

# Human Smooth Muscle VLA-1 Integrin: Purification, Substrate Specificity, Localization in Aorta, and Expression during Development

Vladimir M. Belkin\*, Alexey M. Belkin<sup>‡</sup>, and Victor E. Koteliansky<sup>‡</sup>

\* Institute of Biological and Medical Chemistry, Academy of Medical Sciences, Moscow 109801, Union of Soviet Socialist Republics; <sup>‡</sup> Institute of Experimental Cardiology, Cardiology Research Center, Academy of Medical Sciences, Moscow 121522, Union of Soviet Socialist Republics

**Abstract.** A membrane glycoprotein complex was isolated and purified from human smooth muscle by detergent solubilization and affinity chromatography on collagen-Sepharose. The complex was identified as VLA-1 integrin and consisted of two subunits of 195 and 130 kD in SDS-PAGE. Liposomes containing the VLA-1 integrin adhered to surfaces coated with type I, II, III, and IV collagens, C1q subcomponent of the first component of the complement, and laminin. The liposomes specifically adhered to these proteins in a Ca<sup>2+</sup>, Mg<sup>2+</sup>-dependent manner, but did not bind to gelatin, fibronectin, and thrombospondin substrates.

The expression of VLA-1 integrin in different human tissues and cell types, and during aorta smooth muscle development was studied by SDS-PAGE, and subsequent quantitative immunoblotting was performed with antibodies recognizing  $\alpha_1$  and  $\beta_1$  subunits of the VLA-1 integrin. A high level of VLA-1 integrin expression was an exceptional feature of smooth muscles. Fibroblasts, endothelial cells, keratinocytes, striated

muscles, and platelets contained trace amounts of VLA-1 integrin. In the 10-wk-old human fetal aorta, VLA-1 integrin was found only in smooth muscle cells whereas mesenchymal cells, surrounding aortic smooth muscle cells, were VLA-1 integrin negative. By the 24th wk of gestation, the amount of VLA-1 integrin was significantly reduced in the aortic media (4.3-fold for  $\alpha_1$  subunit and 2.5-fold for  $\beta_1$  subunit) compared with that in the 10-wk-old aortic smooth muscle cells. After birth, the expression of VLA-1 integrin increased and in the 1.5-yr-old child aorta the VLA-1 integrin level was almost the same as in adult aortic media. Smooth muscle cells from intimal thickening of adult aorta express five times less  $\alpha_1$  subunit of VLA integrin than smooth muscle cells from adult aortic media. In primary culture of aortic smooth muscle cells, the content of the VLA-1 integrin was dramatically reduced and subcultured cells did not contain VLA-1 integrin at all.

**D**URING embryogenesis, postnatal development, and atherogenesis, smooth muscle cells (SMC)<sup>1</sup> undergo remarkable phenotypic transitions (40, 43). These processes are accompanied by a prominent coordinated reorganization of cytoskeleton and extracellular matrix (2, 5, 11-16, 26, 35, 39, 47). Cell surface receptors for extracellular matrix proteins are directly involved in the integration of extracellular matrix components with the cytoskeletal framework (4, 18, 23, 41, 42). These transmembrane receptors have been termed integrins (23). Extracellular domains of integrins mediate cell adhesion to extracellular matrix and basement membrane components and some plasma proteins. The cytoplasmic domains of integrins are able to interact with actin filament-associated proteins (1, 22). The integrins are heterodimers consisting of an  $\alpha$  subunit linked noncovalently to a nonrelated  $\beta$  subunit. Several different  $\beta$  subunits have been identified. All  $\beta$  subunits are structurally related to each other, and they define different subfamilies of integrins (23). In humans, the  $\beta_1$  subfamily of integrins includes very late antigens (VLA), initially discovered on activated human T cells (18, 20). Later it was shown that VLA integrins are present on a wide variety of human cells (18). The VLA integrins share a common  $\beta_1$  subunit and at least six different  $\alpha$  subunits (18, 45, 48). A number of experimental data indicate that members of VLA (or  $\beta_1$ ) integrin subfamily play an important role as mediators of cell adhesion and migration (4, 23, 42). Normal VLA integrin function is critical for differentiation and developmental processes. It has been demonstrated that an antibody to chicken  $\beta_1$  integrin blocks myogenic differentiation at the myoblast stage (32). We are particularly interested in defining mechanisms that control SMC growth and differentiation, with specific emphasis on the role played by VLA integrins in these pro-

1. *Abbreviations used in this paper:* SM, smooth muscle; SMC, smooth muscle cells; VLA, very late antigen.

cesses. Recently, two  $\beta_1$  subfamily integrins were identified and purified from chicken gizzard smooth muscle (SM) and rat arterial SMC (3, 25). The rat arterial SM integrin is probably a homolog of human VLA-5 integrin (3). Immunomorphological data have also demonstrated the presence of VLA-1 and VLA-2 integrins in SMC of skin, lung, and blood vessels (20).

In the present work, we concentrated on the study of VLA-1 integrin. The  $\alpha$  chain of VLA-1 integrin has a relative molecular mass of  $\sim 200,000$  D and is complexed with the  $\beta_1$  chain of integrin to form a functional heterodimer ( $\alpha_1\beta_1$ ) (21). On human melanoma cells VLA-1 integrin acts as a receptor for basement membrane type IV collagen (28), and on neurons, for laminin (24). Laminin-binding integrin  $\alpha_1\beta_1$  was also isolated and characterized from rat hepatocytes (10). Growth signals can readily alter the expression of VLA-1 integrin. The transforming growth factor- $\beta$  markedly increases the expression of VLA-1 integrin in WI-38 human fibroblasts (17). The VLA-1 integrin expression is also enhanced in quiescent fibroblasts (9). In a neuroblastoma GRGDSP peptide-resistant cell line, the VLA-1 integrin was specifically overproduced (8). Based on these observations and previously reported data concerning the ability of VLA-1 integrin to interact with type IV collagen and laminin, we hypothesized that the expression of VLA-1 integrin in human SMC is phenotype dependent and can be altered during development. Herein, we describe the isolation, characterization, intracellular localization, and tissue distribution of human SM VLA-1 integrin. We also provide data showing that the expression of VLA-1 integrin is dramatically changed during SMC cultivation and SM development.

## Materials and Methods

### Materials

Laminin was commercially obtained from Bethesda Research Laboratories (Gaithersburg, MD). Human plasma fibronectin was provided by Dr. M. A. Chernousov (USSR Cardiology Research Center, Moscow, USSR). Purified human type I, II, III, and IV collagens and human plasma C1q protein were kindly provided by Dr. S. Domogatsky (USSR Cardiology Research Center). Human platelet thrombospondin was a generous gift of Dr. G. Samokhin (USSR Cardiology Research Center).

### Antibodies

mAb 102DF7 against  $\beta_1$  subunit of integrin was kindly supplied by Dr. I. Virtanen (Department of Anatomy, Helsinki University, Helsinki, Finland) (49). Rabbit anti- $\beta_1$  cytoplasmic domain polyclonal antibodies were a generous gift of Dr. R. Hynes (Massachusetts Institute of Technology, Cambridge, MA) (31). mAb TS2/7 against  $\alpha_1$  subunit of VLA-1 integrin (20) was from T Cell Sciences (Cambridge, MA). Polyclonal antibodies to  $\alpha_1$  subunit of VLA-1 integrin were prepared as described below. Polyclonal antibodies against COOH terminus (13 mer) of  $\alpha_5$  subunit of VLA-5 integrin (fibronectin receptor) were kindly provided by Dr. E. Ruoslahti (La Jolla Cancer Research Foundation, La Jolla, CA). Polyclonal antibodies to chicken gizzard talin were a generous gift of Dr. K. Burrridge (University of North Carolina, Chapel Hill, NC).

