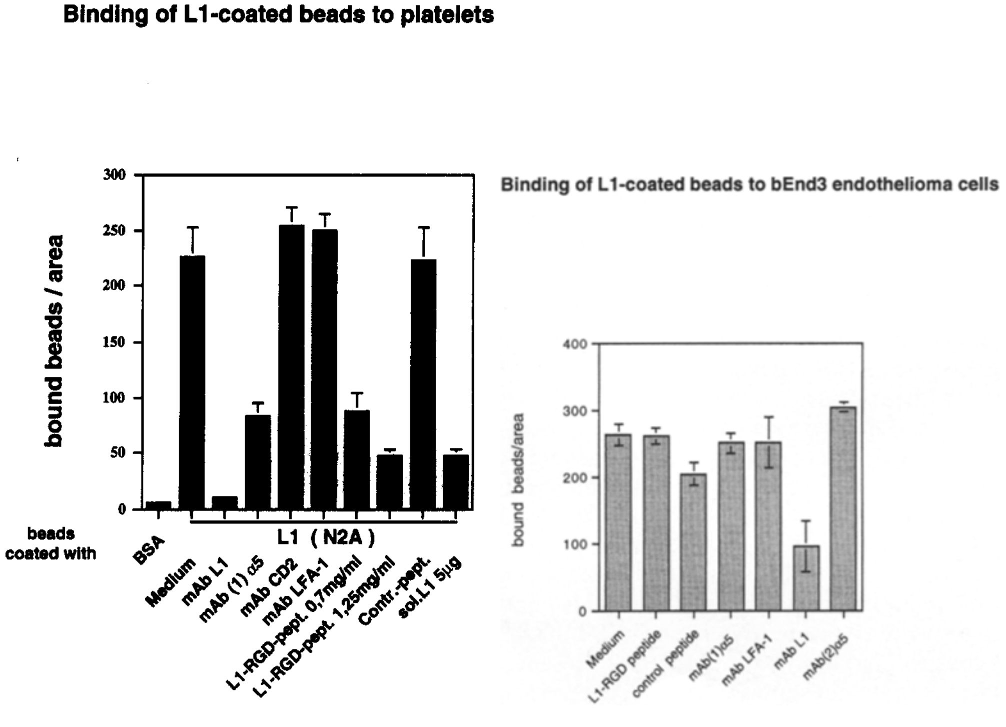

Figure 5. Binding of L1-coated polysterene beads to platelets and bend3 endothelioma cells. (*A*) L1-beads were allowed to bind to platelets immobilized to LAB-TEK chamber glass slides. The assay was carried out in the presence or absence of mAbs, peptides or soluble L1 at the indicated concentration at room temperature. For L1 blocking a mixture of mAbs 324 and 555 was used. (*B*) L1-beads were allowed to bind to bend3 endothelioma cells grown in LAB-TEK chamber glass slides. The assay was carried out in the presence or absence of mAbs, or peptides used at 0.7 mg/ml at room temperature. For L1 blocking a mixture of mAbs 324 and 555 was used. Data are expressed as mean values ± SE. Three independent experiments for each type of binding assay were carried out with similar results. Area = 0.4 mm^2.

