Integrin VLA-3: Ultrastructural Localization at Cell-Cell Contact Sites of Human Cell Cultures

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Abstract. The integrin VLA-3 is a cell surface receptor, which binds to fibronectin, laminin, collagen type I and VI (Takada, Y., E. A. Wayner, W. G. Carter, and M. E. Hemler. 1988. *J. Cell. Biochem.* 37:385–393) and is highly expressed in substrate adherent cultures of almost all human cell types. The ligand specificity of VLA-3 and the inhibition of cell adhesion by anti-VLA-3 monoclonal antibodies suggest its involvement in cell-substrate interaction. In normal tissues, VLA-3 is restricted to few cell types, notably the kidney glomeruli and basal cells of the epidermis. In the epidermis, VLA-3 is generally strongly expressed on the entire plasma membrane of basal cells and is not polarized towards the basement membrane (Klein, C. E., C. Cardon-Cardo, R. Soehnchen, R. J. Cote, H. F. Oettgen, M. Eisinger, and L. J. Old. 1987. *J. Invest. Dermatol.* 89:500–507). Based on this finding we speculated that, in addition to a role of VLA-3 for adhesion of cells to substrate, it could also be relevant for cell-cell interaction. To investigate this, we ultrastructurally localized VLA-3 on the surface of cultured cells by immunoelectron microscopy. In accordance with our concept, we found VLA-3 strongly associated with intercellular contact sites. Interestingly, very little immunoreactivity was detected at the undersurface of cells which had been cultured for 18–32 h. This observation was unexpected but is consistent with previous findings (Kantor, R. R. S., M. J. Mattes, K. D. Lloyd, L. J. Old, and A. P. Albino. 1987. *J. Biol. Chem.* 262:15158–15165) which suggest that the association of VLA-3 with the basal surface of substrate adherent tumor cells is a late event occurring after days of culture under confluent conditions. However, we cannot formally rule out VLA-3 expression at the undersurface of cells under our experimental conditions, since VLA-3 molecules at this location could be inaccessible for in situ labeling of unfixed cells because of spatial interferences.

In conclusion, our results demonstrate the expression of VLA-3 at intercellular contact sites of cultured cells supporting the concept that it may be relevant for intercellular interactions also.

CELLULAR adhesion is known to be mediated by a large number of cell surface molecules functioning as specific receptors for different ligands. This diversity provides a basis for the complex regulatory interactions controlling cell migration and adhesion in embryogenesis, neural development, immune functions, and wound healing (Donaldson and Mahan, 1988; Edelman, 1985; Fraser et al., 1984; Makgoba et al., 1988).

A prominent gene family of cell surface receptors, the integrins, has evolved to mediate both cell-cell and cell-matrix interactions, and recent work has elucidated function and ligands of several proteins of this group (Hynes et al., 1987; Takada et al., 1987*b*). The VLA glycoproteins represent an ubiquitously expressed subgroup of this family (Hemler et al., 1987). Three of them have been identified as receptors with monospecificity for collagens (VLA-2) (Kunicki et al., 1988), fibronectin (VLA-5) (Pytela et al., 1985; Takada et al., 1987*a*), or laminin (VLA-6) (Sonnenberg et al., 1988). The role of VLA-3, a multispecific or promiscuous cell adhesion receptor which binds to four different extracellular matrix proteins (collagen I and VI, fibronectin, laminin) is not clear (Wayner and Carter, 1987; Takada et al., 1987*a*). Although binding of VLA-3 to these ligands has been demonstrated, the biological relevance of this broad specificity remains to be determined (Wayner and Carter, 1987). In this study, we asked the question, whether, in addition to a role in cell-substrate adhesion, VLA-3 could also be relevant for cell-cell interaction. To investigate this, we ultrastructurally localized it on the surface of cultured tumor cells and found it strongly associated with intercellular contact sites. It is well-established that expression of VLA3 can be induced by attachment of cultured cells to extracellular substrate (Rettig et al., 1986; Kantor et al., 1987). Furthermore, monoclonal antibodies directed to the α-chain of VLA-3 inhibit cell attachment to fibronectin, type VI and type I collagen (Wayner and Carter, 1987). These data support its involvement in adhesion to extracellular matrix. Our results, however, show that, in cultured cells, VLA-3 is highly expressed at sites of intercellular contact suggesting that it may also be involved in intercellular interactions.

Material and Methods

Cell Cultures

Epidermal cell cultures were established and maintained as described previously (Eisinger et al., 1979). Human cell lines were obtained from different sources: SV-40-transformed embryonic lung fibroblasts (Wi38 VA13) were obtained from American Tissue Type Culture Collection (Rockville, MD). The melanoma line (Mewo) and the osteosarcoma line (U20S) were provided by Dr. J. Fogh's tumor cell bank (Memorial Sloan Kettering Cancer Center, New York, NY). Lines SCL-I and SCL-II (Tilgen et al., 1983) both derived from squamous cell carcinomas of the skin were a gift from Dr. N. E. Fusenig (German Cancer Research Center, Heidelberg, FRG). Monkey cell line CV-1 and its derivative COS-7 containing a replication-defective SV-40 genome were obtained from Dr. Y. Gluzman (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY; Gluzman, 1981). Cell line SV/HF-5/39 (Neufeld et al., 1987) was derived from the diploid bone marrow fibroblast cell line Hs74 after transformation with a replication-defective SV-40 genome. All cells and short term cultures were maintained in RPMI 1640 containing 10% FBS and supplemented with 2 mM glutamine, 1% nonessential amino-acids, 100 U/ml penicillin, and 100 U/ml streptomycin.

Monoclonal Antibodies

Hybridoma culture supernatant was used for in situ labeling of cells. 1/50 diluted sera from hybridoma bearing nu/nu mice were used for immunoprecipitations. The generation and initial characterization of mAb's J143, Aj2, VM-2, T16, and CNT10 have been described elsewhere (Fradet et al., 1984; Cairncross et al., 1982; Mattes et al., 1983; Morhenn et al., 1985). mAb's J143, Aj2, and T16 were kindly provided by Dr. L. J. Old (Memorial Sloan Kettering Cancer Center, New York, NY). mAb VM-2 and CNT10 were gifts from Dr. V. B. Morhenn (Department of Dermatology, Stanford Medical School, San Francisco, CA) and Dr. M. T. Jennings (Department of Neurology, Memorial Sloan Kettering Cancer Center, New York, NY), respectively. mAb's J143 and VM-2 recognize epitopes on the α chain of the VLA-3 heterodimer (Takada et al., 1987*b*; for VM-2 see our own results). mAb Aj2 defines the β chain common to all VLA proteins (Kantor et al., 1987). mAb CNT10 defining a 80-kD cell surface glycoprotein which is expressed on mesenchymal and neuroectodermal cells was used as negative control antibody for epithelial cells, whereas mAb T16 defining a 40–50-kD surface glycoprotein of human keratinocytes served as negative control for mesenchymal cells.

