

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

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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, endow-

ing 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.

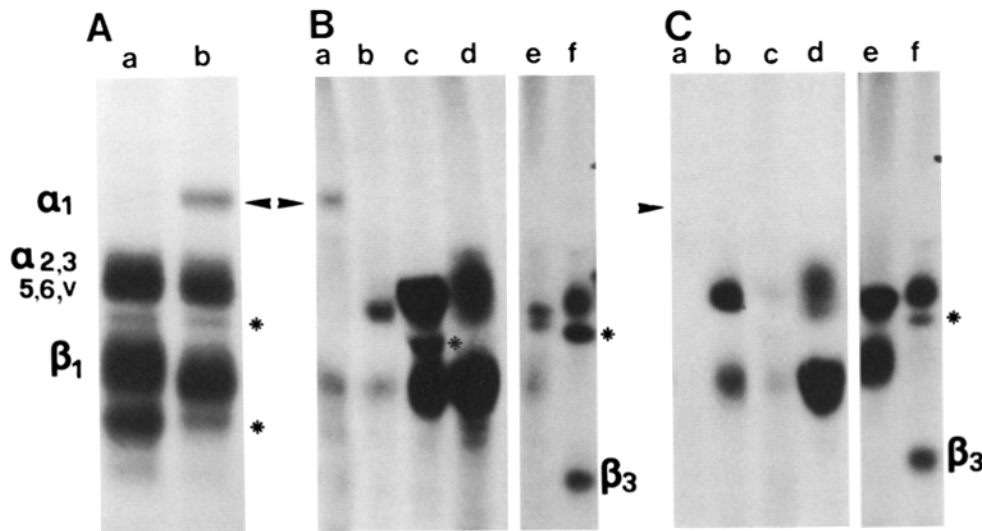


Figure 1. Immunoprecipitation of integrins from microvascular and umbilical EC. Confluent cultures of microvascular EC and umbilical vein EC were labeled with [³⁵S]methionine for 15 h. Cells were detergent extracted and subjected to immunoprecipitation with integrin antibodies. (A) Umbilical vein (lane 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, alpha1 monoclonal antibody TS2/7; lane b, alpha2 mono-

clonal 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 alpha subunit. Note the absence of alpha 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 Dil (3,3'-diiodoacetyl-indocarbocyanine)-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 Dil-acetylated LDL, as previously described (van Hinsberg et al., 1987a). 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., 1987b). 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) (10⁸ cytotoxic U/mg), was a kind gift from Dr. M. Brockhaus (Hoffmann La Roche, Basel, Switzerland). Human recombinant interferon gamma (IFNgamma) (10⁸ 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 × 10⁸ 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 × 10⁷ IU/mg) and granulocyte colony stimulating factor (G-CSF) (10⁷ 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 beta1 and alpha5 subunits (Conforti et al., 1989; Tarone et al., 1990). Its reactivity with beta1 allows the identification of all receptors of group 1 integrins. The polyclonal antisera to the beta1, 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: beta1, CTTVVNPKYEGK; alpha3, CRIQPSETERLTDY; 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 alpha (mAb TS2/7) and to integrin beta1 (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 [³⁵S]methionine or surface labeled with ¹²⁵I. [³⁵S]Methionine labeling was performed by overnight incubation in methionine-free medium (Flow Laboratories) with 5% serum and 40 µCi/ml of [³⁵S]methionine (800 Ci/mM; Amersham International) in the presence of the mediators. ¹²⁵I 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 CaCl₂ (1 mM) and MgCl₂ (1 mM) and labeled with 1 mCi of ¹²⁵I in the presence

