Interaction of urokinase receptor and integrins on endothelial cells and its implication in VEGF-induced migration

Doctoral thesis at the Medical University of Vienna

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Submitted by

Revu Ann Alexander

Supervisor:
Univ.Prof.Dr.Michael Freissmuth,MD
Institute for Vascular Biology and Thrombosis Research
Center for Physiology and Pharmacology
Medical University of Vienna
Schwarzpanierstrasse 17
A-1090 Vienna, Austria.

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My parents. I know that they are under the greatest peer pressure as to where their daughter’s doings are taking her. Though being perplexed to my constant worrying about cells or experiments, they have always expressed a silent faith and support in my endeavours. I hope I won’t let you down.
TABLE OF CONTENTS

LIST OF ABBREVIATIONS 6
LIST OF FIGURES 8
ZUSAMMENFASSUNG 9
ABSTRACT 11

1. INTRODUCTION
1.1 Vascular endothelial growth factor (VEGF) 13
1.2 The plasminogen system 18
1.3 Structure of uPAR and uPA 19
1.4 uPAR in angiogenesis, proteolysis and migration 21
1.5 Integrins 23
1.6 Integrins in angiogenesis 25
1.7 Interactions between uPAR and integrins 27
1.8 The LDL-receptor family 30
1.9 LDL-receptor mediated uPAR interactions 32
1.10 Rationale of the work 35

2. MATERIALS AND METHODS
2.1 Reagents and antibodies 36
2.2 Cell culture 37
2.3 Cytofluorimetric analysis 37
2.4 Surface biotinylation and Western blotting 38
2.5 siRNA treatment of endothelial cells 39
2.6 Immunocytochemistry 39
2.7 Micropatterning 39
2.8 Transwell migration assay 40
2.9 DIVAA in vivo angiogenesis assay 40

3. RESULTS
3.1 Internalization of uPAR and β1 integrin upon VEGF stimulation. 42
3.2 uPAR is not cleaved during short term VEGF stimulation. 45
3.3 uPAR and β1 integrin interact upon VEGF stimulation 47
3.4 uPAR and β1 integrin co-internalize in endocytic vesicles upon VEGF stimulation. 49
3.5 VEGF induced PI3-kinase pathway modulates β1 integrin internalization.
3.6 Domain 3 of uPAR is necessary for β1 integrin endocytosis.
3.7 Integrin α5β1 associates with uPAR for VEGF induced endocytosis.
3.8 UPAR is essential for VEGF induced internalization of α5β1 but not α3β1 integrin.
3.9 The LDL-receptor is necessary for VEGF induced endocytosis of α5β1 integrin.
3.10 Inhibition of uPAR-α5β1 interaction impairs VEGF induced migration of endothelial cells.
3.11 uPAR-integrin interaction is necessary for in vivo angiogenesis.

4. DISCUSSION

5. BIBLIOGRAPHY

6. COPYRIGHT CLEARANCE FOR FIGURES 1,3

7. CURRICULUM VITAE
LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
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<tr>
<td>ATF</td>
<td>amino terminal fragment</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
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<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
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<tr>
<td>HUVEC</td>
<td>human umbilical vein endothelial cell</td>
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<td>LDL-R</td>
<td>low density lipoprotein receptor</td>
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<td>LRP</td>
<td>LDL receptor related protein</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>PAE</td>
<td>porcine aortic endothelial</td>
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<td>PAI-1</td>
<td>plasminogen activator inhibitor-1</td>
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<td>PBS</td>
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<td>PDGF</td>
<td>platelet derived growth factor</td>
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<tr>
<td>PDGFR</td>
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<td>PI-PLC</td>
<td>phosphatidylinositol-specific phospholipase C</td>
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<tr>
<td>PLGF</td>
<td>placental growth factor</td>
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<tr>
<td>PMA</td>
<td>phorbol-12-myristate-13-acetate</td>
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<tr>
<td>RAP</td>
<td>receptor-associated protein</td>
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<tr>
<td>TIR</td>
<td>total internal reflection</td>
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<td>TIMP</td>
<td>tissue inhibitor of metalloproteinases</td>
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<td>tPA</td>
<td>tissue plasminogen activator</td>
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<td>uPA</td>
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<td>uPAR</td>
<td>urokinase plasminogen activator receptor</td>
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<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<td>VEGFR</td>
<td>vascular endothelial growth factor receptor</td>
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<td>VLDLR</td>
<td>very low-density lipoprotein receptor</td>
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## Abbreviations for Amino Acids

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LIST OF FIGURES

Figure 1. The VEGF-receptor signaling pathway.

Figure 2. Schematic representation of the plasminogen system.

Figure 3. The integrin family.

Figure 4. VEGF stimulation induces internalization of β1 integrins in endothelial cells.

Figure 5. uPAR is not cleaved during short term VEGF stimulation.

Figure 6. VEGF-induced interaction of uPAR and β1 integrins.

Figure 7. uPAR and β1-integrins co-internalize in endocytic vesicles upon VEGF stimulation.

Figure 8. VEGF induced β1 integrin internalization requires VEGFR-2 dependent PI3-kinase/MMP-2 activation.

Figure 9. Peptides derived from domain-3 of uPAR block VEGF-induced integrin internalization.

Figure 10. The α5β1 integrin heterodimer specifically interacts with uPAR upon VEGF stimulation.

Figure 11. uPAR is necessary for VEGF induced internalization of α5 integrin subunit but not α3.

Figure 12. VEGF-induced α5β1 integrin internalization requires LDL-receptor-protein-dependent uPAR interaction.

Figure 13. VEGF-induced endothelial migration in vitro requires the interaction of the fibronectin receptor α5β1 integrin with uPAR.

Figure 14. uPAR mediated integrin recycling is necessary for in vivo angiogenesis.

Figure 15. Schematic representation of uPAR mediated integrin internalization for VEGF stimulated endothelial cells.
ZUSAMMENFASSUNG

Mit immobilisierten gegen α5β1-Integrin gerichteten Antikörpern wurden die Integri
nen der endothelialen Zelloberflächen in ein Muster gezwungen. Nach Inkubation der
Endothelzellen mit VEGF verteile sich uPAR in dasselbe Muster. Diese Beobachtung
beweist, dass die beiden Proteine in lebenden Endothelzellen in Abhängigkeit von VEGF
miteinander interagieren. Die Ergebnisse der Experimente liefern daher eine Bestätigung für
die Arbeitshypothese. Darüber hinaus legen die Beobachtungen nahe, dass uPAR ein
wesentlicher Bestandteil des Netzwerks an Signalproteinen, über die VEGF die
Endothelzellmigration kontrolliert: uPAR ist ein Flaschenhals, durch den die VEGF-
induzierte Signale geschleust werden müssen, um sowohl die proteolytische Aktivität an der
invadierenden Membran zu fokussieren als um die Umverteilung und Rezirkulation der
Integri ne zu steuern.
ABSTRACT

Vascular endothelial growth factor (VEGF) is a very potent growth factor that drives both, physiological and pathological angiogenesis. VEGF initiates angiogenesis by activating endothelial cells to migrate and invade surrounding tissues. This requires the coordinated proteolytic degradation of the extracellular matrix by the plasminogen system and regulation of cell-migration provided by integrin-matrix interaction. The cell surface-bound urokinase receptor (uPAR) is a central component of the plasminogen system. The active urokinase:urokinase receptor complex accelerates the conversion of plasminogen to plasmin, initiating a proteolytic cascade leading to extracellular matrix degradation. Previous experiments showed that VEGF-stimulation of endothelial cells induced pro urokinase (pro-uPA) activation; this was accompanied not only by extracellular matrix degradation but also by coordinated internalization of the urokinase receptor complexed to a member of the LDLR family. Internalization is preceded by redistribution of the protease receptor uPAR to focal adhesions. Preventing the internalization of uPAR blocks endothelial cell migration and in vivo angiogenesis. uPAR does not have any primary ligand in the native basement membrane. Hence, internalization is likely to be contingent on the formation of uPAR with another partner. Because of the central role of integrins in matrix interactions, the working hypothesis underlying this thesis posits that uPAR forms a complex and is internalized with integrins. This conjecture was verified by using several approaches: (i) flow cytometry-based internalization experiments allowed for quantifying the VEGF-induced internalization of uPAR in endothelial cells. (ii) Confocal microscopy visualized the co-internalization of uPAR and α5β1 integrins upon angiogenic stimulation of endothelial cells with VEGF. (iii) This retrieval was contingent on uPAR and on receptors of the low density lipoprotein (LDL) receptor family. VEGF-induced integrin redistribution was inhibited by elimination of uPAR from the endothelial cell surface or by inhibitory peptides that block the uPAR/integrin interaction. The absence of uPAR or the presence of the inhibitory peptides also impaired the migratory response of endothelial cells to VEGF. (iv) VEGF-promoted complex formation between uPAR and α5β1 integrin was also visualized on live endothelial cells by using a micropatterning approach: immobilized α5β1 integrin antibody imposed a pattern on the surface-expressed α5β1 integrins. In response to VEGF uPAR was driven into the complex and thus adopted the pattern as dictated by the α5β1 integrin antibody. This observation provided direct and incontrovertible evidence that the two proteins interacted in live VEGF-stimulated endothelial cells. Taken together the observations confirmed the working hypothesis. The findings indicate that uPAR is an essential component of the network
through which VEGF controls endothelial cell migration: uPAR is a bottleneck through which the VEGF-induced signal must be funneled for both, focused proteolytic activity at the leading edge and for redistribution and recycling of integrins.
1. INTRODUCTION

Every blood vessel in our body, starting from the veins, arteries to the very small capillaries is lined by an endothelium. Endothelial cells do not only regulate the exchange of water, electrolytes and nutrients between blood and extracellular space and confine blood cells like the platelets or WBCs, but by their signaling also regulate the structure and function of the blood vessel.

Angiogenesis - by simple definition - is the formation of new blood vessels from preexisting vessels. New blood vessel formation is necessary not only for embryonic growth, but also for wound healing in adults. But angiogenesis also has its deleterious effects. During their evolution from a confined state (“carcinoma in situ”) to an invasive and possibly metastatic stage, tumors must undergo an angiogenic switch to become invasive: some of the cancer cells acquire the ability to produce proangiogenic molecules. These recruit endothelial cells in the nearby blood vessels. The activated endothelial cells proliferate, migrate and form new blood vessels. This process is termed neovascularization. As a result, the tumor has access to the surrounding tissue through these new blood vessels prompting tumor progression and metastasis. Hence, the process is also referred to as tumor angiogenesis (1).