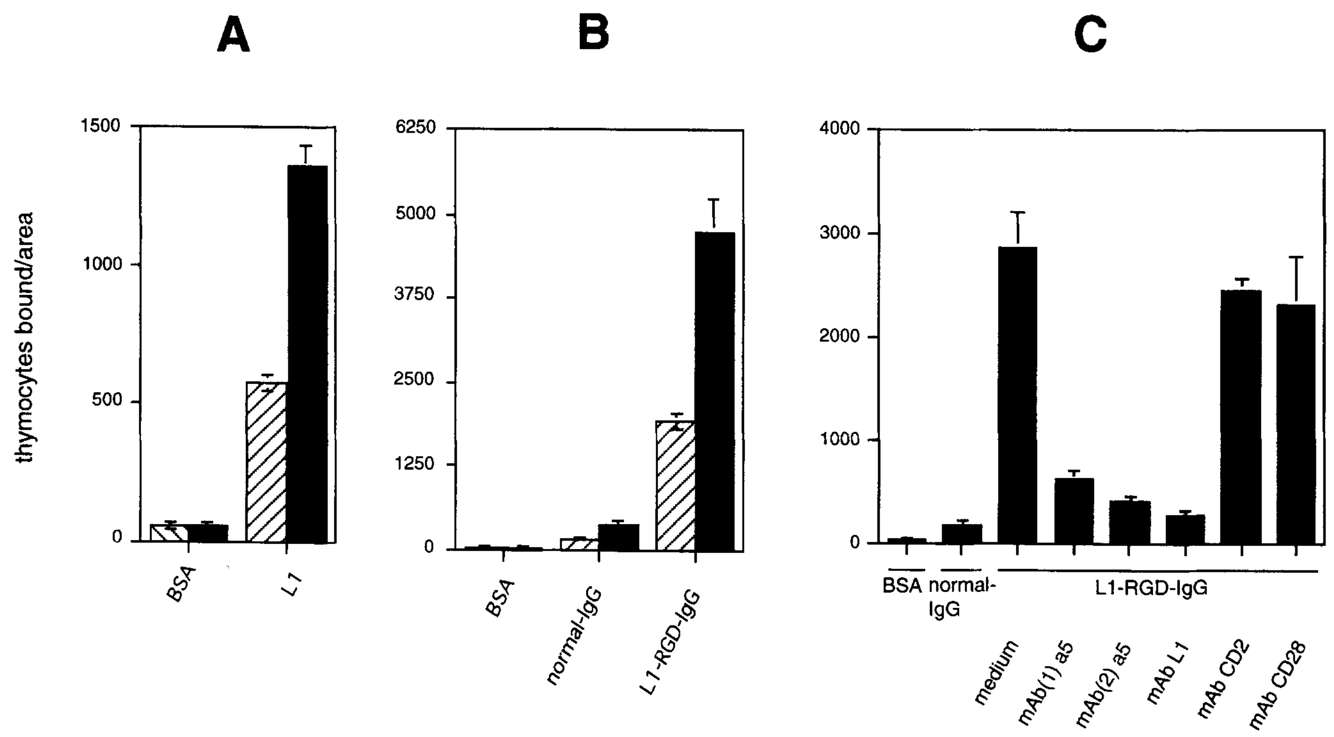

Figure 6. Binding of thymocytes to purified L1 or the L1-RGD peptide. Affinity-purified L1 antigen in BOG or the L1-RGD peptide coupled to carrier IgG (1 mg/ml) were coated to LABTEK chamber glass slides. Thymocytes in HBSS/10 mM Hepes containing 2 mM of each Ca^{2+} and Mg^{2+} (*stripped bars*) or, alternatively, in the presence of 0.5 mM Mn^{2+} (*solid bars*) were tested for binding. Data are expressed as mean values ± SE. Three independent experiments with similar results were done. Area = 0.4 mm^2.

Mn^{2+} ions. The ESb-MP cells also showed weak binding to immobilized L1 that was enhanced by Mn^{2+} treatment of the cells (data not shown). However, due to the ability of these cells to adhere to glass the background binding was much higher than with thymocytes.

To demonstrate direct binding of cells to the L1-RGD site the peptide CW**RGD**GRDLQE**RGD**SDK was conjugated to rabbit IgG as carrier and coated to glass slides in increasing concentrations. As shown in Fig. 6 *B* the L1-RGD peptide was very potent in promoting thymocyte adhesion which was again strongly enhanced by Mn^{2+} activation. Normal IgG (nonconjugated) alone could not support cell adhesion. When the assay was carried out in the presence of Mn^{2+} ions the binding of thymocytes to the L1-RGD–IgG was inhibited by both mAbs to VLA-5 as well as by the mixture of L1 mAbs but not by control mAbs, respectively (Fig. 6 *C*). Additional ELISA data indicated that the mixture of L1 mAbs were indeed able to bind to the immobilized L1-RGD–IgG but not to the control IgG (data not shown).

We also investigated whether L1 or the immobilized L1-RGD peptide could promote the binding of human Nalm-6 cells that are known to express VLA-5 (63). Nalm-6 cells could bind both to the L1 glycoprotein as well as to the L1-RGD peptide after Mn^{2+} activation. The binding was blocked by mAb Sam-1 against human VLA-5 but not by a control mAb to human CD24 (Fig. 7). This suggested, that L1 could promote cell binding even across species barriers.