Immunofluorescence

Monolayer cultures on glass slides were fixed in cold (4°C) acetone. Indirect immunofluorescence tests were carried out as described previously (Klein et al., 1987).

Immunoprecipitation

Cultured cells were metabolically labeled with [^{35}S]methionine (60–200 μCi/ml; New England Nuclear, Boston, MA) for 6–12 h. Glycoproteins were isolated from NP-40 solubilized cell extracts by adsorption to Concanavalin A (Pharmacia Fine Chemicals, Uppsala, Sweden) (Lloyd, 1981). Immunoprecipitation was carried out by incubating a sample of labeled glycoproteins (1.5×10^6 cpm) with 50–250 μl of 1/50 diluted serum or ascites containing mAb for 2 h at 4°C. Immune complexes were then precipitated with protein A–Sepharose CL-4b (Pharmacia Fine Chemicals) preincubated with rabbit anti-mouse Ig (Dako Corp., Santa Barbara, CA). After washes the precipitated glycoproteins were processed by SDS-PAGE on 7.5% and 9% acrylamide gels (Laemmli, 1970). For fluorography, gels were immersed in 0.5 M sodium salicylate for 20 min (pH 5.5).

Immunoelectron Microscopy

Monolayer cultures were in situ labeled and examined by transmission electron microscopy (TEM)[1] and scanning electron microscopy (SEM) of whole mounts and by TEM of vertical sections: Single cell suspensions of the cell types studied were prepared by trypsinisation and plated on coverslips, precoated with an electron-transparent melamine-resin foil as described (Westphal et al., 1988). Briefly, foils for cell cultures were made from a solution of 1% (wt/vol) hexamethylol-melamine ether dissolved in pure, analytical grade ethanol, and polymerized with 0.3% paratoluene sulfonic acid.

Cells were seeded and cultured for 18–36 h. Immunolabeling was performed at 37°C under culture conditions directly or after an earlier mild prefixation with formaldehyde (0.5% in PBS; 15 min). Cells were then incubated for 1 h with tissue culture supernatant containing mAb's. After washes with PBS (3×), goat anti-mouse IgG coupled to 10-, 15-, or 40-nm gold particles (Janssen Pharmaceutica) was added for 1 h. Cells were washed again (3×) and then fixed with glutaraldehyde (2.5% in PBS; 1 h). For whole-mount TEM cells were rinsed with distilled water and dried in a stream of nitrogen gas. The foil with the cells was removed from the glass by treatment with 0.4% hydrofluoric acid and mounted on coppergrids (Plano W. Plannet GmbH, Marburg, FRG). For TEM studies of vertical sections, the cells were washed with PBS, treated with saponin (0.25% in PBS; 30 min), rinsed again, and postfixed with tannic acid (1% in cold PBS; 1 h). After several rinses with distilled water, cells were stained with uranyl acetate (1% in H_2O; 1 h; in the dark). No osmium tetroxide was used. When dehydrated by a graded series of ethanol, a thin layer of Spurr's low viscosity epoxy resin (Plano W. Plannet GmbH) was applied for polymerization. Fragments of the hardened foil-cell layer were removed from the coverslip with a scalpel and reembedded in epoxy resin for vertical sectioning with an ultramicrotome (Ultrotome 3; LKB Produkter, Bromma, Sweden; with a diamond knife). All specimen were examined in a Philips EM 301 or a Philips EM 400T at a voltage of 80 KV.

For TEM and consecutive SEM of identical cell domains, whole mounts were put on lettered copper grids after labeling with 40 nm immunogold particles. In pilot studies, all cell lines had been cultured on melamin coated as well as on uncoated bare coverslips for the comparative assessment of morphology by SEM. They were fixed as described above, dehydrated with ethanol/dimethoxypropane, and critical point dried in Balzers cp-drier operated with liquid CO_2. When sputtercoated with gold-palladium they were imaged in a Philips PSEM 500.

Results

In previous studies, mAb J143 was shown to recognize an epitope on the α^3-chain of VLA-3, whereas mAb Aj2 defines an epitope on the β-chain which is common to all VLA glycoproteins (Takada et al., 1987*b*; Kantor et al., 1987). mAb VM-2 revealed a similar immunoreactivity in normal and diseased skin (Morhenn et al., 1985; our own unpublished results) suggesting that VM-2 also detects the α^3 chain of VLA-3. Sequential immunoprecipitations proved that this was the case (Fig. 1). In preceding experiments, we had studied the expression of VLA-3 in U20S and Wi38 VA13 cells by indirect immunofluorescence tests after permeabilization with acetone (4°C). A specific membrane fluorescence was detectable, which was pronounced at the lateral sides of confluent cultures (see also Kantor et al., 1987) and appeared weaker in subconfluent or sparse cultures. However, when subconfluent cultures were studied to analyze cell–cell contact sites, we were not able to identify them with sufficient certainty by light microscopy. Thus, definite conclusions in respect to the expression of VLA-3 at these sites could not be drawn. This led us to investigate its expression on the ultrastructural level by immunoelectron microscopy.

For the ultrastructural localization of VLA-3 we chose to analyze cultured cells that were seeded on glass coverslips precoated with an electrontransparent melamine-resin foil. This experimental design permitted in situ labeling of live or prefixed cells with mAbs and subsequent analysis of the whole mounts of TEM and SEM. Furthermore, vertical sections of the foils could be prepared and analyzed by TEM. In addition, cells could be seeded on resin foils which had been coated with a variety of extracellular matrix components to study their influence on antigen distribution.

Initial studies of cell morphology by SEM revealed no differences between cells that were cultured on glass and

1. *Abbreviations used in this paper*: SEM, scanning electron microscopy; TEM, transmission electron microscopy.