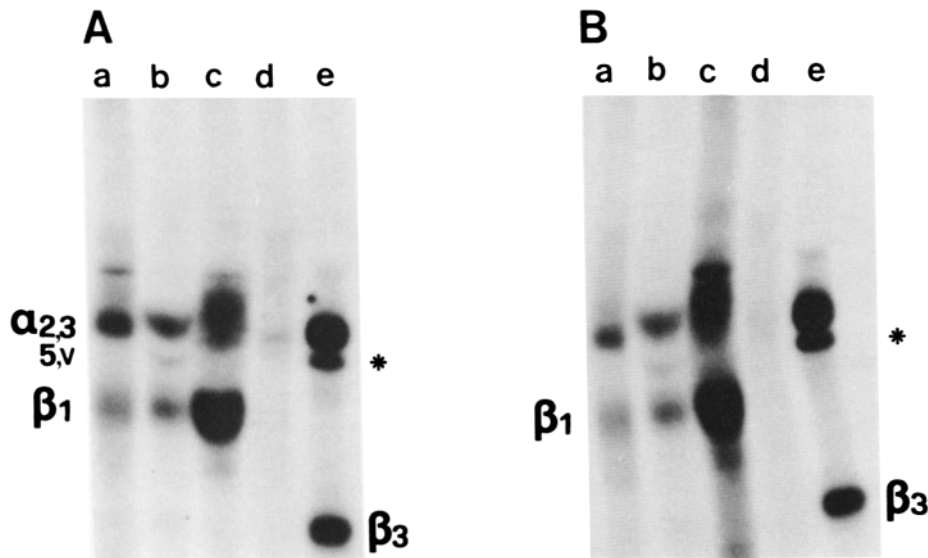


Figure 2. Immunoprecipitation of integrins from aorta (A) and femoral vein (B) EC. Cells were labeled with [³⁵S]methionine for 15 h, extracted with detergent, and immunoprecipitated with antibodies specific for the different alpha subunits. Lanes a, anti-alpha2; lanes b, anti-alpha3; lanes c, anti-alpha5; 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/betal 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 μ g/ml) and H₂O₂ (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 CaCl₂, 1 mM MgCl₂, 10 μ g/ml leupeptin, 4 μ g/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 μ m) were fixed with acetone for 5 min at -20°C, and treated with 3% H₂O₂ 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 μ g/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