Blood vessel formation is a complex response that is driven by a plethora of molecules like chemokines, growth factors, angiopoietins, oxygen sensors, junctional proteins etc. (2). Under the influence of these driving forces endothelial cells have to grow, divide and invade the extracellular matrix. Growth factors and their receptors send signal cues to cells, which then activate the protease system to degrade the extracellular matrix. The cytoskeletal system and the adhesion molecules undergo rearrangements and redistribution facilitating the cell to move forward.

Endothelial cells receive input from many receptors that sense the composition of the extracellular milieu and then recruit intracellular signaling pathways to translate the cue into a biological response. Input from the various receptors must be integrated to allow for a coordinated response. Accordingly, cross-talk and interdependence between various pathways is expected to occur. In my thesis, I look at the interdependence of the protease system and the adhesion molecules under the influence of growth factors, which can drive the process of angiogenesis.
1.1 Vascular Endothelial Growth factor (VEGF)

A large number of mitogens capable of stimulating angiogenesis have been identified over the years; the list includes vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), tumor necrosis factor-α (TNFα), angiogenin etc. Initially bFGF was considered to be the prime angiogenic factor regulating tumor angiogenesis. Several observations, however, questioned this original assumption. The mechanism that allowed for regulated secretion of bFGF was not clear and is still not understood, because bFGF lacks a signal peptide that is characteristic of secreted peptides (3). More importantly, the mitogenic action of bFGF is not restricted to endothelial cells; in fact many other cell types respond to bFGF (4). Finally, it was noted that neutralization of bFGF by antibodies did not affect tumor angiogenesis (5). Thus, a factor other than bFGF must exist to account for the capacity of tumor cells to induce blood vessel sprouting.

Vascular Endothelial Growth Factor or VEGF was first described by Napoleone Ferrara in 1989 (6). It was purified as a secreted protein from the conditioned media of bovine pituitary follicular cells based on its ability to strongly promote endothelial cell growth. The striking property of VEGF was that it was mitogenic specifically for capillary and human umbilical vein endothelial cells. At the same time Connolly et al described that VPF (Vascular Permeability Factor) also increases blood vessel permeability (7). VPF was later established to be identical to VEGF.

In situ hybridization analysis revealed that the expression pattern of VEGF and its receptors is correlated with embryonic angiogenesis. The transcript levels of VEGF and its receptor were abundant during embryonic brain development, but they rapidly declined in adult brain when endothelial cell proliferation had ceased (8,9). If tumors were examined, VEGF was found to be expressed by tumor cells; VEGF-receptor expression, in contrast, was confined to tumor vascular endothelial cells (10,11). Hypoxia or low oxygen tension associated with tumor necrosis also stimulates VEGF secretion by endothelial cells promoting angiogenesis in an autocrine and paracrine manner (12,13).

The mammalian VEGF family consists of 5 members: VEGF-A, B, C, D and PLGF (Placental Growth factor). These VEGF ligands can bind to three structurally similar Type III tyrosine kinase receptors: VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR) or VEGFR-3. VEGF-B and PLGF bind exclusively to VEGFR-1. VEGF-C, D can bind to VEGFR-2 or VEGFR-3, while VEGF-A is the most promiscuous homolog which can bind to all three receptors (14). A structurally related protein, VEGF-E is encoded by the parapoxvirus Orf virus (OV). VEGF-E has bioactive properties similar to VEGF-A but is receptor specific only for
VEGFR-2 (15). VEGF-A exists in several isoforms generated by alternative splicing: VEGF_{165}, VEGF_{121}, VEGF_{189}, VEGF_{206}.

VEGFR-3 is mainly expressed on lymphatic endothelial cells and therefore mostly associated with lymphangiogenesis (16). VEGFR-1 and 2 are mainly expressed by vascular endothelial cells and considered to be key mediators of vascular angiogenesis. Both, VEGFR-2 and VEGFR-1 knockout mice die in the embryonic stage by day 8.5 (17,18). VEGFR-2-deficient mice were found to be devoid of blood vessels, mature endothelial cells and haematopoietic progenitor stem cells (17). In contrast, in VEGFR-1(−/-) mice, differentiated endothelial cells are abundant but disorganized (18). This difference in phenotype documents that the two receptors control endothelial cell proliferation, differentiation and migration in a non-redundant way; this may be accounted for by the recruitment of distinct downstream signaling pathways.

In order to understand the difference in these two receptors, porcine aortic endothelial (PAE) cells were stably transfected with either VEGFR-1 or VEGFR-2. Ligand binding prompts VEGFR activation by receptor dimerization and autophosphorylation at tyrosine residues. For VEGF, VEGFR-1 was found to have a higher binding affinity (16 pM) than VEGFR-2 (760 pM). In spite of this low binding affinity, VEGFR-2 was the receptor responsible for most if not all actions of VEGF that can be observed: VEGF induced actin reorganization, stimulated chemotaxis and elicited a mitogenic response in VEGFR-2-transfected PAE cells. In contrast, PAE cells stably expressing VEGFR-1 did not proliferate in response to VEGF (19). The observation that VEGFR-2 is responsible for mitogenicity was also confirmed by mutating residues in VEGF_{165} necessary for either VEGFR-1 or VEGFR-2 binding. VEGF_{165} mutants with normal VEGFR-2 but reduced VEGFR-1 binding could elicit strong proliferative signals in bovine adrenal cortical capillary endothelial cells. However, VEGF_{165} mutants with normal VEGFR-1 and reduced VEGFR-2 binding were poor mitogenic ligands (20).

The strong binding affinity but weak tyrosine kinase activity of VEGFR-1 suggested that it could be a negative regulator of angiogenesis. In fact, knock out mice which lack the intracellular domain of VEGFR-1 (VEGFR-1 TK−) are perfectly healthy (150) as opposed to lethality and disorganized vasculature observed in VEGFR-1(−/-) mice (18). Therefore, the role of VEGFR-1 in angiogenesis could be to sequester and maintain a physiological balance for VEGF using its ligand binding domain and hence maintain proper blood vessel formation. A role of VEGFR-1 in inflammation has also been described. VEGFR-1 is expressed by macrophage/monocyte lineages and is necessary for differentiation and activation. VEGFR-1 TK− mice are less susceptible to chronic arthritis compared to wild type mice. In the absence
of VEGFR-1 signaling several functions of macrophage are affected resulting in reduced macrophage infiltration and rheumatoid pathology (151). Signaling via VEGFR-2 has been extensively studied (Figure 1). VEGF ligand binding prompts VEGFR-2 receptor dimerization and auto-phosphorylation at several tyrosine residues in the intracellular domain. Y1175 is a crucial auto-phosphorylation site as mice with a phenylalanine substitution in Y1173 (which corresponds to Y1175 in humans) die at E8.5 with similar defects as the VEGFR-2−/− mice (21). Auto-phosphorylation of Y1175 allows for subsequent binding of phospholipase C-γ (PLC-γ) and of several adaptor proteins such as SH2 domain-containing adaptor protein B (SHB) and Shc-like protein (SCK). Binding of PLC-γ to Y1175-P leads to MAPK/ERK1/2 activation and endothelial cell proliferation (22). The MAPK activation is mediated mainly through PLC-γ/protein kinase C (PKC) dependent activation of RAF-MEK pathway but independent of RAS. Phospholipase C-γ generates two second messengers by cleavage of membrane bound PIP_2 (phosphatidylinositol-4,5-bisphosphate) to diacylglycerol and inositoltrisphosphate (IP_3). Diacylglycerol activates the classical and the novel isoforms of protein kinase C (PKC). Inositoltrisphosphate releases calcium from the endoplasmic reticulum by opening its namesake receptor, a ligand-gated channel for calcium. This supports the effective activation of classical (Ca^{2+}-dependent) protein kinase C-isoforms. The VEGF-induced, phospholipase C-γ-mediated accumulation of diacylglycerol leads to a protein kinase C-mediated phosphorylation and hence activation of RAF, the kinase upstream of MEK1 (MAP kinase kinase/activator of ERK1/2). It is somewhat surprising that the small G protein RAS is dispensable in this activation of the cascade that results in the accumulation of active dually phosphorylated ERK1/2 (extracellular signal-regulated kinase-1 and -2 = p44 and p42 mitogen-activated protein kinase/MAP kinase) (23).

Apart from PLC-γ, the adaptor protein SHB also binds to Y1175-P of VEGFR-2. However, there does not seem to be a competition between the two adaptor proteins for the same binding site. siRNA knock down of SHB does not alter PLC-γ phosphorylation upon VEGF stimulation (24). The binding of SHB to phospho-Y1175 activates FAK/focal adhesion kinase and PI3-kinase; these kinases initiate signaling pathways that control endothelial cell migration and survival (24,25). The binding of FAK to phosphorylated SHB also leads to cytoskeletal reorganization and migration: inhibition of PI3-kinase by wortmannin which is activated downstream of FAK also prevents VEGF-A induced endothelial cell migration (26). AKT/PKB (protein kinase B) is a kinase that is activated downstream of PI3-kinase via the action of phosphoinositide-dependent kinase (PDK1). In its activated phosphorylated state, AKT phosphorylates and thereby inhibits the pro-apoptotic activity of BAD and of caspase 9;
this shifts the balance in favor of cell survival (25). The cascade comprising PI3-kinase and Akt was also demonstrated to be activated only upon activation of VEGFR-2 using ligands specific for VEGFR-1 and VEGFR-2 (26). Other phosphorylation sites such as Y1214, Y951 have also been reported to control endothelial cell migration and proliferation (14).

1.2 The plasminogen system

Endothelial cells are surrounded by a three dimensional extracellular matrix, which provides structural support as well as a heterogeneous source of biologically active molecules. The process of angiogenesis is an invasive one, in which first the basement membrane has to be breached. The basement membrane is primarily composed of laminin, type IV collagen and fibronectin. Endothelial cells must then degrade the perivascular extracellular matrix composed of proteoglycans, collagen fibers and elastic fibers. Matrix degradation is facilitated by a fine balance between proteases and protease inhibitors. The two main families of proteases involved in angiogenesis are the serine proteases of the plasminogen system and the matrix metalloproteases.