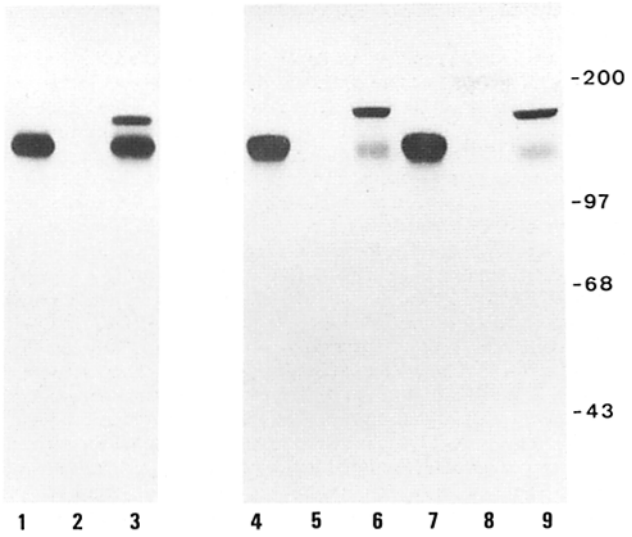


Figure 1. VLA synthesis in substrate-adherent growing human cell cultures. Concanavalin A–bound fractions of NP-40 cell lysates from [^{35}S]methionine-labeled keratinocyte cultures were analyzed by immunoprecipitation and SDS-PAGE under reducing conditions. The fluorogram shows immunoprecipitates obtained with different mAbs: J143 (VLA-3, lane *1*), CNT10 (control, lane *2*), Aj2 (β chain of all VLA heterodimers, lane *3*), VM-2 (lane *4*), J143 after preclearing with VM-2 (lane *5*), Aj2 after preclearing with VM-2 and J143 (lane *6*), J143 (lane *7*), VM-2 after preclearing with J143 (lane *8*), and Aj2 after preclearing with J143 and VM-2 (lane *9*).

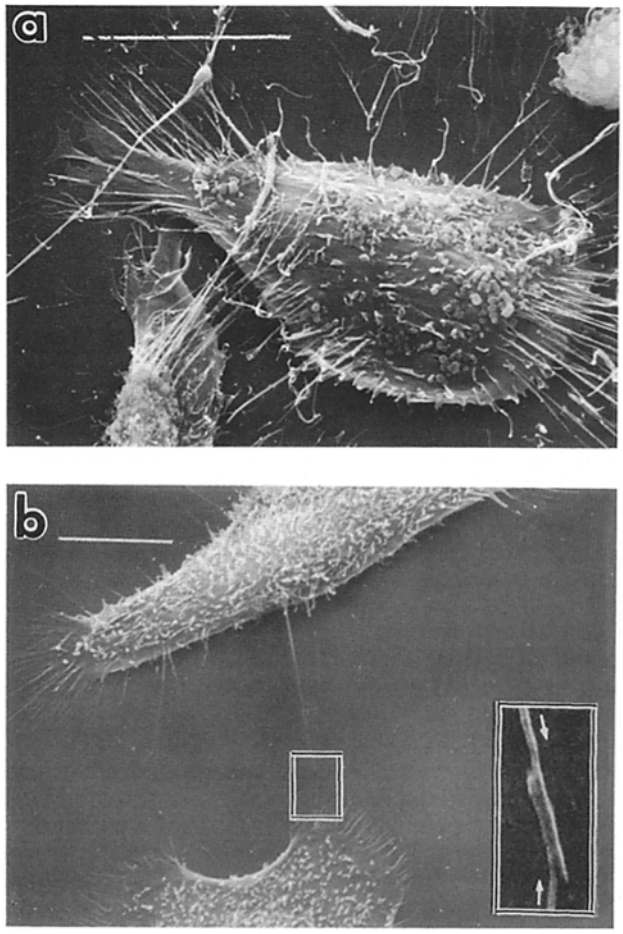


Figure 2. Scanning electron micrographs of Mewo (*a*) and SCL-II (*b*) cultures grown on melamine resin foil. The general growth patterns remained the same whether cells were grown on glass or on foils. Note the different types of cell surface projections and of cell–cell contact formation when comparing the two cell lines Mewo (*a*) and SCL-II (*b*). The cable-like surface projections in *a* overlying the neighboring cell body would correspond to types of cell–cell contacts seen in Figs. 4, 6 *a*, and 7 *e*. The longitudinal attachment of two peripheral cell projections seen in *b* would correspond to types of contacts seen in Figs. 5 (frame *b*) or 6 *c*. Bar, 10 μm.

those that had been seeded on the resin foil (see also Fig. 2). Seven cell lines derived from mesenchymal, epithelial, and neuroectodermal tissues (Wi38 VA13, SV/HF-5/39, U20S, SCL I, SCL II, Mewo, COS-7) were studied. We first assessed the overall distribution of VLA-3. Cells were labeled with mAbs J143 and VM-2 and analyzed by TEM of whole mounts. Both antibodies showed an identical staining pattern: Single immunogold particles indicating VLA-3 reactivity were evenly distributed over the whole cell at low density. However, a major part of the gold particles were associated in clusters and were predominantly localized at the sites of intercellular contact (Figs. 3, 4, and 5). No differences of the reactivity pattern were observed when immunogold particles of different sizes (10, 15, 40 nm) were used. In experiments using negative control antibodies (mAbs CNT10 and T16) virtually no immunogold particles were detectable. Intercellular contacts formed between peripheral projections of different cells showed clustered VLA-3 reactivity which was strictly confined to the site of close contact and was not detectable in adjacent structures. The close association of VLA-3 reactivity with contact sites was also observed, when peripheral cell processes attached more proximally to the plasma membrane of a neighbor cell in the perinuclear region. Here, cell–cell contacts often ranged over a distance of 0.5–1.5 μm leading to a "streetlight" pattern of VLA-3 reactivity (Fig. 3 *b*, Fig. 4). The same pattern of reactivity was found when mAb Aj2 detecting the β chain of the VLA's was used for in situ labeling. In control experiments we tested whether the clustering of VLA-3 reactivity at cell–cell contact sites represented an experimental artefact due to antigen redistribution analogous to the capping of cell surface antigen in lymphocytes. A mild fixation preserving the J143 epitope (0.5–1.0% formaldehyde, 20 min) was applied before labeling. No difference of antigen expression at intercellular contact sites was found in comparison to labeling the living cells at 37°C (Fig. 4). Furthermore, other unrelated cell surface glycoproteins which were studied in parallel (gp 130, mAb Q14, [Klein et al., 1988] gp 92, transferrin receptor, mAb OKT9) showed a diffuse distribution and revealed no preferential expression at sites of intercellular contact (data not shown).