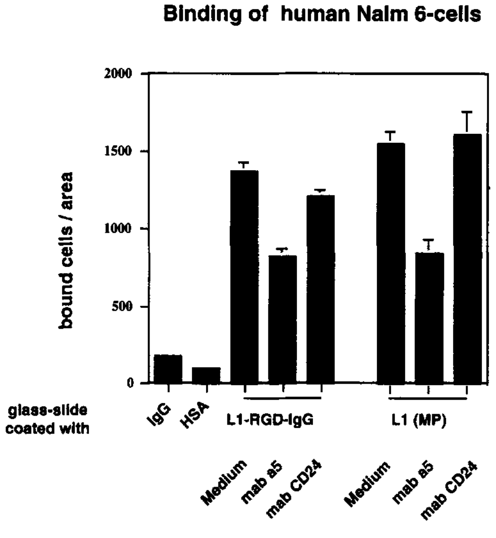

Figure 7. Binding of human Nalm-6 cells to purified L1 and the L1-RGD peptide. The L1 antigen in BOG and the L1-RGD peptide coupled to carrier IgG (1 mg/ml) were coated to LABTEK chamber glass slides. Purified CD24 (HSA) or normal unconjugated IgG served as control antigens. Human Nalm-6 pre-Bcells in HBSS/10 mM Hepes containing 0.5 mM Mn^{2+} were tested for binding in the presence or absence of the indicated antibodies. Data are expressed as mean values ± SE. Two independent experiments with similar results were done. Area = 0.4 mm^2.

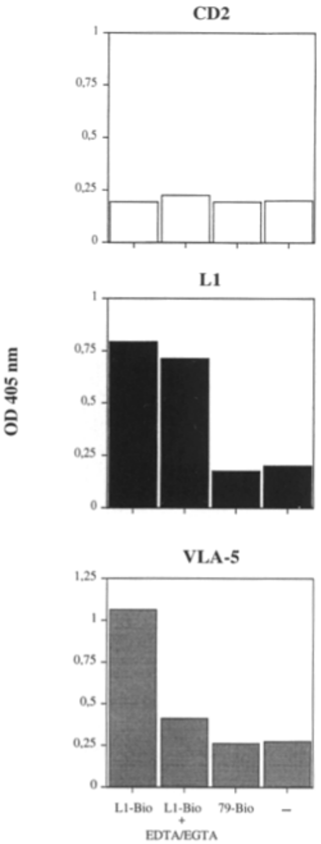

Figure 8. Demonstration of L1–VLA-5 interaction by ELISA. Biotinylated L1 was allowed to bind to immobilized CD2 (*top*), L1 (*middle*), or VLA-5 (*bottom*) in the presence or absence of divalent cations (1 mM of each Ca^{2+}, Mg^{2+}, and Mn^{2+} ions). EDTA and EGTA were used at a final concentration of 10 mM. The presence of coated CD2 on the plate was verified by ELISA using the CD2 mAb 12-15. Biotinylated mAb 79 (10 μg/ml) was used as control for nonspecific binding. Data are expressed as mean values of triplicate determinations. SE of the mean was less than 5% and is not indicated. Three independent experiments with similar results were done.

Demonstration of a Direct VLA-5–L1 Interaction

To demonstrate a direct interaction of VLA-5 and L1 an ELISA-based assay was employed. Soluble L1 was biotinylated and allowed to interact with immobilized VLA-5 in the presence of divalent cations. As shown in Fig. 8, the soluble L1 was able to bind to immobilized VLA-5 or L1 antigen. The heterophilic binding to VLA-5 was dependent on divalent cations since it was not seen in the presence of EDTA/EGTA. In contrast, the homophilic L1–L1 binding was rather insensitive to EDTA/EGTA treatment in agreement with the notion that the L1–L1 binding is Ca and Mg independent. In the presence of the L1-RGD peptide (at 500 μg/ml) the binding of L1 to VLA-5 was inhibited by ~80%. Biotinylated L1 did not bind to immobilized CD2. Conversely, a biotinylated CD2-Cκ fusion protein was unable to bind VLA-5 (data not shown). These results confirmed the functional data on a level of protein–protein interaction.

