The EMBO Journal Vol.18 No.4 pp.882–892, 1999

Role of $\alpha_v\beta_3$ integrin in the activation of vascular endothelial growth factor receptor-2

Raffaella Soldi[1,2], Stefania Mitola[1,2], Marina Strasly[1,2], Paola Defilippi[2], Guido Tarone[2] and Federico Bussolino[1,2,3]

[1]Institute for Cancer Research and Treatment (IRCC) and [2]Department of Genetics, Biology and Biochemistry, School of Medicine, University of Torino, 10100 Torino, Italy

[3]Corresponding author
e-mail: Bussol@molinette.unito.it

R.Soldi and S.Mitola contributed equally to this work

Interaction between integrin $\alpha_v\beta_3$ and extracellular matrix is crucial for endothelial cells sprouting from capillaries and for angiogenesis. Furthermore, integrin-mediated outside-in signals co-operate with growth factor receptors to promote cell proliferation and motility. To determine a potential regulation of angiogenic inducer receptors by the integrin system, we investigated the interaction between $\alpha_v\beta_3$ integrin and tyrosine kinase vascular endothelial growth factor receptor-2 (VEGFR-2) in human endothelial cells. We report that tyrosine-phosphorylated VEGFR-2 co-immunoprecipitated with β3 integrin subunit, but not with β1 or β5, from cells stimulated with VEGF-A_{165}. VEGFR-2 phosphorylation and mitogenicity induced by VEGF-A_{165} were enhanced in cells plated on the $\alpha_v\beta_3$ ligand, vitronectin, compared with cells plated on the $\alpha_5\beta_1$ ligand, fibronectin or the $\alpha_2\beta_1$ ligand, collagen. BV4 anti-β3 integrin mAb, which does not interfere with endothelial cell adhesion to vitronectin, reduced (i) the tyrosine phosphorylation of VEGFR-2; (ii) the activation of downstream transductor phosphoinositide 3-OH kinase; and (iii) biological effects triggered by VEGF-A_{165}. These results indicate a new role for $\alpha_v\beta_3$ integrin in the activation of an *in vitro* angiogenic program in endothelial cells. Besides being the most important survival system for nascent vessels by regulating cell adhesion to matrix, $\alpha_v\beta_3$ integrin participates in the full activation of VEGFR-2 triggered by VEGF-A, which is an important angiogenic inducer in tumors, inflammation and tissue regeneration.

Keywords: angiogenesis/endothelial cells/integrin/tyrosine kinase receptor/vascular endothelial growth factor

Introduction

Integrin-mediated cell–matrix interactions regulate often divergent biological events including cell adhesion, migration, proliferation, differentiation and survival (Giancotti, 1997; Schwartz, 1997). Upon binding to matrix proteins, integrins transmit 'outside-in' signals to the cell, which trigger a large array of intracellular signaling events. These include (i) activation of kinases, such as focal adhesion kinase, $pp60^{src}$, mitogen-activated protein (MAP) kinase, Jun kinase, protein kinase C; (ii) changes in cytosolic ions, such as proton and calcium; and (iii) production of lipid mediators (Clark and Brugge, 1995; Schwartz *et al*., 1995; Yamada and Miyamoto, 1995; Parsons, 1996; Frisch and Ruoslahti, 1997). Many of the integrin-induced signaling pathways can also be activated by binding of soluble growth factors to their receptors, which suggests the existence of co-ordinate mechanisms between integrins and growth factors in the control of cellular functions. Cell adhesion has been shown to increase autophosphorylation of the epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors in response to their cognate ligands (Miyamoto *et al*., 1996). Ligation of specific integrins is followed by activation of the MAP kinase through the engagement of the adaptor molecule, Shc, and the Ras pathway (Wary *et al*., 1996), of the focal adhesion kinase (Schlaepfer *et al*., 1994), and engagement of phosphoinositide 3-OH kinase (PI 3-kinase) (King *et al*., 1997). Cell adhesion is also specifically required for the induction of cyclin D1 (Zhu *et al*., 1996). Within the integrins, $\alpha_v\beta_3$ engagement in cell–matrix interactions seems to be strictly involved in the co-ordinated activation of tyrosine kinase receptors. Therefore, $\alpha_v\beta_3$ associates with the tyrosine-phosphorylated PDGF and insulin receptor (Vuori and Ruoslahti, 1994; Rousseau *et al*., 1997) and its natural ligand vitronectin enhances the biological activity of PDGF-β (Schneller *et al*., 1997; Woodard *et al*., 1998). Alternatively, this integrin complex can interact with tenascin-C, thus favoring the clustering of EGF receptors and EGF-dependent growth (Jones *et al*., 1997).

Interaction between integrin $\alpha_v\beta_3$ and the extracellular matrix has been identified as the most important survival system for nascent vessels. Integrin $\alpha_v\beta_3$ is expressed in high quantities on angiogenic endothelial cells (Brooks *et al*., 1994a), where it suppresses the activity of p53 and the p53-inducible cell-cycle inhibitor $p21^{WAF1/CIP1}$ and increases the Bcl-2:Bax ratio, with a consequent anti-apoptotic effect (Stromblad *et al*., 1996). It is also involved in a late and sustained activation of mitogen-activated protein kinase in the chorionallantoic membrane stimulated by basic fibroblast growth factor (Eliceiri *et al*., 1998). Thus, both the cell cycle and apoptotic signaling cascades are balanced by this integrin complex. Furthermore, $\alpha_v\beta_3$ antagonists induce apoptosis of growing endothelial cells and reduce the invasive behavior of breast carcinoma in human skin by blocking tumor angiogenesis (Brooks *et al*., 1994b, 1995). During vasculogenesis, angioblasts and early endothelial cells secrete into extracellular matrix Del1, which is a recently discovered ligand for $\alpha_v\beta_3$ involved in vascular remodeling (Hidai *et al*., 1998). In addition, ligation of this integrin complex also promotes a calcium influx required for endothelial motility (Leavesley

et al., 1993). The expression of $\alpha_v\beta_3$ integrin is induced in microvascular endothelial cells by vascular endothelial growth factor-A (VEGF-A) (Senger *et al*., 1997), a highly specific activator of *in vitro* endothelial cell migration and proliferation and *in vivo* angiogenesis (Thomas, 1996; Ferrara and Davis-Smyth, 1997).

VEGF-A is a 40–45 kDa homodimer belonging to the cysteine knot family of growth factors. It exists as five different isoforms of 121, 145, 165, 189 and 206 amino acids (Thomas, 1996; Ferrara and Davis-Smyth, 1997). On adult endothelial cells it exhibits high-affinity binding sites corresponding to two distinct tyrosine kinase receptors, the VEGF receptor (VEGFR)-1 encoded by *Flt-1* (De Vries *et al*., 1992) and VEGFR-2 encoded by *KDR/Flk-1* (Terman *et al*., 1991; Millauer *et al*., 1993). The latter is also specifically activated by HIV-1-Tat protein (Albini *et al*., 1996b; Ganju *et al*., 1998), a viral molecule which contributes to angiogenesis associated with Kaposi's sarcoma (Ensoli *et al*., 1994). Although endothelial cells express both receptors, recent findings suggest that VEGFR-2, but not VEGFR-1, is able to mediate the mitogenic and motogenic effect of VEGF-A (Millauer *et al*., 1993; Waltenberger *et al*., 1994).

In this report we present evidence that β3 integrin subunit associates with stimulated-VEGFR-2 and a mAb directed to β3 molecule blocks signaling events elicited by VEGF-A critical to the induction of the angiogenic program in endothelial cells.

Results

Tyrosine phosphorylation of VEGFR-2 is increased in endothelial cells adherent on vitronectin

To examine the role of cell adhesion on VEGFR-2 activation, we evaluated the phosphorylation of the receptor in adherent or suspended endothelial cells treated with VEGF-A_{165}. Confluent and quiescent cells were detached from the plastic surface by calcium chelant or left adherent and challenged with VEGF-A_{165}. Proteins were immunoprecipitated with an anti-VEGFR-2 antibody and then subjected to Western blotting using an anti-phosphotyrosine mAb (Figure 1). The results show that the phosphorylation of the receptor was enhanced in adherent cells. In suspended cells, the effect of VEGF-A_{165} was markedly lower. The phosphorylation level of CD31, a cell–cell interaction protein which is phosphorylated on tyrosine residues (Sagawa *et al*., 1997), was not increased by VEGF-A_{165} in adherent or suspended cells (Figure 1). Subsequent experiments have been performed to test whether a specific matrix protein has a role in VEGFR-2 activation. Endothelial cells were plated on vitronectin, fibronectin or collagen I, which are the ligands for $\alpha_v\beta_3$, $\alpha_5\beta_1$and $\alpha_2\beta_1$, respectively, (Kuhn and Eble, 1994), or on the irrelevant substrate poly-L-lysine, and quiescent cells were stimulated with VEGF-A_{165}. Cell lysates were immunoprecipitated by an anti-VEGFR-2 antibody. Proteins were separated by SDS–PAGE and then blotted with anti-phosphotyrosine mAb. Figure 2 shows that VEGFR-2 is more phosphorylated in VEGF-A_{165}-stimulated endothelial cells plated on vitronectin, than in cells plated on fibronectin, collagen or poly-L-lysine, suggesting a specific involvement of $\alpha_v\beta_3$ integrin in the activation of VEGFR-2. The adhesion of endothelial cells to the different substrates was similar (not shown), thus excluding the possibility that the effect of vitronectin on VEGFR-2 phosphorylation was caused by differences in cell attachment.