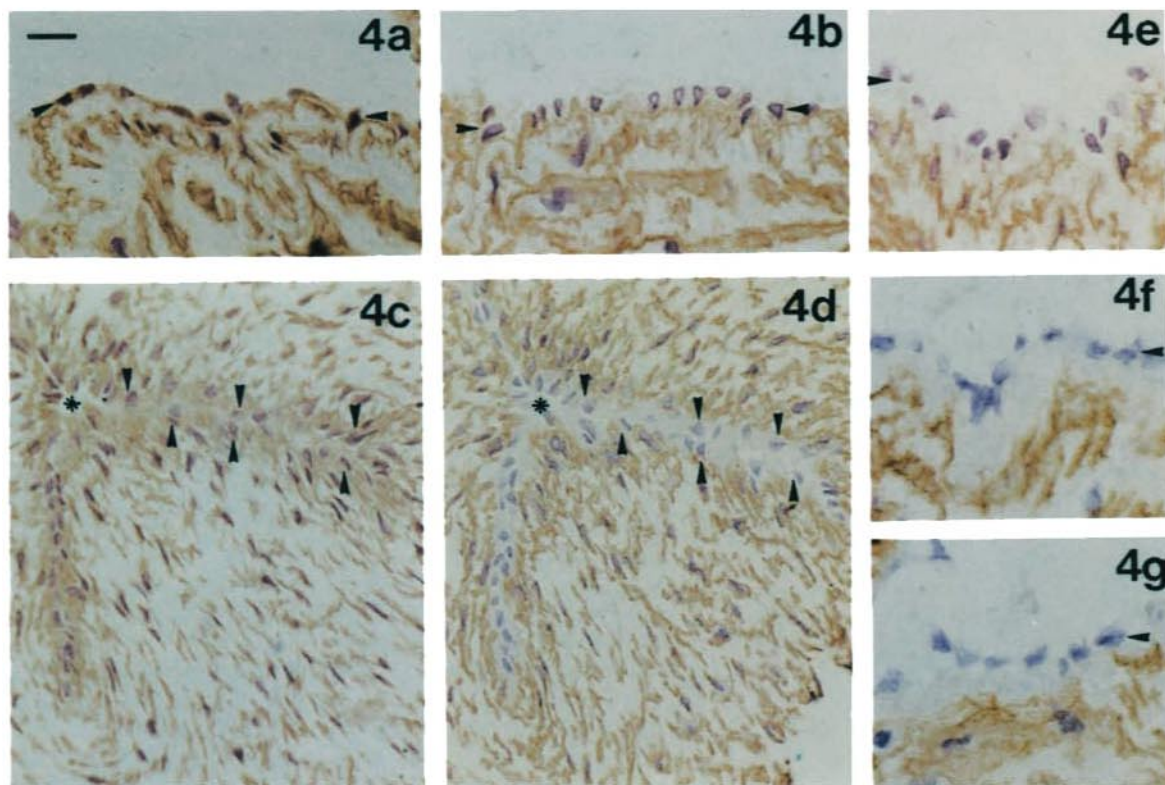
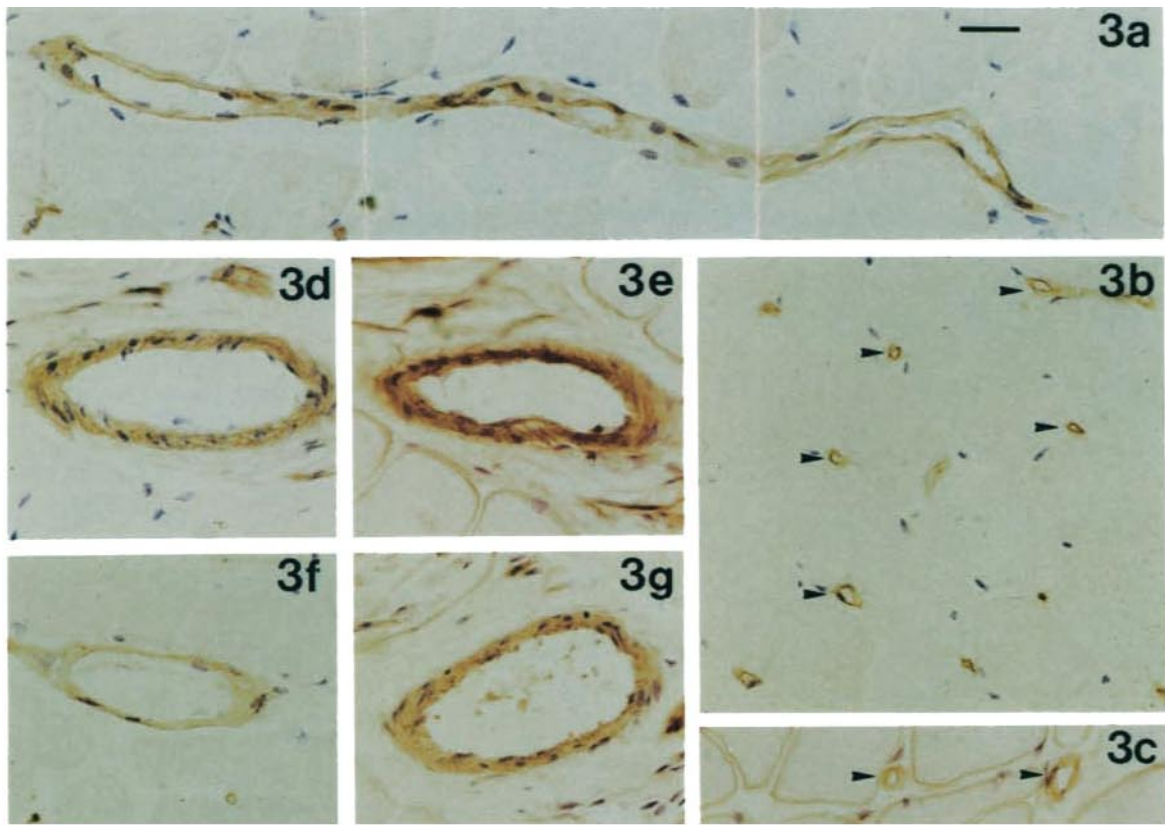
Fibronectin was purified from human plasma by affinity chromatography on gelatin-Sepharose as previously described (Engvall and Ruoslahti, 1977; Tarone et al., 1982). Laminin from EHS tumor and its cell binding fragments P1 and E8 were a kind gift of Dr. R. Timpl (Max Planck Institut für Biochemie, Martinsried, 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 μ M monensin (Sigma Chemical Co.) and 20 μ M 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 μ l 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 μ l 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

