Differential Distribution and Modulation of Expression of Alpha1/Beta1 Integrin on Human Endothelial Cells

Paola Defilippi, Victor van Hinsbergh,* Antonio Bertolotto,‡ Paola Rossino, Lorenzo Silengo, and Guido Tarone

Department of Genetics, Biology and Medical Chemistry, ‡Neurology Clinic, University of Torino, Torino, Italy; and *TNO Gaubius Institute, Leiden, The Netherlands

Abstract. In this paper we report that the integrin complex alpha1/beta1, a laminin/collagen receptor, is expressed on cultured foreskin microvascular endothelium, but is absent on endothelial cells from large vessels such as the aorta and umbilical and femoral veins. The restricted expression of integrin alpha1/beta1 to microvascular endothelium was also demonstrated in vivo, by immunohistochemical staining of human tissue sections. Alpha1 specific antibodies reacted strongly with endothelial cells of small blood vessels and capillaries in several tissues, but not with endothelium of vein and arteries of umbilical cord. Expression of integrin alpha1 can be induced in cultured umbilical vein endothelial cells by treatment with 5 ng/ml tumor necrosis factor alpha (TNFalpha). Induction of alpha1 subunit expression also occurred after treatment of umbilical vein endothelium with 10^{-5} M retinoic acid or with 10 nM PMA; Maximal induction of alpha1 integrin was reached after 48 h of treatment and costimulation with TNFalpha and PMA resulted in a synergistic effect. The induction of alpha1 integrin changed the adhesive properties of umbilical vein endothelial cells, by increasing the adhesiveness to collagen, laminin, and laminin fragment P1, while adhesion to fibronectin and laminin fragment E8 remained constant. The alpha1 integrin is thus a marker of a specific population of endothelial cells and its expression confers distinctive properties of interaction with the underlying basal membrane.

ENDOTHELIAL cells form a continuous layer covering the blood vessel luminal surface and represent the physical barrier between the blood stream and tissues. The mechanical and functional integrity of this barrier is assured by interactions among endothelial cells as well as by their adhesion to the basal membrane, an extracellular matrix structure consisting predominantly of collagen type IV, laminin, nidogen, and heparansulphate. The interaction of endothelial cells with the basal lamina components is mediated by specific cell surface receptors belonging to the integrin family. Integrins are a class of glycoprotein heterodimers of alpha and beta subunits spanning the plasma membrane and connecting the extracellular matrix framework to the intracellular cytoskeletal structures (Ruoslahti and Pierschbacher, 1987; Hynes, 1987; Hemler, 1990). Endothelial cells from umbilical vein have been shown to express several integrin complexes at their surface. These include the fibronectin receptor (alpha5/beta1) (Conforti et al., 1989), the vitronectin receptor (alphaV/beta3) (Cheresh, 1987; Charo et al., 1987; Dejana et al., 1988; Albelda et al., 1990; Tarone et al., 1990), as well as three other complexes that function as collagen and laminin receptors (alpha2/beta1, alpha3/beta1, and alpha6/beta1) (Albelda et al., 1989; Languino et al., 1989; Tarone et al., 1990). All five integrin complexes are simultaneously expressed by umbilical vein endothelium, endowing these cells with the ability to recognize and adhere to both basal membrane and stromal matrix components. Analysis of umbilical vein endothelial cells exposed to several different inflammatory cytokines and growth factors showed that tumor necrosis factor alpha (TNFalpha)[1] and interferon gamma (IFNgamma) induce a selective downregulation of specific integrin complexes (Defilippi et al., 1991), thus indicating that endothelial cells can modulate the integrin profile depending on their functional state.

In this study we show that endothelial cells from different types of blood vessels express different integrin patterns, both in vitro and in vivo. In particular, alpha1/beta1 integrin, known to bind both laminin and collagens, is selectively expressed on endothelial cells from microvasculature, and not on endothelial cells from large vessels such as the aorta and femoral and umbilical veins. Moreover, endothelium from umbilical vein can express integrin alpha1/beta1 when exposed to TNFalpha, retinoic acid, or PMA. The restricted expression of alpha1/beta1 complex and its induction by TNFalpha suggests a specific role of this integrin in endothelial cell function.

1. *Abbreviations used in this paper*: EC, endothelial cells; FN-R, human fibronectin receptor; IFNgamma, interferon gamma; PAP, peroxidase anti-peroxidase; TNFalpha, tumor necrosis factor alpha.

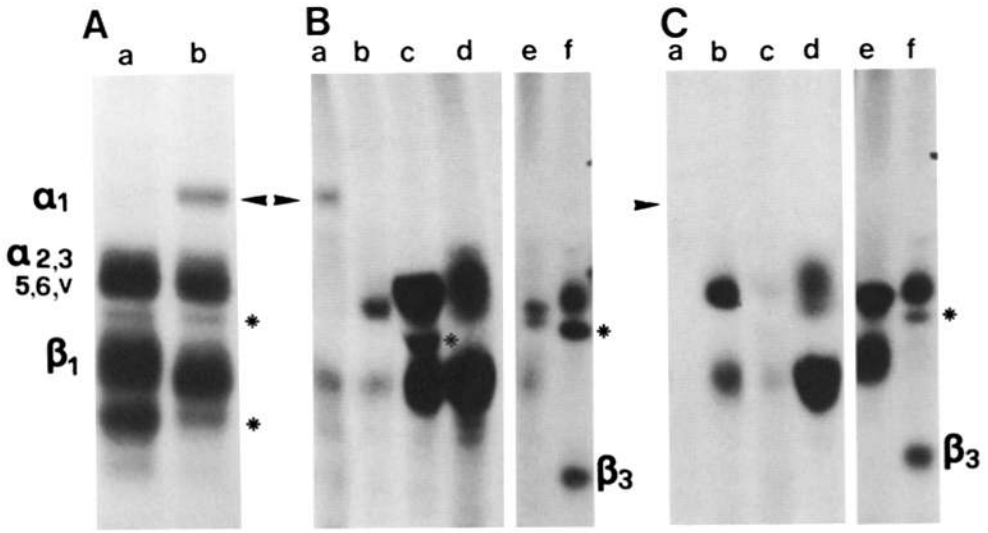


Figure 1. Immunoprecipitation of integrins from microvascular and umbilical EC. Confluent cultures of microvascular EC and umbilical vein EC were labeled with [^{35}S]methionine for 15 h. Cells were detergent extracted and subjected to immunoprecipitation with integrin antibodies. (*A*) Umbilical vein (lane *a*) and microvascular EC (lane *b*) were immunoprecipitated with FN-R antibodies. (*B*) Microvascular EC and (*C*) umbilical vein EC were immunoprecipitated with antibodies specific for the different alpha subunits. Lanes *a*, alphal monoclonal antibody TS2/7; lane *b*, alpha2 monoclonal antibody 10G11; lanes *c*, alpha3 polyclonal antibody; lanes *d*, alpha5 polyclonal antibody; lanes *e*, alpha6 monoclonal antibody GOH3; lanes *f*, alphaV monoclonal antibody NKI-M9. The radioactive antigens were separated by 6% SDS-PAGE in nonreducing conditions and visualized by fluorography. Arrowheads indicate the position of the alphal subunit. Note the absence of alphal in *A*, lane *a* and *C*, lane *a*. Asterisks indicate the immature forms of the beta and alpha subunits as determined by previous pulse-chase experiments.