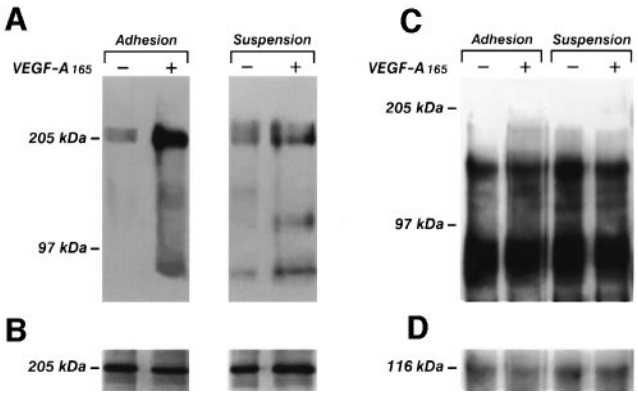

Fig. 1. VEGF-A_{165}-induced phosphorylation in adherent and suspended endothelial cells. Quiescent and confluent or suspended endothelial cells obtained as detailed in the Materials and methods were stimulated for 10 min with VEGF-A_{165} (10 ng/ml) or vehicle alone for 10 min at 37°C. Cells were lysed and immunoprecipitated with anti-VEGFR-2 Ab (**A** and **B**) or with BV10 anti-CD31 mAb. Immunoprecipitate was analyzed by SDS–PAGE followed by immunoblotting with anti-phosphotyrosine mAb (A and **C**). Subsequently, blots were re-probed with anti-VEGFR-2 Ab (B) or with BV10 mAb anti-CD31. (**D**) Immunoreactive bands were detected by ECL technique. The results are representative of three identical experiments.

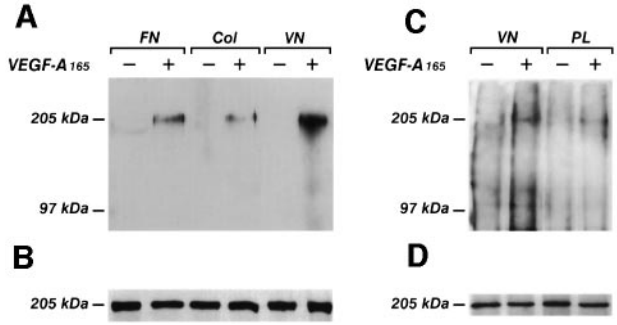

Fig. 2. Effect of different endothelial cell adhesion substrates on the phosphorylation of VEGFR-2. Endothelial cells plated at confluence on vitronectin, fibroncetin or collagen I (**A** and **B**) were made quiescent and then stimulated with VEGF-A_{165} as in Figure 1. For the experiments with poly-L-lysine, quiescent endothelial cells were incubated for 2 h with cycloheximide (1 μM) and then for 1 h with monensin (2 μM) to block the synthesis of extracellular matrix. Then they were detached in cold PBS containing 2 mM EGTA, washed twice in M199 containing 1% FCS and then plated for 1 h on poly-L-lysine (PL) (**C** and **D**). Cells were lysed and immunoprecipitated with anti-VEGFR-2 Ab. Immunoprecipitates were analyzed by SDS–PAGE followed by immunoblotting with anti-phosphotyrosine mAb (A and C). Subsequently, blots were re-probed with anti-VEGFR-2 Ab (B and D). Immunoreactive bands were detected by ECL technique. The results are representative of two identical experiments. The different degree of receptor phosphorylation of cells plated on vitronectin between (A) and (C) was caused by the treatment with cycloheximide and monensin.

The mitogenic activity of VEGF-A_{165} is enhanced by $\alpha_v\beta_3$ ligand substrate

The evidence that vitronectin facilitates the phosphorylation of VEGFR-2 prompted us to investigate whether the activation of endothelial cells by VEGF-A, as well as by HIV-1-Tat, another ligand of VEGFR-2 which stimulates migration and proliferation of endothelial and Kaposi's

Table I. Effect of extracellular matrix and BV4 mAb antiβ3 integrin on BrdU incorporation in endothelial cells stimulated by VEGF-A_{165} and HIV-1-Tat

Conditions	Unstimulated cells OD_{450}	VEGF-A_{165}-stimulated cells OD_{450}	HIV-1-Tat-stimulated cells OD_{450}
Cells plated on BSA	47 ± 4	62 ± 8[a,b]	60 ± 4[a,b]
Cells plated on fibronectin	107 ± 10	198 ± 8[c]	186 ± 3[c]
Cells plated on collagen	114 ± 5	227 ± 37[c]	180 ± 8[c]
Cells plated on fibrinogen	112 ± 9	288 ± 10[a,b,c]	258 ± 5[a,b,c]
Cells plated on vitronectin	119 ± 9	292 ± 21[a,b,c]	280 ± 11[a,b,c]
Cells plated on vitronectin + mAb BV4 anti-β3 integrin	115 ± 31	156 ± 18	145 ± 20
Cells plated on vitronectin + mAb anti-MHC class I antigen	110 ± 7	291 ± 16[a,b,c]	277 ± 9[a,b,c]
Cells plated on poly-L-lysine	85 ± 3	116 ± 10[a,b]	100 ± 5[a,b]

Endothelial cells (2.5×10^3) were plated in 96-well plates coated with different proteins and grown for 12 h in Medium 199 containing 5% FCS. Cells were then washed, starved for 12 h in Medium 199 containing 5% BSA, and then stimulated with VEGF-A_{165} or HIV-1-Tat (both used at 10 ng/ml) for 24 h in presence or absence of mAb (20 μg/ml), and during the last 3 h in the presence of BrdU. Mean ± SD of three experiments done in quadruplicate. Statistical analysis were performed by one-way analysis of variance ($F = 302.99$) and by Student–Newman-Keuls test.
[a] $p < 0.05$ versus stimulated cells plated on fibronectin.
[b] $p < 0.05$ versus stimulated cells plated on collagen I.
[c] $p < 0.05$ versus unstimulated cells.

sarcoma cells (Albini *et al*., 1996b; Ganju *et al*., 1998), was enhanced by specific substrates. After plating on several matrix proteins, quiescent sub-confluent cells were stimulated with either VEGF-A_{165} or HIV-1-Tat and the mitogenic activity was evaluated by 5-bromo-2′-deoxyuridine (BrdU) incorporation. Table I shows that the proliferation of endothelial cells after challenge with the two VEGFR-2 ligands was higher on vitronectin (145 and 135% stimulation with VEGF-A_{165} and HIV-1-Tat, respectively) and on fibrinogen, another specific ligand of $\alpha_v\beta_3$ (Kuhn and Eble, 1994) (168 and 141% stimulation with VEGF-A_{165} and HIV-1-Tat, respectively) than on collagen I (99 and 57% stimulation with VEGF-A_{165} and HIV-1-Tat, respectively), or on fibronectin (85 and 73% stimulation with VEGF-A_{165} and HIV-1-Tat, respectively) or poly-L-lysine (36 and 17% stimulation with VEGF-A_{165} and HIV-1-Tat, respectively).

VEGFR-2 phosphorylation is inhibited by anti-α_v and anti-β_3 antibodies

Next we evaluated whether mAbs raised against integrin subunits β_3 and α_v could interfere with the VEGFR-2 phosphorylation stimulated by VEGF-A_{165}. We used BV4, an anti-β_3 subunit mAb. [This antibody was developed in the laboratory of Dr E.Dejana (Mario Negri Insitute, Milano, Italy) by immunizing mice with human endothelial cells.] BV4 is able to immunoprecipitate the 93 kDa β3 integrin subunit in human endothelial cells (G.Tarone, unpublished results; see also Figure 11). The effect of BV4 mAb on endothelial cell adhesion to vitronectin was compared with the effects elicited by mAbs B212 (anti-β3; Thiagarajan *et al*., 1985), LM609 (anti-$\alpha_v\beta_3$ integrin complex; Brooks *et al*., 1994a) and 0.165 [anti-major histocompatibility complex (MHC) class I antigen]. Cells were incubated with increasing concentrations of mAbs for 20 min at 4°C and then were plated in the adhesion assay. Alternatively, confluent endothelial cells grown on vitronectin were incubated for 6 h with increasing concentrations of the mAbs. The results reported in Table II show that BV4 does not inhibit cell adhesion to substrate nor does it cause cell detachment, suggesting that it does not interfere in the mechanisms involved in endothelial cell adhesion to vitronectin.

Table II. Effect of anti-β3 integrin subunit mAbs on endothelial cell adhesion to and detachment from vitronectin

mAb	Cell adhesion OD_{540}	Cell detachment OD_{540}
BV4 1 μg/ml	338 ± 23	412 ± 25
BV4 10 μg/ml	344 ± 17	400 ± 29
BV4 20 μg/ml	355 ± 29	425 ± 45
B212 1 μg/ml	158 ± 78[a]	390 ± 23
B212 10 μg/ml	192 ± 12[a]	206 ± 34[a]
B212 20 μg/ml	155 ± 10[a]	190 ± 23[a]
LM609 1 μg/ml	195 ± 30[a]	345 ± 34
LM609 10 μg/ml	153 ± 12[a]	164 ± 25[a]
LM609 20 μg/ml	156 ± 17[a]	134 ± 43[a]
0.165 20 μg/ml	345 ± 35	420 ± 33
None	385 ± 15	453 ± 27

Endothelial cell adhesion to vitronectin was assayed as described previously (Defilippi *et al*., 1991a). Cells were detached in PBS containing 2 mM EGTA, washed twice in M199 containing 1% FCS and then incubated with increasing concentrations of mAbs for 20 min at 4°C. Cells (3×10^4) were plated in 96-well plates and after 1 h incubation at 37°C were fixed, stained by crystal violet and the absorbance was read at 540 nm in microtiter plate spectrophotometer (EL340, Bio-tek Instruments, Highland Park, VT). The adhesion of endothelial cells to BSA, used as negative control, was 23 ± 5. To study cell detachment, confluent endothelial cells grown on vitronectin in 96-well plates were incubated for 6 h in M199 medium containing 5% human serum albumin at 37°C with the mAbs. After two washes with pre-warmed M199 medium, cells were processed as described above. Mean ± SD of four samples in one typical experiment out of three performed with similar results.
[a] $p < 0.05$ versus untreated cells, evaluated by one-way analysis of variance ($F =$ in 201.12) and Student–Newman-Keuls test.

Confluent and quiescent endothelial cells were pre-incubated for 20 min at 4°C with mAbs against αv, β3 and β1 integrin subunits, washed and then stimulated with VEGF-A_{165} at 37°C. Cell lysates were immunoprecipitated by anti-VEGFR-2 Ab, and the proteins separated by SDS–PAGE were probed with anti-phosphotyrosine mAb. Anti-α_v and anti-β3 mAbs inhibited the VEGFR-2 phosphorylation, but BV7 (anti-β1 mAb) was ineffective (Figure 3).