Alpha/Betal 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 betal 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 betal subunit as determined by pulse-chase experiments (Defilippi, P., and G. Tarone, unpublished data; Heino et al., 1989). The coprecipitation of alpha subunits with betal 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 alpha 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 alpha monoclonal antibody TS2/7 (*a*, *b*, *d*, and *f*), with beta 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 alpha-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.

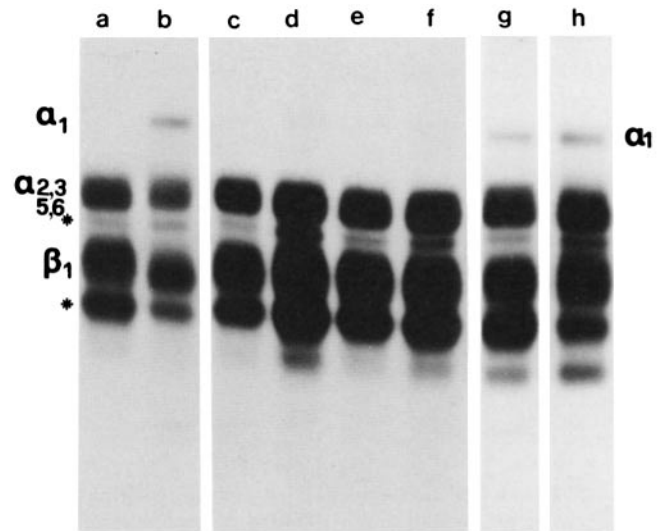


Figure 5. Induction of alpha1 subunit expression on umbilical vein EC. Confluent cultures of umbilical vein EC were treated for 72 h with different mediators, and labeled with [^{35}S]methionine in the last 15 h of treatment. Cells were detergent extracted and subjected to immunoprecipitation with FN-R 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 .

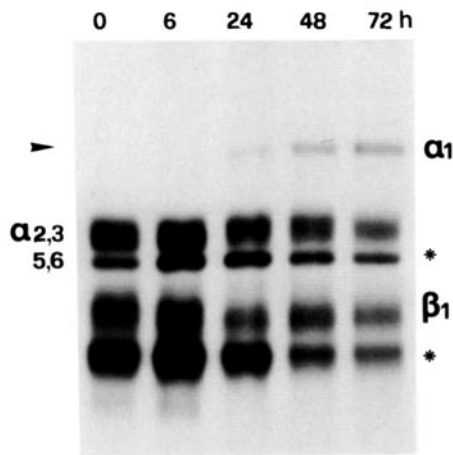


Figure 6. Kinetics of induction of alpha subunit by TNFalpha in umbilical vein EC. Umbilical vein EC were treated for the indicated times with 5 ng/ml TNFalpha, and labeled with [³⁵S]methionine in the last 6 h of treatment. Cells were detergent extracted and immunoprecipitated with FN-R antibodies. The radioactive antigens were separated by 6% SDS-PAGE in nonreducing conditions and visualized by fluorography. Arrowhead indicates the position of alpha 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 alpha 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 μ M, and the optimal dose was found to be 10 nM. When PMA was used at concentrations above 500 nM, a progressive decrease in alpha induction was observed, without any toxic effect on cell viability, as measured by methionine incorporation in TCA-precipitable materials. The level of induced alpha subunit in umbilical vein EC treated with optimal doses of TNFalpha or PMA, was comparable to that found in microvascular EC.