Materials and Methods

Cell Culture and Treatments

Endothelial cells (EC), were isolated by collagenase (Boehringer Mannheim GmBH, Mannheim, Germany) treatment from human umbilical vein according to the method of Gimbrone (1976). Cells pooled from three to six cords were cultured on gelatin coated 90 mm plastic tissue culture dishes (Falcon Labware, Oxnard, CA), in medium 199 (Flow Laboratories, Irvine, Ayrshire, Scotland) supplemented with 20% FCS (Flow Laboratories), 100 μg/ml bovine brain extract, 100 μg/ml porcine heparin (Sigma Chemical Co., St. Louis, MO), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were used for the experiments between passages 2 and 6. Human foreskin microvascular EC were isolated as described by Davison et al. (1980) and cultured on fibronectin coated dishes in medium 199 supplemented with 20% human serum, 20 mM Hepes, and penicillin/streptomycin. Primary cultures were incubated for 4 h with DiI (3,3'-dioctadecylindocarbocyanine)-labeled acetylated LDL, subsequently detached from the dish and separated by fluorescence activated cell sorting, as described by Voyta et al. (1984). After purification the cells were propagated on gelatin coated dishes in M199 medium with 10% human serum, 10% newborn calf serum, 150 μg/ml bovine brain extract, and 100 μg/ml porcine heparin, 20 mM Hepes, penicillin, and streptomycin. The medium was replaced every 2–3 d. At confluence the cells were divided with a split ratio 1:2.5. The cells were characterized for the presence of von Willebrand factor, the binding of Ulex europeus lectin I, the binding of the endothelium-specific monoclonal antibody Pal-E and the uptake of DiI-acetylated LDL, as previously described (van Hinsberg et al., 1987*a*). Confluent cells between passages 7 and 11 were used for the experiments. Endothelial cells from human aorta and femoral vein were isolated as previously described (van Hinsberg et al., 1987*b*). They were grown on gelatin coated dishes in the same medium as given above. Confluent cells at passage 8 were used for the experiments.

Treatments with the mediators were performed on confluent cultures. Human recombinant tumor necrosis factor alpha (TNFalpha) (10^8 cytotoxic U/mg), was a kind gift from Dr. M. Brockhaus (Hoffmann La Roche, Basel, Switzerland). Human recombinant interferon gamma (IFNgamma) (10^8 IU/mg), a kind gift from Dr. G. Garotta (Hoffmann La Roche, Basel, Switzerland), was used at 100 IU/ml. Human recombinant interleukin 6 (IL-6) (2×10^8 IU/mg), a kind gift from Dr. J. Content (Institut Pasteur du Brabant, Bruxelles, Belgium), was used at 5 ng/ml. Human recombinant IL-1beta (Immunex Corp., Seattle, WA) (5×10^7 IU/mg) and granulocyte colony stimulating factor (G-CSF) (10^7 IU/mg) (Bachem Feinchemikalien AG, Bubendorf, Switzerland) were used at 5 ng/ml and 1,000 U/ml, respectively. Retinoic acid and PMA were from Sigma Chemical Co. During treatment the medium was changed every day. All the treatments of EC were performed in medium 199 supplemented with 20% FCS, in the presence of 5 ng/ml of recombinant basic fibroblast growth factor (bFGF) (Amersham International, Buckinghamshire, England) as trophic factor.

Antibodies and Immunoprecipitation of Integrins

The polyclonal antibody to the human fibronectin receptor (FN-R), was prepared by immunizing a goat with affinity-purified fibronectin receptor from human placenta (Pytela et al., 1985). The antiserum was previously characterized and shown to react with both betal and alpha5 subunits (Conforti et al., 1989; Tarone et al., 1990). Its reactivity with betal allows the identification of all receptors of group 1 integrins. The polyclonal antisera to the betal, alpha3, and alpha5 integrin subunits were prepared in our laboratory by immunizing rabbits against synthetic peptides reproducing amino acid sequences from the cytoplasmic domains of each subunit. The following peptides, obtained from Multiple Peptide System (San Diego, CA), were used: betal, CTTVVNPKYEGK; alpha3, CRIQPSETERLTDDY; alpha5, KRSLPYGTAMEKAQLKPPATSDA. Peptides were coupled to hemocyanin with glutaraldehyde (approximate peptide to carrier molar ratio of 50:1) and rabbits were injected with 500 μg of the conjugate in complete Freund adjuvant. Antibodies reacted specifically with the peptide sequence used for immunization as determined by ELISA assay on peptide-BSA conjugates. The antibodies were specific to the appropriate subunit and did not show cross-reaction with other integrin subunits as demonstrated by immunoprecipitation assays on different cell lines.

Mouse monoclonal antibodies to human integrin alphal (mAb TS2/7) and to integrin betal (mAb A1A5) were a kind gift from Dr. Martin Hemler (Dana-Farber Cancer Institute, Boston, MA). The monoclonal antibody NKI-M9 to integrin alphaV subunit, the monoclonal antibody 10G11 to integrin alpha2 and the monoclonal antibody GOH3 to integrin alpha6 subunit were kind gifts from Dr. A. Sonnenberg (Central Laboratory of the Netherlands Red Cross, Amsterdam, The Netherlands). Since the integrin alpha/beta complexes are not dissociated under the experimental conditions used, antibodies, although specific for one subunit, immunoprecipitate both of them.

Umbilical vein and microvascular EC were grown at confluence, treated as indicated and metabolically labeled with [^{35}S]methionine or surface labeled with ^{125}I. [^{35}S]Methionine labeling was performed by overnight incubation in methionine-free medium (Flow Laboratories) with 5% serum and 40 μC/ml of [^{35}S]methionine (800 Ci/mM; Amersham International) in the presence of the mediators. ^{125}I labeling was performed as described previously (Rossino et al., 1990). Briefly, cells were released from culture dishes by 5 mM EDTA treatment in PBS and washed three times by centrifugation with culture medium. Cells were resuspended in PBS containing $CaCl_2$ (1 mM) and $MgCl_2$ (1 mM) and labeled with 1 mCi of ^{125}I in the presence

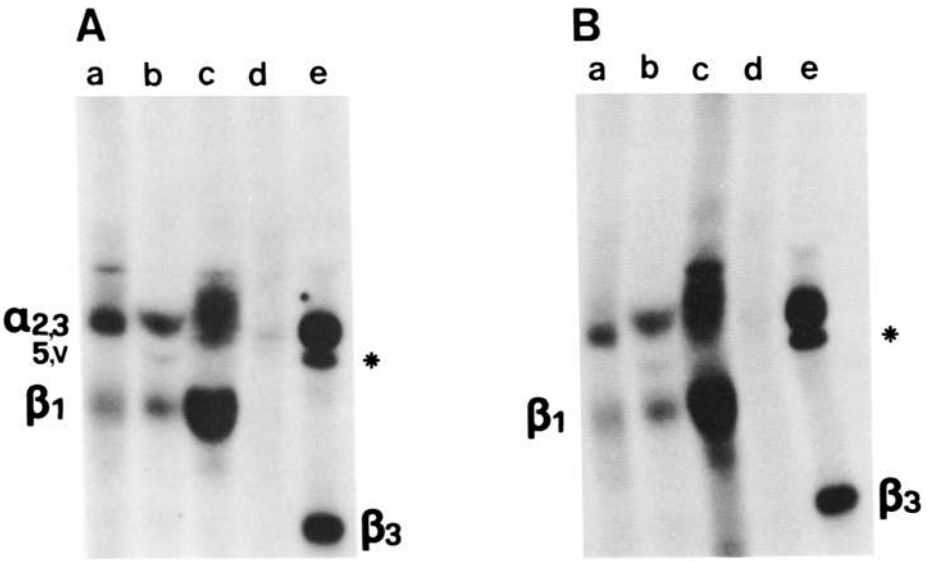


Figure 2. Immunoprecipitation of integrins from aorta (*A*) and femoral vein (*B*) EC. Cells were labeled with [^{35}S]methionine for 15 h, extracted with detergent, and immunoprecipitated with antibodies specific for the different alpha subunits. Lanes *a*, anti-alpha2; lanes *b*, anti-alpha3; lanes *c*, anti-alpha5; lanes *d*, anti-alpha1; lanes *e*, anti-alphaV. The radioactive antigens were separated by 6% SDS-PAGE in nonreducing conditions and visualized by fluorography. Antibodies to alpha6 are missing from this panel. A separate experiment, however, showed that the relative amount of alpha6/beta1 complex in these two EC types is comparable to that detected in umbilical EC. Asterisks indicate the immature form of the alphaV subunit as determined by previous pulse-chase experiments.

of lactoperoxidase (200 μg/ml) and H_2O_2 (0.002%). To analyze integrins from cell extracts, labeled cells were washed with cold PBS and the cells were extracted for 20 min at 4°C with 0.5% Triton X-100 (BDH Chemicals Ltd., Essex, England) in 20 mM Tris-HCl pH 7.4, 150 mM NaCl (TBS) with 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 μg/ml leupeptin, 4 μg/ml pepstatin, and 0.1 TIU/ml aprotinin (all from Sigma Chemical Co.) After centrifugation at 10,000 *g* for 10 min, extracts were incubated with the specific antibodies for 1 h at 4°C with gentle agitation. Soluble immunocomplexes were bound to protein A–Sepharose beads (Pharmacia, Uppsala, Sweden) and recovered by centrifugation. After washing, bound material was eluted by boiling beads in 1% SDS (Pierce Chemical Co., Rockford, IL), analyzed by 6% SDS-PAGE (Laemmli, 1976), and fluorographed (Chamberlain, 1979).