### Purification of Total SM $\beta_1$ Class Integrins

All procedures were done at  $+4^\circ\text{C}$  with exceptions specifically noted. 400 g of SM from human uterus was homogenized in a Waring blender with 2.5 liters of deionized water containing 0.5 mM PMSF. After centrifugation (10 min at 9,000 rpm in JA-10 rotor; Beckman Instruments, Inc., Berkeley, CA) the supernatant was discarded and the pellet was extracted in water with 0.5 mM PMSF. For extraction of peripheral membrane proteins the pellet was

resuspended in 2.5 liters of buffer, containing 20 mM Tris, 1 mM EGTA, 0.5 mM PMSF, pH 9.0 (25, 27). The suspension was stirred for 60 min at  $37^\circ\text{C}$ . Centrifugation was repeated and the supernatant was discarded. To solubilize myosin, tissue pellet was dissolved in 2 liters of buffer, containing 20 mM Tris-HCl, 0.6 M KCl, 0.5 mM PMSF, pH 7.4, stirring constantly for 60 min (27). The homogenate was centrifuged (as above) and the pellet was washed with 2.5 liters of water with 0.5 mM PMSF. After centrifugation, the pellet was extracted in 2 liters of buffer, containing 20 mM Tris-HCl, 0.5% Triton X-100, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 0.5 mM PMSF, pH 7.4, for 15 h with constant stirring. The detergent extract was cleared by centrifugation and then applied onto a column with 50 mg of anti- $\beta_1$  mAb 102DF7 coupled to 5 ml of cyanogen bromide-activated Sepharose. The column was washed with 10 vol of extraction buffer, and then with 10 vol of buffer, containing 20 mM Tris-HCl, 0.1% Triton X-100, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 1 M NaCl, pH 7.4. Bound proteins were eluted with 200 mM glycine-HCl, 0.1% Triton X-100, 1 mM PMSF, pH 2.2, and immediately neutralized with 0.1 vol of 1 M Tris-HCl, pH 8.5

### Purification of VLA-1 and VLA-5 Integrins

Type I collagen, type IV collagen, and fibronectin were coupled to cyanogen bromide-activated Sepharose (Pharmacia, Uppsala, Sweden) by standard technique. Triton X-100 detergent extract of uterus SM (20 mM Tris-HCl, 0.5% Triton X-100, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 0.5 mM PMSF, pH 7.4), obtained exactly as described above, was applied to columns with corresponding ligands. In some experiments, 1 mM  $\text{CaCl}_2$  and 1 mM  $\text{MgCl}_2$  were substituted for 1 mM  $\text{MnCl}_2$  in the column buffer. Nonbound material was removed by washing with 10 vol of buffer, containing 20 mM Tris-HCl, 0.1% Triton X-100, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 0.2 M NaCl, pH 7.4. Elution of bound material was performed by washing the columns with 2 vol of 20 mM Tris-HCl, 0.1% Triton X-100, 10 mM EDTA, pH 7.4.

For purification of  $\alpha_1$  subunit of VLA-1 integrin, 3–4 mg of eluate from collagen type I-Sepharose was subjected to preparative electrophoresis, and gel pieces, corresponding to 195-kD protein, were excised. Rabbits were immunized subcutaneously with 1 mg of highly purified  $\alpha_1$  subunit in complete Freund's adjuvant. 4 wk later, the rabbits were boosted with 0.5 mg of the antigen. Rabbit sera were collected and the IgG fraction purified by standard chromatography on a column of DE-52 cellulose.

### Immunoprecipitation and Immunoblotting

Immunoprecipitation with anti- $\alpha_1$  subunit mAb TS2/7 was performed on crude Triton X-100 extracts and eluates from anti- $\beta_1$  antibody-Sepharose or collagen type I-Sepharose. All protein preparations were extensively dialyzed against buffer, containing 20 mM Tris-HCl, 0.1% Triton X-100, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 150 mM NaCl, 0.5 mM PMSF, pH 7.4, before precipitation. 4  $\mu\text{g}$  of anti- $\alpha_1$ -specific antibodies was added to each sample, containing an excess of VLA-1 integrin. After incubation for 18 h at  $+4^\circ\text{C}$ , 100  $\mu\text{l}$  of goat anti-mouse IgG coupled to Sepharose CL-4B (5 mg/ml of the gel) was added for 2 h. Immunoprecipitates were thoroughly washed five times in the immunoprecipitation buffer by centrifugation, eluted in SDS gel sample buffer, and subjected to electrophoresis.

Immunoblotting was done according to the procedure of Towbin et al. (46). Electrotransfer was carried out for 3 h at 400 mA current. The blots were revealed by the indirect immunoperoxidase technique or by immunoradiolabeling. 4-Chloro-1-naphthol (Sigma Chemical Co., St. Louis, MO) was used as a substrate for peroxidase.  $^{125}\text{I}$ -Labeled goat anti-rabbit IgG (1  $\mu\text{g}/\text{ml}$ ;  $10^7$  cpm/ $\mu\text{g}$  sp act) was used as a secondary antibody for the study of the distribution of  $\alpha_1$  and  $\beta_1$  integrin subunits in human tissues and cell types. Immunoblots were dried and exposed to X-ray films (Tasma, USSR) for 48–96 h at  $-70^\circ\text{C}$  to determine the positions of the polypeptides. Then the strips of nitrocellulose corresponding to  $\alpha_1$  or  $\beta_1$  subunits were cut out and radioactivity was measured in a  $\gamma$  counter. The method was reproducible with repeated measurements on the same sample (i.e., the ratio of the SD to the mean was  $<5\%$  of the mean). Radioactivity values changed linearly over the range of protein loadings used.

SDS-PAGE was done on 7.5 or 5–15% polyacrylamide slab gels with Laemmli's buffer system (29). Tissue or cell samples were dispersed immediately in Laemmli's electrophoresis sample buffer, without  $\beta$ -mercaptoethanol, and protein concentration was determined with BCA Protein Assay Reagent (Pierce Chemical Co., Rockford, IL). Each electrophoretic sample contained 0.15 mg of total protein. After protein content estimation, 5%  $\beta$ -mercaptoethanol was added and tissue or cell samples were boiled for 5 min before electrophoresis.

## Reconstitution of Purified SM VLA-1 Integrin into Liposomes and Liposome Binding Assay

Liposomes were prepared as described in references 33 and 37. VLA-1 integrin of 95–98% purity, as judged by SDS-PAGE, was eluted with 20 mM Tris-HCl, 100 mM octylglucoside, 10 mM EDTA, pH 7.4, from collagen I-Sepharose and used for incorporation into liposomes. A mixture of egg yolk phosphatidilcholine (Sigma Chemical Co.) (800  $\mu$ g for liposome-binding experiments) and 200  $\mu$ g of cholesteryl-[1- $^{14}$ C]-oleate (53.1 mCi/mmol sp act) was dried onto a glass tube under a stream of N<sub>2</sub> and dissolved in TBS (25 mM Tris-HCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, pH 7.5), containing 100 mM octylglucoside or protein fractions (20  $\mu$ g of VLA-1) in the same buffer. Detergent was removed by extensive dialysis for 24 h at +4°C. To purify liposomes, the suspension was made 45% in sucrose, overlaid with 2 ml of 30% sucrose and 1 ml of 10% sucrose, and centrifuged at +4°C for 18 h at 45,000 rpm in an SW 50.1 rotor (Beckman Instruments, Inc.). The liposomes were recovered at the top of the 10% sucrose layer, 75–90% of VLA-1 was incorporated into lipid vesicles in standard experiments. In separate experiments both COOH- and NH<sub>2</sub>-terminal domains of the VLA-1 integrin  $\beta_1$  subunit were shown to be exposed on the surface of liposomes, since polyclonal antibodies against COOH-terminal (intracellular)  $\beta_1$  peptide and mAb 102DF7, which recognizes the NH<sub>2</sub>-terminal (extracellular) part of the  $\beta_1$  integrin molecule, were able to precipitate liposomes containing VLA-1 integrin.

For liposome-binding experiments type I, II, III, and IV collagens, Clq, laminin, fibronectin, trombospondin, gelatin, and BSA were diluted in carbonate buffer, pH 8.6, to a concentration of 25  $\mu$ g/ml and 200  $\mu$ l was used to coat microtiter wells (0.28 cm<sup>2</sup> surface area; Linbro, Flow Laboratories, Hamden, CT) by incubation overnight at +4°C. Wells were washed twice with TBS and blocked for 1 h with TBS plus 10 mg/ml BSA. Labeled liposomes (100 or 200  $\mu$ l; 10<sup>5</sup> cpm/well sp act) suspended in TBS with 2 mg/ml BSA were allowed to attach for 16 h at +4°C. Then the supernatants were removed and the wells were washed three times with TBS. Bound liposomes were dissolved in 1% SDS and radioactivity was counted in a scintillation counter. In some experiments, 10 mM EDTA or synthetic peptides GRGDSP and GRGESP (1 mg/ml final concentration) were added to the wells.

## Human Tissue Samples

Human fetal tissue was obtained from fetuses aborted either spontaneously or on medical grounds. Child and adult tissues were obtained at autopsies taken within 2–4 h of death. At least five independent samples were analyzed for each tissue. SD to the mean of  $\alpha_1\beta_1$  ratios, were <10% of the mean. Normal segments of the aorta from 35–45-yr-old donors were analyzed. For immunoblotting analysis, aortas were longitudinally opened, adventitia were discarded, and the endothelium was removed mechanically. Normal segments were separated and divided into three layers: medial and two intimal, the subendothelial and the muscular-elastic (adjacent to the media) sublayers.