Discussion

The analysis of homotypic cell aggregation, either spontaneous or induced, has been helpful for the elucidation of general adhesion mechanisms. For the integrin-mediated homotypic aggregation of lymphocytes the interaction of LFA-1 with ICAMs and the α4-integrin–mediated binding pathway have been identified (18). A counter ligand for α4-integrins on lymphocytes has not been found, and there is suggestive evidence that a homophilic α4–α4 integrin binding may be involved (4, 68). In the present report we analyzed the homotypic aggregation induced in ESb-MP cells by treatment with mAbs to the glycosylphosphatidylinositol (GPI)-anchored molecule CD24. These cells can undergo spontaneous aggregation that occurs slowly, requires low divalent cation concentrations and is completely blocked by polyclonal antibodies to L1 (40, 50). In the presence of mAbs to CD24 the aggregation was drastically increased and was detectable in much shorter time. The available data suggested a novel mechanism of homotypic cell binding by VLA-5 integrin and L1 via RGD epitope(s) which were also involved in the heterotypic binding of bone marrow cells or ESb-MP cells to immobilized platelets.

Additional proof for a VLA-5–L1 binding came from studies in which the purified L1 antigen was coated to polysterene beads or immobilized to glass slides. In the latter case a VLA-5–mediated binding to L1 as well as to the L1-derived RGD peptide was seen with mouse thymocytes and human Nalm-6 cells. In thymocytes the level of binding to these substrates was poor however was drastically enhanced after Mn^{2+} treatment of the cells. The L1 antigen-coated polysterene beads were able to bind to platelets and bend3 endothelioma cells expressing VLA-5. The binding to both substrates was dependent on L1 and could be blocked by mAbs to L1. Binding to platelets was also inhibited by mAbs to VLA-5 and by the L1-RGD peptide whereas the binding to the endothelioma cells was clearly not affected. This was surprising since VLA-5 was detectable at the luminal cell surface of endothelioma cells and was therefore accessible for the binding of the beads. It is likely that in polarized cells like endothelial cells VLA-5 may be in different affinity states dependent on the localization of the integrin (22). Cultured human endothelial cells express integrins including VLA-5 on the luminal and basolateral site however assemble fibronectin matrix at their basolateral surface (52). Also differentiated monocytic cells express two populations of fibronectin receptors: a minority in a high affinity state and the majority in a low affinity state (25). This could explain why in endothelioma cells the VLA-5 was unable to bind to L1. However, the L1-dependent binding of the beads observed suggested an additional ligand distinct from VLA-5 in these cells which has not yet been identified.

Previously we characterized CD24 as a ligand for P-selectin on ESb-MP cells, granulocytes, and monocytes (2, 73). MAbs to CD24 could block P-selectin–mediated binding of myeloid cells to platelets or endothelioma cells in the cold (2). In this report we show that at room temperature or above CD24-specific mAbs could specifically enhance homotypic aggregation of ESb-MP cells by a VLA-5–L1 pathway. The mechanism of this effect is presently unknown. It is likely that the slow spontaneous aggregation of ESb-MP cells is due to the previously described homotypic L1–L1 interaction (42, 50). The role of CD24 mAbs could be to activate an additional VLA-5–L1 heterotypic binding pathway leading to the observed enhanced cell clustering. Such activation of cell adhesiveness could happen by either stimulating the α5-integrin via signaling or by the induction of conformational changes in the VLA-5 integrin. Similar activation of fibronectin receptors including VLA-5 has been reported after treatment of cells with stem cell factor (49), mAbs to ICAM-3 (19) or CD9 (60). A second possibility is that the binding of CD24 mAbs may lead to a conformational change of the L1 adhesion molecule. Indeed, studies by Kadmon et al. (44) have implicated a *cis*-interaction and functional cooperation of CD24 and L1 in neurones but not in B lymphoblasts. Interestingly, recent structural studies of integrin-ligand interaction have indicated that amino acids adjacent to the integrin-binding sequence may be critical for ligand recognition (for review see reference 32). A given RGD site could be cryptic and only exposed after conformational changes. This concept of ligand activation emphazises the importance of local secondary structure and protein folding in regulating the functional state of integrin ligands. We can not exclude at present whether such a model may be applicable to at least some forms of L1 or not.

Evidence for a role of CD24 in the regulation of the cell adhesion comes also from other systems. In B lymphoblasts different mAbs to CD24 could either activate LFA-1–dependent cell aggregation (43) or could inhibit cell aggregation (42). In a study on pre-B cell lines in which either CD24 was inactivated by gene targeting or expression restored by transfection it was shown that CD24 influenced the avidity of α4-integrins (31, 45). In cells of the myeloid lineage CD24 appears to have two roles: a ligand for P-selectin and a signaling molecule for the activation of the VLA-5 integrin.