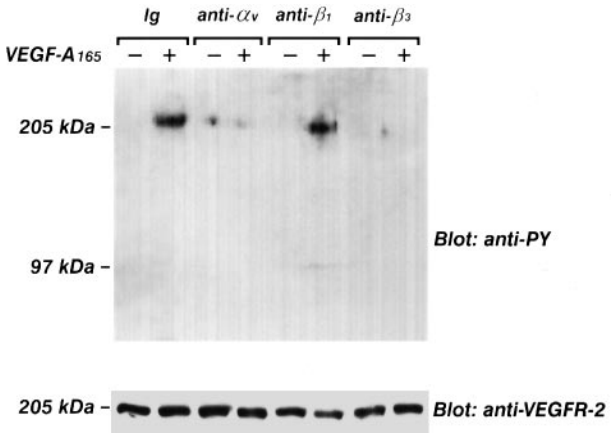

Fig. 3. Effect of BV4 mAb anti-β3, BV7 mAb anti-β1, and mAb anti-α_v on VEGF-A_{165}-induced phosphorylation of VEGFR-2. Quiescent, confluent endothelial cells were pre-incubated for 20 min at 4°C with specific or irrelevant Abs, washed and then stimulated with VEGF-A_{165} as in Figure 1. Cells were lysed and immunoprecipitated with anti-VEGFR-2 Ab. Immunoprecipitate was analyzed by SDS–PAGE followed by immunoblotting with anti-phosphotyrosine mAb. Subsequently, blots were re-probed with anti-VEGFR-2 Ab. Immunoreactive bands were detected by ECL technique. The results are representative of six similar experiments.

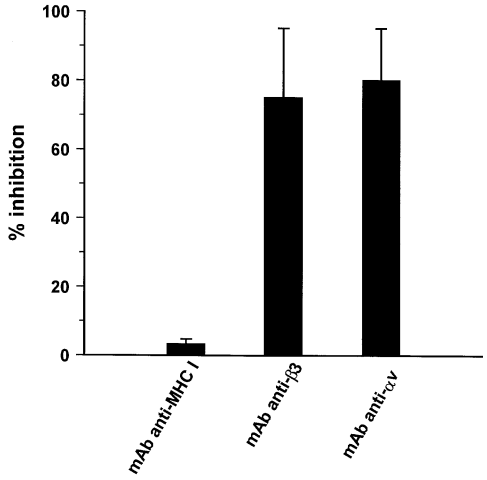

Fig. 4. Densitometric analysis of the inhibitory effect of BV4 mAb anti-β3 and mAb anti- α_v on VEGF-A_{165}-induced phosphorylation of VEGFR-2. The phosphorylated protein recognized by anti-phosphotyrosine mAb corresponding to VEGFR-2 in the experiments detailed in Figure 3 were analyzed by densitometric analysis performed with a GS250 Molecular Imager (Bio-Rad, Hercules, CA). Results (mean ± SD) indicate % inhibition induced by mAbs of the phosphorylation in tyrosine residues of VEGFR-2 in endothelial cells stimulated by VEGF-A_{165} in the presence of irrelevant Ig.

The inhibition of VEGFR-2 phosphorylation caused by anti-β3 and anti-α_v ranged from 100 to 50% (Figure 4). As a positive control, a neutralizing anti-VEGFR-2 antibody inhibited the receptor phosphorylation induced by VEGF-A_{165} (data not shown). Thus, these experiments indicate that $\alpha_v\beta_3$ integrin, but not β1, is crucial in mediating tyrosine phosphorylation of VEGFR-2.

The β3 integrin is necessary for the activation of PI 3-kinase in endothelial cells stimulated by VEGF-A_{165}

PI 3-kinase belongs to the intracellular signals triggered by VEGF-A in bovine endothelial cells, as demonstrated

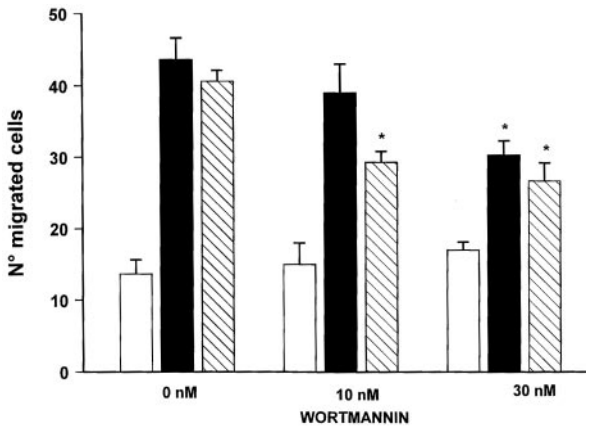

Fig. 5. Dose-dependent effect of wortmannin on VEGF-A_{165}-induced endothelial cell migration. Suspended endothelial cells were incubated for 15 min at 37°C with increasing concentrations of wortmannin and their ability to migrate across a 5 μm pore-size polycarbonate filter in response to VEGF-A_{165} (10 ng/ml) (black bar), HIV-1-Tat (hatched bar) or vehicle (open bar) was evaluated in a Boyden's chamber. At the end of the incubation (37°C, 4 h), filters were removed, stained with Diff-Quik (Baxter Spa, Rome, Italy) and five high power oil-immersion fields were counted. Results (mean ± SD) of one experiment (performed in triplicate) representative of at least three independent experiments are shown. *p <0.05 versus wortmannin-untreated cells.

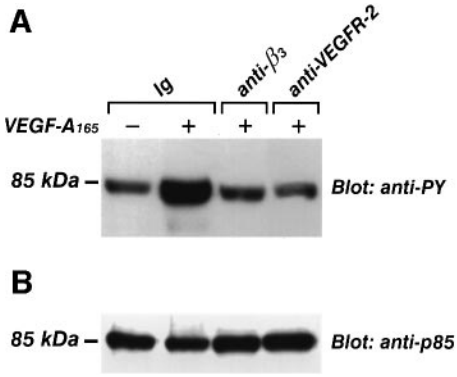

Fig. 6. Inhibitory effect of BV4 anti-β3 integrin subunit mAb and anti-VEGFR-2 Ab on VEGF-A_{165}-induced p85 phosphorylation. Quiescent, confluent endothelial cells were pre-incubated for 20 min at 4°C with specific or irrelevant Ig, washed and then stimulated with VEGF-A_{165} (10 ng/ml) for 15 min at 37°C. Cells were lysed and immunoprecipitated with anti-p85 mAb. Immunoprecipitate was analyzed by SDS–PAGE followed by immunoblotting with anti-phosphotyrosine mAb (**A**). Subsequently, blots were re-probed with anti-p85Ab (**B**). Immunoreactive bands were detected by ECL technique. The results are representative of two similar experiments.

by the phosphorylation of its regulatory protein p85 (Guo *et al.*, 1995). As shown in Figure 5, wortmannin used at nanomolar concentrations specific for PI 3-kinase inhibition (Arcaro and Wymann, 1993), reduced the human endothelial cells migration induced by VEGF-A_{165}. To determine whether β3 integrin interferes in PI 3-kinase activation occurring after VEGFR-2 stimulation, we evaluated the effect of BV4 (anti-β3 subunit mAb) on the phosphorylation in tyrosine residues of the p85 subunit and on the catalytic activity of the enzyme. After pre-incubation with BV4 or with anti-VEGFR-2 Ab for 20 min at 4°C, cells were stimulated with VEGF-A_{165}. Cell lysates were immunoprecipitated with anti-p85 mAb, and the separated proteins by SDS–PAGE were probed with

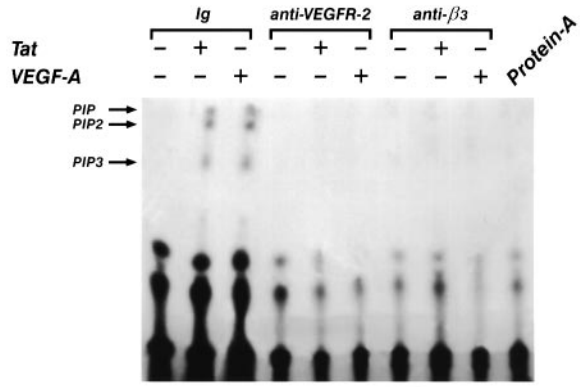

Fig. 7. Inhibitory effect of BV4 anti-β3 mAb and anti-VEGFR-2 Ab on VEGF-A_{165}-induced PI 3-kinase activation. Quiescent, confluent endothelial cells were pre-incubated for 20 min at 4°C with specific or irrelevant mAb, washed and then stimulated with VEGF-A_{165} (10 ng/ml) or with HIV-1-Tat (10 ng/ml) for 15 min at 37°C. PI 3-kinase assay was performed on immune complexes done with anti-phosphotyrosine mAb from lysates of endothelial cells. PIP, phosphatidylinositol; PIP2, phosphatidylinositol (4)P; PIP3, phosphatidylinositol (3,4)P2. Protein A indicates a PI 3-kinase assay done on protein A alone.

anti-phosphotyrosine mAb. VEGF-A_{165} increased the tyrosine phosphorylation of p85, but it failed to phosphorylate the PI 3-kinase subunit when endothelial cells were pre-incubated with anti-β3 or anti-VEGFR-2 antibodies (Figure 6). Similarly, BV4 and anti-VEGFR-2 inhibited the catalytic activity of PI-3 kinase induced by VEGF-A_{165} and by HIV-1-Tat (Figure 7). BV7, anti-β1 mAb (not shown) and irrelevant Ig did not affect p85 phosphorylation and the increase of PI 3-kinase activity triggered by both ligands (Figures 6 and 7).