## Immunofluorescence

Freshly isolated human aortic segments were immersed in freezing isopentane. 3–4- $\mu$ m-thick longitudinal and transverse cryostat sections were prepared at –20°C. Sections were mounted on glass slides. For double-indirect immunofluorescence, sections were incubated with mouse monoclonal anti- $\alpha_1$  TS2/7 antibody (1:20) and rabbit anti- $\beta_1$  antibodies (1:100) for 1 h at 37°C. Sections were washed in PBS for 30 min and then fluorescein-conjugated goat anti-mouse IgG (1:50) and rhodamine-conjugated goat anti-rabbit IgG (1:50) were added. After a further incubation for 45 min, the sections were washed and mounted in PBS with 50% (vol/vol) glycerol. The stained sections were examined under a Zeiss epifluorescent photomicroscope III. Photographs were taken with Kodak Tri-X-film. In all experiments, immunofluorescence was specific: no staining was observed when antibodies were preabsorbed with corresponding antigens or when nonimmune IgG was used.

## Cell Culture

SMC from human aortic media were isolated by collagenase-elastase digestion of the tissue (6) and cultured in DME (Flow Laboratories, Inc., McLean, VA) supplemented with 10 mM HEPES, 100  $\mu$ g/ml sodium pyruvate, 50  $\mu$ g/ml ascorbic acid, 0.6 mg/ml L-glutamine, 50  $\mu$ g/ml gentamicin sulfate, and 10% heat-inactivated human serum. For immunofluorescence, sterile glass coverslips were coated with type I and IV collagens and

fibronectin (50  $\mu$ g/ml) in PBS for 1 h at 37°C. After removing excess protein solutions, the coverslips were treated with 0.2% glutaraldehyde in PBS for 15 min at 37°C, washed with sterile PBS, and used for cell plating. Human skin fibroblasts (growing or quiescent), human umbilical vein endothelial cells, and human keratinocytes (34) were kindly provided by Drs. O. Ornatsky and M. Lukashev (USSR Cardiology Research Center). Platelets were isolated and purified from human plasma.

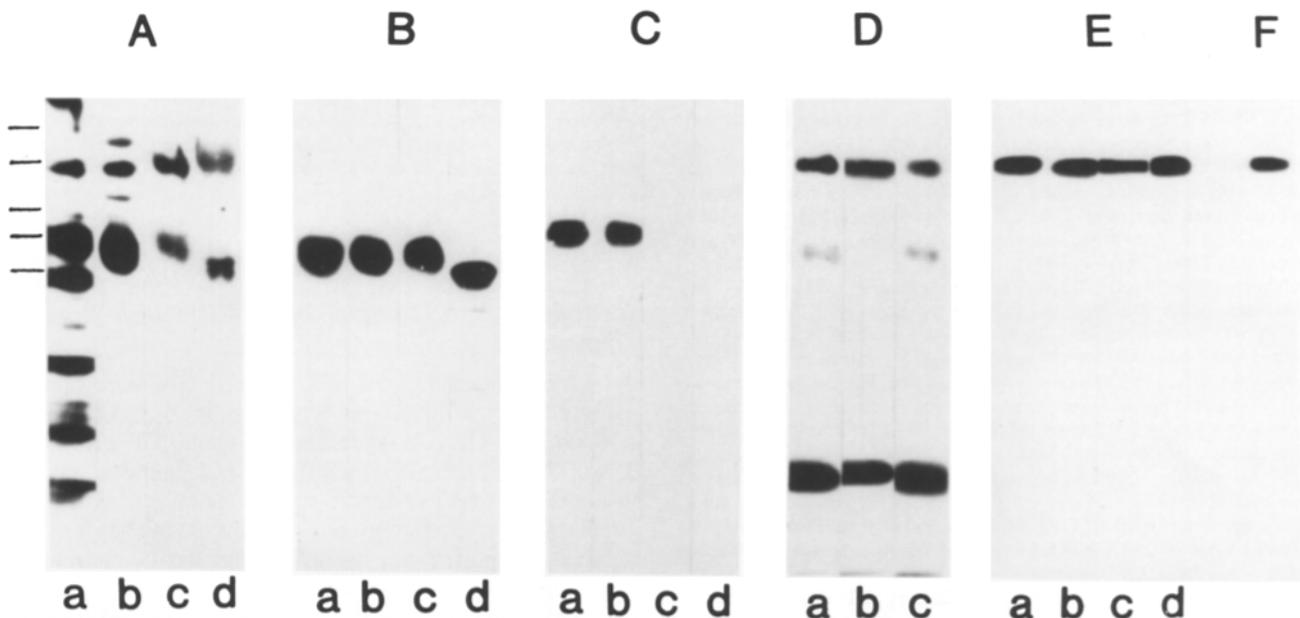
## Results

### Identification and Isolation of VLA-1 Integrin from Human SM

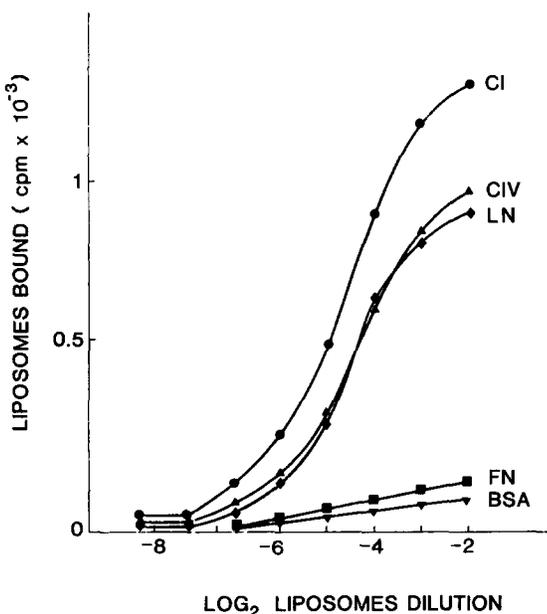
Total VLA integrins from human uterus SM were purified using affinity chromatography on an anti- $\beta_1$  mAb coupled to Sepharose 4B. A crude extract of membrane proteins in Triton X-100 contained a number of polypeptides, ranging in molecular masses from 20 to 250 kD (Fig. 1 A, lane a). After affinity chromatography on anti- $\beta_1$ -Sepharose, four major polypeptides were bound and specifically eluted from the column. Apparent  $M_r$  values of 130, 165, 195, and 235 kD were determined for these proteins, as judged by SDS-PAGE under reduced conditions (Fig. 1 A, lane b). Based on its electrophoretic mobility and immunoblotting results (Fig. 1 B, lanes a and b) the lower band was identified as the  $\beta_1$  integrin subunit. A major integrin subunit of 195 kD, which eluted together with  $\beta_1$  subunit from the column, was shown to be an  $\alpha_1$  integrin, since this polypeptide was precipitated from a crude Triton X-100 extract or from an anti- $\beta_1$ -Sepharose eluate when anti- $\alpha_1$  subunit mAb TS2/7 was used (Fig. 1 D, lanes a and b). The presence of  $\alpha_5$  integrin subunit in Triton X-100 extract and anti- $\beta_1$ -Sepharose eluate was also verified by immunoblotting (Fig. 1 C, lanes a and b). These results indicate that human SM contains several different  $\alpha$  subunits of  $\beta_1$  subfamily ( $\alpha_1$ ,  $\alpha_5$ , and probably others), and  $\alpha_1$  integrin was shown to be a predominant subunit among  $\beta_1$ -associated SM integrins. We were unable to determine the identity of two other integrin bands of 165 and 235 kD. The 165-kD polypeptide was not recognized by a polyclonal antibody against  $\alpha_2$  integrin subunit (data not shown).

Ligand affinity chromatography was used to purify functional VLA-1 and VLA-5 SM integrins. Based on the previously obtained data concerning the ligand specificity of VLA-1 integrin, we used collagen type IV-Sepharose column for the affinity purification of the integrin receptor. Two polypeptides of 195 and 130 kD, as well as some other minor bands, were seen on SDS-PAGE of collagen type IV-Sepharose eluate (data not shown). When the same affinity chromatography experiment was done using collagen type I-Sepharose matrix, only two components migrating on SDS-PAGE as 195- and 130-kD bands were revealed (Fig. 1 A, lanes c and d). Molecular masses of these polypeptides, immunoblotting (Fig. 1 B, lanes c and d), and immunoprecipitation data (Fig. 1 D, lane c), confirmed the correspondence of purified proteins to  $\alpha_1$  and  $\beta_1$  integrin subunits of the VLA-1 complex. Total yield of 5–6 mg of VLA-1 integrin was purified from 400 g of uterus SM tissue. When the same procedure was performed with fibronectin-Sepharose, 1.5–2 mg of VLA-5 was obtained (data not shown).