It is at present difficult to assess in which cellular interactions a VLA-5–L1 binding could be involved. The role of VLA-5 has been studied in aspects of myeloid cell differentiation (8, 25), interaction with bone marrow stromal cells (72), the growth and differentiation of hematopoietic cells (6, 23, 78, 82), embryogenesis (87) and cell migration (7, 63) mainly focussing on the ability of this receptor to bind to fibronectin. Some reports have also described a potential function for VLA-5 in cell–cell interactions (5, 57). Since the L1 adhesion molecule is expressed by a wide variety of cells including neural cells, intestinal epithelium (81) and leukocytes (50), putative interactions with VLA-5 being also expressed on many cells would be possible. At present we can not exclude that other integrins which are known to recognize the RGD sequence in a variety of extracellular matrix proteins may also be able to interact with the L1 adhesion molecule. It should also be mentioned that in contrast to the mouse the human L1 homologue has only one RGD site in the VI. Ig domain. At present we therefore can not extrapolate from our findings to the role of L1 in other species.

In the nervous system L1 is involved in granule neuron migration in the developing mouse cerebellar cortex (58), the fasciculation of neurites (27) and neurite outgrowth on other neurites and Schwann cells (21, 74). Neurite outgrowth in vitro can be stimulated by purified L1 (55), by transfected L1 expressed on monolayers of 3T3 fibroblasts (83) or with an L1-Fc–chimera in solution (24). Preincubation of neurones with antibodies to L1 could inhibit the stimulated outgrowth of neurites implicating a predominantly homotypic L1–L1 interaction (24). Neurite outgrowth is influenced by the substrate and occurs also on extracellular matrix components (1, 11, 85) as well as on for example tissue sections of embryonic muscle where it is largely integrin-dependent (33). Expression of fibronectin receptors has been demonstrated in the mouse nervous system including some neurons (66). Although VLA-5 on the nerve growth cone has not formally been demonstrated, its expression is not unlikely since VLA-5 can participate in the endocytotic cycle and can be brought to the leading edge of a moving cell (14). It is therefore possible that neurite outgrowth on L1 substrate involves VLA-5 integrin. A role of β1-integrins (which includes VLA-5) in the neurite outgrowth on CAMs was indeed suggested in studies on TAG-1. TAG-1 is structurally related to L1 and comprises six Ig domains and four fibronectin type III repeats with an RGD site located in the second repeat (28). Although TAG-1 can mediate homophilic binding, the neurite outgrowth on TAG-1 required an L1-like molecule and β1-integrins on the neurites (26). Antibodies to β1-integrins could block the neurite extension on TAG-1 substrate but not on NgCAM, a putative chick homologue to L1. These data do not support the assumption of an important role for the VLA-5/L1 pathway in neurite growth. However, structurally NgCAM and L1 show significant differences in overall sequence and are also different in the localisation of RGD sequences (16). VLA-5 could be involved in cases where the growth of neurones is studied on other cells i.e. fibroblasts or neural cells. Fibroblasts do express VLA-5 and in earlier studies fibronectin receptors (likely to include VLA-5) were also shown to be expressed by neural cells (66).

The signaling via CAMs like L1, N-CAM, or N-cadherin that ultimately leads to the growth of neuronal processes has been recently linked to the FGF-receptors (84). The VLA-5 integrin has also been implicated, at least in part, to play a role in growth regulation (70). For tumor cells it has been shown that cells selected for low expression of α5β1 are more tumorigenic than higher expressors (77) and that transfection of α5β1 into CHO cells suppresses the growth, migration and tumorigenicity of these cells (29). The mechanism of this growth regulation is not entirely clear however may be related to the ligand occupancy of the VLA-5 integrin (70, 71). In a previous study we investigated the tumorigenicity of $L1^{hi}$ and $L1^{lo}$ subclones of ESb-MP in syngeneic mice (51). We found that animals bearing $L1^{lo}$ subclone tumors died significantly earlier than $L1^{hi}$ animals. The earlier death was due to a much faster growth of the primary tumor and an earlier onset of metastasis. The finding presented in this paper that L1 and VLA-5 are receptor and ligand on these cells that are used for homotypic cell interaction could be relevant to explain the tumor growth results. In this context it is interesting to note, that in resting lymphocytes after the induction of proliferation in vivo or in vitro the L1 molecule is rapidly downregulated (35). This suggests that the VLA-5–L1 interaction may be more important in non-proliferating cells.

In summary, we have shown that L1 adhesion molecule is a ligand to VLA-5 integrin and that this newly established interaction may be important in diverse cell–cell interactions.

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