The analysis of a large number of cell–cell contacts showed that virtually every intercellular contact was associated with VLA-3 expression. However, contacts that were formed between cell projections derived from the same cell or cell processes which twisted back and attached to their own cell body, did not express VLA-3 reactivity suggesting that VLA-3 is specifically expressed at contact sites which are formed between different cells. In mixed cultures of mesenchymal,

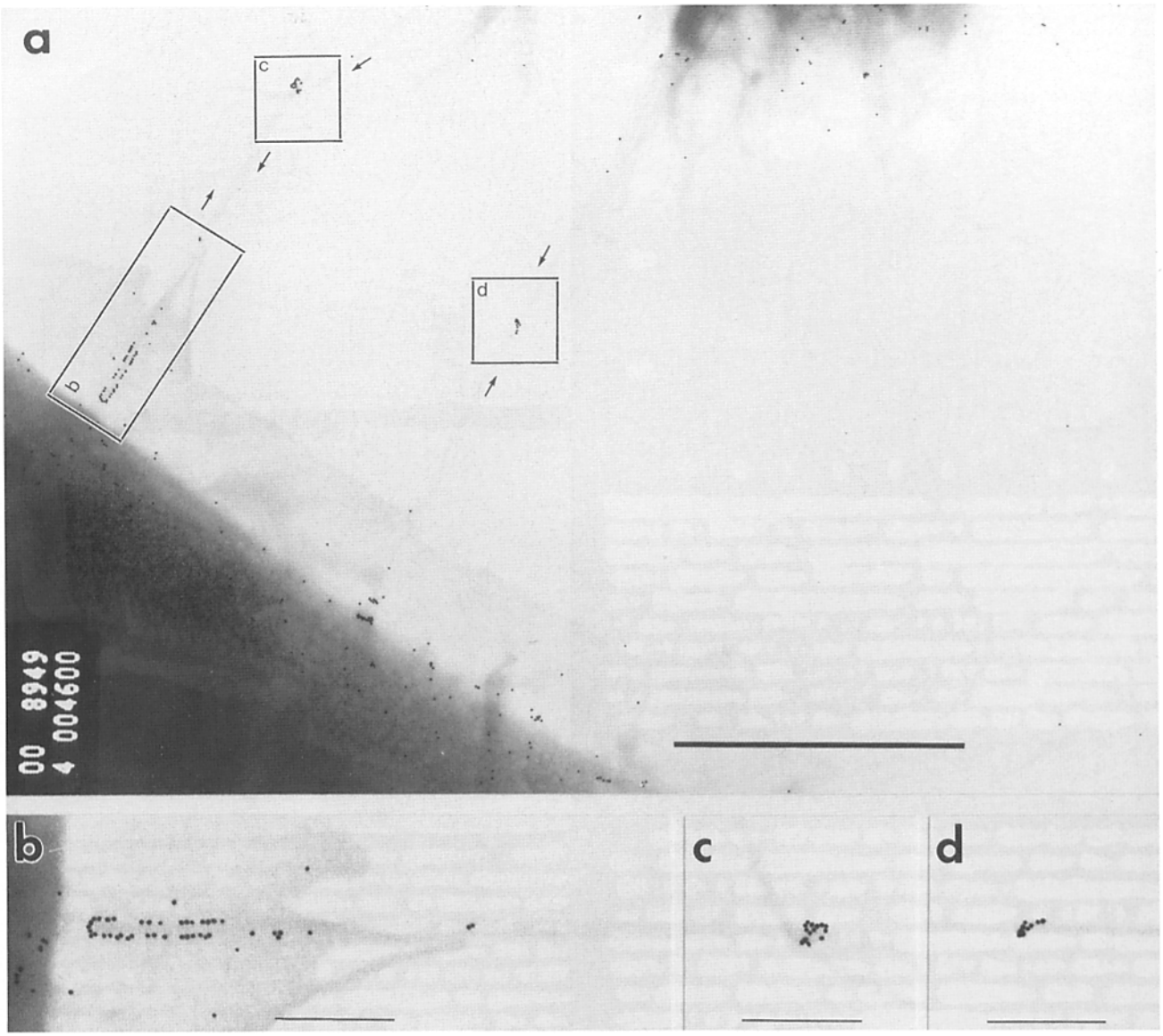


Figure 3. Transmission electron micrograph of U20S cells (whole mount) after labeling with mAb J143 and goat anti-mouse IgG coupled to 40 μm gold. The rectangular frame (frame *b*) encloses a linear contact between a cell body and a filopodium of a neighbor cell. The square frames (frames *c* and *d*) enclose focal contacts between the tips of filopodia of the two cells. Arrows point towards the distal ends of the filopodia. Philips EM 400 T, unstained, airdried preparation. Bars: (*a*) 5 μm; (*b–d*) 1 μm.

neuroectodermal, and epithelial cells, VLA-3 was also associated with intercellular contacts formed between cells of different histogenesis. In contrast, when human cells were cocultured with mouse L cells, no VLA-3 reactivity was detectable at contact sites between mouse and human cells.

For the further characterization of antigen expression on the cell surface, we examined identical cell microdomains of whole mount preparations by TEM and subsequent SEM. The combination of both techniques allowed us to exactly determine whether gold particles are localized on the apical cell surface or not. Gold particles which are seen by TEM and are also detected by SEM are located on the apical surface, whereas gold particles seen by TEM but not by SEM are either internalized or localized at the undersurface of cells. For several cell–cell contact sites (n = 3) formed between peripheral cell projections we demonstrated that VLA-3 reactivity resided on the apical cell surface directly at the cell–cell interface (see Fig. 6, *a–d*). Also the "streetlight" reactivity of VLA-3 was clearly assigned to cell projections which attached to the upper plasmamembrane of a neighbor cell (n = 7). Thus, its association with structures located at the basal cell surface was excluded.