To raise polyclonal antibodies to  $\alpha_1$  subunit of SM integrins, suitable for immunoblotting experiments, we im-



**Figure 1.** Analysis of human uterus SM VLA integrins. (A) Coomassie blue-stained SDS-polyacrylamide gels of (lane a) crude Triton X-100 extract from uterus SM; (lane b) eluate from anti- $\beta_1$ -Sepharose; (lane c) eluate from collagen type I-Sepharose in reducing conditions; (lane d) eluate from collagen type I-Sepharose in nonreducing conditions. (B) Immunoblots of the gels (lanes a-d) from A with polyclonal anti- $\beta_1$ -integrin antibody. (C) Immunoblots of the gels (lanes a-d) from A with polyclonal anti- $\alpha_5$ -integrin antibody. (D) Immunoprecipitation analysis of  $\alpha_1$  subunit of VLA integrins with TS2/7 anti- $\alpha_1$  mAb. Immunoprecipitated proteins were visualized by Coomassie blue staining of SDS-polyacrylamide gels. (Lane a)  $\alpha_1$  and  $\beta_1$  subunits of VLA integrin were precipitated from crude Triton X-100 extract of human uterus SM; (lane b)  $\alpha_1$  subunit of VLA integrin was precipitated from anti- $\beta_1$ -Sepharose eluate. Absence of  $\beta_1$  subunit in immunoprecipitate is due to dissociation of  $\alpha_1$  and  $\beta_1$  subunits during the elution of VLA-1 integrin from anti- $\beta_1$ -Sepharose (pH < 3.5) (21); (lane c)  $\alpha_1$  and  $\beta_1$  subunits of VLA integrin were precipitated from collagen type I-Sepharose eluate. The lower bands in all gels are IgG heavy chains. (E) Immunoblots of the gels (lanes a and d) from A with polyclonal anti-195-kD protein antibody. (F) Immunoblots of anti- $\alpha_1$  subunit TS2/7 antibody immunoprecipitate from crude Triton X-100 extract of human uterus SM with polyclonal anti-195-kD protein antibody. Molecular mass markers of 250, 200, 150, 130, and 100 kD are denoted to the left of the gels.



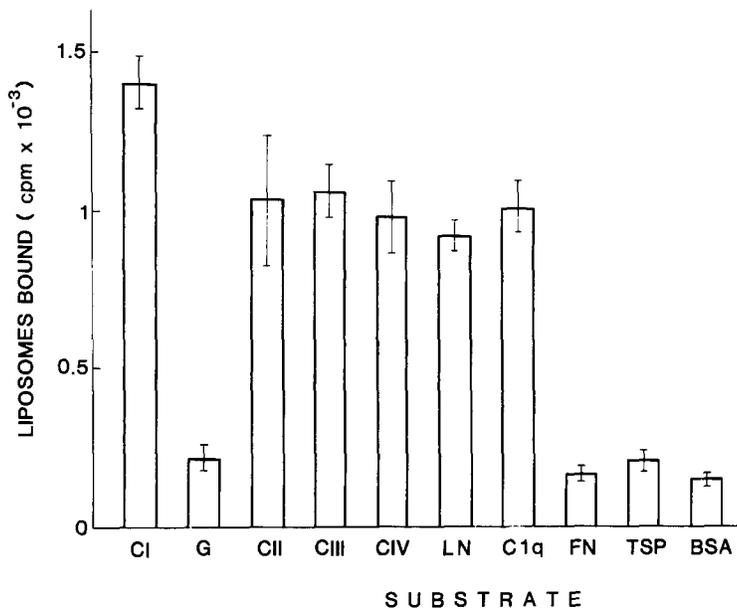
**Figure 2.** Binding of VLA-1 integrin-containing liposomes to adhesive proteins. Microtiter wells were coated with type I collagen (CI), type IV collagen (CIV), laminin (LN), fibronectin (FN), and bovine serum albumin (BSA). The binding of VLA-1 integrin-containing liposomes was determined in the presence of 1 mM  $\text{Ca}^{2+}$  and 1 mM  $\text{Mg}^{2+}$ .

munized rabbits with pure  $\alpha_1$  subunit obtained by preparative electrophoresis. The resulting polyclonal antibodies recognized only a protein of 195 kD in immunoblotting of crude Triton X-100 extract of uterus SM and eluates from anti- $\beta_1$ -Sepharose and collagen type I-Sepharose (Fig. 1 E, lanes a-d). Finally, the specificity of the polyclonal antibodies was confirmed by their reaction with the polypeptide precipitated from the crude Triton X-100 extract with anti- $\alpha_1$  subunit mAb (Fig. 1 F). Further, we used these antibodies for the investigation of VLA-1 distribution in human tissues and cultured cells.

#### Substrate Specificity of Human SM VLA-1 Integrin

To investigate ligand binding of the SM VLA-1 integrin,  $^{14}\text{C}$ -labeled liposomes containing the VLA-1 integrin were prepared by detergent dialysis. As shown in Fig. 2, VLA-1 integrin-containing liposomes were able to bind to type I and IV collagens and laminin substrates immobilized on polystyrene flat-bottom wells, and these interactions were concentration dependent. At the same time, VLA-1 incorporated in liposomes did not interact markedly with other adhesive proteins, e.g., fibronectin or BSA.

Next, we tested different extracellular matrix proteins and plasma components as putative ligands for VLA-1 integrin. VLA-1-containing liposomes were capable of binding to type I, II, III, and IV collagens, laminin, and Clq subcompo-

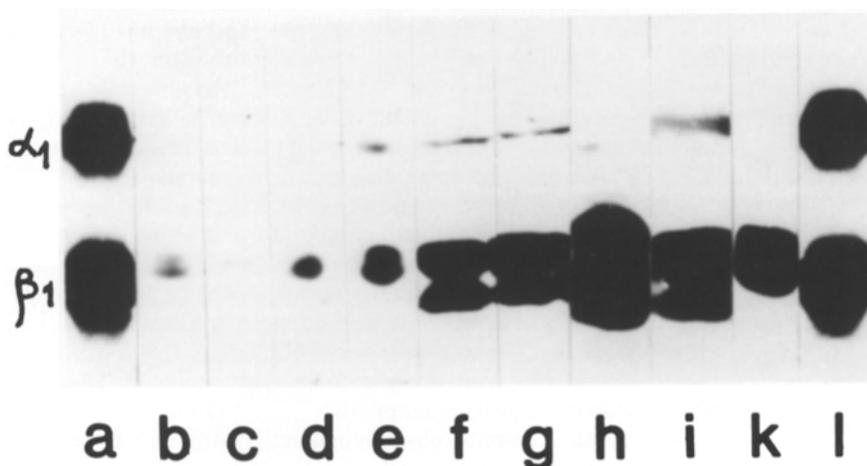


**Figure 3.** Substrate specificity of liposomes containing human SM VLA-1 integrin. Substrates were prepared with type I, II, III, and IV collagens (*CI*, *CII*, *CIII*, and *CIV*), gelatin (*G*), laminin (*LN*), C1q subcomponent of the first component of the complement (*C1q*), fibronectin (*FN*), thrombospondin (*TSP*), and BSA. Binding to all other substrates was determined in the presence of 1 mM Ca<sup>2+</sup> and 1 mM Mg<sup>2+</sup>.

ment of the complement cascade, which contains a collagen-like domain (Fig. 3). In our experimental conditions, type I collagen was the best substrate for VLA-1-bearing liposomes. In contrast, VLA-1 integrin, integrated in liposomes, did not bind to fibronectin and thrombospondin. Negative controls showed that omission of the VLA-1 integrin almost completely eliminated the binding of liposomes to any substrate (data not shown). VLA-1-containing liposomes were unable to interact with gelatin, or BSA-coated wells. VLA-1 liposome binding to corresponding ligands was observed only in the presence of 1 mM Mn<sup>2+</sup> (or 1 mM Mg<sup>2+</sup> and Ca<sup>2+</sup>) in the incubation buffer. EDTA-containing medium strongly (7–10 times) inhibited interactions of VLA-1 integrin with type I, II, III, and IV collagens, laminin, and C1q protein. Using GRGDSP peptide in these experiments, we found that SM VLA-1 integrin interacts with different adhesive proteins in an RGD-independent manner (data not shown).