Immunoperoxidase Procedure

Immunohistochemical stainings were performed using the peroxidase anti-peroxidase (PAP) procedure (Sternberger et al., 1970). Muscle tissue was obtained by quadriceps biopsies of adult subjects with normal histological pattern. Umbilical cords were obtained from the delivery room of the local hospital. Dermis samples were obtained by skin biopsies from volunteer healthy donors. Histologically normal parts of brain and kidney were obtained by surgery from patients with brain or renal cancer and immediately frozen.

Cryostat sections (7 μm) were fixed with acetone for 5 min at −20°C, and treated with 3% H_2O_2 for 5 min at room temperature to block endogenous peroxidase. Sections were incubated overnight at 4°C with optimal dilution of the primary antibody followed by 30 min at room temperature with the secondary antibody. Antibodies to synthetic peptides were affinity purified on BSA-peptide conjugates coupled to Sepharose and used at concentrations ranging from 1 to 10 μg/ml. Samples were then incubated with peroxidase anti-peroxidase complex (Sigma Chemical Co.) and the reaction was developed with diaminobenzidine tetrahydrochloride (0.05%).

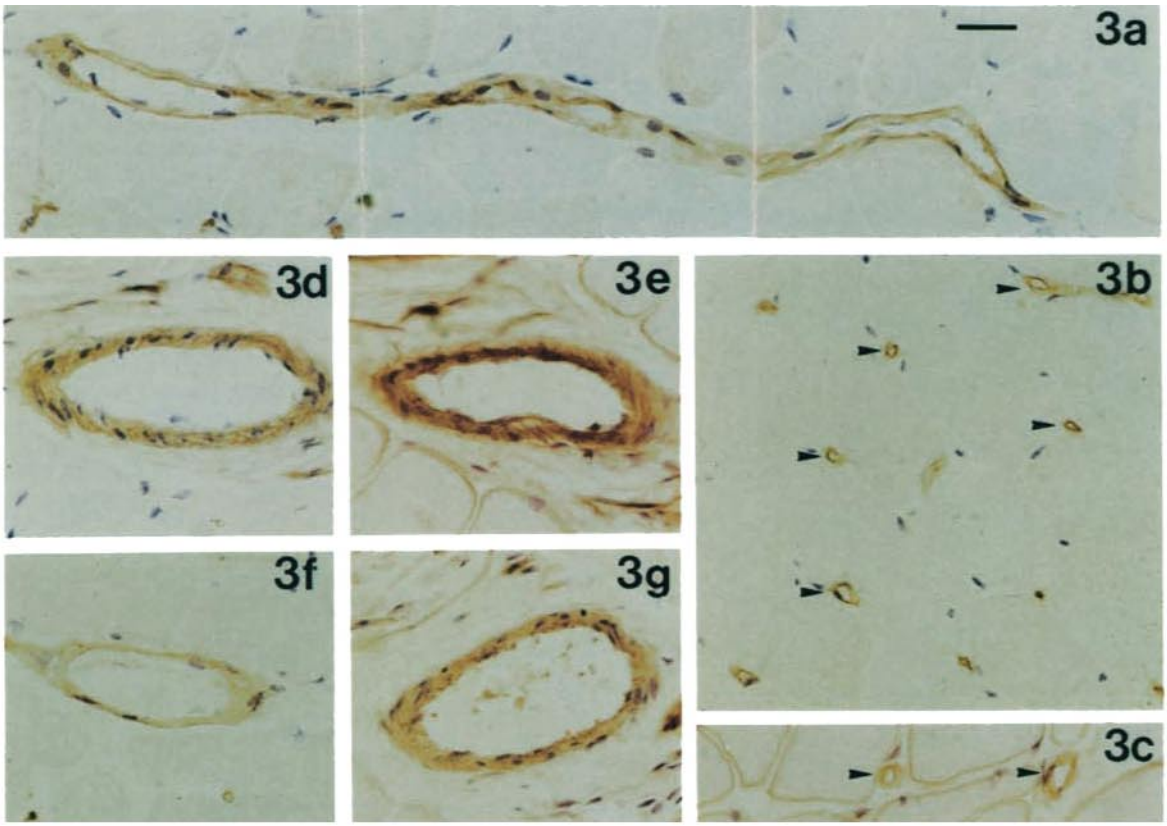
Adhesion Assay

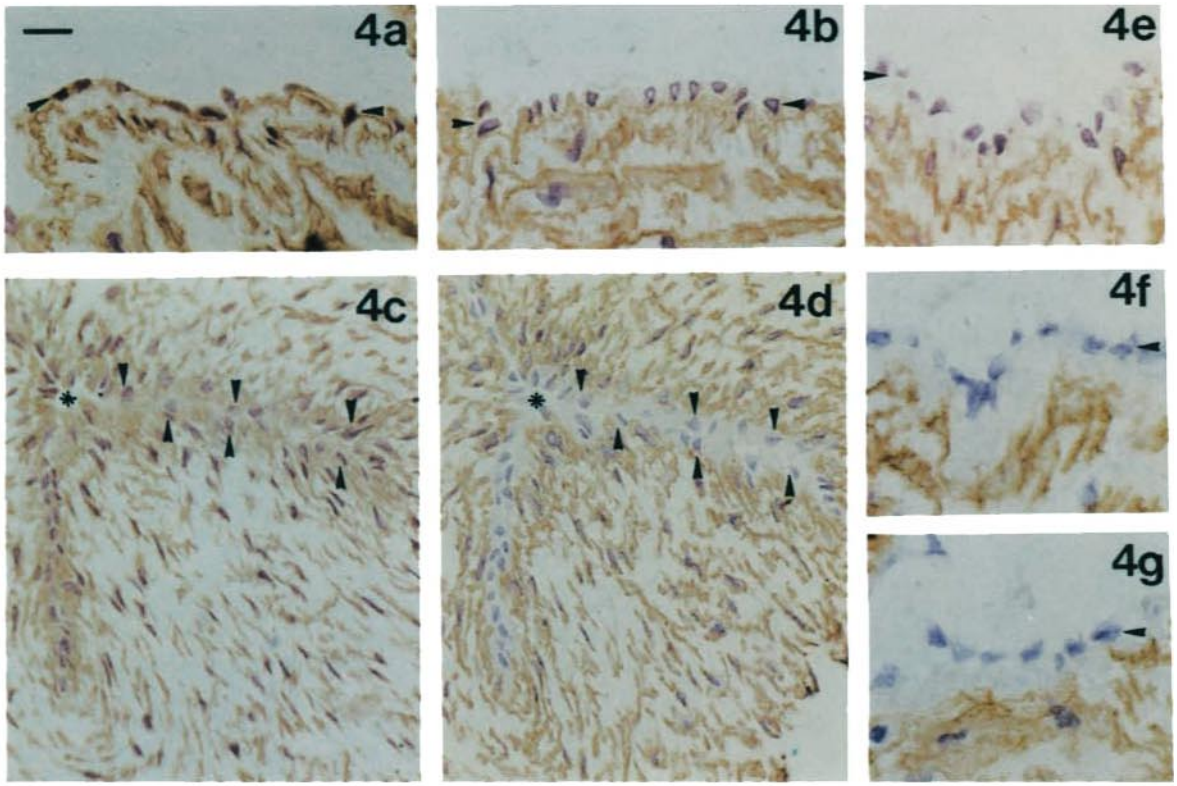
Fibronectin was purified from human plasma by affinity chromatography on gelatin-Sepharose as previously described (Engvall and Ruoslahti, 1977; Tarone et al., 1982). Laminin from EHS tumor and its cell binding fragments P1 and E8 were a kind gift of Dr. R. Timpl (Max Planck Institut fur Biochemie, Martinsried, Germany). Collagen type IV was from Sigma Chemical Co. Adhesion assay was performed as described previously (Defilippi et al., 1991). 96-well microtiter dishes (Costar, Cambridge, MA) were coated with optimal concentrations of various substrates and post-coated with BSA (Eurobio, Paris, France). Cells were released from culture dishes by gentle 5 mM EDTA treatment in PBS and plated in serum-free DMEM medium (Flow Laboratories) on coated wells for different times at 37°C. To eliminate the contribution of protein synthesis and secretion in cell adhesion, cells were pretreated 2 h before detachment with the protein synthesis inhibitor cycloheximide (20 mM) (Sigma Chemical Co.), and plated in the adhesion assay in the presence of 1 μM monensin (Sigma Chemical Co.) and 20 μM cycloheximide. Cell adhesion was evaluated by a colorimetric assay for acid phosphatase activity, by modifying the method described by Connolly et al. (1986). Adherent cells were washed two times with PBS, and acid phosphatase activity in each well was measured by incubating cells for 90 min at 37°C, in the presence of 200 μl of 10 mM *p*-nitrophenyl phosphate (Sigma Chemical Co.), 0.1% Triton X-100, 0.1 M sodium acetate, pH 5.5. The colorimetric reaction was developed by adding 20 μl of 1 N NaOH. The optical absorbencies at 405 nm were read with a Microplate Reader (BioRad 450; Bio-Rad Laboratories, Richmond, CA). A preliminary experiment showed that the optical density was linearly correlated with the number of viable cells.