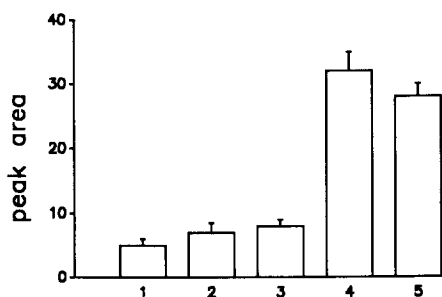


Figure 7. Synergistic induction of alpha subunit. Umbilical vein EC were treated for 48 h with 1 nM PMA (1), 0.5 ng/ml TNFalpha (2), 10^{-6} M retinoic acid (3), 1 nM PMA plus 0.5 ng/ml TNFalpha (4), and 1 nM PMA plus 10^{-6} M retinoic acid (5). The bars represent the absolute values of the peak area,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

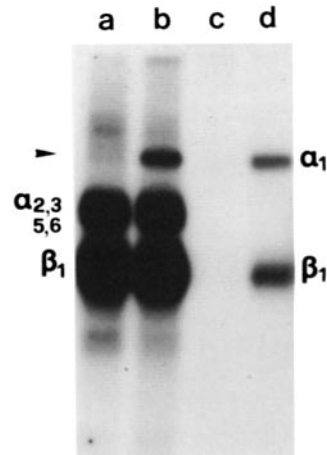


Figure 8. Expression of alpha subunit on the cell surface. Confluent cultures of untreated umbilical vein EC (lanes a and c), and of umbilical vein EC treated for 72 h with 5 ng/ml TNFalpha (lanes b and d), were harvested and labeled with ¹²⁵I, as indicated in Materials and Methods. Labeled cells were detergent extracted and subjected to immunoprecipitation with FN-R antibodies (lanes a and b) or alpha 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 alpha subunit.

ing conditions and visualized by fluorography. Arrowhead indicates the position of the alpha 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 alpha subunit was observed in cells costimulated with PMA and TNFalpha or PMA and retinoic acid. The induced alpha subunit was exposed at the cell surface, as indicated by immunoprecipitation of surface iodinated cells (Fig. 8, lanes b and d).

Alpha Integrin Expression Increases Adhesiveness of Umbilical Vein EC

To test whether the described induction of alpha 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 alpha, because TNFalpha and PMA treatments induced modifications of expression of other integrin complexes, besides alpha, 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 alpha/beta1 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 alpha/beta1 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.