The diverse roles of the plasminogen system from embryogenesis, wound healing, tissue remodeling, angiogenesis to inflammation and cancer is well documented and appreciated. The pivotal step in the plasminogen (fibrinolytic) system is the conversion of plasminogen to plasmin. This is primarily mediated by the two serine proteases - urokinase plasminogen activator (uPA) or tissue plasminogen activator (tPA). While uPA is involved in cell migration and tissue remodeling, tPA is involved with fibrin homeostasis (27). uPA is secreted as a single chain 54 kDa pro-enzyme, referred to as pro-uPA. Pro-uPA can convert plasminogen to plasmin in trace amounts. Plasmin in turn cleaves the K158-I159 peptide bond of pro-uPA, converting it to active uPA (28). This active uPA then accelerates the plasmin generation by 200-fold (29,30). A proteolytic cascade is activated whereby plasmin that is formed degrades fibrin, vitronectin, fibronectin and activates matrix metalloproteases (MMPs) (31) (Figure 2).

Endothelial cells express MMP-1, -2, -3, -9, -14 and their inhibitors, tissue inhibitor of metalloproteinases (TIMP1 and TIMP2) (32). Activated MMP-2 degrades the basement membrane type IV collagen while, MMP-1 cleaves collagen fibrils and MMP-9 degrades proteoglycans and elastins of the perivascular ECM (33). Stimulation of human umbilical vein endothelial cells with VEGF and other growth factors like bFGF, EGF can induce the transcription factor ETS-1, which in turn induces the expression of MMP-1, -3 and -9, TIMP-1 and uPA within 2 hours (34,35). The proteolytic activity of this cascade is subject to inhibition at different levels (Fig. 2): the serpins (serine protease inhibitors) PAI-1 and PAI-2 suppress plasmin formation by inhibiting uPA and tPA, α2-antiplasmin blocks the action of plasmin that was generated in the circulation; TIMPs and α2-antiplasmin preclude the formation of active matrix metalloproteases.
1.3 Structure of uPAR and of uPA

uPA is composed of three domains, the amino terminal epidermal growth factor-like domain, the kringle domain and carboxy terminal protease domain. The epidermal growth factor-like domain and the kringle domain together form the aminoterminal fragment. Both, pro-uPA and active uPA are ligands for the urokinase receptor (uPAR) with a very high binding affinity in the sub nanomolar range ($K_d$ 0.5 nM) (36). Complex formation between pro-uPA or uPA and uPAR on the cell surface enhances plasminogen activation by about 16-fold (28). Thus, uPAR is a key player because it allows to focus plasminogen activation at a given area of the cell surface.

The urokinase receptor uPAR is also referred to as CD87; uPAR is composed of 283 amino acids and has 3 internally homologous domains of approximately 90 amino acids each, which are designated domain-1, -2 and -3 starting from the amino terminal end. The domains are separated by short linker regions. The carboxyl terminal ends with a GPI anchor, which is added to amino acid 283 during translation. Accordingly, uPAR is devoid of any intracellular
domains. Site-directed photoaffinity labeling of uPAR shows that a close spatial proximity between domain-1 and domain-3 of intact uPAR is necessary for binding of uPA. The residues R53 and L66 of domain-1 and H251 of domain-3 must be brought together to form the composite ligand binding site for uPA (39). These biochemical observations were confirmed when the X-ray crystal structure of the soluble form of uPAR was solved in 2005: uPAR was found to be organized as a globular receptor with a deep central cavity (19 Å deep) and a large external surface. The amino terminal fragment of uPA occupies this cavity leaving the entire external surface open for interactions with a variety of other surface proteins; in fact, uPAR is known to form heteromultimeric complexes (40).

uPAR belongs to the Ly-6/uPAR superfamily and is heavily glycosylated with 5 potential N-linked glycosylation sites. The glycan moieties account for nearly 50% of its molecular mass of about 55-60 kDa (37). Except for N52, the other four glycosylation sites are necessary for proper folding and membrane localization of uPAR. Site directed mutagenesis of these four sites results in ER retention and no surface expression of uPAR. In contrast, if glycosylation at N52 is precluded a conformationally stable and active uPAR is expressed on the cell surface. However; due to the location of N52 in the ligand binding domain uPA binding affinity to uPAR is reduced by 4-5 fold (38).

A non-ligand binding variant of uPAR has also been identified on the cell surface. This variant lacks domain 1; uPA and plasmin can cleave cell surface-associated uPAR between domain-1 and domain-2 generating uPAR(D2+D3) (41). Cleavage by uPA can occur at two positions; between R83 and A84 and between R89 and S90 (42). Cleavage at R83/A84 exposes a chemotactic peptide SRSRY (88-92), in the linker region of uPAR. The peptide SRSRY in its soluble form is a potent chemoattractant for monocytic cells. Migration is mediated by the binding of the peptide to the G-protein coupled receptor: low affinity receptor for formyl-peptide (FPRL1), and ensuing p56/p59^{lck} tyrosine phosphorylation (43,152). Cell surface bound uPAR(D2+D3) devoid of domain-1 and hence proteolytic capabilities is still capable of inducing cell migration. This is because fMLP induced chemotaxis requires the interaction of it’s receptor- FPRL1 with the SRSRY peptide region of uPAR(D2+D3) (153).

Intact uPAR may also be released from the cell surface by cleavage of the glycolipid anchor. The resulting soluble form of intact uPAR has also been found in blood sera of healthy people in very low levels (2.71±1.12 µg/l). However patients with malignant cancers have elevated levels of soluble uPAR (5.82±2.27 µg/l) in serum (154). Elevated levels of soluble uPAR are found in blood plasma and tumor tissue of many malignant cancers such as colorectal cancer (44), squamous cell lung carcinoma (45), acute myeloid leukemia (46) and breast cancer
patients (47). These increased levels of soluble uPAR were found to be inversely related to patient survival; hence, cleaved soluble uPAR may be of clinical significance as a prognostic marker. The source for this cleaved soluble uPAR forms in blood plasma is most likely the tumor tissue per se: increased expression of uPAR was observed in metastases; these finding can be rationalized by taking into account that uPAR supports invasive growth of cells and that the metastatic burden is a prognostic determinant in many types of cancer. However, the enzymes responsible for the cleavage of soluble uPAR are not known.

1.4 uPAR in angiogenesis, proteolysis and migration

The presence of the urokinase receptor on cultured endothelial cells was first described in 1990; this discovery provided an explanation for the mechanism by which the soluble proteins uPA and tPA regulated plasminogen activation at the interface between vessel wall and blood or the subendothelium (48). A balloon catheter induced carotid injury model in rats showed very little or no mRNA expression of uPA, tPA or uPAR in the quiescent endothelium but a strong expression of all three plasminogen activator molecules in vivo in the regenerating endothelium at the wound edge (49). A similar expression pattern was also observed in human glioblastoma tumors in vivo. In situ hybridization localized uPAR mRNA to endothelial cells at sites of vascular proliferation in the leading edge of the tumor. With increasing malignancy uPAR expression also increased in the glioblastoma tumor cells. (50) It was later appreciated that blood and lymphatic vascular endothelial cells respond to specific stimulation with angiogenic growth factors like VEGF and bFGF by increased expression of uPA and uPAR, which in turn promoted plasminogen activation (51,52).

A wide variety of human cancers also express elevated levels of uPAR which is indicative of their capacity to invade tissues and to metastasize (53). This observation and the current mechanistic model of the plasminogen activation system predicts that uPAR antagonists – i.e., agents that suppress binding of uPA to uPAR and hence plasmin formation - inhibit tumor angiogenesis and metastasis formation in vivo. Several studies have provided a proof-of-principle by examining the action of uPAR antagonists in tumor models. An antibody that competitively inhibited uPA binding to uPAR substantially reduced bFGF-induced neovascularization and B16 melanoma growth in mice (54). Injection of PC3 prostate cancer cells stably transfected to express a catalytically inactive but receptor bound uPA lead to reduced metastasis compared to wild type PC3 cells in mice (55,56).

The generation of uPAR-deficient mice allowed for examining the function of uPAR in vivo. Surprisingly, homozygous uPAR−/− mice are fertile and viable (57). In vitro studies
demonstrated a delay (3 h) in uPA mediated plasminogen activation of peritoneal macrophages isolated from uPAR\(^{-/-}\) mice. This was attributed to the absence of uPAR-bound uPA on macrophage cell surfaces. uPAR\(^{-/-}\) macrophages were also as efficient as wild type macrophages in matrix degradation (57). Several in vivo studies were carried out with uPAR\(^{-/-}\) mice to examine the role of uPAR in disease models: when assessed three weeks after injury of the arterial wall, the absence of uPAR did not affect arterial neointima formation or smooth muscle cell migration (vascular wound healing) (58,59); it also did not impede fibrin clearance (60) or the formation of new blood vessels, if examined two weeks following injection of malignant keratinocytes (61). The observation of pericellular localization of uPA around uPAR\(^{-/-}\) cells (58) implied that plasminogen activation could take place in a manner independent of binding to uPAR. This combined with defects in fibrin clearance (60) and neointima formation (62,63) in uPA\(^{-/-}\) mice led to conclusion that uPAR seemed to have a redundant role for in vivo uPA-dependent plasminogen activation. However, in vivo, uPAR also supports cellular migration by a mechanism that is not mediated by activation of the proteolytic cascade (May et al. 64). Leukocytes adhere on the vascular endothelium in a manner dependent on β2 integrins. This is an essential step in transendothelial migration. In uPAR\(^{-/-}\) mice, migration of leukocytes to the inflamed peritoneum is significantly impaired compared to the wild-type mice. The impaired migration in uPAR\(^{-/-}\) mice suggests that β2 integrin-dependent leukocyte migration is regulated by uPAR (64). The urokinase receptor was also found to be necessary for neutrophil recruitment in response to pulmonary infection with Pseudomonas aeruginosa, a gram-negative bacterium that elicits a β2 integrin-dependent inflammatory response. While neutrophil recruitment is impaired in mice deficient in uPAR, this is not seen in uPA\(^{-/-}\) mice. This finding is formal proof that the recruitment of neutrophils is independent of uPA (65, 66). The modulation of β2 integrin dependent migration of leukocytes by uPAR can be rationalized by assuming that these two proteins interact directly. This conjecture was verified by immunoaffinity purification: Bohuslav et al used uPA as bait to isolate a large receptor complex comprising uPAR, β2 integrin (LFA-1) and phosphorylated SRC kinase (67). While – as mentioned earlier (see section 1.3) - uPAR lacks an intracellular domain, its large external surface provides many potential binding sites. In fact, uPAR was found to interact with different integrin family members such as β1, β2 and β3 integrins, with the extracellular matrix ligand vitronectin, with GPCRs, with GP130, with mannose-6-phosphate/insulin-like growth factor receptor-2 and also with receptor tyrosine kinases like the PDGF- and the EGF-receptor. These observations supported the development of a concept in which uPAR was viewed as multifunctional receptor capable of influencing cellular events like migration,
proliferation, differentiation and adhesion apart from its cognate downstream proteolytic cascade.