Results

Alpha1/Beta1 Integrin Is Expressed on Microvascular but Not on Large Vessel Endothelium in Culture

Integrin complexes of human foreskin microvascular EC were immunoprecipitated with antibodies to the FN-R which react predominantly with the beta1 subunit (Tarone et al., 1990). As shown in Fig. 1 *A* (lane *b*) these antibodies precipitate the beta subunit (120 kD) as well as associated alpha subunits that are resolved in two bands with molecular masses of 180 and 160 kD. The fourth band of 100 kD represent an immature form of the beta1 subunit as determined by pulse-chase experiments (Defilippi, P., and G. Tarone, unpublished data; Heino et al., 1989). The coprecipitation of alpha subunits with beta1 antibodies is due to association of these molecules in noncovalent complexes at the cell surface (Ruoslahti and Pierschbacher, 1987; Hynes, 1987; Hemler, 1990). The integrin pattern described above is identical to that of umbilical vein EC (Fig. 1 *A*, lane *a*) except for the 180-kD band which is unique to microvascular EC. This molecule has a molecular mass corresponding to that of the alpha1 subunit (Hemler, 1990). To more precisely identify this component, as well as the other alpha subunits that comigrate in the 160-kD band, antibodies specific for each known alpha subunit were used. As shown in Fig. 1 *B* (lane





Figures 3 and 4. (Fig. 3) Immunohistochemical staining of human skeletal muscle tissue sections with antibodies to integrins. Frozen sections of striated skeletal muscle tissue were incubated with alphal monoclonal antibody TS2/7 (*a*, *b*, *d*, and *f*), with betal monoclonal antibody A1A5 (*c* and *e*) or with polyclonal antibodies to von Willebrand factor (*g*), followed by the appropriate secondary antibodies

a) the 180-kD subunit is recognized by alpha1-specific monoclonal antibodies. In addition, microvascular EC also express alpha2, alpha3, alpha5, alpha6, and alphaV (Fig. 1 *B*, lanes *b–f*). AlphaV subunit associates with beta3 to form the vitronectin receptor, while the other alphas are all associated with beta1. Preliminary experiments showed that EC do not express alpha4 subunit and this antibody was thus omitted from the panels in Fig. 1.

The use of alpha subunit specific antibodies confirmed the absence of alpha1 subunit from umbilical vein EC (Fig. 1 *C*, lane *a*). These cells, like microvascular EC, express alpha2, alpha3, alpha5, alpha6, and alphaV (Fig. 1 *C*). Quantitative differences were detected in the relative amount of alpha3 and alpha6 subunits between the two EC types: while alpha3 is abundant in microvascular EC, it is only poorly expressed in umbilical vein endothelium; the opposite is true for alpha6.

To determine whether the lack of expression of alpha1 integrin was a property unique to endothelium from umbilical vein, we also analyzed EC isolated from human aorta and femoral vein. As shown in Fig. 2, these EC do not express the alpha1 subunit and display an integrin pattern similar to that of umbilical vein EC. Thus, the lack of alpha1 expression is typical of various large blood vessels.

Alpha1 Integrin Is Expressed in Microvascular, but Not in Umbilical Vessel Endothelium In Situ

To test whether the differential expression of alpha1 integrin described above was also detectable in situ, we stained frozen human tissue sections with alpha1 integrin specific antibodies. As shown in Fig. 3 (*a*, *b*, *d*, and *f*), the antibodies intensely stained capillaries as well as vessels with larger diameters (50–150 μm). These structures were also positive with integrin beta1 antibodies (Fig. 3, *c* and *e*). In addition to skeletal muscle (Fig. 3) capillaries and small vessels in the dermis, kidney, and brain were also positive for both alpha1 and beta1 antibodies.

Different results were obtained with endothelium from umbilical cord sections. As shown in Fig. 4 (*d–g*), the endothelial layer of umbilical vein and arteries was negative for alpha1 integrin antibodies, while the underlying smooth muscle cells of the vessel wall were strongly positive. Similar sections were also stained with beta1 antibodies. In this case, strong positive reaction was visible in the endothelial layer as well as in the underlying smooth muscle (Fig. 4, *a–c*). These data fully confirmed the results observed with in vitro cultured EC.

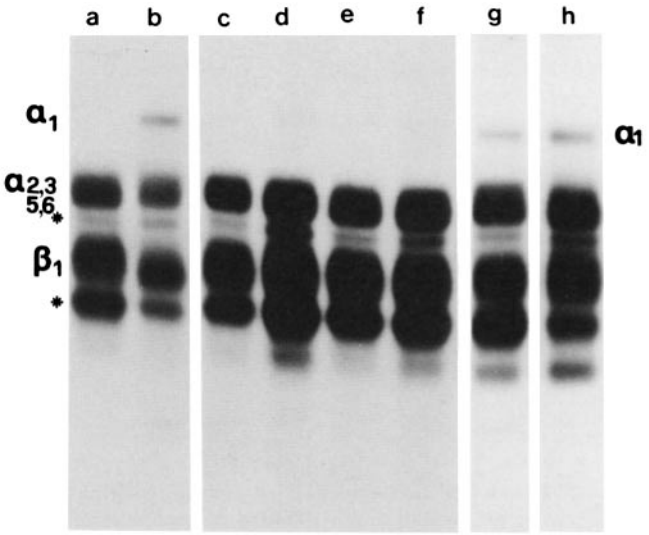


Figure 5. Induction of alpha1 subunit expression on umbilical vein EC. Confluent cultures of umbilical vein EC were treated for 72 h with different mediators, and labeled with [^{35}S]methionine in the last 15 h of treatment. Cells were detergent extracted and subjected to immunoprecipitation with FN-R polyclonal antibodies. Untreated cells (lane *a*); cells treated with: 5 ng/ml TNFalpha (lane *b*); 100 IU/ml IFNgamma (lane *c*); 5 ng/ml IL-1beta (lane *d*); 5 ng/ml IL-6 (lane *e*); 1,000 U/ml G-CSF (lane *f*); 10^{-5} M retinoic acid (lane *g*); 10 nM PMA (lane *h*). The radioactive antigens were separated by 6% SDS-PAGE in nonreducing conditions and visualized by fluorography. Asterisks indicate immature forms of alpha and beta subunits.