#### **Distribution of VLA-1 Integrin in Human Tissues and Cell Types**

To determine the distribution of VLA-1 integrin in various human tissues and cultured cells, the samples were examined by SDS-PAGE and quantitative immunoblotting was done with antibodies recognizing  $\alpha_1$  and  $\beta_1$  subunits of SM VLA-1 integrin. Experimental results are shown in Fig. 4 and Table I. Various amounts of  $\alpha_1$  and  $\beta_1$  subunits were found in different human tissues and cultured cells. The largest amount of VLA-1 integrin appeared to be present only in SM. Striated muscles, skin, endothelial cells, fibroblasts, platelets, and keratinocytes contained trace amounts of VLA-1 integrin. Therefore, we conclude that (a) SM can be the best source for VLA-1 integrin isolation and (b) high levels of VLA-1 integrin expression are a characteristic property of SMC.



**Figure 4.** Quantitative analysis of  $\alpha_1$  and  $\beta_1$  VLA integrin subunit content in different human tissues and cultivated cells by SDS-PAGE, immunoblotting, and subsequent autoradiography. (Lane *a*) Uterus SM; (lane *b*) cardiac muscle; (lane *c*) skeletal muscle; (lane *d*) skin; (lane *e*) brain; (lane *f*) skin fibroblasts (growing); (lane *g*) skin fibroblasts (quiescent); (lane *h*) endothelial cells; (lane *i*) platelets; (lane *k*) keratinocytes; and (lane *l*) purified VLA-1 integrin.

**Table I. Relative Amounts of  $\alpha_1$  Subunit of VLA-1 Integrin in Human Tissues and Cultured Cells**

Source	$\alpha_1$	$\beta_1$	$\alpha_1/\beta_1$
	$cpm \times 10^{-3}$	$cpm \times 10^{-3}$	
Uterus smooth muscle	47.5	101.0	0.47
Cardiac muscle	0.4	ND	ND
Skeletal muscle	0.2	ND	ND
Skin	0.24	9.8	0.02
Brain	1.3	9.2	0.14
Skin fibroblasts (growing)	3.6	108.0	0.03
Skin fibroblasts (quiescent)	4.5	102.0	0.04
Endothelial cells	3.2	233.0	0.01
Platelets	0.8	56.0	0.01
Keratinocytes	9.0	186.0	0.05
Purified VLA-1 integrin	74.6	94.2	0.79

**Expression of VLA-1 Integrin in Human Aortic SM**

**Fetal Aorta.** The distribution of  $\alpha_1$  and  $\beta_1$  subunits of VLA integrin was studied in aortas of 9–10- and 24-wk-old human fetuses and in the mesenchyme surrounding the 10-wk-old aorta. Cryostat sections of the fetal aorta were double stained with mouse mAb TS2/7 to  $\alpha_1$  subunit of VLA integrin and with rabbit polyclonal antibody to  $\beta_1$  subunit of integrin (Fig. 5). The whole aortic wall of 10-wk-old fetuses was stained with both antibodies (Fig. 5, *a* and *b*), thus revealing the expression of VLA-1 integrin in the 10-wk-old fetal vascular SM. On the contrary, very weak staining with antibodies against  $\alpha_1$  subunit of VLA integrin was observed in the aorta of the 24-wk-old and in the mesenchyme of the 10-wk-old fetuses. At the same time the sections were positively stained with antibodies to  $\beta_1$  subunit of integrin (Fig. 5, *c* and *d*). Immunoblotting data were consistent with the results of immunofluorescence studies (Table II and Fig. 6 *A*, lanes *a* and *b*). The content of VLA-1 integrin in the extract of 9–10-wk-old fetal aorta was obviously higher than in the extract of 24-wk-old fetal aorta. Thus, in aortic samples of the 24-wk-old fetus the  $\alpha_1$  subunit content was 4.3-fold lower and the  $\beta_1$  subunit content, only 2.5-fold lower as compared with samples of the 9–10-wk-old embryos.

**Child Aorta.** Immunoblotting analysis of extracts from child aorta indicates that during the postnatal period of human vascular SM development the content of VLA-1 integrin increases (Table II and Fig. 6 *A*, lanes *c–e*). The content of  $\alpha_1$  subunit of VLA integrin in 3-mo-old child aortic media was three times higher compared with 24-wk-old fetus aorta. In 12-yr-old aortic media the amount of  $\alpha_1$  subunit was two times greater than in 3-mo-old child aorta. The  $\alpha_1/\beta_1$  ratio also increased 1.6-fold in the aorta from 3-mo-old child, 2.4-fold in the aorta from 1.5 yr-old child, and 3.4-fold in the aortic media from 12-yr-old child as compared to that in the 24-wk-old fetus. Only in 1.5-yr-old child aorta the share of  $\alpha_1$  subunit approached the level characteristic of adult aortic medial SM. Thus, the significant changes of VLA-1 integrin content occurred not only during prenatal development, but also during several months after birth (Table II and Fig. 6 *A*).

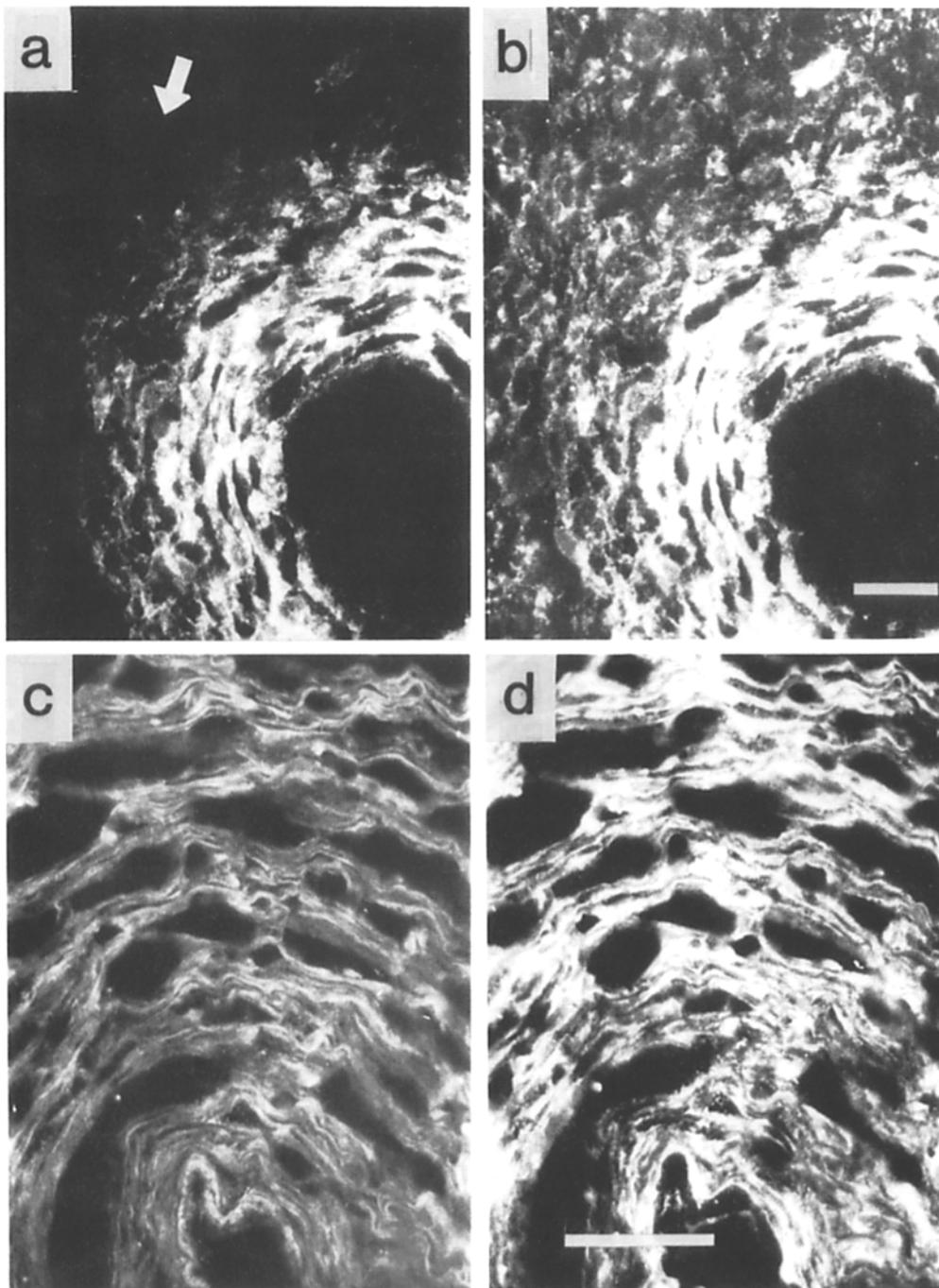
**Adult Aorta.** In the adult human aorta, SMC occupy two anatomical layers; tunica media and tunica intima (38). Tunica intima is formed during postnatal growth, and the adult