1.5 Integrins

Adhesion molecules mediate the anchorage of cells to the extracellular matrix. The integrin adhesion molecules are found as heterodimers, each consisting of a non-covalently linked α-subunit and β-subunit. Both subunits are type I transmembrane proteins with multiple extracellular domains and a short cytoplasmic tail. The diversity of the family is evident by the fact that there are 18 α-subunits and 8 β-subunits, which can form 25 different heterodimers (Figure 4). Each heterodimer binds to its own specific set of matrix ligands: in addition, multiple integrins share the same matrix ligand. By binding to matrix ligands, integrins relay information on the outside environment through their cytoplasmic tails into the cell. Many adaptor proteins like talin, vinculin and actin-binding proteins connect the cytoplasmic tail of the integrins to the cytoskeleton and to signaling molecules. Integrins exist in a latent form and are subject to activation by signals arising on the cytoplasmic side (a process referred to as “inside-out signaling”). Thus integrins are bidirectional signaling molecules, which mediate cell adhesion and support migration, proliferation, survival and differentiation.

Cell migration is contingent on the regulated association and dissociation of adhesion receptors, i.e., integrins must disengage from ECM contacts at the trailing end and form new focal contacts at the leading edge (68). This requires continuous and vectorial integrin redistribution, which occurs by both, clathrin-dependent and clathrin-independent mechanisms (69). The cytoplasmic domain of β1 integrins contains two conserved NXXY-motifs responsible for recruitment of endocytotic adaptor proteins. Mutation of these motifs prevents clathrin-dependent endocytosis (70). As mentioned above, integrins undergo a transition from a latent form that does not recognize cognate ligands to a binding-competent conformation. To date it is not clear if the active and inactive forms of integrin follow the same route of endocytosis and which regulatory mechanisms define differences in endocytotic routing. This may be due to association with different surface molecules: NRP1, a co-receptor for VEGF and semaphorin 3A, controls endothelial cell adhesion to fibronectin in a manner independent of its ligands. Through its cytoplasmic association with the endocytic adaptor GIPC1, NRP-1 mediates its co-internalization with active α5β1 integrin into RAB5-positive early endosomes. Inactive α5β1 also undergoes endocytosis, albeit independently of NRP-1 (71). Migration also requires formation and disassembly of focal adhesions. In HT1080
fibrosarcoma cells, focal adhesions disassembly requires the internalization of active β1 integrins in a clathrin-dependent manner (72). The transmembrane 4 superfamily member PETA-3/CD151 colocalizes with many integrin chains such as β3, α2, α3, α5, α6 on the cell surface and in endocytic vesicles. Depletion of CD151 impedes endothelial migration and tube formation. Accordingly, CD151 has been proposed to play a major role in regulating integrin trafficking or function (73).

Figure 3. The integrin family.
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1.6 Integrins in angiogenesis

The state of endothelial cells (i.e., quiescent, migrating or proliferating) determines the pattern of integrin expression (see below). The activation of proteases during angiogenesis creates an enormous change to the extracellular matrix. Proteases remodel the ECM to present a promigratory environment which supports cell invasion, migration and survival. VEGF in particular increases vascular permeability (7). Thereby blood-borne provisional ECM proteins like fibrinogen, fibronectin and vitronectin gain access to the perivascular space and generate an ECM conducive to vascular remodelling. The MMP-mediated degradation of ECM is not only restricted to the clearing of the surrounding matrix; the degradation of type IV collagen and laminin exposes proangiogenic cryptic binding sites for integrins. Using cell adhesion and affinity chromatography experiments αvβ3 integrin was found to bind to the RGD sites exposed by denatured but not native type IV collagen (74). In vivo, MMP-2 mediates type IV collagen denaturation to expose these cryptic sites for αvβ3 integrin. These in turn promote angiogenesis and growth of tumour cells (75).

The major integrins expressed on the quiescent vascular endothelium are α1β1, α2β1, α3β1, α5β1, α6β4, α6β1, α8β1, and αvβ5 (76). The majority of these integrins are either collagen- or laminin-binding receptors, i.e., components of the basal basement membrane. Integrins α5β1, α8β1, and αvβ5 are fibronectin-binding integrins. The provisional ECM is formed from the existing ECM by three processes: (i) the partial degradation of the existing ECM, (ii) the resulting exposure of cryptic binding sites in components of the existing ECM and (iii) the deposition of blood-borne soluble matrix ligands. These reactions create an ECM favourable for fibronectin- and vitronectin-binding integrins during angiogenesis and lead to increased expression of integrins α5β1, α8β1, and αvβ5 and de novo expression of integrin αvβ3 (77,78).

Because of their importance in supporting endothelial function, the role of integrins has been extensively studied in angiogenesis using in vivo as well as in vitro systems. Antibodies against αvβ3 and αvβ5 integrin and antagonistic peptides effectively blunt angiogenesis. Specifically, inhibition of αvβ3 integrin blunted bFGF- or TNFα-induced angiogenesis; inhibition of αvβ5 integrin suppressed VEGF-induced angiogenesis in an in vivo rabbit corneal or chick chorioallantoic membrane assay (80). Based on these and related findings, integrins α5β1, αvβ3/αvβ5 are considered as candidate drug targets and their suitability for the treatment of different types of cancer is being explored in clinical trials (79). However, studies on mice, in which integrin subunits were ablated by gene targeting, cast doubt on the viability of this concept: embryonic angiogenesis is not impaired in mice rendered genetically
deficient in integrin subunit β3 or β5 (81,82). In fact, tumor growth and angiogenesis are significantly enhanced upon subcutaneous injection of tumor cells in these mice. Importantly, in β3 integrin-deficient mice, VEGFR-2 expression was enhanced in endothelial cells (83). Eighty percent of the αv integrin-deficient mice die in gestation due to placental defects; the remaining 20%, which are born alive, exhibit intracerebral and intestinal hemorrhages but notably no vascular defects (84). Thus embryonic angiogenesis proceeds normally in the absence of αvβ3 and αvβ5.

The β1 integrin subfamily is essential for early angiogenesis in mice: a deletion of β1 integrin in vascular endothelial cells is embryonically lethal, the mice pups die around embryonic day E9.5-E10.5 with defects in angiogenic sprouting and vessel branching (85). Global deletion of α5 integrin is also embryonically lethal at embryonic day E10-E11: the mice pups with the homozygous deletion displayed mesodermal defects with a distended and leaky vascular system (86). Mice with conditional α5 integrin gene knock out were generated by crossing α5 integrin floxed mice with a Tie2-Cre transgene mouse line expressed in endothelial and hematopoietic cells. However, endothelial knock out of α5 caused no obvious defects in embryonic vasculogenesis or angiogenesis as the mice were viable (87) In contrast, a double knock-out of α5 and αv integrin in endothelial cells causes lethality at around embryonic day E14.5 with extensive defects in remodelling of the heart and vessels (87). The most plausible interpretation of these observation is a model in which embryonic angiogenesis does not rely on a single β1 integrin heterodimer but is rather supported by several integrins that functionally overlap. This redundancy allows for compensation in the absence of a single integrin by related integrin heterodimers or even by other proteins. However, from the perspective of drug development, it would be interesting to know how tumour angiogenesis is affected in mice in which α5-integrins is specifically ablated in the vascular endothelium.

Growth factors and receptor tyrosine kinases can control integrin recycling to regulate cell migration. Stimulation of fibroblasts with PDGF results in increased recycling of αvβ3-integrins through RAB4-positive, early endosome; this promotes cell adhesion and spreading (88). Stimulation of endothelial cells with angiopoietin-2 induces complex formation of its receptor - Tie2 with αvβ3 integrins at cell junctions. Upon this interaction αvβ3 integrin adaptor proteins – talin and p130cas - are dissociated followed by αvβ3 integrin internalization and lysosomal degradation (89). Conversely integrins can influence the recycling of other integrins and receptor tyrosine kinases: αvβ3 integrin is a negative regulator of VEGFR-2 recycling. Inhibition of αvβ3 in vivo leads to increased recycling of VEGFR-2 and thereby promotes angiogenesis (90). This finding also provides a plausible explanation
for the aggravated tumor angiogenesis observed in β3 integrin-deficient mice (83). Thus integrin trafficking is a key modulator that can govern cell migration and signaling.

1.7 Interactions between uPAR and integrins

The consequences of uPAR-integrin interaction have been much under scrutiny since Bohuslav et al purified a complex containing uPAR and β2 integrin (LFA-1) from monocytes (67). Wei et al explored the functional significance for complex formation between integrins and uPAR by using a HEK293 cell line that overexpressed uPAR: uPAR bound to activated β1 integrins, and promoted adhesion and migration on a vitronectin matrix (96). However contradictory observations concerning uPAR and integrin interaction on a vitronectin matrix have been subsequently reported.

Vitronectin, a prototypical protein of the ECM, is a high-affinity ligand of uPAR; this was first suggested by the observation that uPA induced adhesion of anchorage-independent myeloid cell lines to purified vitronectin (91). Related findings were also observed with adherent cells: HEK293 cells over expressing GPI-anchored uPAR but not soluble uPAR strongly adhered to vitronectin in the presence of uPA (92). Using an alanine scanning library of single site uPAR mutants, the functional epitope necessary for interaction with vitronectin was found to be located outside the uPA binding cavity involving residues in domain 1 and residues in the linker peptide connecting domain 1 and 2 (93). Mutation of residues W32 and R91, which are located in domain 1 and 2, respectively, reduces vitronectin affinity five-fold but does not affect binding of uPA. HEK293 cells expressing the mutant uPAR also exhibited reduced adhesion and ERK1/2 phosphorylation when seeded on a vitronectin matrix as opposed to HEK293 cells with uPAR either intact or with mutations in integrin binding sites. Therefore the study claimed that a uPAR-vitronectin interaction is sufficient to induce adhesion and signaling in HEK293 cells (94). If microvascular endothelial cells are plated on vitronectin, its binding to both integrins and uPAR is necessary to localize uPA to the focal adhesions. Treatment of endothelial cells with an inhibitory peptide that disrupts uPAR-integrin interaction before plating on a vitronectin matrix did not affect the localization of uPA to focal adhesions (95). These studies indicate that uPAR and integrins sequentially ligate to vitronectin to mediate adhesion or uPA localization on a vitronectin matrix.