Umbilical Vein EC Express Alpha1 Integrin When Exposed to TNFalpha, Retinoic Acid, or PMA

To test whether large vessel endothelium can be induced to express alpha1 integrin, umbilical vein EC were exposed to various treatments in vitro. As shown in Fig. 5 (lane *b*), exposure of the cells to TNFalpha resulted in synthesis of alpha1 subunit. Other inflammatory cytokines and growth factors, including IL-1beta, IFNgamma, IL-6, G-CSF, and bFGF, were inactive (Fig. 5), indicating a rather specific effect of TNFalpha. When screening several compounds for their ability to affect alpha1 integrin expression, we found that, in addition to TNFalpha, retinoic acid and the tumor promoter PMA were also active (Fig. 5, lanes *g* and *h*). The induction of alpha1 integrin with TNFalpha, retinoic acid or

and the PAP complex. The sections were counterstained with hematoxylin. Note the positive staining with alpha1 antibody of microvessels in longitudinal (*a*) and transverse (*b*) sections (*arrowheads*). Arterioles (*d*) and venules (f) were also positive with alpha1 antibody. Serial sections of the vessel in *d* were also stained with beta1 (*e*) or von Willebrand (*g*) antibodies for comparison. Muscle fibers were stained only with beta1 antibody (*c* and *e*). Bar, 30 μm. (Fig. 4) Immunohistochemical staining of umbilical vein and artery sections with integrin antibodies. Frozen sections of umbilical cord were incubated with beta1 polyclonal antibodies (*a–c*), or with alpha1 monoclonal antibody TS2/7 (*d–g*), followed by the appropriate secondary antibodies and the PAP complex. The sections were counterstained with hematoxylin. Note the lack of reaction of alpha1 antibody with EC in the vein (*e–g*) and in the artery (*d*). This is particularly evident by comparison with sections stained with beta1 antibodies where EC from both vein (*a* and *b*) and artery (*c*) are strongly stained. Arrowheads point to the endothelial cell layer. Note that the smooth muscle cells underneath EC and in the underlying layers of the vessel walls are strongly positive with both alpha1 and beta1 antibodies. The lumen of the artery in *c* and *d* (*asterisks*), can be barely appreciated due to the collapsed vessel walls. Bars: (*a*, *b*, and *e–g*) 45 μm; (*c* and *d*) 30 μm.

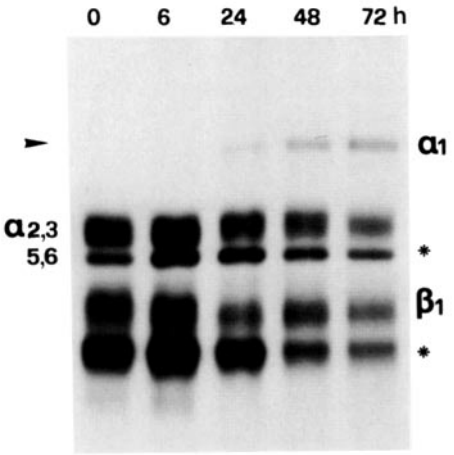


Figure 6. Kinetics of induction of alphal subunit by TNFalpha in umbilical vein EC. Umbilical vein EC were treated for the indicated times with 5 ng/ml TNFalpha, and labeled with [^{35}S]methionine in the last 6 h of treatment. Cells were detergent extracted and immunoprecipitated with FN-R antibodies. The radioactive antigens were separated by 6% SDS-PAGE in nonreducing conditions and visualized by fluorography. Arrowhead indicates the position of alphal subunit. Asterisks indicate immature forms of beta and alpha subunits.

PMA followed rather slow kinetics as the protein was appreciable at 24 h and reached maximal levels after 48–72 h of treatment (Fig. 6). Expression of alphal remained constant in the presence of the mediators for at least 120 h. A dose-response analysis was performed with the three active agents at 48 h of treatment. Retinoic acid was used from 10^{-4} to 10^{-9} M, and the response reached maximal levels at 10^{-5} M. TNFalpha was used between 0.1 and 50 ng/ml and the response reached a plateau at 5 ng/ml. PMA was used at concentrations between 0.5 nM and 1 uM, and the optimal dose was found to be 10 nM. When PMA was used at concentrations above 500 nM, a progressive decrease in alphal induction was observed, without any toxic effect on cell viability, as measured by methionine incorporation in TCA-precipitable materials. The level of induced alphal subunit in umbilical vein EC treated with optimal doses of TNFalpha or PMA, was comparable to that found in microvascular EC.

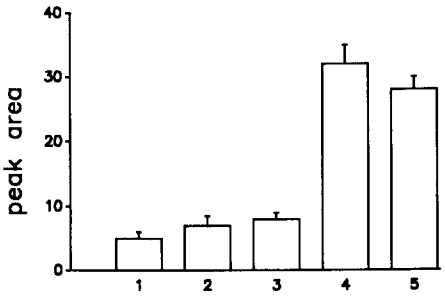


Figure 7. Synergistic induction of alphal subunit. Umbilical vein EC were treated for 48 h with 1 nM PMA (*1*), 0.5 ng/ml TNFalpha (*2*), 10^{-6} M retinoic acid (*3*), 1 nM PMA plus 0.5 ng/ml TNFalpha (*4*), and 1 nM PMA plus 10^{-6} M retinoic acid (*5*). The bars represent the absolute values of the peak area, expressed in arbitrary units, obtained by densitometric analysis of the autoradiograms.

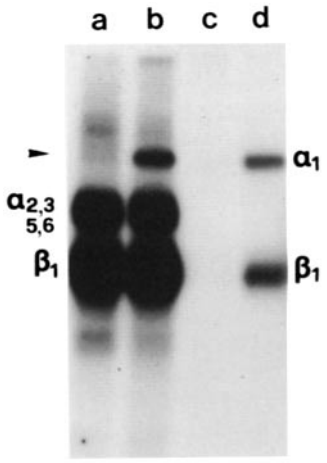


Figure 8. Expression of alphal subunit on the cell surface. Confluent cultures of untreated umbilical vein EC (lanes *a* and *c*), and of umbilical vein EC treated for 72 h with 5 ng/ml TNFalpha (lanes *b* and *d*), were harvested and labeled with ^{125}I, as indicated in Materials and Methods. Labeled cells were detergent extracted and subjected to immunoprecipitation with FN-R antibodies (lanes *a* and *b*) or alphal monoclonal antibody (lanes *c* and *d*). Radioactive antigens were separated by 6% SDS-PAGE in nonreducing conditions and visualized by fluorography. Arrowhead indicates the position of the alphal subunit.

Retinoic acid was slightly less potent compared with TNFalpha and PMA. To identify a possible cooperation between the active mediators, combined treatments were tested. As shown in Fig. 7, when suboptimal doses were used, a synergistic induction of alphal subunit was observed in cells costimulated with PMA and TNFalpha or PMA and retinoic acid. The induced alphal subunit was exposed at the cell surface, as indicated by immunoprecipitation of surface iodinated cells (Fig. 8, lanes *b* and *d*).

Alphal Integrin Expression Increases Adhesiveness of Umbilical Vein EC

To test whether the described induction of alphal integrin on umbilical vein EC has any effect on the adhesive properties of these cells, adhesion experiments were performed. The adhesive response of umbilical vein EC treated for 48 h with retinoic acid were compared to that of untreated cells. Retinoic acid was chosen to induce alphal, because TNFalpha and PMA treatments induced modifications of expression of other integrin complexes, besides alphal, and this could complicate the interpretation of the results (Defilippi, P., and G. Tarone, unpublished data). As shown in Fig. 9, retinoic acid–treated EC showed increased adhesion to microtiter plates coated with collagen IV (*a*), and laminin (*b*), while they retained normal adhesion to fibronectin (*d*). When cells were plated on laminin fragments P1 and E8, treated cells adhered faster to P1 fragment compared to untreated cells, while they behaved similarly on E8 fragment (Fig. 9 *c*). These data are consistent with the ligand specificity of alphal/betal integrin (Ignatius and Reichardt, 1988; Kramer and Marks, 1989; Rossino et al., 1990; Hall et al., 1990).

Discussion

The data reported in this paper show that integrin alphal/betal is selectively expressed by EC from microvasculature but not from large blood vessels such as the aorta, femoral vein, and umbilical arteries and vein. Moreover, the expression of this integrin can be induced in large vessel endothelium by the inflammatory cytokine TNFalpha as well as by other selected agents.