Previous studies of Rettig et al. (1984, 1986), and Takada et al. (1987*a*,*b*) suggested that VLA-3 is involved in adhesion of cells to substrate. We have studied whether VLA-3 reactivity can also be demonstrated at the undersurface of cultured cells. Since TEM of whole mount preparations does not allow the precise identification of cell substrate adhesion sites we prepared sections of in situ labeled cells which were cut perpendicular to the cell layer. Single gold particles or

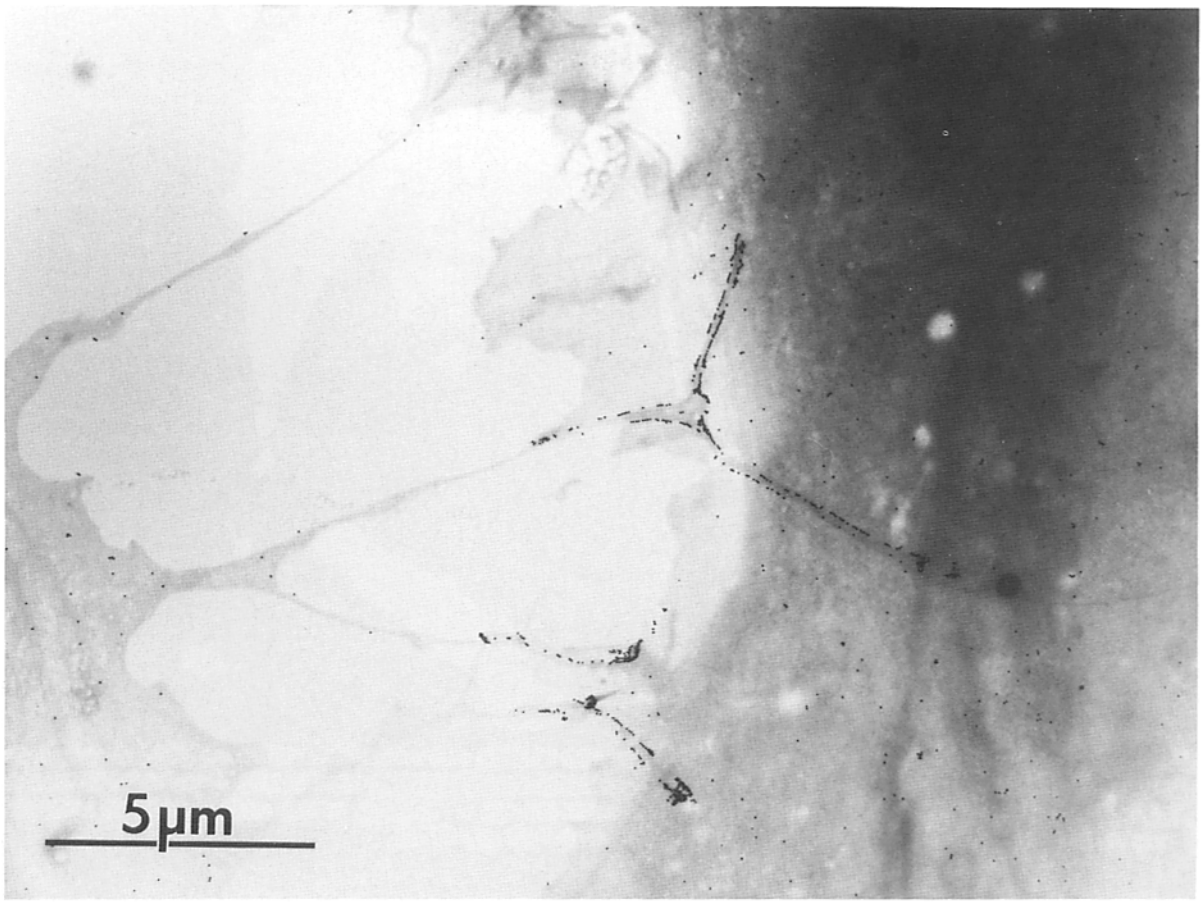


Figure 4. Transmission electron micrograph of Wi38 VA13 cells, which were fixed with 0.5% formaldehyde (15 min) before labeling with mAb J143 and 40 µm gold goat anti-mouse IgG. Note, the "street-light" expression of VLA-3 along branching filopodias overlying the body of a neighbor cell. Compare also Fig. 3 (frame *b*).

small clusters were occasionally found at the undersurface of cells but they were not clustered at sites of close contact to the substratum. However, clustered VLA-3 reactivity was regularly found at sites of cell–cell contact (Fig. 7). Vertical sectioning of in vivo labeled cells also showed that VLA-3 reactivity was endocytosed via coated pits and finally concentrated in large vesicles (Fig. 8). In some cases, coated pits containing VLA-3 reactivity were found in close vicinity of cell–cell contact sites (Fig. 8 *b*).

We also studied the influence of natural extracellular matrix components on VLA-3 expression. Cells were seeded on melamine-resin foils, which had been precoated with fibronectin or, alternatively, with an extract of the mouse EHS sarcoma containing the basement membrane constituents laminin, collagen IV, entactin, and mucopolysaccharides. No changes of VLA-3 reactivity were noted when compared to the reactivity in cells cultured on the plain foil (data not shown).

Discussion

Members of the integrin gene family have been identified as important receptors involved in cell–cell or cell–substrate adhesion mediating functions such as platelet aggregation, T lymphocyte help, lymphocyte cytotoxicity, and complement binding (Sonnenberg et al., 1988; Hynes et al., 1987). Recently, the ligand specificities of several receptors of the VLA subgroup have been defined. One of them, the integrin VLA-3 binds to fibronectin, laminin, collagen I and VI (Wayner and Carter, 1987), and is highly expressed in all human cell cultures which grow substrate adherent (Fradet et al., 1984; Rettig et al., 1984, 1986; Takada et al., 1987*b*; Kantor et al., 1987). In this study, we have characterized its relationship to cell adhesion on the ultrastructural level by immunoelectron microscopy and found VLA-3 strongly associated with intercellular contact sites of cultured human cells. The use of two independently derived mouse mAbs recognizing the α^3 chain of VLA-3 led to identical results. Moreover, the use of mouse mAb Aj2 detecting the β_1 chain of the VLAs revealed the same pattern of reactivity, suggesting that a functional VLA-3 heterodimer is indeed expressed at cell–cell contacts. To further analyze this observation we performed two different sets of experiments. First, coculture experiments showed that VLA-3 expression was associated with cell–cell contacts formed between human cells of the same and of different cell types, whereas no association was observed with contacts formed between mouse and human cells. The latter observation indicates that the expression of VLA-3 at intercellular contact sites between human cells is specific and is not due to experimental artefacts