Aguirre Ghiso et al extended the notion that it is the overexpression of uPAR in tumor cells that drives the physical association of uPA, uPAR and α5β1 integrins. This coincides with increased levels of phosphorylated and hence activated FAK, SRC and ERK1/2, which
support sustained tumor growth. In contrast, low levels of uPAR are associated with reduced activation of ERK1/2 and tumor dormancy (97,98). Upon testing CHO cells expressing human α or β integrin subunits for their ability to bind immobilized soluble uPAR, the interaction of uPAR with integrins was not limited to α5β1 but to also encompassed the β3 integrin family, in particular αvβ3, α3β1, α6β1 and α9β1 (99). Indirect immunofluorescence in HT1080 cells showed colocalization between uPAR and alpha integrin subunits confirming the adhesion assay studies. α5 and αv integrin colocalized with uPAR on a fibronectin matrix and α3 and α6 colocalized with uPAR on laminin. Furthermore, FRET studies verified the physical interaction between uPAR and integrins. HT1080 cells labeled with TRITC conjugated uPAR mAb and FITC-conjugated β1 or β3 mAb detected FRET values on a fibronectin, vitronectin or laminin matrix but not on polylysine. There is a critical distance for Förster resonance energy transfer (FRET): for the pair of donor fluorphore FITC and acceptor fluorphore TRITC, this distance is 4.9-5.5 nm. Therefore, uPAR and integrin β1 or -β3 are within 5.5 nm of each other, distances at which proteins can have direct physical contact.

Wei et al showed that soluble uPAR binds to immobilized α3β1 integrin but not α5β1 or α2β1 integrins in the presence of uPA (101). The interaction between uPAR and α3β1 integrin was demonstrated using a 17-mer integrin α3β1 inhibitor peptide (α325). UPA-mediated uPAR binding to α3β1 integrin was inhibited in the presence of α325 peptide in binding assays. In the MDA-MB-231 tumor cell line, which expresses little if any αvβ3, the addition of uPA drives complex formation between uPAR and α3β1. Concomitantly, α5β1 and α2β1 integrins are activated in a Goi protein-dependent manner. This promotes adhesion on fibronectin and collagen. The study demonstrated that uPA and uPAR can preferentially complex with one integrin heterodimer and influence the signaling of other integrin family proteins. However a drawback is the lack of biochemical studies in the MDA-MB-231 tumor cell line ruling out possible uPA mediated uPAR and α5β1 or α2β1 integrin interaction on the cell surface (101).

UPAR is overexpressed in kidney podocytes of patients suffering from progressive kidney diseases. LPS administration leads to proteinuria in wildtype but not uPAR−/− mice. Using a transient in vivo gene delivery system, which restored uPAR expression in uPAR−/− mice, Changli et al demonstrated that uPAR activates αvβ3 integrin in a uPA independent manner to promote podocyte motility and proteinuria (102). Mechanistically, activated integrin αvβ3 and uPAR complexes drive tyrosine phosphorylation of the adaptor protein p130Cas. The adaptor protein CRK binds to the phosphorylated p130Cas through its SH2 domain. Through its SH3 domain, CRK recruits Dock180- a guanine nucleotide exchange factor (GEF). Once the p130Cas-CRK-Dock180 complex is formed, Dock180 activates the Rho GTPase RAC by
catalyzing the exchange of GDP for GTP. Activated RAC accumulates at the cell front promoting actin polymerization and cell migration (103,104). The work summarized above highlighted the importance of complex formation between uPAR and integrin subunits demonstrating that these complexes gave rise to intracellular signals that were translated into cellular responses. Accordingly, these insights stimulated research into the nature of the interaction site. 

Wei et al also identified a peptide of 25 amino acids in a phage display library which inhibited the interaction of uPAR with both, β2 and β1 integrins (96,105). Using CD11b α-integrin chimeras, Simon et al uncovered that the exposed loop in the highly conserved non-I-domain region at the upper surface of the αM chain of β2 integrin interacts with uPAR (105). Interestingly the peptide identified by phage display and the amino acid stretch in the αM (termed M25) chain showed sequence homology. The CD11b interacting region was also compared for their homology with sequences of other integrin α chains. Particularly the corresponding amino acid stretch of integrin α3 and α6 showed 40% sequence similarity; however, only a single amino acid residue is identical, i.e., the histidine residue corresponding to H245 (101,106). The importance of this residue is highlighted by the following findings: epithelial cells deficient in α3 integrins do not form dense clusters in cell culture. This can be restored by reintroducing α3 integrins in these cells (106). In these cells, uPAR engages α3 integrin and this results in activation of ERK1/2 via SRC and the subsequent transcriptional induction of uPA. However, this response is abrogated, if a α3 integrin harboring the point mutation H245A is expressed in α3-deficient epithelial cells (98,107).

Two separate groups worked on elucidating the uPAR interacting region with integrin. Chaurasia et al immobilized synthetic peptides corresponding to regions in uPAR on nitrocellulose membranes and checked for their ability to bind purified α5β1 integrin in a solid phase dot-ELISA assay. A peptide sequence of 9 contiguous amino acids in domain III of uPAR (240-248) bound to α5β1 integrin. This was followed by dot-ELISA of single site soluble uPAR mutants for the various amino acids; revealing a single amino acid – S245 whose mutation completely abrogated α5β1 integrin binding. The introduction of this single mutation in HEP3 cells as opposed to wild-type uPAR did not restore ERK activation or their growth on a chorioallantoic membrane model (108). Wei et al introduced point mutations for charged amino acids into the cDNA encoding uPAR and expressed the resulting mutants in HEK293 cells. These transfected cells were then probed by co-immunoprecipitation for α5β1 and α3β1 integrin binding. This approach identified the single residues H249 and D262 in domain III of uPAR capable of disrupting its interaction with α5β1 and α3β1, respectively (109). HT1080 fibrosarcoma cells and H1299 lung carcinoma cells utilize α5β1 integrins to
adhere on fibronectin. These tumor cell lines were stably silenced of their endogenous uPAR and transfected with either H249 or D262 mutant uPAR. ERK and MMP-9 activation was markedly reduced in tumor cells with the H249 mutation. uPAR-α5β1 integrin complexes engage with the fibronectin matrix triggering a FAK and SRC activation. SRC activates the RAC-1 pathway which leads to induction of ERK activation and MMP-9 production; disruption of the pathway leads to reduced tumor invasion (109). Similarly, H1299 cells with a (H249A-D262A) mutation in uPAR failed to induce tumor growth in an orthotopic lung tumor model (110).

Several proteins have been shown to either positively or negatively regulate the uPAR-integrin interaction and its ensuing effect on cell adhesion and migration. The mannose 6-phosphate/insulin-like growth factor 2 is considered to have tumor suppressing abilities as its absence is recorded in tumor malignancies. Schiller et al described a mechanism whereby mannose 6-phosphate receptor expression promotes uPAR cleavage and down regulates αvβ3 integrin surface expression in human kidney carcinoma cell lines. Abolition of mannose 6-phosphate by RNA interference results in increased uPAR mediated plasminogen activation, αvβ3 mediated cell adhesion and cell invasion. However, the molecular mechanism as to how mannose 6-phosphates regulates uPAR expression is not properly understood (111). ECRG2 - a serine protease inhibitor binds to the kringle domain of uPA without interfering with uPA and uPAR interaction. But the presence of ECRG2 in this complex impairs the interaction of uPAR with α5β1 and α3β1 integrin. Consequently SRC and ERK activation is down regulated with concomitant reduced cell invasion and migration (112). Tumorigenicity of human epidermoid carcinoma (Hep3) cells is driven by uPAR expression. Excess uPAR bridges the association between α5β1 integrin and the receptor tyrosine kinase epidermal growth factor receptor. α5β1 integrin is activated and epidermal growth factor receptor is ligand independently phosphorylated resulting in ERK activation and tumor proliferation (113).

1.8 The LDL-receptor family

LDL-Receptor gene family members are scavenger receptors which transport their bound ligand to lysosomes for degradation. Their extracellular domain consists of epidermal growth factor (EGF) precursor like repeats and complement type cysteine-rich repeats responsible for ligand binding. Depending on the type of the LDL-receptor family member the intracellular domain consists of at least one or three NPXY motifs - responsible for clathrin mediated endocytosis. LDL-Receptors bind to a variety of diverse ligands for eg; proteinases, proteinase inhibitor complexes, lipoproteins, viral proteins or tyrosine kinase receptors.
One of the members of the LDL-receptor family, LDL receptor-related protein (LRP) regulates extracellular proteolysis by mediating the catabolism of uPA-PAI-1 complexes and tPA-PAI-1 complexes. LRP is necessary for embryonic development as gene deletion in mice resulted in developmental arrest at day 13.5 (114). Inflammatory stimuli activate macrophages to migrate in a CD11b dependent manner. Macrophages require tPA, its inhibitor PAI-1 and LRP for the different steps of CD11b dependent migration on fibrin rich provisional matrix. tPA interacts with CD11b and enhances macrophage adhesion on fibrin. tPA is inactivated by PAI-1 which promotes the association of the complex of tPA, PAI-1 and CD11b with LRP. LRP mediated endocytosis of the complex switches the macrophage to a de-adhesive state promoting forward migration (115). Circulating tPA catalyzes plasmin activation which is necessary for dissolving fibrin clots in blood. Hepatic parenchymal cells of the liver are responsible for regulating tPA levels to maintain plasma fibrinolysis. This is achieved by the LRP receptor, which binds and clears tPA/PAI-1 complexes by endocytosis and lysosomal degradation (116). It has to be noted that tPA maintains fibrin homeostasis in the circulating system thereby circumventing cell anchoring for it’s function. Therefore uPAR is not involved in LRP mediated clearance of tPA. Various members of the matrix metalloproteinase family MMP-13, MMP-9 and MMP-2 in complex with their inhibitors TIMP are also endocytotically cleared by LRP (114).