The β3 integrin is required for the activation of the angiogenic program in endothelial cells

Through activation of VEGFR-2, endothelial cells change their phenotype and start to proliferate and migrate (Thomas, 1996). To verify the biological relevance of the β3 integrin subunit on VEGF-A_{165}-mediated endothelial cell activation, we have studied the effect of the anti-β3 subunit mAb, BV4, on polarization, migration and proliferation of endothelial cells challenged with either VEGF-A_{165} or HIV-1-Tat. BV4 inhibited by 40, 32 and 47%, respectively, the migration, polarization (Figure 8) and proliferation (Table I) induced by VEGF-A_{165}, and to a similar extent the activities triggered by HIV-1-Tat (Figure 8; Table I). BV7 (anti-β1 integrin mAb) did not inhibit migration and polarization induced by both ligands (Figure 8). An anti-MHC class I mAb was ineffective in all experimental conditions tested (Figure 8; Table I), whereas a neutralizing anti-VEGFR-2 antibody inhibited migration, polarization and proliferation (data not shown).

Effects of BV4 on binding displacement of [I^{125}]VEGF-A_{165} on endothelial cells

In order to assess the mechanisms leading to the inhibitory effect of BV4, we have evaluated the effect of this mAb on the binding of [I^{125}]VEGF-A_{165} on endothelial cells. Figure 9 shows that the pre-incubation of endothelial cells with increasing concentrations of BV4 mAb or anti-MHC class I mAb did not inhibit the binding of labeled VEGF-A_{165} to the surface of endothelial cells, indicating that β3 integrin is not involved directly in the binding of VEGF-A_{165} to its specific binding sites, nor did it cause a steric hindrance. In contrast, cold VEGF-A_{165} inhibited the binding to endothelial cells with an apparent IC_{50} of 60 pM.

Integrin β3 becomes associated with VEGFR-2 upon VEGF-A_{165} stimulation

We next tested the possibility that VEGFR-2 stimulation by its ligand induces the formation of a complex with β3 integrin. The membrane surface of endothelial cells was biotinylated and then cells were challenged with VEGF-A_{165}. Only in the β3 immune complexes from VEGF-A_{165}-stimulated cells was a 205 kDa band detected (Figure 10), this being the molecular weight of VEGFR-2 (Thomas, 1996). To identify this protein, cell lysates were immunoprecipitated with the BV4 mAb. The presence of VEGFR-2 in the immune complexes was investigated by performing a second immunoprecipitation on solubilized proteins with an anti-VEGFR-2 antibody and immunoblotting with anti-VEGFR-2 or with anti-phosphotyrosine Abs. As shown in Figure 11, in absence of the ligand, VEGFR-2 was not present in the anti-β3 immunoprecipitate. However, the receptor was associated in phosphorylated form with β3 integrin in the presence of VEGF-A_{165}. The β1 or β5 immunoprecipitates from stimulated and unstimulated endothelial cells, did not contain VEGFR-2 (Figure 11). The amount of tyrosine-phosphorylated receptor associated with β3 integrin visualized in double-immunoprecipitation experiments seemed to be lower than the overall amount of phosphorylated receptor (i.e. Figure 1). A quantitative investigation of the amount of VEGFR-2 associated with β3 integrin has been done on biotinylated endothelial cells. Figure 12 shows that the amount of a 205 kDa protein immunoprecipitated by anti-VEGFR-2 or by BV4 antibodies in VEGF-A_{165}-stimulated cells was similar to that associated with β3 integrin. This experiment suggests that themajor fraction of VEGFR-2 is associated with β3 integrin after ligand challenge. The discrepancy observed in the level of tyrosine phosphorylation studied by immunoprecipitating VEGFR-2 from cell lysate or from β3 integrin, in double-immunoprecipitation experiments, could be caused by a poor recovery of the receptor during the second step of immunoprecipitation.

Discussion

VEGFR-2 is a tyrosine kinase receptor which is responsible for the angiogenic activity of VEGF-A (Millauer *et al*., 1993; Waltenberger *et al*., 1994). The tyrosine kinase activity of the receptor and its autophosphorylation begin when two molecules of VEGFR-2 bind the N-terminal 110 amino acids of two dimerized monomers of VEGF-A (Keyt *et al*., 1996; Muller *et al*., 1997). At least for the interaction between VEGF-A_{165} and VEGFR-2, it has been demonstrated that neuropilin-1 acts as a co-receptor which enhances the affinity of the receptor for the ligand (Soker *et al*., 1998). Through its phosphorylated tyrosine residues, the activated VEGFR-2 associates with the adapter molecules Shc, Grb2 and Nck, to Ras GTPase activating protein, $p59^{fyn}$, $pp62^{yes}$ and phospholipase Cγ, and to the tyrosine phosphatases SHP-1 and SHP-2

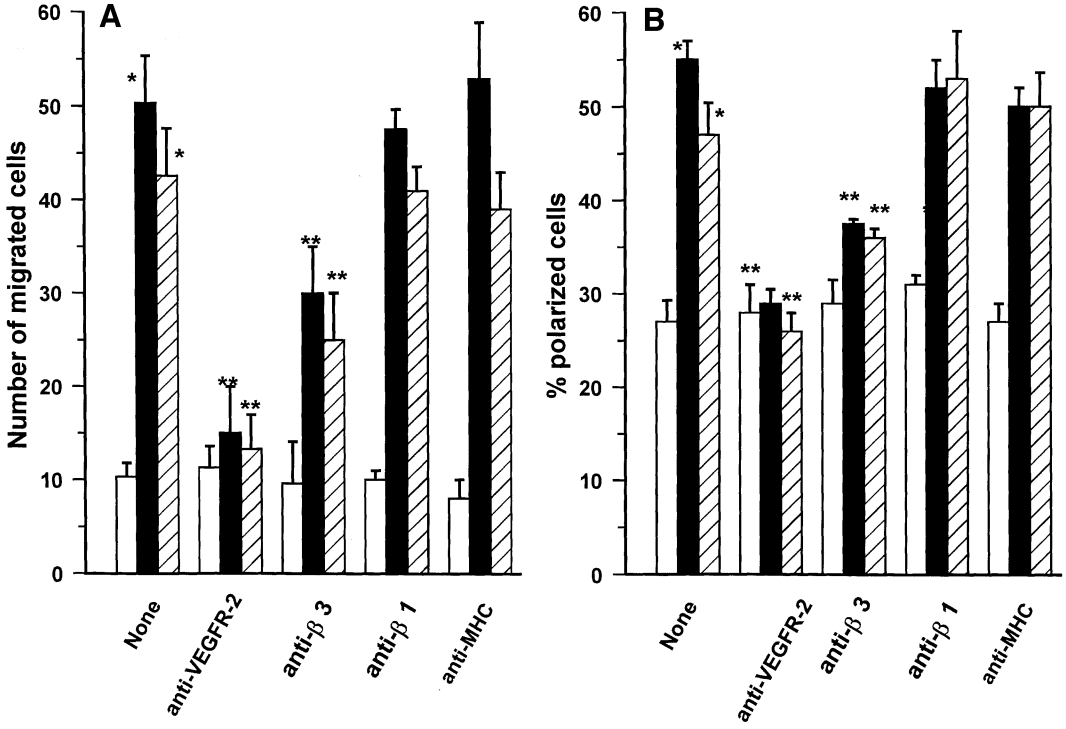

Fig. 8. Effect antibodies specific for integrin subunits or VEGFR-2 on migration (**A**) and polarization (**B**) of endothelial cells induced by VEGF-A_{165} or HIV-1-Tat. Suspended endothelial cells were incubated for 20 min at 4°C with BV4 (anti-β3 integrin mAb), BV7 (anti-β1 integrin mAb), 0.165 (anti-MHC class I mAb) and anti-VEGFR-2 antibody. Cells were washed and then stimulated with VEGF-A_{165} (10 ng/ml) (black bar), HIV-1-Tat (10 ng/ml) (hatched bar) or vehicle alone (open bar). Migration was evaluated by Boyden's chamber technique as shown in detail in Figure 5. The percentage of polarized cells was obtained by counting bipolar cells in suspension by phase-contrast microscopy at 400× magnification. Mean ± SD of four experiments performed in triplicate. Data were analyzed by one-way analysis of variance [experiments shown in (A): $F = 63.35$; experiments shown in (B): $F = 50.01$) and Student–Newman-Keuls test. *$p < 0.05$ between unstimulated and stimulated cells; **$p < 0.05$ between stimulated cells and cells stimulated after mAb-pretreatment.

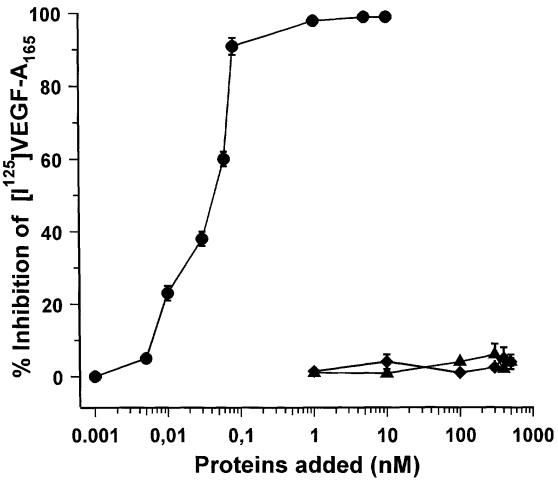

Fig. 9. Effect of BV4 mAb anti-β3 integrin subunit on the binding at equilibrium of [^{125}I]VEGF-A_{165} to endothelial cells. Cell monolayers were incubated for 90 min at room temperature in 0.2 ml of binding buffer with 0.05 nM [^{125}I]VEGF, with or without the indicated concentrations of unlabeled VEGF-A_{165} (●), BV4 mAb (◆) and anti-MHC class I antigen (▲). At the end of incubation, the cells were washed, solubilized with 2% SDS and the radioactivity was counted. Data are the mean ± SD of three determinations in one experiment out of two with similar results.

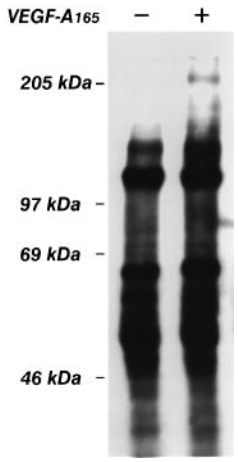

Fig. 10. Effect of VEGF-A_{165} on association of biotinylated membrane proteins to β3 integrin subunit. Surface membrane proteins of confluent and quiescent endothelial cells were labelled with the membrane-impermeable NHS.biotin and then incubated for 10 min at 37°C with VEGF-A_{165} (10 ng/ml). Cells were lysed and immunoprecipitated with BV4 mAb anti-β3 integrin. The biotinylated proteins were analyzed by SDS–PAGE followed by blotting stained with avidin–peroxidase and enhanced chemiluminescence technique. The figure is representative of two experiments which gave similar results.