aortic intima can be divided into two sublayers: subendothelial and muscular-elastic (adjacent to the media) (38). Both intimal sublayers and media of the human aorta were mechanically separated and assayed for the content of  $\alpha_1$  and  $\beta_1$  VLA integrin subunits. In media and in both intimal sublayers,  $\alpha_1$  subunit, as well as  $\beta_1$  subunit of VLA integrin were detected (Table II and Fig. 6 *B*). However, the content of  $\alpha_1$  subunit of VLA integrin differed depending on the layer. The amount of  $\beta_1$  subunit of integrin in all layers was practically the same (Table II). In subendothelial intima,  $\alpha_1$  subunit content was significantly decreased as compared with that in adjacent muscular-elastic intima and media (Table II). The  $\alpha_1/\beta_1$  ratio was 0.11 in subendothelial SMC and  $\sim 0.5$  in muscular-elastic intimal and medial SMC.

For detailed investigation of VLA-1 integrin distribution in adult human aorta SMC, cross sections of grossly normal segments of the aorta (from 35–45-yr-old donors) were double stained with antibodies to  $\alpha_1$  and  $\beta_1$  subunits of VLA-1 integrin. A positive staining of SMC with anti- $\beta_1$  antibody was observed in all layers of adult human aorta (Fig. 7). A strong positive staining was also observed with anti- $\alpha_1$  antibody in medial and muscular-elastic intimal SMC. On the contrary, immunofluorescence of subendothelial intimal SMC stained by anti- $\alpha_1$  antibody was rather weak (Fig. 7, *e* and *f*). The majority of subendothelial intimal SMC demonstrated a low level of VLA-1 integrin expression; however, some cells were VLA-1 integrin negative (Fig. 7 *e*). In other experiments it was shown that VLA-1 integrin-negative cells were positively stained with SM isoactin-specific antibody, proving the SM origin of these cells (data not shown).

**VLA-1 Integrin in Cultured SMC from Human Adult Aorta**

To determine cellular localization of the VLA-1 integrin, freshly isolated human aortic SMC, grown for 1 wk on collagen-coated coverslips, were examined by indirect immunofluorescence microscopy. Earlier, it was shown that on the seventh day of cultivation, SMC have a well-developed fibronectin-containing extracellular matrix (13). The cells were stained using antibodies against human  $\alpha_1$  and  $\alpha_5$  subunits of VLA integrin, against  $\beta_1$  subunit of integrin, and against talin. In general, two main types of  $\alpha_1$  integrin localization were found in primary SMC cultured on type I collagen substrate. The  $\alpha_1$  subunit of VLA-1 integrin had a patchlike distribution on the ventral cell surface (these sites correspond to focal adhesion sites [28]) and was revealed as a streaklike pattern that coincided with extracellular matrix attachment sites (Fig. 8, *a*, *c*, and *e*). The intracellular localization of the  $\beta_1$  subunit of VLA integrin was very similar, but in addition some parts of the protein were randomly distributed on the upper cell surface membrane (Fig. 8, *a* and *b*). We always found a good correspondence in the distribution patterns of  $\alpha_1$  and  $\alpha_5$  subunits, and between  $\alpha_1$  subunit of VLA-1 integrin and talin (Fig. 8, *c–f*). We were unable to identify any subcellular sorting of the  $\alpha_1$  and  $\alpha_5$  subunits of VLA integrins on type I collagen substrate, most likely because of a well-developed fibronectin-rich extracellular matrix characteristic of primary SMC cultures (28). Thus, cultivated SMC contain VLA-1, VLA-5 integrins, and talin at the same cell–substrate and cell–matrix attachment sites.



**Figure 5.** Immunofluorescence staining of human fetal aorta sections with antibodies to VLA-1 integrin subunits. Cross sections of 10-wk-old fetal aorta (*a* and *b*) and 24-wk-old fetal aorta (*c* and *d*) were double labeled with fluorescein anti- $\alpha_1$ -subunit and rhodamine anti- $\beta_1$ -subunit of VLA-1 integrin. (*a* and *c*) anti- $\alpha_1$  subunit; (*b* and *d*) anti- $\beta_1$  subunit. In 10-wk-old fetal aorta (*a*),  $\alpha_1$  subunit staining was seen only in SMC, while the surrounding mesenchyme was  $\alpha_1$ -negative (indicated by the arrow). Bars, 20  $\mu\text{m}$ .

To determine whether the amount of VLA-1 integrin was altered during SMC cultivation, we estimated the content of  $\alpha_1$  and  $\beta_1$  subunits of VLA integrin in primary culture and subcultures of SMC by quantitative immunoblotting. In our experimental conditions, SMC isolated from tunica media of human aorta did not proliferate up to 10–12 d in culture. In the course of primary culture, the content of  $\alpha_1$  subunit of VLA integrin significantly decreased (Table II and Fig. 6 C). At the same time, the share of  $\beta_1$  subunit of integrin did not change. The  $\alpha_1/\beta_1$  ratio of VLA integrin in cells cultured for 2 d was 0.41, and in cells grown in culture for 10 d the

$\alpha_1/\beta_1$  ratio was 0.06 (Table II). The  $\alpha_1/\beta_1$  ratio of VLA integrin in subcultured SMC from media of human aorta (8 passage) was very low and did not differ from that of proliferating cells in primary culture.

### Discussion

In this report, we describe the purification of a complex consisting of two polypeptides of 195 and 130 kD from human uterus SM. The following independent criteria show that this complex might be VLA-1 integrin. First, the electrophoretic

**Table II. Relative Amounts of  $\alpha_1$  Subunit of VLA-1 Integrin in Human Aorta and Cultured Aortic SMC**

Source of smooth muscle	$\alpha_1$	$\beta_1$ cpm $\times 10^{-3}$	$\alpha_1/\beta_1$
9-10-wk-old fetal aorta	13.0	48.3	0.27
24-wk-old fetal aorta	3.0	19.0	0.16
3-mo-old child aorta	9.1	35.0	0.26
1.5-yr-old child aorta	14.3	38.1	0.38
12-yr-old aortic media	18.4	33.6	0.54
Adult aorta wall layer			
Media	14.9	32.4	0.46
Muscular-elastic intima	16.3	28.8	0.57
Subendothelial intima	3.8	35.7	0.11
Cultured aortic medial cells			
Primary culture (2 d)	77.6	190.0	0.41
Primary culture (10 d)	10.1	174.0	0.06
Subculture (8th passage)	2.0	159.0	0.01

mobilities of the polypeptides in reduced and nonreduced conditions correspond to  $\alpha_1$  and  $\beta_1$  subunits of classical VLA-1 integrin found in T cells. Second, the 130-kD polypeptide interacts specifically with defined monoclonal and polyclonal antibodies to human  $\beta_1$  subunit of integrin. Polyclonal antibody used in the present study is directed against the COOH-terminal domain of the  $\beta_1$  subunit in human tissue (31). The 195-kD polypeptide was able to interact with a well-characterized mAb, TS2/7, which was originally developed for the identification and isolation of  $\alpha_1$  subunit of VLA-1 integrin from T cells (20). Third, the 195- and 130-kD polypeptides were able to form a stable heterodimer solubilized in nonionic detergents. This heterodimer, precipitated in the absence of detergent, could be effectively incorporated into liposomes, thus acting as an integral membrane protein. Finally, this heterodimer, isolated from human SM, exhibited functional properties characteristic of receptors to extracellular matrix proteins.