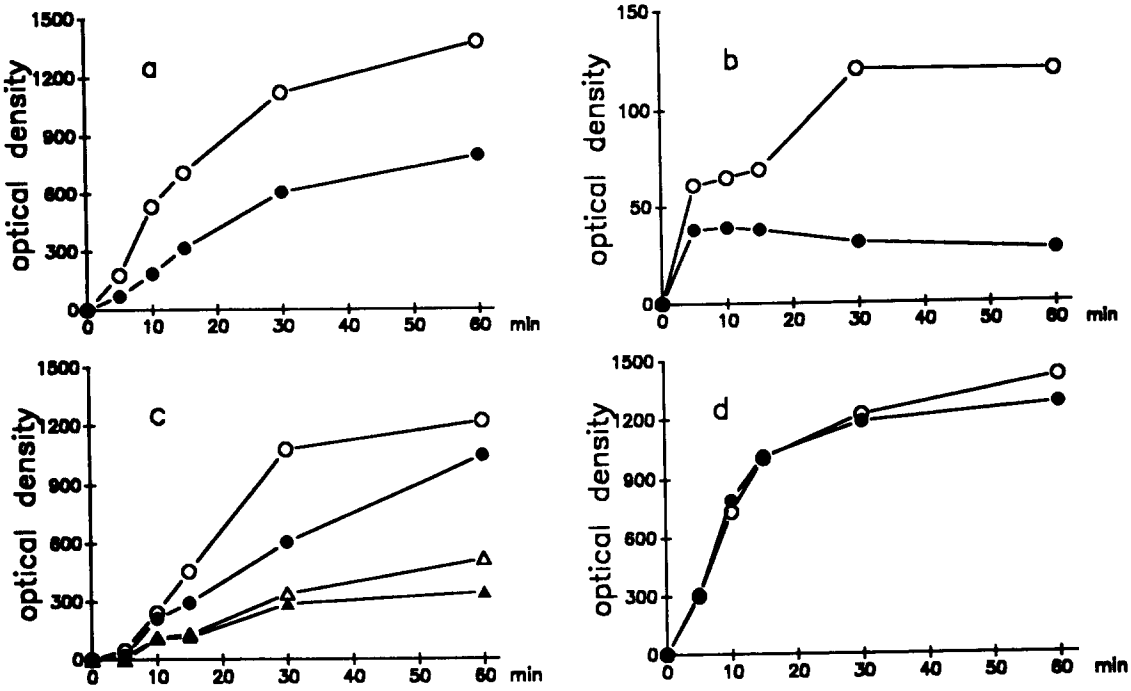


Figure 9. Kinetics of adhesion of untreated and retinoic acid treated umbilical vein EC to matrix proteins. Untreated umbilical vein EC (●,▲) and umbilical vein EC treated with 10^{-5} M retinoic acid for 72 h (○,△), were incubated in serum free medium at 37°C for different lengths of time on dishes coated with: (*a*) 0.5 μg/ml collagen IV; (*b*) 6 μg/ml laminin; (*c*) 2 μg/ml laminin fragment P1 (●,○), 5 μg/ml laminin fragment E8 (▲,△); (*d*) 3 μg/ml fibronectin. At the indicated times, adherent cells were washed two times with PBS, and the number of cells was determined by assaying the acid phosphatase activity (see Materials and Methods). Each point is a mean value of a triplicate.

Expression of alphal/betal seems to be a general property of microvascular endothelium in different tissues. We have detected positive capillary and microvascular endothelium in skeletal muscle, foreskin, dermis, brain, and kidney. Alphal positive capillary endothelium in dermis, brain, and kidney has also been reported by others (Konter et al., 1989; McGeer et al., 1990; Korhonen et al., 1990). The strong staining of microvascular endothelium in situ also excluded the possibility that synthesis and expression of alphal subunit in cultured microvascular EC was due to artifacts or contamination of cell culture. At the same time, lack of alphal integrin on EC from large vessels also seems to be a general property. Negative results were obtained on cultured cells from aorta, femoral vein and umbilical vein, and absence of alphal in both arteries and veins was confirmed by tissue staining of the blood vessels in the umbilical cord. Thus our results demonstrate that alphal/betal integrin clearly labels only EC in microvasculature.

The absence of alphal/betal complex in large vessel EC is not a constitutive property, since we show that different mediators can induce alphal integrin expression in umbilical vein EC. Among those tested, the inflammatory cytokine TNFalpha, the tumor-promoter PMA, and retinoic acid were found to be active. Retinoic acid is a naturally occurring morphogen involved in tissue differentiation and pattern formation during vertebrate development (Brockees, 1989). It is thus possible that retinoic acid affects endothelial cell function during development and its ability to induce alphal integrin in these cells should probably be considered in the context of its morphogenic activity. Retinoic acid was also found to induce increased expression of alphal integrin in neuroblastoma and teratocarcinoma cell lines (Rossino, P., and G. Tarone, unpublished results), thus indicating that this integrin alpha subunit is upregulated by retinoic acid in several cell types.

A second stimulus for alphal subunit induction is TNFalpha, a well characterized cytokine that causes several biochemical and morphological changes in EC in response to inflammatory stimuli (Pober, 1988). In particular TNFalpha increases expression of class I histocompatibility antigens (Collins et al., 1986; Lapierre et al., 1988) and of three adhesive proteins, ICAM-1 (Pober et al., 1986), ELAM-1 (Pober et al., 1986; Bevilacqua et al., 1987), and VCAM-1 (Osborn et al., 1989). The modulation of the latter proteins and of alphal integrin by TNFalpha differs by several criteria. Maximal levels of alphal integrin are reached after 48 h of TNFalpha treatment, while maximal expression of ICAM-1 and VCAM-1 occurs within 20 h (Pober et al., 1986; Osborn et al., 1989) and within 6 h for ELAM-1 (Pober et al., 1986; Bevilacqua et al., 1987). Furthermore, induction of ELAM-1 is transient as the levels of the molecule decline after 6–8 h of exposure to TNFalpha despite the continuous presence of the cytokine, while the alphal subunit remains constant over the time period measured (120 h). The kinetics of induction of alphal integrin is comparable to that observed for the induction of class I major histocompatibility antigens (Collins et al., 1986) and indicates that these two molecules have a role in later stages of the inflammatory response, as compared with ICAM-1, VCAM-1, and ELAM-1. The latter three molecules are located at the apical surface of endothelial cell, and mediate adhesion of leukocytes to the vessel wall at inflammatory sites. The alphal/betal integrin

is likely to be located on the basal surface of the cells to mediate interaction with basal lamina.

The pathway of intracellular signaling that leads to modulation of alphal integrin expression is likely to involve activation of protein kinase C (Castagna et al., 1982; Nishizuka, 1984). This is suggested by the fact that low doses of PMA (10 nM), known to activate protein kinase C (Nishizuka, 1984), induce the expression of alphal integrin. The activation of protein kinase C has been implicated in the signal transduction by TNFalpha in several cell types (Johnson and Baglioni, 1990; Schutze et al., 1990). In line with this we found that PMA potentiates the effect of TNFalpha on alphal induction, when the two agents are administered simultaneously. Furthermore, treatment of umbilical vein EC with doses of PMA above 500 nM, known to deplete cells of protein kinase C activity (Adams and Gullick, 1989), results in a decreased level of induction of alphal integrin.

We have previously shown that treatment of umbilical vein EC with a combination of both TNFalpha and IFNgamma alters the expression of the vitronectin receptor (integrin beta3) (Tarone et al., 1990; Defilippi et al., 1991). In this case the two cytokines caused a downregulation of this receptor by specifically affecting the synthesis of the beta3 subunit. A third integrin complex, alpha6/beta1, is downregulated by TNFalpha (Defilippi, P., and G. Tarone, unpublished results), thus showing that alteration of the endothelium–matrix interactions is likely to play an important role in the vascular response during inflammation.