However, the functions of LDL-receptor family members are not limited to endocytosis but they also participate in signaling. An example of LDL-receptor mediated signaling is observed in breast cancer cell lines such as MCF-7 and MDA-MB-435 which express very low-density lipoprotein receptor (VLDLR) instead of LRP (117). Treatment of MCF-7 cells with uPA induces a transient ERK activation, which can be made sustained by the simultaneous addition of PAI-1. PAI-1 mediated sustained ERK activation in turn activates MCF-7 cell proliferation and growth. RAP – a 39 kDa protein is a high affinity ligand for VLDLR and other LDL receptor family members. The addition of RAP converts the uPA and PAI-1 mediated sustained ERK activation to a transient one; demonstrating the presence and requirement of VLDLR for PAI-1 mediated signaling (118). A similar observation was made by us in low PAI-1 expressing HT1080 fibrosarcoma cells. Addition of exogenous PAI-1 induces sustained ERK activation which can be inhibited by the addition of RAP. In addition the role of MAP kinase phosphatases was also investigated as their function is to deactivate MAPK signaling. Indeed, upon PAI-1 treatment MAP kinase phosphatases are down regulated with the concomitant ERK activation leading to increased metastatic potential.
(Mihaly-Bison et al, unpublished). These observations prove that PAI-1 can modulate uPA-uPAR signaling in cohorts with a LDL receptor family member.

LDL receptor family members can also modulate the signaling of tyrosine kinase receptors. Simultaneous stimulation of tyrosine kinases receptor like epidermal growth factor receptor, VEGF-R or FGF-R and LRP transforms the transient ERK activation of the tyrosine kinase receptor into a sustained one. However, the uPAR tri-molecular complex is necessary for sustained ERK activation as it acts as a bridging complex that links together the receptor tyrosine kinase and LRP receptor (119). LRP-1 can modulate the PDGFR-β signaling pathway by acting as a co-receptor. Activated PDGFR-β is endocytosed into early endosomes by LRP-1. Once within the endosomes, PDGFR-β kinase domain catalyzes the tyrosine phosphorylation of NPXY motif in the cytoplasmic domain of LRP-1. Phosphorylated LRP-1 is then necessary to modulate PDGFR-β mediated activation of ERK; as LRP⁻/⁻ mouse embryonic fibroblasts (MEF) exhibit hindered ERK phosphorylation and cell growth upon PDGF stimulation (120,121).

1.9 LDL-Receptor-mediated uPAR interactions.

The principal regulator of plasminogen activation - PAI-1, binds and deactivates active uPA-uPAR complexes on the cell surface. The lack of cysteine residues makes PAI-1 unstable at 37°C rendering it to convert into an inactive form. Therefore in the blood stream active PAI-1 is found bound to the somatomedin B domain (SMB) of vitronectin (123,124). Vitronectin is a major ligand for PAI-1 as it aids in its localization in the extracellular matrix and hence pericellular proteolysis. The binding region for uPAR in vitronectin is also located on the SMB domain of vitronectin (125). Both PAI-1 and uPAR share overlapping residues for their binding to vitronectin; both require the eight cysteine amino acids in the SMB domain. However uPAR in addition also requires the negatively charged Glu23 in the SMB domain; the mutation of which prevents uPAR binding to PAI-1 (125,126). PAI-1 has the upper hand in competing with uPAR for binding to vitronectin as the Kd for PAI-1/Vn is ~0.3nM and the Kd for PAI-1/uPAR is ~10nM. On a vitronectin matrix the balance between PAI-1 and uPA-uPAR govern cell adhesion and detachment. When uPA is present in excess of PAI-1, uPA governs uPAR mediated cell attachment to vitronectin. If PAI-1 is in excess it competes with uPAR for vitronectin binding prompting cell adhesion. Integrins bind to the RGD domain of vitronectin which is adjacent to the SMB domain. Deng et al further explained that PAI-1 hindered not only uPAR binding to vitronectin but also integrin binding to the RGD domain.
promoting cell detachment particularly in U937, HeLa, MCF7 and HT1080 cells (124, 126, 127).

Czekay et al provided a new light on the PAI-1 mediated cell detachment mechanism. According to them PAI-1 competes with uPAR; but rather than binding to vitronectin binds to uPA. When PAI-1 binds to the uPA, uPAR and integrin complex; integrins are inactivated and uPAR is disengaged from vitronectin. This is followed by internalization of the whole complex mediated by LDL receptor related protein (LRP) leading to cell de-adhesion. By this mechanism PAI-1 can detach cells from matrices such as fibronectin and collagen too, provided there is a high pool of uPA-uPAR-integrin complexes on the cell surface. If the ratio of matrix ligated integrins are high compared to matrix ligated integrin uPAR complexes - such as in cell lines with very little uPAR (MCF-7) - the de-adhesive effect of PAI-1 is diminished (128,129). Thus PAI-1 regulates cells adhesion independent of regulating plasmin production.

Czekay et al hypothesize that PAI-1 interaction with uPA bound uPAR inactivates and may also cause a conformational change to uPAR. This seems to trigger the deactivation of the integrin. The studies on integrin conformation changes upon uPAR interaction are limiting. uPAR on interaction with integrins modulates integrin signaling to cause cytoskeletal changes and promote cell migration (96-106). Therefore integrins interacting with uPAR alone or the uPA and uPAR complex must be in the active conformation. But in studies conducted by Wei et al activation specific integrin antibodies could not recognize α5β1 in complex with uPAR. Wei et al proposed a notion that it was difficult to envisage the activated extended conformation of α5β1 interacting with the relatively small uPAR. Therefore α5β1 adopts a modified “bent” but still active conformation not recognized by activation specific integrin antibodies on HT1080 fibrosarcoma cells (130).

The presence of the uPA-PAI-1 complex and not uPA alone initiates uPAR internalization. Mouse uPA cannot bind to human uPAR. By exploiting this species specificity Olson et al demonstrated that mouse LB6 cells can internalize human uPA-PAI-1 complex only if they are transfected with human uPAR. (131,132). Treatment of cells with PIPLC which cleaves all GPI-anchored proteins including uPAR or competitive treatment with amino-terminal fragment of uPA which cannot bind PAI-1 blocked internalization (131). Nykjaer et al demonstrated that the internalization of the tri-molecular complex is mediated by the endocytic receptor; LRP-1. As it was the addition of PAI-1 complex that triggered LRP-1 to initiate internalization, it was presumed PAI-1 would have the binding site for LRP (133,134). PAI-1 complexed with different proteinases such as high or low molecular weight uPA or trypsin was tested for LRP dependent endocytosis in MEF cells. Irrespective of the proteinase
the presence of PAI-1 was necessary to initiate endocytosis. Hence PAI-1 was proposed to undergo a conformational change when bound to a proteinase and in effect reveal a high affinity binding site for LRP. Utilizing mutational studies this binding site was uncovered to locate in the heparin binding domain at R76 (135). However Czekay et al showed that pretreatment with purified domain III of uPAR inhibits the internalization of the tri-molecular complex; suggesting a direct binding of uPAR to LRP (136). The tri-molecular complex is internalized by LRP via clathrin coated vesicles into early endosomes (136). uPA and PAI-1 are degraded in the lysosomes and fresh uPAR is recycled to the leading edge of the cell surface (137). It is necessary to maintain this cycle of internalization and recycling in order to regulate plasmin activity, cell migration and invasion. This is so, as domain III of uPAR which hindered uPAR tri molecular complex internalization inhibited HT1080 fibrosarcoma cell migration on matrigel (136).
1.10 Rationale of the work

We have previously shown that the angiogenic response of endothelial cells to VEGF depends on the urokinase receptor. Stimulation of endothelial cells with VEGF induces rapid activation of pro-uPA bound uPAR within minutes (138). Pro-uPA activation is dependent on the VEGF-receptor-2 mediated PI3kinase signalling pathway. The PI3kinase signalling pathway is necessary for pro-MMP2 activation which in turn accelerates the conversion of pro-uPA to uPA. In order to regulate the fibrinolytic activity PAI-1 binds to the active uPA:uPAR complexes. This triggers internalization of the trimolecular complex mediated by the VLDL Receptor (131,132). Vascular cells express the VLDL Receptor and not LRP of the LDL-Receptor family (139). uPA:PAI:1 are degraded while uPAR receptor is redistributed to the focal adhesions. We have also shown that treatment with LRP abrogates VEGF induced internalization of uPAR migration on a vitronectin matrix. Importantly, treatment with RAP also significantly reduces VEGF induced \textit{in vivo} angiogenesis on a matrigel model (139). This observation suggested that VEGF relied – at least in part – on uPAR to induce angiogenesis. Here we addressed the question, how uPAR was necessary for VEGF-induced endothelial migration on fibronectin and other typical extracellular matrix components that are not ligands for uPAR. That integrins are principal modulators of cell migration and the vast literature on integrin-uPAR interactions is already discussed here. Therefore, we initiated this work by examining for changes in integrins, uPAR-integrin interactions, and their functional influence under VEGF stimulation in endothelial cells.
2. MATERIALS AND METHODS

2.1 Reagents and Antibodies

VEGF\textsubscript{165} was from Promocell GmbH (Heidelberg, Germany); VEGF-E and PIGF were from RELIATech (Wolfenbüttel, Germany); recombinant mouse VEGF\textsubscript{164} and (2R)-2-[(4-biphenylylsulfonyl)amino]-3-phenylpropionic acid (matrix metalloprotease-2/-9 = MMP-2/MMP-9 inhibitor) from Merck (Darmstadt, Germany); wortmannin was from Sigma Chemicals (St. Louis, MO). The rat receptor-associated protein (RAP, 39kD Protein) was made as a recombinant fusion protein with glutathione S-transferase as previously described (21). The synthetic human uPAR inhibitory peptides were from Genosphere Biotechnologies (Paris, France). The sequences of the peptides are: peptide 243-251 (TASMCQHAH), Control Peptide 240-247 (GCATASMCQ) and scrambled peptide 244-252 (SCAAMHQHT). The murine uPAR- derived peptide m.P243-251 (TASWCQGSH) was purchased from piChem (Graz, Austria). Matrigel solution was from Becton-Dickinson (San Jose, CA). DIVAA kit was purchased from Trevigen (Gaithersburg, MD).

Antibodies: uPAR - 3937 monoclonal was from American Diagnostica (Greenwich, CT); the anti uPAR monoclonal antibody R2 was a kind gift of Gunilla Hoyer-Hansen (Finsen Laboratory, Copenhagen, Denmark) (41); the P4C10 mouse monoclonal antibody against β1 integrin was from Sigma (St.Louis, MO) a biotinylated version (MEM-101A) was from LifeSpan Biosciences (Seattle, WA); alternatively we also used the I2G10 from Novus Biologicals (Littleton, CO). The rat monoclonal antibody against mouse integrin subunit beta-1 was from Becton Dickinson GmbH (Heidelberg, Germany).