(Waltenberger *et al*., 1994; Kroll and Waltenberger, 1997; Takahashi and Shibuya, 1997). The formed transductosome mediates the activation of MAP kinase, PI 3-kinase, Jun kinase, cGMP-dependent kinase and focal adhesion kinase (Guo *et al*., 1995; Abedi and Zachary, 1997; Kroll and Waltenberger, 1997; Rousseau *et al*., 1997; Hood and Granger, 1998; Pedran *et al*., 1998), which are putative mediators of chemotaxis, mitogenicity, actin reorganization and gross morphology changes in endothelial cells.

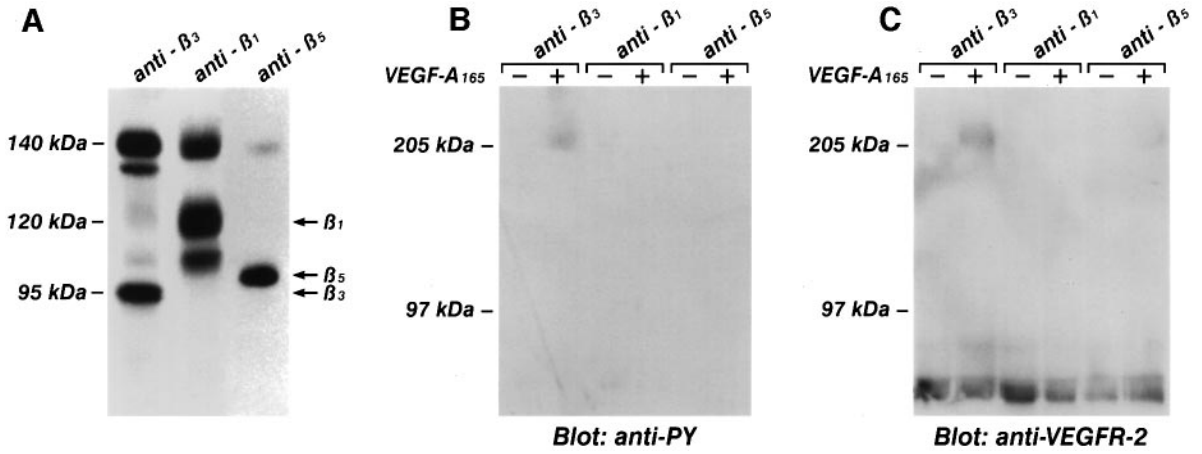

Fig. 11. Phosphorylated VEGFR-2 becomes associated with β3, but not with β1 and β5 integrin subunits. (**A**) Cell lysate from quiescent and confluent endothelial cells labeled with [^{35}S]methionine were immunoprecipitated with mAbs BV4 anti-β3, BV7 anti-β1 or with Ab anti-β5 integrins. Soluble immune complexes were eluted by boiling beads in 1% SDS and analyzed by 6% SDS–PAGE in non-reducing conditions. The pattern shown by this picture is not modified by cell treatment with VEGF-A_{165} (not shown). (B and C) Quiescent and confluent endothelial cells were incubated for 10 min at 37°C with VEGF-A_{165} (10 ng/ml) and cell lysate were immunoprecipitated with mAb BV4 anti-β3, BV7 anti-β1 or Ab anti-β5. After protein solubilization from protein A–Sepharose, samples were subjected to a second immunoprecipitation with anti-VEGFR-2 antibody. Samples were analyzed by SDS–PAGE followed by immunoblotting with anti-phosphotyrosine antibody (**B**). Subsequently, the blot was reprobed with anti-VEGFR-2 (**C**). The figure is representative of three similar experiments. For each experiment, the same batch of endothelial cells was used.

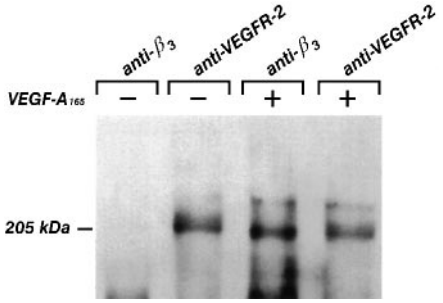

Fig. 12. Recovery of VEGFR-2 expressed on endothelial cell surface in immunocomplexes anti-β3 and anti-VEGFR-2 antibodies. Surface membrane proteins of confluent and quiescent endothelial cells were labeled with the membrane-impermeable NHS.biotin and then incubated for 10 min at 37°C with VEGF-A_{165} (10 ng/ml). Cells were lysed and immunoprecipitated with BV4 mAb anti-β3 integrin or with anti-VEGFR-2 Ab. The biotinylated proteins were analyzed by SDS–PAGE followed by blotting stained with avidin–peroxidase and enhanced chemiluminescence technique. The picture is representative of two experiments performed with similar results.

The findings in this work add new insights to the mechanism of activation of an angiogenic program in vascular endothelial cells stimulated by VEGF-A. Cell adhesion is critical for receptor activation based on two experimental observations: (i) VEGF-A_{165}-dependent VEGFR-2 phosphorylation was strongly activated in adherent cell, but showed a substantially reduced response in suspended cells; and (ii) the ligand-induced phosphorylation of VEGFR-2, migration and proliferation of endothelial cells were enhanced when the cells were plated on vitronectin or fibrinogen, which bind to $\alpha_v\beta_3$. Furthermore, β3 integrin is required for the full activation of VEGFR-2, by a mechanism possibly independent of its role in cell adhesion. The BV4 anti-β3 integrin mAb, which does not inhibit cell adhesion (see Table II and Materials and methods), markedly reduced the VEGFR-2 phosphorylation triggered by VEGF-A_{165}, the activation of PI 3-kinase, which is a downstream event to VEGFR-2 dimerization (Guo *et al*., 1995). Furthermore, this mAb reduced the mitogenic and the motogenic effects of two ligands of VEGFR-2: VEGF-A_{165} and HIV-1-Tat. The lack of effect of the BV4 mAb on endothelial cell adhesion to vitronectin (Table II), and the observed inhibitory effects on VEGFR-2, suggest that the β3 integrin subunit is involved directly in VEGFR-2 activation. This hypothesis is strongly supported by the result that VEGF-A_{165}-stimulated, tyrosine phosphorylated VEGFR-2 was associated with β3 integrin. The specificity of the model was restricted to $\alpha_v\beta_3$ integrin. In fact, we did not observe anti-β1 mAb inhibition of endothelial cell migration and polarization induced by VEGF-A_{165} or HIV-1-Tat. Fibronectin, the ligand of $\alpha_5\beta_1$, and collagen I, the ligand of $\alpha_2\beta_1$, were also unfavorable substrates for the ligand-dependent phosphorylation of VEGFR-2. Furthermore, the observed selectivity of VEGFR-2 for β3 integrin is not simply due to its high level of expression in endothelial cells. In our experimental conditions, the levels of β3 and β5 were similar and lower than that of β1 (Figure 11). Furthermore, it has been reported previously that the amount of $\alpha_v\beta_3$ in cultured endothelial cells is lower than that of $\alpha_2\beta_1$ and $\alpha_5\beta_1$(Defilippi *et al*., 1991b) and similar to that of $\alpha_v\beta_5$ (Bhattacharya *et al*., 1995).

The relevance of $\alpha_v\beta_3$ integrin in the activation of tyrosine kinase receptors has been achieved in three different models. In rat vascular smooth muscle cells, the clustering of β3 integrin favor the EGF-dependent tyrosine phosphorylation of EGF receptor and cell growth (Jones *et al*., 1997). Furthermore, a neutralizing anti-$\alpha_v\beta_3$ mAb inhibits the effect of EGF in terms of receptor phosphorylation and proliferation induction (Jones *et al*., 1997). In human foreskin fibroblasts, $\alpha_v\beta_3$ is associated with activated insulin and platelet-derived growth factor (PDGF)-β receptors and potentiates the growth and the motility response to PDGF-BB (Schneller *et al*., 1997). Similar results have been reported by Woodard and colleagues on rat endothelial cells (Woodard *et al*., 1998).