Integrin alphal/beta1 is expressed on several cell types and binds both collagens and laminin (Ignatius and Reichardt, 1988; Kramer and Marks, 1989; Turner et al., 1989; Rossino et al., 1990; Hall et al., 1990). Recently Kramer et al. (1990) reported the expression of alphal/beta1 integrin in microvascular EC and showed that this receptor can bind laminin. Moreover, previous data indicate that the interaction of EC with laminin and collagens involves multiple receptors, including alpha2/beta1, alpha3/beta1, alpha6/beta1, and alphaV/beta3 (Languino et al., 1989; Kramer et al., 1990), and the precise role of each of them is not yet determined. An interesting functional property of the alphal/beta1 integrin is its ability to bind to a laminin region distinct from that recognized by other integrin complexes. Laminin structure has been extensively investigated using the protein produced by the mouse EHS tumor. This protein is a cross-shaped molecule of 900 kD, consisting of three polypeptide chains, A, B1, and B2 (Timpl, 1989), and can be split into two major fragments by proteolysis. These are the E8 fragment, obtained by elastase digestion and corresponding to the lower portion of the long arm of the cross (Paulsson et al., 1985), and the P1, or E1, fragment, obtained by either pepsin or elastase digestion, and corresponding to the central region of the molecule at the junction of the three short arms (Timpl, 1989). The three laminin receptors, alpha2/beta1, alpha3/beta1, and alpha6/beta1 all bind to the long arm region (Fragment E8) (Languino, L., and E. Ruoslahti, personal communication; Gehlsen et al., 1989; Sonnenberg et al., 1990; Hall et al., 1990), while the binding site of the recently identified laminin binding alphaV/beta3 complex (Kramer et al., 1990) has not yet been mapped. The alphal/beta1 receptor, on the other hand, binds to the central cross region of laminin (P1 fragment) (Hall et al., 1990; Rossino et al., 1990). In agreement with the binding specificity, we found that expression of alphal integrin on umbilical vein EC, potentiates the adhesion of EC to collagen, laminin, and laminin fragment P1, but not to laminin fragment E8 or to fibronectin. It is also possible that the alphal/beta1 receptor recognizes a specific laminin isoform different from the A, B1, B2 molecule used in our experiments. In fact, in addition to the mouse EHS laminin, different isoforms of the molecule have been described, with distinct subunit composition and tissue distribution (Edgar et al., 1988; Leivo and Engvall, 1988; Hunter et al., 1989). These isoforms, however, are not yet available in pure form for experimental test.

The adhesive behavior of alphal-expressing EC may be functionally important under specific circumstances, such as angiogenesis (new blood vessel formation). TNFalpha and PMA, that induce alphal expression, were also found to stimulate angiogenesis in an in vitro assay where endothelial cells are induced to penetrate a three-dimensional collagen gel (Leibovich et al., 1987; Montesano and Orci, 1987). Angiogenetic stimuli should induce several biochemical changes in endothelium, including proliferation, cytoskeletal assembly and protease secretion. During this process, EC should switch from a stationary to a migratory behavior and it is quite likely that such transition in adhesive properties requires changes of integrin expression such as those described here and in previous work (Defilippi et al., 1991).

We would like to thank Martin Hemler and Arnoud Sonnenberg for the generous gift of the monoclonal antibodies. We also thank Nino Ferraro for helping in the photography of histological slides and Angioletta Navini for helping in the experiments.

P. Defilippi was recipient of a postdoctoral fellowship from Fidia Pharmaceuticals Co. This work was supported by grants from the National Research Council "Progetto Finalizzato Biotecnologie." A. Bertolotto is partially supported by a grant from the Associazione Italiana Sclerosi Multipla.

Received for publication 9 November 1990 and in revised form 15 April 1991.

References

Adams, J. C., and W. J. Gullick. 1989. Differences in phorbol-ester-induced down-regulation of protein kinase C between cell lines. *Biochem. J.* 257: 905–911.

Albelda, S. M., M. Daise, E. M. Levine, and C. A. Buck. 1989. Identification and characterization of cell–substratum adhesion receptors on cultured human endothelial cells. *J. Clin. Invest.* 83:1992–2002.

Bevilacqua, M. P., J. S. Pober, D. L. Mendrick, R. S. Cotran, and M. A. Gimbrone, Jr. 1987. Identification of an inducible endothelial leukocyte adhesion molecule, ELAM-1. *Proc. Natl. Acad. Sci. USA.* 84:9238–9242.

Brockes, J. P. 1989. Retinoids, homeobox genes, and limb morphogenesis. *Neuron.* 2:1285–1294.

Castagna, M., Y. Takai, K. Kaibuchi, K. Sano, V. Kikkawa, and Y. Nishizuka. 1982. Direct activation of calcium-activated phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.* 257:7847–7851.

Chamberlain, J. P. 1979. Fluorographic detection of radioactivity in polyacrylamide gels with water-soluble fluor, sodium salicylate. *Anal. Biochem.* 98:132–135.

Charo, I. F., L. S. Bekeart, and D. R. Phillips. 1987. Platelet glyco-protein IIb/IIIa-like proteins mediate endothelial cell attachment to adhesive proteins and the extracellular matrix. *J. Biol. Chem.* 262:9935–9938.

Cheng, Y.-F., and R. H. Kramer. 1989. Human microvascular endothelial cells express integrin-related complexes that mediate adhesion to the extracellular matrix. *J. Cell. Physiol.* 139:275–286.

Cheresh, D. A. 1987. Human endothelial cells synthesize and express an Arg-Gly-Asp directed adhesion receptor involved in attachment to fibrinogen and Von Willebrand factor. *Proc. Natl. Acad. Sci. USA.* 84:6471–6475.

Collins, T., L. A. Lapierre, W. Fiers, J. L. Strominger, and J. S. Pober. 1986. Recombinant tumor necrosis factor increases mRNA levels and surface expression of HLA-A,B antigens in vascular endothelial cells and dermal fibroblasts in vitro. *Proc. Natl. Acad. Sci. USA.* 83:446–450.

Conforti, G., A. Zanetti, S. Colella, M. Abbadini, P. C. Marchisio, R. Pytela,

F. G. Giancotti, G. Tarone, L. R. Languino, and E. Dejana. 1989. Interaction of fibronectin with cultured human endothelial cells: characterization of the specific receptor. *Blood.* 73:1576–1585.

Connolly, D. T., M. B. Knight, N. K. Harakas, A. J. Wittwer, and J. Feder. 1986. Determination of the number of endothelial cells in culture using an acid phosphatase assay. *Anal. Biochem.* 152:136–140.

Davison, P. M., K. Bensch, and M. A. Karasek. 1980. Isolation and growth of endothelial cells from the microvessels of the newborn human foreskin in cell culture. *J. Invest. Dermatol.* 75:316–321.

Defilippi, P., G. Truffa, G. Stefanuto, F. Altruda, L. Silengo, and G. Tarone. 1991. Tumor necrosis factor alpha and interferon gamma modulate the expression of the vitronectin receptor (integrin beta3) in human endothelial cells. *J. Biol. Chem.* 266:7638–7645.

Dejana, E., L. R. Languino, S. Colella, G. C. Corbascio, E. Plow, M. Ginsberg, and P. C. Marchisio. 1988. The localization of a platelet GpIIb-IIIa-related protein in endothelial cell adhesion structures. *Blood.* 71:556–572.

Edgar, D., R. Timpl, and H. Thoenen. 1988. Structural requirements for the stimulation of neurite outgrowth by two variants of laminin and their inhibition by antibodies. *J. Cell Biol.* 106:1299–1306.

Elices, M. J., and M. E. Hemler. 1989. The human integrin VLA-2 is a collagen receptor on some cells and a collagen/laminin receptor on others. *Proc. Natl. Acad. Sci. USA.* 86:9906–9910.

Engvall, E., and E. Ruoslahti. 1977. Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. *Int. J. Cancer.* 20:1–5.

Gehlsen, K. R., F. G. Klier, K. Dickerson, W. S. Argraves, E. Engvall, and E. Ruoslahti. 1989. Localization of the binding site for a cell adhesion receptor in laminin. *J. Biol. Chem.* 264:19034–19038.

Gimbrone, M. A. 1976. Culture of vascular endothelium. *In* Progress in Hemostasis and Thrombosis. T. H. Spaet, editor. Vol. 3. Grune & Stratton, Inc., New York. 1–28.

Hall, D., L. F. Reichardt, E. Crowley, B. Holley, H. Moezzi, A. Sonnenberg, and C. H. Damsky. 1990. The alpha1/beta1 and alpha6/beta1 integrin heterodimers mediate cell attachment to distinct sites on laminin. *J. Cell Biol.* 110:2175–2184.