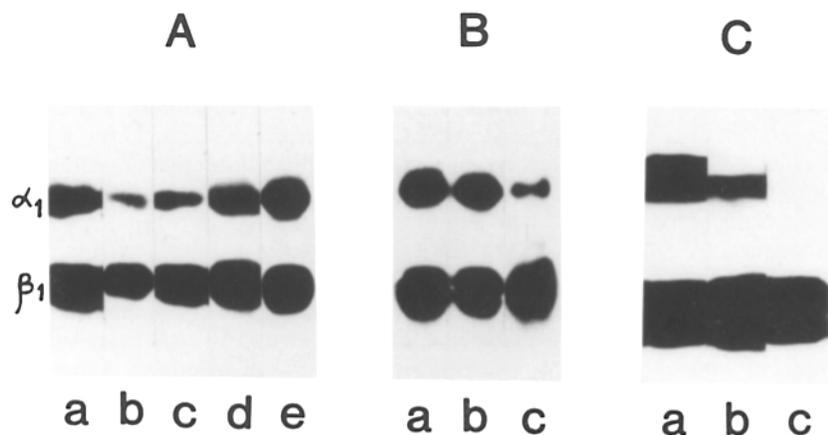
#### Functional Properties of Human SM VLA-1 Integrin

Our results show that human SM VLA-1, being incorporated into liposomes, specifically interacts with basement membrane components, laminin, and type IV collagen, as well as with interstitial type I, II, and III collagens. It ought to be

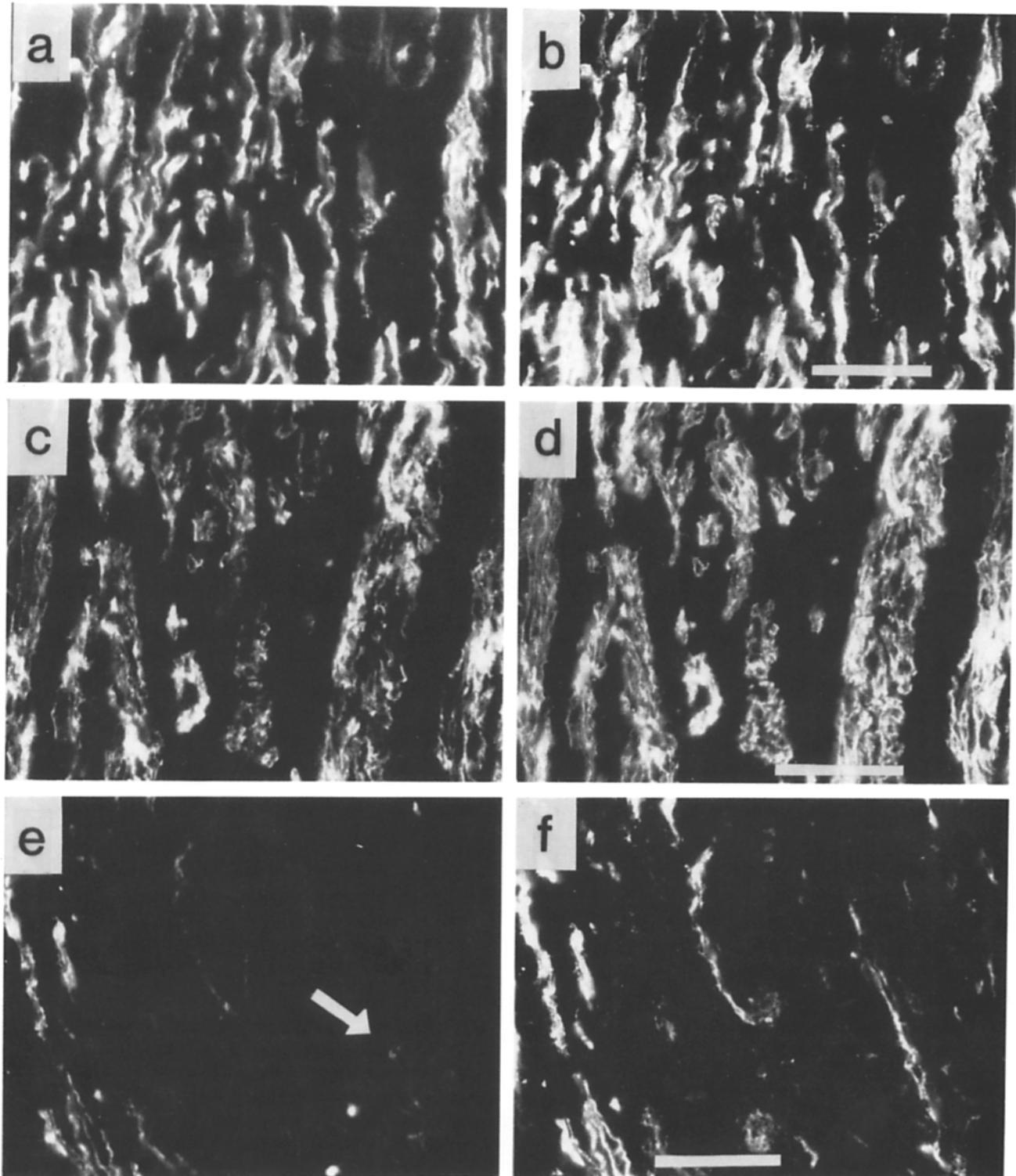
noted, that in contrast to previously published data (28), the affinity of SM VLA-1 integrin to type I collagen was the highest and exceeded that to type IV collagen. Thus, these data contradict the widely held concept that differentiated human SMC are predominantly surrounded by type IV collagen and laminin. However, the possibility that VLA-1 binding to type I collagen takes place at the early stages of embryogenesis, when SM precursor cells are in close contact with interstitial collagens, cannot be excluded. Furthermore, since we were unable to identify any VLA-2 integrin (considered to be the major integrin receptor for type I collagen [28]) in total SM extract, it seems to us that high affinity of SM VLA-1 integrin to type I collagen is important for the interaction of SMC with extracellular matrix. Affinity chromatography of SM integrins on collagen type I-Sepharose failed to detect any other collagen-binding integrins besides VLA-1. Thus, VLA-1 integrin has functional properties characteristic of integrins with a broad spectrum of specificity, interacting with extracellular matrix proteins in an RGD-independent manner.

Data reported in this study, as well as those published by other groups (3, 25, 48), indicate that VLA-1 and VLA-5 integrins are the major VLA integrins in human SM and can serve as receptors for laminin, fibronectin, basement membrane, and interstitial collagens. Apart from VLA integrins, SMC also express the  $\beta_3$  subfamily of integrins (7), which potentially promote the adhesion of SMC to vitronectin, thrombospondin, fibrinogen, and von Willebrand factor. Thus, at least three different integrins, VLA-1, VLA-5, and the  $\beta_3$  subclass integrin(s), are needed to provide the interactions of SMC with all known extracellular matrix proteins.