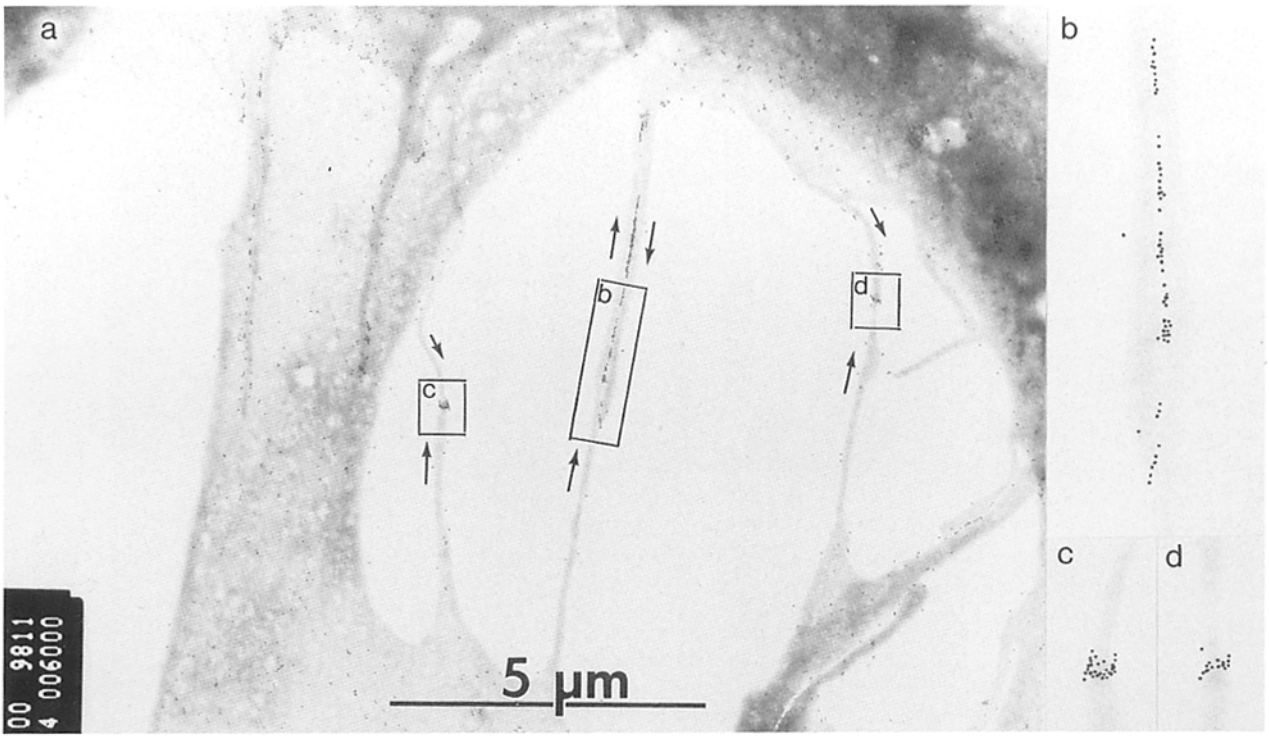


Figure 5. Transmission electron micrograph of COS cells (whole mount) after labeling with mAb VM-2 and 10 μm gold goat anti-mouse IgG. The rectangular frame (frame *b*) shows VLA-3 expression at a longitudinal contact between two filopodia of two different cells and the square frames (frames *c* and *d*) demonstrate VLA-3 at contacts between the tips of filopodia. Arrows point towards the distal ends of filopodia. Note the identical staining pattern, as seen in Fig. 3 with mAb J143 and 40 nm.

such as "antigen capping." Moreover, this possibility was excluded since cell cultures that were labeled at 4°C or after fixation with formaldehyde showed the same characteristic expression pattern of VLA-3 as cultures, which had been labeled as live cells at 37°C under culture conditions. Furthermore, peripheral cell processes of human cells, which turned back to their own cell body or which attached to other peripheral cell processes of the same cell did not show VLA-3