The nature of the connection between $\alpha_v\beta_3$ and VEGFR-2 remains to be elucidated. The β3 integrin subunit is associated with the phosphorylated form of VEGFR-2. This effect is specific, because β5, which has been demonstrated to be involved in angiogenic response to VEGF-A (Friedlander *et al*., 1995), and β1 integrins did not associate to VEGFR-2. Furthermore, the pre-treatment of quiescent endothelial cells with BV4 anti-β3 mAb, but not with BV7 anti-β1 mAb, reduces the receptor activation as well as the activation of downstream signals (i.e. activation of PI 3-kinase). These data suggest that $\alpha_v\beta_3$ is a molecular component specifically required for a correct function of the receptor. The lack of inhibition exerted by BV4 on the binding of VEGF-A_{165} to endothelial cells and on cell adhesion excludes the possibility that the inhibitory effect of the BV4 mAb was due to interference in ligand-receptor interaction or to a block of adhesion machinery. The chemical basis of the association between β3 integrin subunit and VEGFR-2 is unknown. We can not exclude the possibility that molecules associated with VEGFR-2, such as a insulin receptor substrate-1-like molecule that has been demonstrated to bind $\alpha_v\beta_3$ (Vuori and Ruoslahti, 1994), or that neuropilin-1, the co-receptor of VEGFR-2 (Soker *et al*., 1998), have docking sites for β3 integrin. It has been reported that β3 integrin can be phosphorylated on tyrosine residues (Blystone *et al*., 1996; Law *et al*., 1996). Indeed the SH2 or PTB domains of different signaling molecules associated with VEGFR-2 (Waltenberger *et al*., 1994; Kroll and Waltenberger, 1997; Takahashi and Shibuya, 1997) may also bind directly to β3 cytoplasmic tail. In endothelial cells, an anti-apoptotic signal is started by $\alpha_v\beta_3$ clustering (Stromblad *et al*., 1996). However, we have shown that the clustering of β3 integrin obtained by subsequent cell incubation with a specific mAb followed by a secondary goat anti-mouse IgG did not enhance the VEGFR-2 phosphorylation induced by VEGF-A_{165} (S.Mitola and F.Bussolino, unpublished results). This suggests that in our model the role of $\alpha_v\beta_3$ can not be limited to the formation of specialized cytoskeleton structures enriched with signaling molecules, which collaborate with growth factor-dependent signaling (Miyamoto *et al*., 1996; Giancotti, 1997; Jones *et al*., 1997; Schwartz, 1997). One possibility is that β3 association might protect VEGFR-2 against the activity of phosphatases. Consistent with this, protein tyrosine phosphatases SHP-1 and SHP-2 are associated with VEGFR-2 (Kroll and Waltenberger, 1997). Furthermore, it is possible that β3 is needed to ensure the correct subcellular juxtaposition of cytoplasmic tails of the dimerized VEGFR-2. Both hypotheses agree with the observation that the activation of the catalytic activity of PI 3-kinase and the tyrosine phosphorylation of its regulatory subunit p85 induced by VEGF-A_{165} and HIV-1-Tat also required β3 integrin, as inferred by the inhibitory role of BV4 mAb. PI 3-kinase is at least involved in cell survival and in cytoskeleton rearrangement by co-operating with the integrin system (Khwaja *et al*., 1997; Toker and Cantley, 1997), and therefore the observed activation in endothelial cells challenged with VEGF-A_{165} and HIV-1-Tat agrees with its known functions. In this study, a relationship between PI 3-kinase activation and biological activation of endothelial cells stimulated by VEGFR-2 ligands has been achieved by the use of wortmannin, employed at low doses considered specific for PI 3-kinase inhibition (Arcaro and Wymann, 1993). In bovine aortic endothelial cells, Guo and co-workers reported that VEGF-A was capable of inducing the phosphorylation of PI 3-kinase (Guo *et al*., 1995), but successively it has been reported that VEGF-A did not activate the catalytic activity of the enzyme in human endothelial cells (Abedi and Zachary, 1997). We believe that this discrepancy could be due to differences in experimental conditions, in particular to a different time-course or to a difference in conditions of cell culture.

Consistent results have demonstrated a crucial role for extracellular matrix in the development of vasculature in physiological and pathological conditions (Ingber and Folkman, 1989; Bussolino *et al*., 1997), and in particular, a prominent role is attributed to $\alpha_v\beta_3$ integrin (Brooks *et al*., 1994a,b; Friedlander *et al*., 1996; Stromblad *et al*., 1996; Senger *et al*., 1997; Eliceiri *et al*., 1998), at least in adult life (Bader *et al*., 1998). Besides being expressed *in vivo* on endothelial cells of angiogenic vessels (Brooks *et al*., 1994a; Friedlander *et al*., 1996) and *in vitro* by microvascular endothelial cells after treatment with VEGF-A (Senger *et al*., 1997), $\alpha_v\beta_3$ integrin takes part in the *in vitro* endothelial angiogenic program with different and temporally distinct roles. First, as demonstrated here, $\alpha_v\beta_3$ co-operates with an angiogenic receptor (i.e. VEGFR-2) for its full activation triggered by the ligand; next it is necessary for the second wave of activation of MAP kinase induced by an angiogenic activator (Eliceiri *et al*., 1998); and finally, most probably through the MAP kinase pathway, it regulates the expression of genes which control the cell cycle (Stromblad *et al*., 1996).

Materials and methods

Cells

Human endothelial cells from umbilical cord veins, prepared and characterized as previously described (Bussolino *et al*., 1992), were grown in M199 medium (Gibco-BRL, Grand Island, NY) supplemented with 20% fetal calf serum (FCS) (Irvine, Santa Ana, CA), endothelial cell growth factor (100 μg/ml) (Sigma Chemical Co., St Louis, MO) and porcine heparin (Sigma) (100 μg/ml). Cells were used at second passage and growth on a plastic surface coated with porcine gelatin (Sigma), unless specified.

Antibodies

The rabbit polyclonal antibody against the C-terminus peptide of VEGFR-2 was purchased from Santa Cruz Biotecnology Inc. (Santa Cruz, CA; C-1158), the rabbit neutralizing (Albini *et al*., 1996a) polyclonal anti-VEGFR-2 antibody was kindly provided by Dr H.Weich, (Gesellschaft fuer Biotechologische Forschung, Braunschweig, Germany). The rabbit polyclonal anti-p85 antibody and the anti-phosphotyrosine mAb (clone G410) were purchased from Upstate Biotechnology Incorporated (Lake Placid, NY). mAb BV7 to β1 integrin (class IgG_1) (Martin-Padura *et al*., 1994), BV4 to β3 integrin (class IgG_1) and BV10 to CD31 (class IgG_1) (Lampugnani *et al*., 1992) were originally described by Dr E.Dejana (Istituto Mario Negri, Milano, Italy) and obtained by Bioline (Torino, Italy). B212 mAb to β3 integrin (Thiagarajan *et al*., 1985) was a gift from Dr P.Thiagarajan (Jefferson Medical Collge, PA). Anti-α_v integrin subunit mAb was purchased from American Type Cell Collection (Bethesda, MD). The polyclonal anti-β5 integrin subunit Ab was raised against the C-terminal sequence (NH_2-KTFNKFNKSYNGTVD-COOH) (Bioline). A mAb anti-MHC class I antigen (0.165 mAb, class IgG_1) was kindly provided by Professor F.Malavasi (Istituto di Biologia e Genetica, Ancona, Italy). LM609, which recognizes $\alpha_v\beta_3$ integrin complex, was a gift from Dr D.A.Cheresh (The Scripps Research Institute, La Jolla, CA).

Endothelial cell labeling with [^{35}S]methionine

Endothelial cell were grown at confluence and labeled with [^{35}S]methionine (800 Ci/mmol; Amersham, UK) by overnight incubation in methionine-free medium (Sigma) with 5% FCS and 40 μCi/ml of radioisotope.

Endothelial cell surface biotinylation

Confluent endothelial cells were incubated for 20 min at room temperature with Sulfo-NHS-biotin (Pierce, Rockford, IL) dissolved in phosphate-buffered saline (PBS) at 1 mg/ml. After washing the cells once with PBS, the residual NHS groups were reacted with 0.1 M glycine in PBS on ice for 15 min. After several washes, the cells were processed for specific immunoprecipitation followed by electrophoresis and blotting as specified below. Biotinylated proteins were visualized with avidin-peroxidase and developed using the enhanced chemiluminescence technique (Amersham).

Immunoprecipitation and immunoblotting

Confluent endothelial cells (1×10^7 cells/ 150 cm^2 dish) were made quiescent by 20 h starvation in M199 containing 0.5% FCS and 0.1% human serum albumin (Farma Biagini, Lucca, Italy), pre-incubated for 15 min at 37°C with 1 mM Na_3V and then stimulated with VEGF-A_{165} (Dr H.Weich, Gesellschaft für Biotechologische Forschung, Braunschweig, Germany) or with HIV-1-Tat (Intracel, London, U.K.) in presence of heparin (1 U/ml). Cells were lysed in a 50 mM Tris–HCl buffer pH 7.4, containing 150 mM NaCl, 1% Triton X-100, and protease and phosphatase inhibitors (50 μg/ml pepstatin, 50 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF; 500 μg/ml soybean trypsin inhibitor, 100 μM $ZnCl_2$, 1 mM Na_3VO_4, Sigma). After centrifugation (20 min, 10 000 *g*), supernatants were pre-cleared by incubation for 1 h with protein A–Sepharose or with anti-mouse Ig-agarose (Sigma). Samples (1 mg of protein) were incubated with rabbit polyclonal anti-VEGFR-2 (Santa Cruz), or BV4 (anti-β3 integrin subunit) mAbs, BV7 (anti-β1 integrin), anti-p85, anti-phosphotyrosine, anti-CD31 (5–10 μg/ml) and anti-β5 integrin for 1 h at 4°C and immune complexes were recovered on protein A–Sepharose or anti-mouse Ig–agarose. Immunoprecipitates were washed four times with lysis buffer, twice with the same buffer without Triton X-100 and once with TBS. In some experiments, anti-β3 or anti-β1 integrin immune complexes recovered on agarose beads were boiled for 2 min in a solubilization buffer containing 0.4% SDS, 50 mM triethanolamine chloride (pH 7.4), 100 mM NaCl, 2 mM EDTA, 10% glycerol and protease and phosphatase inhibitors, and 2 mM 2β-mercaptoethanol. After boiling, iodacetamide was added to a concentration of 10 mM (Soldi *et al*., 1997). These extracts were again immunoprecipitated with anti-VEGFR-2 antibody (Santa Cruz). Proteins were solubilized under reducing or non-reducing conditions (Laemmli, 1970), separated by SDS–PAGE (8 or 10%), and transferred to Immobilon-P sheets (Millipore, Bedford, MA) and probed with anti-phosphotyrosine, or with anti-VEGFR-2 mAbs (Santa Cruz). The enhanced chemiluminescence technique (Amersham) was used for detection.

PI 3-kinase

PI 3-kinase assay was performed directly on anti-phosphotyrosine immunoprecipitates as described before (Auger *et al*., 1989; Soldi *et al*., 1994) except that the beads were also washed twice with Tris-buffered saline containing 0.5 M LiCl. Briefly, immunoprecipitates were incubated with 40 μM ATP, 50–100 μCi [γ-^{32}P]ATP (Amersham), and a presonicated mixture of phosphatidylinositol (4,5)P_2 and phosphatidylserine (50 μg/ml final concentration of both lipids, Sigma), in 25 mM HEPES pH 7.4, and 1 mM EGTA. The reaction was stopped after 10 min incubation at room temperature by the addition of 1 vol. of 1 M HCl and 2 vol. chloroform/methanol (1:1). The lipids in the organic phase were separated by thin-layer chromatography (Merck, Darmstadt, Germany, Silica Gel 60) in 1-propanol:2 M acetic acid 65:35 (v/v) and visualized by autoradiography.