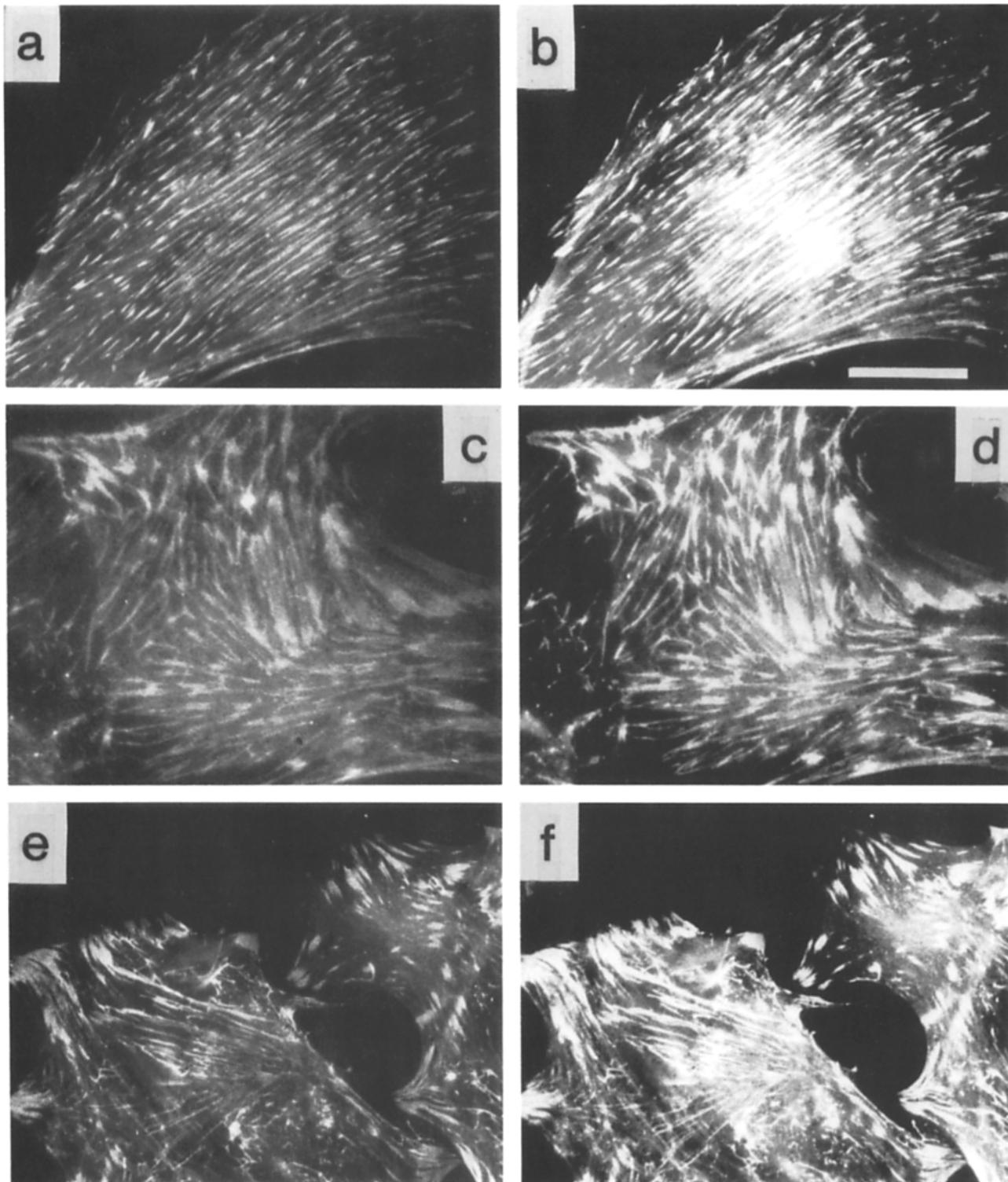
Based on the fact that VLA-1 integrin has a high affinity to native collagens, we tested the interaction between VLA-1 integrin-containing liposomes and C1q subcomponent of the first component of the complement. A possible interaction between these two proteins was predicted by the presence of collagen-like domains in the C1q subcomponent molecule (36). We were able to demonstrate specific interaction between VLA-1 integrin-containing liposomes and C1q-coated surfaces. As known, VLA-1 integrin expression is dramatically enhanced in very late activated peripheral blood T cells (19). It has been suggested that in activated T lymphocytes VLA integrins are involved in cell-matrix interactions (18). The data obtained in the present study led us to speculate



**Figure 6.** Quantitative analysis of  $\alpha_1$  and  $\beta_1$  VLA integrin subunit content in human aorta and cultivated aortic SMC by SDS-PAGE, immunoblotting, and subsequent autoradiography. (A) Developing human aorta: (lane a) 9-10-wk-old fetal aorta; (lane b) 24-wk-old fetal aorta; (lane c) 3-mo-old child aorta; (lane d) 1.5-yr-old child aortic media; (lane e) 12-yr-old aortic media. (B) Adult human aorta: (lane a) media; (lane b) muscular-elastic intima; (lane c) subendothelial intima. (C) Cultivated aortic SMC: (lane a) primary culture, 2 d; (lane b) primary culture, 10 d; (lane c) subculture, 8th passage.



**Figure 7.** Immunofluorescence staining of human adult aortic intima and media sections with antibodies to VLA-1 integrin subunits. Transverse sections of adult aortic media (*a* and *b*), and muscular-elastic (*c* and *d*) and subendothelial (*e* and *f*) intima were double labeled with fluorescein anti- $\alpha_1$ -subunit and rhodamine anti- $\beta_1$ -subunit of VLA-1 integrin. (*a*, *c*, and *e*) Anti- $\alpha_1$  subunit; (*b*, *d*, and *f*) anti- $\beta_1$  subunit. In the subendothelial intima (*e*), SMC negatively stained with antibodies to  $\alpha_1$  subunit were seen (indicated by the arrow). Bars: (*a*, *b*, *e*, and *f*) 20  $\mu\text{m}$ ; (*c* and *d*) 10  $\mu\text{m}$ .



**Figure 8.** Immunofluorescent analysis of  $\alpha_1$ ,  $\alpha_5$ ,  $\beta_1$  integrin subunit and talin localization in primary cultured human aortic SMC, grown on type I collagen. Cells were double labeled with fluorescein anti- $\alpha_1$  subunit of VLA integrins (*a*, *c*, and *e*) and with rhodamine anti- $\beta_1$  subunit of VLA integrins (*b*); with rhodamine anti- $\alpha_5$  subunit of VLA integrins (*d*); with rhodamine anti-talin (*f*). Bar, 10  $\mu\text{m}$ .

that at least one member of the VLA family, namely VLA-1 integrin, is responsible for the involvement of T cells in the complement pathway via the interaction between T cells and C1q subcomponent of the first component of the complement. Therefore, T cell interaction with target cell antigens

may be dependent on the presence of some complement system components such as the C1q subcomponent. The appearance of C1q subcomponent on the cell surface can probably promote formation of complexes between T lymphocytes and target cells.

### ***VLA-1 Integrin Is a Biochemical Marker of Human Aortic SMC Development and Differentiation***

We have been interested in the mechanism(s) by which components of the extracellular matrix and the cytoskeleton were involved in the development and differentiation of human aortic SMC (2, 11-16). The evidence presented in this paper suggests that VLA-1 integrin is a differentiation marker of human aortic SMC and can play an important regulatory role in the control of SM development. The following facts substantiate our conclusion: (a) Human SMC express very high levels of VLA-1 integrin. Mesenchymal cells and cells derived from the mesenchyme, such as fibroblasts and endothelial cells, contain relatively small amounts (compared to SMC) of VLA-1 integrin on their surface. (b) Expression of VLA-1 integrin significantly changes during prenatal and postnatal development of the human aortic SM. (c) Phenotypic modulation of SMC in culture (during transition from the contractile to the synthetic state [6]) is accompanied by a dramatic drop in the VLA-1 integrin expression. In differentiated (contractile) SMC the VLA-1 integrin content is remarkably high, whereas in dedifferentiated (synthetic) SMC, the amount of VLA-1 integrin is rather low.

The development of the human aorta is characterized by nondifferentiated SMC becoming fully differentiated and altering their phenotype several times (14, 15, 26, 35). Signals controlling SMC differentiation remain as yet unknown. Recent studies have shown that VLA integrins may play an important role as mediators of signal transduction controlling the differentiation of skeletal muscles (32). Our results clearly demonstrate that during development vascular SMC alter the expression of VLA-1 integrin at least four times. During the prenatal period, expression of VLA-1 integrin changes twice: before 8-10 wk of gestation, conceivably, when mesenchymal cells begin to differentiate into SMC, and between the 10th and 20th weeks of gestation. Another change in VLA-1 integrin expression occurs during postnatal development, during the first year after birth, and in the adult aorta, when SMC are recruited into the intimal cell population.

Various signals from the cell's environment can alter the expression of VLA integrins. Transforming growth factor- $\beta_1$  elevates the synthesis of VLA-1 integrin in WI-38 lung fibroblasts and in Hep G2 hepatoma cells (17). Growth conditions also affect VLA-1 integrin expression. In rapidly growing normal human fibroblasts the level of VLA-1 integrin is decreased, whereas in quiescent fibroblasts, the expression of VLA-1 integrin is greatly enhanced (9). All these experimental data might be of significance in the understanding of the regulation of VLA-1 integrin expression in human aortic SMC. A large body of evidence indicates that during vessel development the expression of growth factors is varied. Thus, fetal SMC produce PDGF while the adult cells do not (30, 44). Otherwise, in adult media, SMC are present in a quiescent, contractile state (43). On the other hand, the functional relevance of differential VLA-1 integrin expression during SM development might be consistent with the fact that the expression of VLA-1 integrin ligands changes also. For instance, during vessel wall formation fibronectin appears before laminin in the SMC extracellular matrix (39). Arrangement of the basement membrane around SMC often correlates with SM differentiation and

maturation. Basement membrane proteins such as laminin and type IV collagen, being ligands for VLA-1 integrin, appear at the last stages of SM development and possibly promote terminal differentiation of SMC and/or the maintenance of differentiated state through the interaction with VLA-1 integrin.

In conclusion, we have shown that (a) human SM contains high amounts of VLA-1 integrin; (b) VLA-1 integrin is able to interact with all types of collagens assayed, with laminin and C1q subcomponent of the first component of the complement system; (c) in primary cultured SMC VLA-1 integrin was found to be present in focal contacts and extracellular matrix attachment sites; (d) the expression of VLA-1 integrin in human aortic SMC is developmentally regulated and is characteristic only of differentiated SMC.

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