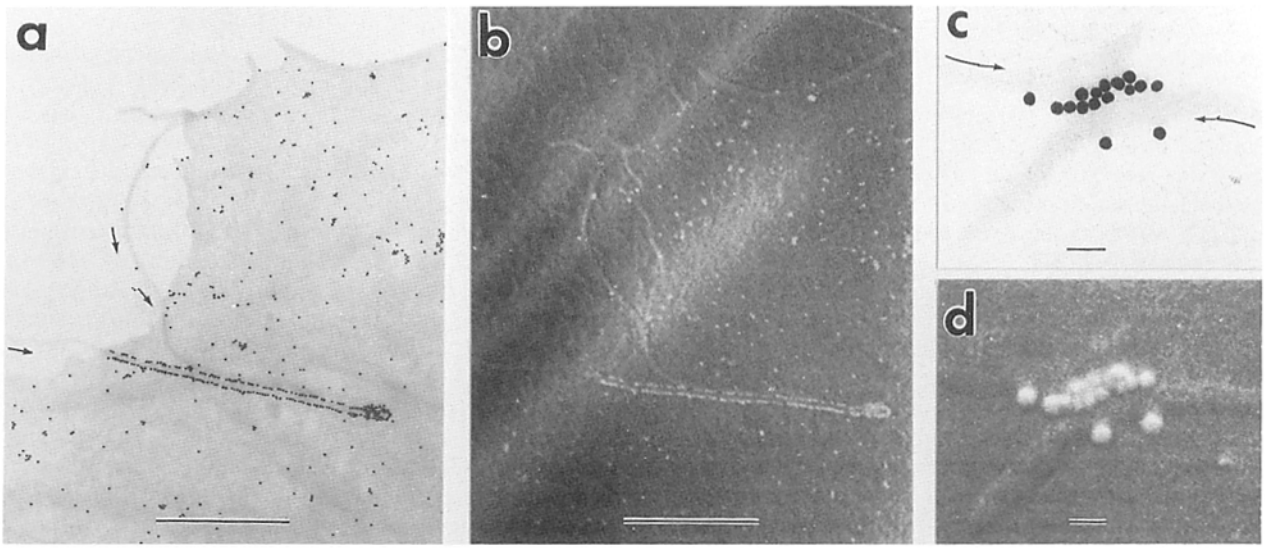


Figure 6. Two pairs of micrographs of U20S cells (whole mount) taken by consecutive TEM (*a* and *c*) and SEM (*b* and *d*) of identical cell domains, respectively. In *a* a single arrow points to intercellular contact of a peripheral cell projection attaching to the plasma membrane of a neighbor cell in the perinuclear region. Note the "street-light" VLA-3 reactivity. An autologous cell-cell contact, however, formed between a peripheral cell projection and its own cell body (*two arrows*) is devoid of reactivity (*c*) and (*d*) show higher magnification transmission and scanning micrographs of an intercellular contact between two filopodia. Distinct secondary electron emission signals were recorded from 40-nm gold particles which had been sputtered with a 5-nm gold layer and scanned with an electron beam measuring 16 nm in diameter. The arrows point toward the distal ends of the filopodia. Bars: (*a* and *b*) 2 μm; (*c* and *d*) 200 μm.

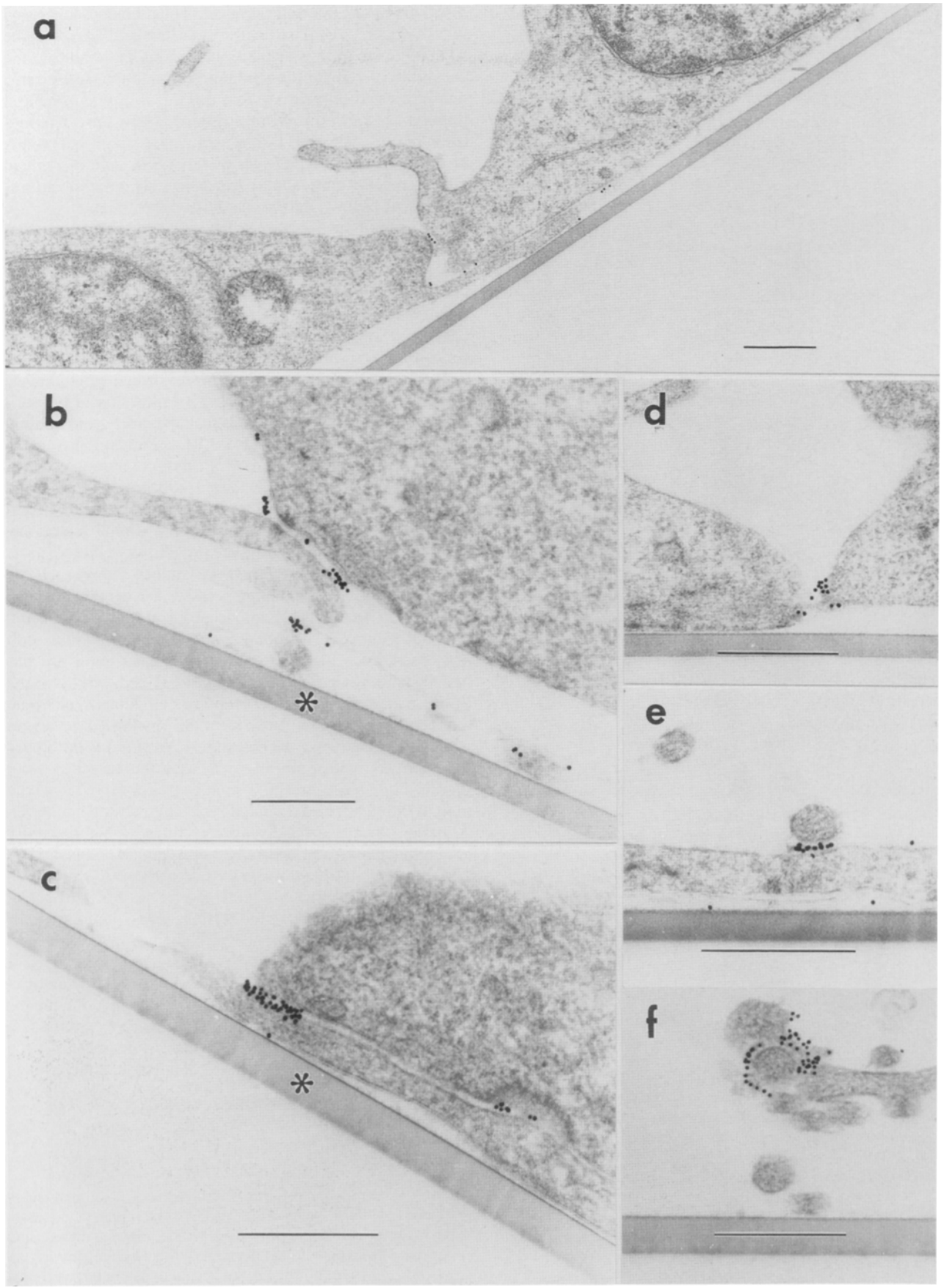
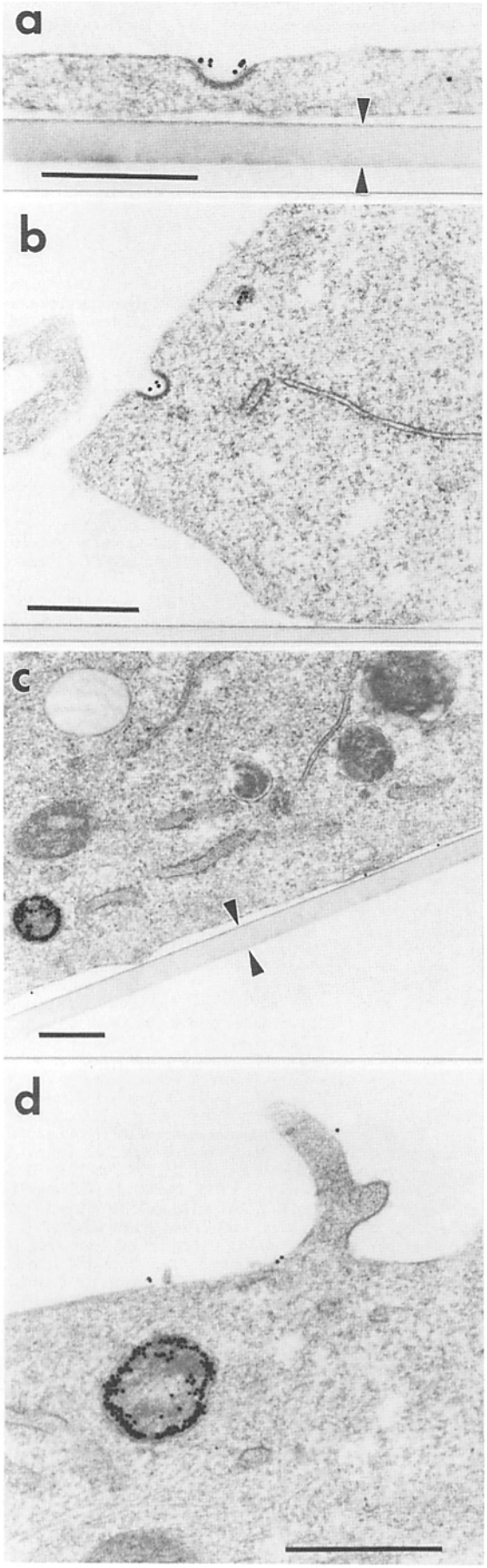