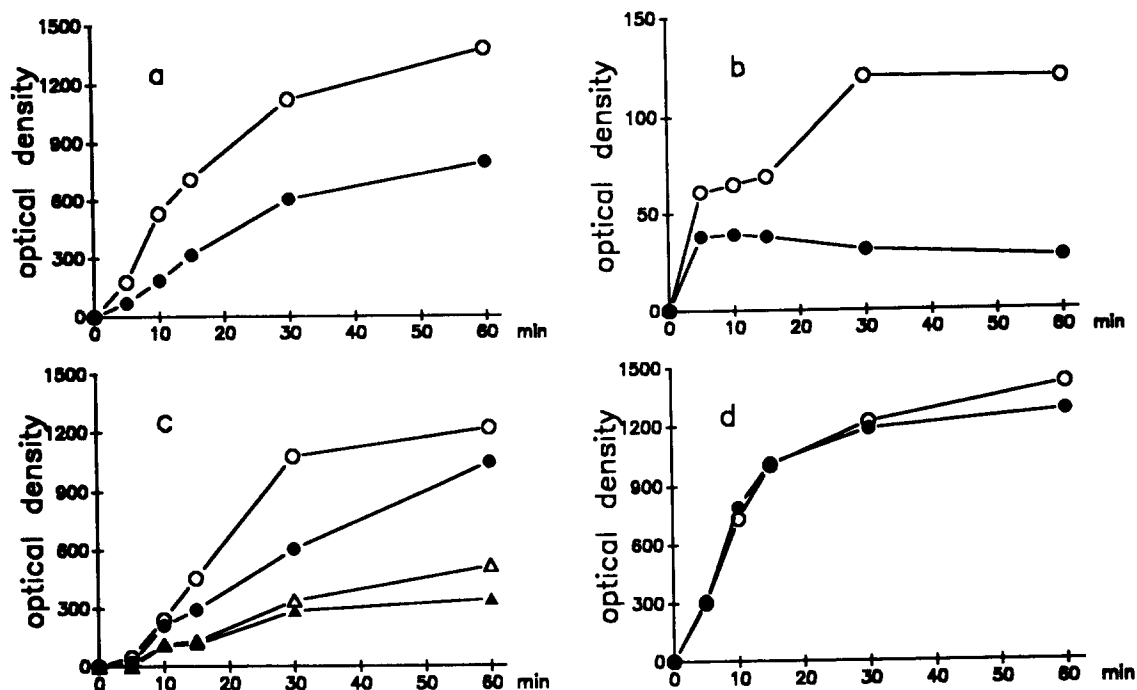


Figure 9. Kinetics of adhesion of untreated and retinoic acid treated umbilical vein EC to matrix proteins. Untreated umbilical vein EC (\bullet , \blacktriangle) and umbilical vein EC treated with 10^{-5} M retinoic acid for 72 h (\circ , \triangle), were incubated in serum free medium at 37°C for different lengths of time on dishes coated with: (a) $0.5 \mu\text{g/ml}$ collagen IV; (b) $6 \mu\text{g/ml}$ laminin; (c) $2 \mu\text{g/ml}$ laminin fragment P1 (\bullet , \circ), $5 \mu\text{g/ml}$ laminin fragment E8 (\blacktriangle , \triangle); (d) $3 \mu\text{g/ml}$ 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 α 5/ β 1 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. α 5 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 α 5 subunit in cultured microvascular EC was due to artifacts or contamination of cell culture. At the same time, lack of α 5 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 α 5 in both arteries and veins was confirmed by tissue staining of the blood vessels in the umbilical cord. Thus our results demonstrate that α 5/ β 1 integrin clearly labels only EC in microvasculature.

The absence of α 5/ β 1 complex in large vessel EC is not a constitutive property, since we show that different mediators can induce α 5 integrin expression in umbilical vein EC. Among those tested, the inflammatory cytokine TNF α , 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 (Brockeys, 1989). It is thus possible that retinoic acid affects endothelial cell function during development and its ability to induce α 5 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 α 5 integrin in

neuroblastoma and teratocarcinoma cell lines (Rossino, P., and G. Tarone, unpublished results), thus indicating that this integrin α subunit is upregulated by retinoic acid in several cell types.

A second stimulus for α 5 subunit induction is TNF α , a well characterized cytokine that causes several biochemical and morphological changes in EC in response to inflammatory stimuli (Pober, 1988). In particular TNF α increases expression of class I histocompatibility antigens (Collins et al., 1986; Lapiere 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 α 5 integrin by TNF α differs by several criteria. Maximal levels of α 5 integrin are reached after 48 h of TNF α 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 TNF α despite the continuous presence of the cytokine, while the α 5 subunit remains constant over the time period measured (120 h). The kinetics of induction of α 5 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 α 5/ β 1 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 alpha1 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 alpha1 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 alpha1 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 alpha1 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 alpha1/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 alpha1/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 alpha1/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 alpha1/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 alpha1 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 alpha1/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 alpha1-expressing EC may be functionally important under specific circumstances, such as angiogenesis (new blood vessel formation). TNFalpha and PMA, that induce alpha1 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). Angiogenic 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).

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