The antibodies P1D6 and P1B5 against the α5 integrin and α3 integrin subunits were also from Chemicon International; a biotinylated version against the integrin α5 subunit (X-6) was from Acris (Rancho Santa Margarita, CA). The Clathrin heavy chain rabbit monoclonal antibody and rabbit polyclonal antibody against P-FAK (Tyr397) was from Cell Signaling Technology (Beverly, MA). The rabbit polyclonal antibody against Beta3 was from Millipore (Billerica, MA) and FAK rabbit polyclonal antibody was from Calbiochem (Darmstadt, Germany). The biotinylated anti mouse antibody from Sigma (St.Louis, MO) and rat monoclonal antibody against mouse CD31 antibody from Dianova (Hamburg, Germany).

Rabbit antiserum 7 raised against amino acids 8 to 23 of the G protein β1/β2-subunits was used as loading control for surface biotinylation experiments (141). Alexa Fluor 488 or 568-conjugated anti-mouse or anti-rabbit IgG antibodies and the Zenon One Mouse IgG1 Labeling Kit were purchased from Molecular Probes (Leiden, The Netherlands). Alexa Fluor 488
conjugated Fab-fragments (Zenon kit) were from Molecular Probes (Leiden, The Netherlands); Streptavidin 647 from Molecular Probes (Leiden, The Netherlands). Goat serum was from (Dako Diagnostics, Vienna, Austria).
M199 (Sigma-Aldrich, St. Louis, MO), Bovine endothelial cell growth supplement (Technoclone, Vienna, Austria), Dulbecco modified Eagle medium (Invitrogen, Carlsbad, CA), heparin (PromoCell; Heidelberg, Germany), Triton X-100 (Sigma, MO), sulfo-NHS-LC-biotin (Pierce, Rockford, IL), Complete™ protease inhibitors, Roche Diagnostics GmbH, Vienna, Austria), streptavidin-coated Sepharose 4B beads (Zymed Laboratories, San Francisco, CA), Femto-ECL reagents for enhanced chemiluminescence from Pierce (Rockford, IL), Adhesive silicone masks from (Secure Seal, Grace Biolabs, Germany), Transwell™ inserts from Corning LifeSciences (Lowell, MA).

2.2 Cell Culture

HUVECs (human umbilical vein endothelial cells) of up to passage 5 were used for all experiments. The cultures were maintained in M199 (Sigma M5017) supplemented with 20% FBS, 3 mg/ml ECGS and 22.5 mg/ml heparin (PromoCell C-30140, Germany). Cells were maintained at at 37°C with 5% CO2. Experiments were performed using subconfluent cultures, which were rendered quiescent by incubation for 24 hr in medium containing 5% FCS, followed by serum-deprivation for additional 4 h in M199 containing 1% BSA.

2.3 Cytofluorimetric Analysis

Monolayers of subconfluent endothelial cells were treated as indicated in the various experiments with growth factors (VEGF165, VEGF-E) for the appropriate time at 37°C. Following stimulation, the cells were transferred to ice, washed once with ice-cold PBS, and harvested with 3 mM EDTA. The harvested cells were fixed with 4% paraformaldehyde (Sigma, MO) for 15 min, and – in those instances where surface-bound and total levels of integrins were assessed - aliquots were permeabilized with 0.2% Triton X-100 (Sigma,MO) for 10 min. Non-specific binding sites were blocked with 2% goat serum (Dako) followed by incubation with the appropriate primary antibody for 60 min at room temperature. After a PBS wash to remove unbound antibodies, the samples were incubated with an Alexa Fluor 488-conjugated (H+L) secondary antibody (Invitrogen, CA), washed and analyzed with FACSsort (Becton-Dickinson). For the cell surface α integrin screening, cells were treated as above, and stained using primary antibodies from alpha integrin IHC kit. In the case of experiments using inhibitors or peptides, the cells were pretreated with the appropriate
amounts for 10 min prior to stimulation. For the PI-PLC treatment, endothelial cells were treated either with 5U ml⁻¹ PI-PLC at 37°C for 15 min or with buffer as control. After treatment the supernatants were removed and cells were gently washed with M199 containing 10 mg ml⁻¹ BSA.

2.4 Surface Biotinylation and Western Blotting

For surface biotinylation experiments, cells were grown in 100 cm² dishes and serum starved as above. Either before or after stimulation with VEGF, cells were washed three times with ice-cold PBS (pH 8) and incubated with 2 mM Sulfo-NHS-LC-Biotin (Pierce, IL) for 30 min on ice to biotinylate surface proteins. The reaction was quenched by the addition of an ice-cold solution containing 200 mM glycine in PBS (pH 7.0) to consume excess biotinylation reagent followed by two PBS washes. Subsequently, the cells were scraped off in 1 ml of prechilled PBS containing a cocktail of protease inhibitors (Complete, Roche), and briefly spun at 4°C. The pellets were then lysed in RIPA Buffer containing 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 1% deoxycholate, 0.15M NaCl and 0.1M Tris-HCl ph 7.6 and protease inhibitors for 30 min on ice. The cell extracts were centrifuged at 13,000 rpm for 10 min at 4°C, and clear supernatants were collected. The biotinylated surface proteins were adsorbed from the lysates by overnight incubation with Sepharose 4B Streptavidin beads (Zymed Laboratories, CA). The beads were washed three times with RIPA buffer and the adsorbed material eluted by boiling in non-reducing sample buffer. Proteins were separated using SDS polyacrylamide gels, transferred to PVDF membrane and probed with relevant antibodies—mouse monoclonal (R2) for uPAR and mouse monoclonal (I2G10) for integrin β1 subunit. Immunoreactive bands were detected by enhanced chemiluminescence according to manufacturer’s instructions (Pierce, IL).

For analysis of uPAR cleavage experiments, endothelial cells were seeded at a density of 9x10⁵ in 6 well plates and grown to confluence. Following serum starvation as above, samples were stimulated with either VEGF₁₆₅ (50ng/ml) or PMA (100nM) for the indicated time periods. In some cases, protease inhibitor cocktail (Complete, Roche) was also added. Following stimulation, samples were transferred to ice and washed once with cold PBS. Cells were lysed using lysis buffer containing 4% SDS and 10% β-mercaptoethanol. ~20µg protein per sample was separated using SDS polyacrylamide gels and probed for uPAR as described above.
2.5 siRNA treatment of endothelial cells

The predesigned uPAR siRNA and the scrambled siRNA was from Ambion (LifeTech, Austria). Endothelial cells were transfected with siRNA at a concentration of 100nM using X-tremeGENE siRNA transfection reagent from Roche, (Diagnostics GmbH, Vienna, Austria) in serum free medium according the manufacturer’s instructions. 36 hours after transfection, cells were starved and subjected for FACS analysis as above.

2.6 Immunocytochemistry

Serum-starved endothelial cells seeded on gelatin-coated cover slips were stimulated as described. Following stimulation with VEGF, cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton-X for 10 min and blocked with 5% goat serum in primary antibody dilution buffer (DAKO) for 60 min at room temperature. The samples were incubated with the appropriate primary antibodies in 2% goat serum overnight at 4°C. After washing, cells were incubated for 45 min with secondary antibodies conjugated to appropriate fluorophores. Following washes, samples were mounted in Vectashield (Vector Laboratories; Burlingame, CA). The primary antibodies used were mouse anti-human uPAR antibody (3937) and rat anti-human β1 integrin antibody (Mab13). Secondary antibodies were goat anti-mouse Alexa Flour 568 and goat anti-rat Alexa Flour 488 (1:250) respectively. The images were captured using a Zeiss Axiovert 200 LSM 500 microscope at 60-fold magnification. Colocalization was quantified after image thresholding and background substraction using the colocalization threshold plugin of ImageJ program. Twenty to 25 individual cells were analyzed.

2.7 Micropatterning

The micropatterning experiment was done as described previously by Schwarzenbacher et al. (142) with modifications. Poly (dimethylsiloxane) stamps containing the microarray (square of size and depth 3 µm) were generated by standard photolithography. Stamps were rinsed with 100% ethanol and distilled H2O, dried under as tream of N2, and incubated with 100 mg/ml Cy5 labelled BSA for 30 min at room temperature. The stamps were then extensively washed with PBS and distilled H2O and dried under N2. The stamps were then placed under their own weight onto epoxy-derivatized glass cover slips for 30 min. Upon removing the stamps, the cover lips were sealed with adhesive silicone masks (Secure Seal, Grace Biolabs, Germany). The Cy5-labelled BSA micropatterned glass cover slips were then incubated for 1 h at room temperature with 50 µg/ml streptavidin dissolved in PBS, rinsed with PBS and
finally incubated with 10 µg/ml biotinylated monoclonal antibody. BSA efficiently blocks unspecific adsorption of both streptavidin and mAB, thereby providing a well-defined 3 µm micro pattern. Serum-starved endothelial cells were incubated with or without VEGF\textsubscript{165} were seeded on micropatterns and allowed to adhere. Following stimulation for 60 min, the cells were fixed with paraformaldehyde at a final concentration of 4%. The samples were stained for uPAR with the monoclonal mouse 3937 antibody (prelabeled with Alexa Fluor 488-conjugated Fab-fragments (Zenon kit, Molecular Probes) for 60 min. Images were acquired using an epi-fluorescence microscope (Axiovert 200/M, Zeiss, Germany) in the total internal reflection (TIR) configuration using an oil immersion objective (100-fold magnification). Images were analyzed using a program that allowed for semi-automated image and data analysis that accomplished the following tasks: an automatic gridding algorithm calculated the grid-size and the rotation angle \( \varphi \) of the image. The grid subdivided the total image into adjacent squares, which were quantified according to the average specific signal with a central circle (\( F^+ \)) and unspecific background outside the circle. (\( F^- \)). This information was used to calculate fluorescence (F) and contrast (C).

2.8 Transwell Migration Assay

Transwell inserts (8 µm pore, Costar, Inc.) were coated with 5 µg/ml fibronectin for 1 h and were blocked with 2% bovine serum albumin in phosphate-buffered saline for 1 h. Serum starved endothelial cells (25,000) were added to the upper chamber with or without the inhibitory peptides (25 µM) or RAP (40µg/ml) and allowed to migrate for 4 h at 37 °C. The cells that had not migrated were then removed from the upper chamber by wiping the upper surface with an absorbent tip. Cells that had migrated to the lower side of the Transwell insert were then fixed for 15 min with 4% paraformaldehyde and stained with DAPI 1:1000 (Pierce) for 15 min. After extensive PBS washing, the number of cells that had migrated was counted by using fluorescence microscopy with a 10× objective of an Olympus AX70 microscope. The assay was performed in triplicates as data is expressed as percentage of migration.