Migration, polarization and proliferation assays

Endothelial cell motility was studied using a modified Boyden's chamber technique as previously described (Bussolino *et al*., 1992; Albini *et al*., 1996b). To study endothelial cell polarization, cells were detached in cold PBS containing 2 mM EGTA and washed twice in M199 containing 1% FCS. Endothelial cells (10^6/ml M199 containing 1% human serum albumin) were pre-warmed in polypropilene tubes at 37°C for 5 min and then exposed to 10 ng/ml of VEGF-A_{165} or HIV-1-Tat for 10 min. The reaction was stopped by adding an equal volume (1 ml) of ice-cold phosphate-buffered formaldehyde (10% v/v; pH 7.2). The percentage of cells with bipolar configuration (front-tail) was determined in at least 200 cells for each tube by phase-contrast microscopy at 400× magnification (Mitola *et al*., 1997).

The evaluation of endothelial cells proliferation was performed with Biotrak cell proliferation ELISA system (Amersham). Cells (2.5×10^3) were plated in 96-well plate coated with human serum albumin (0.1%), or human vitronectin, or human fibronectin, or with poly-L-lysine (Sigma) or human fibrinogen (Kabivitrum Stockholm, Swedish) (0.001%), and grown for 12 h in M199 containing 5% FCS. Then, they were washed and maintained for 12 h at 37°C in serum-free M199 containing 5% human serum albumin, and subsequently stimulated with 10 ng/ml of VEGF-A_{165} or HIV-1-Tat for 24 h. BrdU was added to the cells for an additional time of 3 h. Fixed cells were treated according to the manufacturer's instruction and the resultant color developed by the anti-BrdU peroxidase-labeled immune complexes was read at 450 nm in microtiter plate spectrophotometer (EL340, Bio-tek Instruments).

Treatment of endothelial cells with specific antibodies

To study the effect of specific antibodies on the experimental designs above (experiments of VEGFR-2 phosphorylation, migration and polarization), endothelial cells were incubated in M199 with 10 μg anti-β3 (BV4 mAb), anti-β1 (BV7 mAb) or anti-MHC class I antigen (0.165 mAb), or with a neutralizing anti-VEGFR-2 antibody, or with irrelevant mouse or rabbit IgG (Sigma) at 4°C for 20 min. Cells were washed twice in cold M199 containing 1% human serum albumin and then exposed to stimuli. To evaluate the effect of the above mAbs in BrdU incorporation, the antibodies (20 μg/ml) were added in the last 27 h.

Binding displacement assay

In a previous study, we demonstrated that the equilibrium of binding of labelled VEGF-A with its high affinity sites on endothelial cells is reached after 90 min at room temperature (Albini *et al*., 1996b). On the basis of this data, binding displacement studies with mAb anti-β3 integrin were performed. Cell monolayers on 24-well plates were incubated for 90 min at room temperature in 0.2 ml M199 containing 20 mM (*n*-[2-hydroxyethyl]piperazine-*N*′-[4-butanesulfonic acid]), pH 7.4, 0.1% BSA, 100 μg/ml soybean trypsin inhibitor (Sigma) with 0.05 nM [^{125}I]VEGF (specific activity 140 μCi/μg) (Amersham) and increasing concentrations of cold BV4 mAb anti-β3, mAb anti-MHC class I or VEGFA_{165}. The cells were washed twice with PBS, solubilized with 2% SDS in PBS and the radioactivity was measured.

Acknowledgements

We wish to thank Dr Elisabetta Dejana (Istituto 'Mario Negri', Milano, Italy) and Dr Andrea Graziani for helpful criticisms, and Dr Herbert Weich (GBF, Braunschweig, Germany) who provided anti-VEGFR-2 antibody and recombinant VEGF-A_{165}. This study was supported by grants from European Community (Biomed-2 Project: BMHL-CT96-0669), Italian Association for Cancer Research (A.I.R.C.), Istituto Superiore di Sanità (X AIDS Project, Program on Tumor Therapy), Centro Nazionale delle Ricerche (Progetto Finalizzato Biotecnologie) and Ministero dell' Università e della Ricerca Scientifica e Tecnologica (60% and Programmi di Ricerca di Rilevante Interesse Nazionale-1998). S.M. and M.S. are supported by grants from FIRC.

References

Abedi,H. and Zachary,I. (1997) Vascular endothelial growth factor stimulates tyrosine phosphorylation and recruitment to new focal adhesions of focal adhesion kinase and paxillin in endothelial cells. *J. Biol. Chem.*, **272**, 15442–15451.

Albini,A., Benelli,R., Presta,M., Rusnati,M., Ziche,M., Rubartelli,A., Paglialunga,G., Bussolino,F. and Noonan,D. (1996a) HIV-tat protein is a heparin-binding angiogenic growth factor. *Oncogene*, **12**, 289–297.

Albini,A. *et al.* (1996b) The angiogenesis induced by HIV-1 tat protein is mediated by the Flk-1/KDR receptor on vascular endothelial cells. *Nature Med.*, **2**, 1371–1375.

Arcaro,A. and Wymann,M.P. (1993) Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-triphosphate in neutrophil responses. *Biochem. J.*, **296**, 297–301.

Auger,K.R., Serunian,L.A., Soltoff,S.P., Libby,P. and Cantley,L.C. (1989) PDGF-dependent tyrosine phosphorylation stimulates production of novel phosphoinositides in intact cells. *Cell*, **57**, 167–175.

Bader,B.L., Rayburn,H., Crowley,D. and Hynes,R. (1998) Extensive vasculogenesis, angiogenesis and organogenesis precede lethality in mice lacking all α_v integrins. *Cell*, **95**, 507–519.

Bhattacharya,S., Fu,C., Bhattacharya,J. and Greenberg,S. (1995) Soluble ligands of the integrin mediate enhanced tyrosine phosphorylation of multiple proteins in adherent bovine pulmonary artery endothelial cells. *J. Biol. Chem.*, **270**, 16781–16787.

Blystone,S.D., Lindberg,F.P., Williams,M.P., McHugh,K.P. and Brown, E.J. (1996) Inducible tyrosine phosphorylation of the β3 integrin requires α_v integrin cytoplasmic tail. *J. Biol. Chem.*, **271**, 31458–31462.

Brooks,P.C., Clark,R.A.F. and Cheresh,D.A. (1994a) Requirement of vascular integrin $\alpha_v\beta_3$ for angiogenesis. *Science*, **264**, 569–571.

Brooks,P.C., Montgomery,A.M.P., Rosenfeld,M., Reisfeld,R.A., Hu,T., Klier,G. and Cheresh,D.A. (1994b) Integrin $\alpha_v\beta_3$ antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell*, **79**, 1157–1164.

Brooks,P.C., Stromblad,S., Klemke,R., Visscher,D., Sarkar,F.H. and Cheresh,D.A. (1995) Antiintegrin $\alpha_v\beta_3$ blocks human breast cancer growth and angiogenesis in human skin. *J. Clin. Invest.*, **96**, 1815–1822.

Bussolino,F. *et al*. (1992) Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. *J. Cell Biol.*, **119**, 629–641.

Bussolino,F., Mantovani,A. and Persico,G. (1997) Molecular mechanisms of blood vessel formation. *Trends Biochem. Sci.*, **22**, 251–256.

Clark,E.A. and Brugge,J.S. (1995) Integrins and signal transduction pathways: the road taken. *Science*, **268**, 233–239.

De Vries,C., Escobedo,J.A., Ueno,H., Houck,K., Ferrara,N. and Williams,L.T. (1992) The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science*, **255**, 989–991.

Defilippi,P., Truffa,G., Stefanutto,G., Altruda,F., Silengo,L. and Tarone,G. (1991a) Tumor necrosis factor α and interferon γ modulates the epression of the vitronectin receptor (integrin β3) in human endothelial cells. *J. Biol. Chem.*, **266**, 7638–7645.

Defilippi,P., van Hinsberg,V., Bertolotto,A., Rossino,P., Silengo,L. and Tarone,G. (1991b) Differential distribution and modulation of expression of α1/β1 integrin on human endothelial cells. *J. Cell Biol.*, **114**, 855–863.

Eliceiri,B.P., Klemke,R., Stromblad,S. and Cheresh,D.A. (1998) Integrin $\alpha_v\beta_3$ requirement for sustained mitogen-activated protein kinase activity during angiogenesis. *J. Cell Biol.*, **140**, 1255–1263.

Ensoli,B. *et al*. (1994) Synergy between basic fibroblast growth factor and HIV-1 Tat protein in induction of Kaposi's sarcoma. *Nature*, **371**, 674–680.

Ferrara,N. and Davis-Smyth,T. (1997) The biology of vascular endothelial growth factor. *Endocrinol. Rev.*, **18**, 4–25.

Friedlander,M., Brooks,P.C., Shaffer,R.W., Kincaid,C.M., Varner,J.A. and Cheresh,D.A. (1995) Definition of two angiogenic pathways by distinct α_v integrins. *Science*, **270**, 1500–1502.

Friedlander,M., Theesfeld,C.L., Sugita,M., Fruttiger,M., Thomas,M.A., Chang,S. and Cheresh,D.A. (1996) Involvement of integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ in ocular neovascular diseases. *Proc. Natl Acad. Sci. USA*, **93**, 9764–9769.

Frisch,S.M. and Ruoslahti,E. (1997) Integrins and anoikis. *Curr. Opin. Cell Biol.*, **9**, 701–706.

Ganju,R.K., Munshi,N., Nair,B.C., Zy,L., Gill,P. and Groopman,J.E. (1998) Human immunodeficiency virus Tat modulates the Flk-1/KDR receptor, mitogen-activated protein kinases and components of focal adhesion in Kaposi's sarcoma cells. *J. Virol.*, **72**, 6131–6137.

Giancotti,F.G. (1997) Integrin signaling: specificity and control of cell survival and cell cycle progression. *Curr. Opin. Cell Biol.*, **9**, 691–700.

Guo,D., Jia,Q., Song,H.Y., Warren,R.S. and Donner,D.B. (1995) Vascular endothelial cell growth factor promotes tyrosine phosphorylation of mediators of signal transduction that contain SH2 domains. Association with endothelial cell proliferation. *J. Biol. Chem.*, **270**, 6729–6733.