Heino, J., R. A. Ignotz, M. A. Hemler, C. Crouse, and J. Massagué. 1989. Regulation of cell adhesion receptors by transforming growth factor beta. *J. Biol. Chem.* 264:380–388.

Hemler, M. E. 1990. VLA proteins in the integrin family: structures, functions and their role on leukocytes. *Annu. Rev. Immunol.* 8:365–400.

Hunter, D. D., V. Shah, J. P. Merlie, and J. R. Sanes. 1989. A laminin-like adhesive protein concentrated in the synaptic cleft of the neuromuscular junction. *Nature (Lond.).* 338:229–234.

Hynes, R. O. 1987. Integrins: a family of cell surface receptors. *Cell.* 48: 549–554.

Konter, U., I. Kellner, E. Klein, R. Kaufmann, V. Mielke, and W. Sterry. 1989. Adhesion molecule mapping in normal human skin. *Arch. Dermatol. Res.* 281:454–462.

Korhonen, M., J. Ylanne, L. Laitinen, and I. Virtanen. 1990. The alpha1–alpha6 subunits of integrins are characteristically expressed in distinct segments of developing and adult human nephron. *J. Cell Biol.* 111: 1245–1254.

Kramer, R. H., and N. Marks. 1989. Identification of integrin collagen receptors on human melanoma cells. *J. Biol. Chem.* 264:4684–4688.

Kramer, R. H., Y. F. Cheng, and R. Clyman. 1990. Human microvascular endothelial cells use beta1 and beta3 integrin receptor complexes to attach to laminin. *J. Cell Biol.* 111:1233–1243.

Ignatius, M. J., and L. F. Reichardt. 1988. Identification of a neuronal laminin receptor: an Mr 200K/120K integrin heterodimer that binds laminin in a divalent cation-dependent manner. *Neuron.* 1:713–725.

Johnson, S. E., and C. Baglioni. 1990. Positive and negative regulation of a tumor necrosis factor response in melanoma cells. *J. Biol. Chem.* 265:6642–6649.

Laemmli, U. K. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Languino, L. R., K. R. Gehlsen, W. G. Carter, E. Engvall, and E. Ruoslahti. 1989. Endothelial cells use alpha2/beta1 integrins as laminin receptor. *J. Cell Biol.* 109:2455–2462.

Lapierre, L. A., W. Fiers, and J. S. Pober. 1988. Three distinct classes of regulatory cytokines control endothelial cell MHC antigen expression: interaction with immune (gamma) interferon differentiate the effects of tumor necrosis factor and lymphotoxin from those of leukocyte (alpha) and fibroblastic (beta) interferons. *J. Exp. Med.* 167:794–804.

Leibovich, S. J., P. J. Polverini, H. M. Shepard, D. M. Wiseman, V. Shively, and N. Nuseir. 1987. Macrophage-induced angiogenesis is mediated by tumor necrosis factor-alpha. *Nature (Lond.).* 329:630–632.

Leivo, I., and E. Engvall. 1988. Merosin, a protein specific for basement membranes of Schwann cells, striated muscle and trophoblast, is expressed late in nerve and muscle development. *Proc. Natl. Acad. Sci. USA.* 85:1544–1548.

McGeer, P. L., S. G. Zhu, and S. Dedhar. 1990. Immunostaining of human brain capillaries by antibodies to very late antigens. *J. Neuroimmunol.* 226:213–218.

Montesano, R., and L. Orci. 1987. Phorbol esters induce angiogenesis in vitro from large-vessel endothelial cells. *J. Cell. Physiol.* 130:284–291.

Nishizuka, Y. 1984. The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature (Lond.).* 308:693–696.

Osborn, L., C. Hession, R. Tizard, C. Vassallo, S. Luhowskyj, G. Chi-Rosso, and R. Lobb. 1989. Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. *Cell.* 59:1203–1211.

Pober, J. S. 1988. Cytokine-mediated activation of vascular endothelium. *Am. J. Pathol.* 133:426–433.

Pober, J. S., M. A. Gimbrone, Jr., L. A. Lapierre, D. L. Mendrick, W. Fiers, R. Rothlein, and T. A. Springer. 1986. Overlapping patterns of activation of human endothelial cells by interleukin 1, tumor necrosis factor and immune interferon. *J. Immunol.* 137:1893–1896.

Pytela, R., M. D. Pierschbacher, and E. Ruoslahti. 1985. Identification and isolation of a 140 kd cell surface glycoprotein with properties expected for a fibronectin receptor. *Cell.* 40:191–198.

Rossino, P., I. Gavazzi, R. Timpl, M. Aumailley, M. Abbadini, F. Giancotti, L. Silengo, P. C. Marchisio, and G. Tarone. 1990. Nerve growth factor induces increased expression of a laminin-binding integrin in rat pheochromocytoma PC12 cells. *Exp. Cell Res.* 189:100–108.

Ruoslahti, E., and M. D. Pierschbacher. 1987. New perspectives in cell adhesion: RGD and integrins. *Science (Wash. DC).* 238:491–497.

Santoro, S. A., S. M. Rajpara, W. D. Staatz, and V. L. Woods. 1988. Isolation and characterization of a platelet surface collagen binding complex related to VLA-2. *Biochem. Biophys. Res. Commun.* 153:217–223.

Schutze, S., S. Nottrott, K. Pfizenmaier, and M. Kronke. 1990. Tumor necrosis factor signal transduction. Cell-type-specific activation and translocation of protein kinase C. *J. Immunol.* 144:2604–2608.

Sonnenberg, A., P. W. Modderman, and F. Hogervorst. 1988. Laminin receptor on platelets is the integrin VLA-6. *Nature (Lond.).* 336:487–489.

Sonnenberg, A., C. J. T. Linders, P. W. Modderman, C. H. Damsky, M. Aumailley, and R. Timpl. 1990. Integrin recognition of different cell-binding fragments of laminin (P1, E3, E8) and evidence that alpha6 beta1 functions as a major receptor for fragment E8. *J. Cell Biol.* 110:2145–2155.

Staatz, W. D., S. M. Rajpara, E. A. Wayner, W. G. Carter, and S. A. Santoro. 1989. The membrane glycoprotein Ia-IIa (VLA-2) complex mediates the Mg^{2+}-dependent adhesion of platelets to collagen. *J. Cell Biol.* 108:1917–1924.

Sternberger, L. A., P. H. Hardy, J. J. Cuculis, and H. G. Meyer. 1970. The unlabeled antibody enzyme method for immunohistochemistry. Preparation and properties of soluble antigen-antibody complexes (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. *J. Histochem. Cytochem.* 18:315–333.

Tarone, G., G. Galetto, M. Prat, and P. M. Comoglio. 1982. Cell surface molecules and fibronectin-mediated cell adhesion: effect of proteolytic digestion of membrane proteins. *J. Cell Biol.* 94:179–186.

Tarone, G., G. Stefanuto, P. Mascarello, P. Defilippi, F. Altruda, and L. Silengo. 1990. Expression of receptors for extracellular matrix proteins in human endothelial cells. *J. Lipid Mediators.* 2:S45–S53.

Turner, D. C., L. A. Flier, and S. Carbonetto. 1989. Identification of a cell-surface protein involved in PC12 cell-substration adhesion and neurite outgrowth on laminin and collagen. *J. Neurosci.* 9:3287–3296.

van Hinsbergh, V. W. M., E. D. Sprengers, and T. Kooistra. 1987*a*. Effect of thrombin on the production of plasminogen activators and PA inhibitor-1 by human foreskin microvascular endothelial cells. *Thromb. Haemostasis.* 57:148–153.

van Hinsbergh, V. W. M., D. Binnema, M. A. Scheffer, E. D. Sprengers, T. Kooistra, and D. C. Rijken. 1987*b*. Production of plasminogen activators and inhibitor by serially propagated endothelial cells from adult human blood vessels. *Arteriosclerosis.* 7:389–400.

Voyta, J. C., D. P. Via, C. E. Butterfield, and B. R. Zetter. 1984. Identification and isolation of endothelial cells based on their increased uptake of acetylated-low density lipoprotein. *J. Cell Biol.* 99:2034–2040.