Figure 7. Transmission electron micrographs of U20S (*a–d*) and Wi38 VA13 (*e* and *f*) cells. Perpendicular sections of cells, which were cultured on the melamine-resin foil (*asterisk*) and had been labeled with mAb J143 and 40-nm gold particles. *a* and *d* give a survey of contact areas between two neighboring cells. *b* and *c* show tips of filopodias underlying and attaching to the neighbor cells. *e* represents a cross section through a linear contact between a filopodium and a cell body (see also frame *b* in Fig. 3). *f* demonstrates a contact between different cross-sectioned filopodial tips. Bar, 0.5 μm.



reactivity at sites of close contact. Thus, contact formation with autologous cell structures was not sufficient for the expression of VLA-3 at these sites. This may indicate the capability of cells to discriminate self and non-self plasma membranes. It is plausible that this capability is of central importance for complex multicellular organisms. The molecular basis, however, is not understood. Our observation of the differential expression of VLA-3 at autologous and nonautologous intercellular contacts may provide an experimental basis to further study the phenomenon.

In a second set of experiments, we investigated whether culturing the cells on foils which had been precoated with fibronectin or basement membrane extracts containing laminin would influence the distribution of VLA-3 on the cell surface. No changes of VLA-3 reactivity were observed in comparison to cells seeded on the plain foils. Particularly, there was no increase of VLA-3 reactivity at the undersurface of cells, indicating that exposure of transformed cells to these compounds did not polarize VLA-3 expression to the substrate. This observation suggests that fibronectin and laminin may not be the relevant ligands for VLA-3 under these conditions. This is also supported by our indirect immunofluorescence experiments using cell line U20S in which we found that among the three ligands tested (laminin, fibronectin, and collagen VI) only collagen VI showed a similar membrane reactivity as VLA-3 implying the possibility of its coexpression at intercellular contact sites (data not shown).

The ultrastructural assignment of VLA-3 to cell–cell contacts was based on electron microscopical evaluations using different protocols. Combining whole mount studies with that of sections which were cut perpendicular to the foil was helpful in assigning VLA-3 reactivity to cell–cell contacts. Furthermore, the analysis of identical cell domains of whole mounts by TEM and subsequent SEM allowed to assess whether immunogold particles were located on the upper plasma membrane or not. In some cases, this served as proof that clustered VLA-3 reactivity which was observed in whole mounts could not be associated with cell–substrate contacts. Although, we have found only very little VLA-3 reactivity at the undersurface of cells and no clusters at cell–substrate contact sites, we cannot formally rule out VLA-3 expression at these sites, since VLA-3 molecules at the undersurface of cells could be inaccessible for in situ labeling of unfixed cells because of spatial interferences. This question has to be addressed in future studies using postembedding labeling of ultrathin cryosections. However, previous data by Kantor et al. (1987) suggest that the association of VLA-3 with the basal surface of substrate adherent tumor cell cultures is a late event occurring between 16 h and 5 d after plating of a confluent culture. We have studied cells under subconfluent conditions after culturing them for 18–36 h. With reference

Figure 8. Transmission electron micrographs of U20S cells (perpendicular sections), which were labeled without prefixation under tissue culture conditions with mAb J143 and goat anti-mouse IgG coupled to 40-nm gold. Note, the endocytosis of gold particles via coated pits (*a* and *b*) followed by the accumulation in large vesicles near the lower (*c*) and the upper (*d*) surface of cells. In *a* and *c* the cross-sectioned melamine substrate foil is indicated by arrowheads. Bar, 0.5 μm.

to the observation of Kantor et al. (1987), it is possible that the experimental conditions which were chosen in our study did not provide enough time for the "late" polarization of VLA-3 to cell–substrate attachment sites.

The TEM analysis of perpendicular sections also showed that VLA-3 reactivity is endocytosed and is associated with coated pits. Since several receptor ligand complexes in eucaryotic cells mediating important extrinsic signals are engulfed by clathrin-coated pits and since coated pits containing VLA-3 reactivity were found in the vicinity of cell–cell contacts, we postulate that VLA-3 not simply binds to its ligands during contact formation but also serves as a mediator of intercellular regulatory signals, which are transduced by receptor internalization. Recently, van Noesel et al. (1988) demonstrated the regulatory properties of integrin LFA-1 in human T-lymphocyte activation.

In conclusion, our data demonstrate that integrin VLA-3, a multispecific receptor for various extracellular matrix proteins, is highly expressed at intercellular contact sites of human tumor cell cultures suggesting that it is not only involved in cell–substrate but also in cell–cell interaction of human cells. Very recently, evidence has been obtained that another member of the VLA-group, the VLA-4, functions in cell–cell interactions (Groux et al., 1989). In future studies, it remains to be shown whether the expression of VLA-3 at cell–cell contacts is mediated by one of the four known, or by another as yet unknown ligand. New experiments designed to analyze the function of VLA-3 should evaluate the possibility of VLA-3 having a role in both cell–substrate and cell–cell interactions.

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