Hidai,C. *et al*. (1998) Cloning and characterization of developmental endothelial locus-1: an embryonic endothelial cell protein that binds the $\alpha_v\beta_3$ integrin receptor. *Genes Dev.*, **12**, 21–33.

Hood,J. and Granger,H.J. (1998) Protein kinase G mediates vascular endothelial growth factor-induced Raf-1 activation and proliferation in human endothelial cells. *J. Biol. Chem.*, **273**, 23504–23508.

Ingber,D. and Folkman,J. (1989) How does extracellular matrix control capillary morphogenesis? *Cell*, **58**, 803–805.

Jones,R.L., Crack,J. and Rabinovitch,M. (1997) Regulation of Tenascin-C, a vascular smooth muscle cell survival factor that interacts with the $\alpha_v\beta_3$ integrin to promote epidermal growth factor receptor phosphorylation and growth. *J. Cell Biol.*, **139**, 279–293.

Keyt,B.A., Berleau,L.T., Nguyen,H.V., Chen,H., Heinsohn,H., Vandlen,R. and Ferrara,N. (1996) The carboxyl-terminal domain (111–165) of vascular endothelial growth factor is critical for its mitogenic potency. *J. Biol. Chem.*, **271**, 7788–7795.

Khwaja,A., Rodriguez-Viciana,P., Wennstrom,S., Warne,P.H. and Downward,J. (1997) Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. *EMBO J.*, **16**, 2783–2793.

King,W.G., Mattaliano,M.D., Chan,T.O., Tsichilis,P.M. and Brugge,P.S. (1997) PI3-kinase is required for integrin-stimulated AKT and Raf-1/MAP kinase pathway activation. *Mol. Cell. Biol.*, **17**, 4406–4418.

Kroll,J. and Waltenberger,J. (1997) The vascular endothelial growth factor receptor KDR activates multiple signal transduction pathways in porcine aortic endothelial cells. *J. Biol. Chem.*, **272**, 32521–32527.

Kuhn,K. and Eble,J. (1994) The structural bases of integrin–ligand interactions. *Trends Cell Biol.*, **4**, 256–261.

Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **230**, 680–682.

Lampugnani,M.G., Resnati,M., Raiteri,M., Pigott,R., Pisacane,A., Houen,G., Ruco,L.P. and Dejana,E. (1992) A novel endothelial-specific membrane protein is a marker of cell–cell contacts. *J. Cell Biol.*, **118**, 1511–1522.

Law,D.A., Nannizzi-Alaimo,L. and Phillps,D.R. (1996) Outside–in integrin signal transduction. Alpha Ib beta3- (GP IIb IIIa) tyrosine phosphorylation induced by platelet aggregation. *J. Biol. Chem.*, **271**, 10811–10815.

Leavesley,P.I., Schwartz,M.A., Rosenfeld,M. and Cheresh,D.A. (1993) Integrin β1- and β3-mediated endothelial cell migration is triggered through distinct signaling mechanisms. *J. Cell Biol.*, **121**, 737–743.

Martin-Padura,I., Bazzoni,G., Zanetti,A., Bernasconi,S., Elices,M.J., Mantovani,A. and Dejana,E. (1994) A novel mechanisms of colon carcinoma cell adhesion to the endothelium triggered by beta 1 integrin chain. *J. Biol. Chem.*, **269**, 6124–6132.

Millauer,B., Wizigmann-Voos,S., Schnurch,H., Martinez,R., Moller,N.P., Risau,W. and Ullrich,A. (1993) High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell*, **72**, 835–846.

Mitola,S., Sozzani,S., Luini,W., Arese,M., Borstatti,A., Weich,H.A. and Bussolino,F. (1997) Tat-HIV-1 induces human monocyte chemotaxis by activation of vascular endothelial growth factor receptor-1. *Blood*, **90**, 1365–1372.

Miyamoto,S., Teramoto,H., Gutkind,J.S. and Yamada,K.M. (1996) Integrins can collaborate with growth factor for phosphorylation of receptor tyrosine kinases and MAP kinase activation: roles of integrin aggregation and occupancy of receptors. *J. Cell Biol.*, **135**, 1633–1642.

Muller,Y.A., Li,B., Christinger,H.W., Cunningham,B.C. and de Vos,A.M. (1997) Vascular endothelial growth factor: crystal structure and functional mapping of the kinase domain receptor binding site. *Proc. Natl Acad. Sci. USA*, **94**, 7192–7197.

Parsons,J.T. (1996) Integrin-mediated signaling-regulation by protein-tyrosine kinases and small GTP-binding proteins. *Curr. Opin. Cell Biol.*, **8**, 146–152.

Pedran,A., Razandi,M. and Levin,E.R. (1998) Extracellular signal-regulated protein kinase/Jun kinase cross-tal underlies vascular endothelial cell growth factor-induced endothelial cell proliferation. *J. Biol. Chem.*, **273**, 26722–26728.

Rousseau,S., Houle,F., Landry,J. and Huot,J. (1997) p38 MAP kinase activation by vascular endothelial growth factor mediates actin reorganization and cell migtaion in human endothelial cells. *Oncogene*, **15**, 2169–2177.

Sagawa,K., Kimura,T., Swieter,M. and Siraganian,R.P. (1997) The protein-tyrosine phosphatase SHP-2 associates with tyrosine phosphorylated adhesion molecules PECAM-1 (CD31). *J. Biol. Chem.*, **272**, 31086–31091.

Schlaepfer,D.D., Hanks,S.K., Hunter,T. and van der Geer,P. (1994) Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature*, **372**, 786–791.

Schneller,M., Vuori,K. and Ruoslahti,E. (1997) $\alpha_v\beta_3$ integrin associates with activated insulin and PDGFβ receptors and potentiates the biological activity of PDGF. *EMBO J.*, **16**, 5600–5607.

Schwartz,M.A. (1997) Integrins, oncogenes and anchorage independence. *J. Cell Biol.*, **139**, 575–578.

Schwartz,M.A., Schaller,M.D. and Ginsberg,M.H. (1995) Integrins: emerging paradigms of signal transduction. *Annu. Rev. Cell Dev. Biol.*, **11**, 549–599.

Senger,D.R., Ledbetter,S.R., Claffey,K.P., Papadopoulos-Sergiou,A., Peruzzi,C.A. and Detmar,M. (1997) Stimulation of endothelial cell migration by vascular permeability factor/vascular endothelial growth factor through cooperative mechanisms involving the $\alpha_v\beta_3$ integrin, osteopontin and thrombin. *Am. J. Pathol.*, **149**, 293–305.

Soker,S., Takashima,S., Miao,H.Q., Neufeld,G. and Klagsbrun,M. (1998) Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell*, **92**, 735–745.

Soldi,R., Graziani,A., Benelli,R., Ghigo,D., Bosia,A., Albini,A. and Bussolino,F. (1994) Oncostatin M activates phosphatidylinositol-3-kinase in Kaposi's sarcoma cells. *Oncogene*, **9**, 2253–2260.

Soldi,R., Primo,L., Brizzi,M.F., Sanavio,F., Polentarutti,N., Pegoraro,P., Mantovani,A. and Bussolino,F. (1997) Activation of JAK2 in human vascular endothelial cells by granulocyte-macrophage colony stimulating factor. *Blood*, **89**, 863–872.

Stromblad,S., Becker,J.C., Yebra,M., Brooks,P.C. and Cheresh,D.A. (1996) Suppression of p53 activity and $p21^{WAF/CIP1}$ expression by vascular cell integrin $\alpha_v\beta_3$ during angiogenesis. *J. Clin. Invest.*, **98**, 426–433.

Takahashi,T. and Shibuya,M. (1997) The 230 Da mature form of KDR/Flk-1 (VEGF receptor-2) activates the PLC-γ pathway and partially induces mitotic sgnals in NIH3T3 fibroblasts. *Oncogene*, **14**, 2079–2089.

Terman,B.I., Carrion,M.E., Kovacs,E., Rasmussen,B.A., Eddy,R.L. and Shows,T.B. (1991) Identification of a new endothelial cell growth factor receptor tyrosine kinase. *Oncogene*, **6**, 1677–1683.

Thiagarajan,P., Shapiro,S.S., Levine,E., De Marco,L. and Yalcin,A. (1985) A monoclonal antibody to human platelet glycoprotein IIIa detects a related protein in cultured human endothelial cells. *J. Clin. Invest.*, **75**, 869–901.

Thomas,K.A. (1996) Vascular endothelial growth factor, a potent and selective angiogenic agent. *J. Biol. Chem.*, **271**, 603–606.

Toker,A. and Cantley,L.C. (1997) Signalling through the lipid products of phosphoinositide-3-OH-kinase. *Nature*, **387**, 673–676.

Vuori,K. and Ruoslahti,E. (1994) Association of insulin receptor substrate-1 with integrins. *Science*, **266**, 1576–1578.

Waltenberger,J., Claesson-Welsh,L., Siegbahn,A., Shibuya,M. and Heldin,C.H. (1994) Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *J. Biol. Chem.*, **269**, 26988–26995.

Wary,K.K., Mainiero,F., Isakoff,S.J., Marcantonio,E.E. and Giancotti,F.G. (1996) The adaptor protein Sch couples a class of integrins to the control of cell cycle progression. *Cell*, **87**, 733–743.

Woodard,A.S., Garcia-Cardena,G., Leong,M., Madri,J.A., Sessa,C.W. and Languino,R.L. (1998) The synergistic activity of $\alpha_v\beta_3$ integrin and PDGF receptor increases cell migration. *J. Cell Sci.*, **111**, 469–478.

Yamada,R.M. and Miyamoto,S. (1995) Integrin transmembrane signaling and cytoskeletal control. *Curr. Opin. Cell Biol.*, **7**, 681–689.

Zhu,X., Otsubo,M., Bohmer,R., Roberts,J.M. and Assoian,R.K. (1996) Adhesion dependent cell cycle progression linked to the expression of cyclin D1, activation of cyclin E-cdk2 and phosphoryation of the retinoblastoma proteins. *J. Cell Biol.*, **133**, 391–403.

Received October 9, 1998; revised December 28, 1998; accepted December 30, 1998