2.9 DIVAA \textit{in vivo} angiogenesis assay

For the DIVAA \textit{in vivo} angiogenesis assay, angioreactors – (silicon cylinders of 1.0 cm length and 0.15 cm diameter) were filled with basement membrane extract either alone or containing 2 µg/ml VEGF\textsubscript{164} and 1mg/ml heparin with or without 250µM of the mouse inhibitory peptide at 4°C. 6-8 week old BL6 mice were anaesthetized with ketamin (100mg/kg) and xylazin (10mg/kg). A reactor each was subcutaneously implanted on either sides of the flank of the
mice. The reactors were excised out on the 11th day after sacrificing the mice and frozen in liqN2. Sections were stained with rat anti-mouse CD31 antibody and Hoechst. Tissue samples were visualized with an AX-70 Olympus microscope, photographed using an OPtronics DEI-750D CCD camera and analysed using the Cell® imaging software (Olympus).

2.10 Statistics

Statistically significant differences were assessed by using Student’s t test for paired observations. Two or more groups were compared with the control group by ANOVA followed by Dunnett’s test for multiple comparisons as post hoc test.
3. RESULTS

3.1 Internalization of uPAR and β1 integrin upon VEGF stimulation.

We first examined VEGF induced changes of β1 integrins in endothelial cells. We focused on the β1 subfamily of integrins, because members of this sub-family are considered to be the adhesion molecules utilized in the unstimulated resting state of endothelial cells. Resting endothelial cells were stimulated with VEGF_{165} for 30 min and immunostained for the β1 integrin subunit. In resting endothelial cells β1 integrin subunit was found mainly at the cell periphery: the immunoreactivity outlined the cell borders (Figure 4A, control). In contrast, in VEGF_{165}-stimulated endothelial cells immunostaining at the cell borders declined and increased in the vicinity of the nuclei (Fig. 4A, VEGF). This shift suggested that VEGF caused internalization of integrins. We verified this by two methods: flow cytofluorometric analysis and cell surface biotinylation.

Serum starved endothelial cells were stimulated with or without VEGF_{165} and analyzed for surface expression by flow cytofluorometric analysis. The surface expression of integrin β1 subunit decreased significantly by ~40% following stimulation with VEGF_{165} for 60 min (Figure 4B). uPAR internalization was confirmed in parallel. uPAR surface expression also decreased by ~35% as reported previously by Prager et al (140). VEGF_{165} dose response curve for integrin β1 subunit internalization shows maximum response at 50 ng/ml (Figure 4C). The biotinylated surface proteins were enriched by affinity purification on streptavidin-coated beads and immunoblotted for both β1 integrin subunit and uPAR. When surface biotinylation was carried out prior to stimulation with VEGF (Figure 4D), the amount of biotinylated receptors recovered from the whole cell lysates did not differ between stimulated or unstimulated cells. This indicates that proteins are not degraded or cleaved from the cell surface during the stimulation time period. However, if biotinylation of surface proteins was done after VEGF stimulation the stimulated cells had approximately ~60% and ~50% of biotinylated β1 integrin and uPAR respectively on their cell surface compared to the unstimulated cells. This reflected the reduction in surface receptor levels of uPAR and of β1 integrins upon VEGF_{165} stimulation of endothelial cells observed before by flow cytometry.
Figure 4A

Figure 4B

Figure 4C
Figure 4. VEGF stimulation induces internalization of β1 integrins in endothelial cells. (A) Serum starved endothelial cells were stimulated for 30 min with VEGF \(_{165}\) (50ng/ml). Samples were fixed, permeabilized and stained for integrin β1 subunit (green). Confocal image were taken at 60x magnification. scale bar 10 µm (B) VEGF \(_{165}\) induces internalization of uPAR and β1 integrins. Representative immuno-cytofluorimetric histograms of endothelial cells, stimulated with or without VEGF \(_{165}\) (50ng/ml) are presented. Upon stimulation under serum starved conditions for 60 min at 37°C, the cells were transferred to ice and harvested with EDTA. The samples were fixed and analyzed for integrin β1 subunit or uPAR surface expression. VEGF stimulated sample-red histogram, Control sample-black histogram, No staining-grey histogram. (C) Dose response curve for VEGF-induced integrin subunit-β1 internalization. Endothelial cells were stimulated with VEGF \(_{165}\) (1-50 ng/ml) for 60 min. Samples were analyzed for integrin subunit β1 surface expression by flow cytometry as above. Line graph summarizes data from 3 experiments. Mean ± SEM, **p< 0.01 (D) Cell surface proteins were biotinylated with 2 mM Sulfo-NHS-LC-Biotin either before or after VEGF \(_{165}\) stimulation for 60 min at 37°C. The labeled surface proteins were enriched by streptavidin affinity purification from the lysates and probed for the integrin β1 subunit (12G10 antibody) and uPAR (R2 antibody) by Western blotting. Loading control: G protein β-subunit. Blots from a typical experiment are shown (n=3). The percentage of biotinylated surface protein on stimulated cells compared to the unstimulated control was quantified from the densitometric values for both uPAR and β1-integrin. Mean ± SD, **p< 0.01
3.2 uPAR is not cleaved during short term VEGF stimulation.

VEGF induces activation of metalloproteases that can cause cleavage of surface molecules (34,35). Proteases and enzymes can either cleave the intact uPAR or only the Domain 1 leaving behind uPAR(D2+D3) (41,44). We checked if stimulation of endothelial cells with VEGF could induce cleavage or shedding. Endothelial cells were stimulated with either VEGF₁₆₅ or phorbol-12-myristate-13-acetate (PMA) for different time periods, and analyzed for total uPAR content by western blot. By using an antibody that detects both forms of uPAR, we observed that intact uPAR levels did not decline but modestly increased over several hours (12 h) in VEGF-stimulated endothelial cells. In the presence of the PMA (phorbol-12-myristate-13-acetate), which was used as positive control, D2D3 fragments accumulated already after 6 h In contrast, VEGF-induced cleavage was modest after 6 h and increased up to 36 h (Figure 5A). We also checked if this increase in uPAR(D2+D3) fragments is due to the activity of proteases present in the culture medium. It was so; as pretreatment with a cocktail of protease inhibitors inhibited the formation of uPAR(D2+D3) fragments (Figure 5B). Thus, short term-stimulation with VEGF (60 min) caused a decline in cell surface levels of both, uPAR and β₁ integrins that was not accounted for by proteolytic degradation but by internalization.

Figure 5A
Figure 5B

**Figure 5. uPAR is not cleaved during short term VEGF stimulation.** (A) Serum starved endothelial cells were stimulated with either VEGF (50ng/ml) or PMA (100 nM) for the indicated time periods. Thereafter, the cells were lysed and probed for uPAR using the R2 antibody which detects both the full length and cleaved forms of uPAR by western blotting. Loading control: Actin. Blots from a typical experiment are shown (n=3). Graphs are plotted from the densitometric values after normalization with actin (ratio of uPAR level to actin; as compared to the control). Mean ± SE. (B) Cleavage of uPAR due to long term VEGF stimulation is protease dependent. Serum starved endothelial cells were stimulated with VEGF (50ng/ml) with or without protease inhibitors for 18 h. Thereafter, the cells were lysed and subjected to western blot as above. (n=3) Mean ± SD, **p< 0.01
3.3 uPAR and β1 integrin interact upon VEGF stimulation.

With both uPAR and β1 integrin internalizing at a similar time interval upon VEGF stimulation, we explored for a possible interaction between these two proteins at the cell surface. We used the technique of micropatterning, which was devised to assess protein-protein interactions at the cell surface, as an independent approach to demonstrate the interaction between uPAR and integrins in VEGF-stimulated endothelial cells (142). In this technique grids of BSA-Cy5 are printed on functionalized glass coverslips, and the interspaces are filled with streptavidin and biotinylated antibody. We used a non functional biotinylated antibody to the integrin subunit β1. Accordingly, β1 integrins on the attaching and spreading cells, were captured at these spots of immobilized antibody. After fixation, the samples were immuno-stained for uPAR. In unstimulated cells uPAR was distributed homogeneously with occasional punctuate staining (Figure 6Aa). In contrast, upon VEGF-stimulation of endothelial cells, uPAR was redistributed to the pattern defined by the immobilized integrin antibody (Figure 6Ac). This patterning documents the interaction between the two cell surface receptors in living cells. The quantitative analysis for the single spot fluorescence yielded a mean contrast of 0.35 for stimulated cells (Figure 6Ad) and a mean contrast 0.07 for unstimulated cells (Figure 6Ac). This higher mean contrast between areas of “antibody-spots” and the background “BSA-grid” provides a quantitative readout for the extent to which uPAR became enriched in areas of immobilized integrins. In contrast, if an activating antibody against integrin-subunit β1 was used as bait, we did not observe any association between uPAR and β1 integrins upon VEGF stimulation (Fig. 6B). These observations are consistent with our hypothesis that the uPAR tri molecular complex interacts with inactive integrins upon VEGF stimulation.

We also tested if PECAM1 (CD31) and VCAM1 (CD106) - two adhesion molecules show redistribution patterns on a β1 integrin micropattern surface. Both PECAM1 and VCAM1 have no reported interaction with integrin β1 (Figure 6C,D). Endothelial cells grown on β1 integrin micropattern surfaces were stimulated with VEGF, fixed and immunostained for either PECAM1 or VCAM1. No difference was observed in the distribution pattern of either of these two proteins between non-stimulated or stimulated cells.
Figure 6. VEGF-induced interaction of uPAR and β1 integrins. (A) Cell surface interaction between uPAR and β1 integrins assessed by micropatterning. Endothelial cells were grown on functionalized glass coverslips, with grids of BSA-Cy5 printed on them and interspaces filled with streptavidin and biotinylated monoclonal antibody against integrin subunit β1. Upon stimulation with VEGF$_{165}$ for 60 min, samples were fixed and immuno-stained with uPAR monoclonal antibody 3937 pre-labeled with Zenon Alexa Fluor 488 Fab fragments. Representative TIRF images were taken using an Axiovert 200/M microscope (100x objective). uPAR is seen redistributed to the β1 antibody pattern, indicating interaction upon VEGF stimulation. Statistical analysis for the single spot fluorescence contrast C vs. brightness F of multiple cells (n= 64) is represented in a color density plot (b,d)...

(B) Endothelial cells were grown on an activating β1 integrin antibody patterned surface and treated as in (A). No interaction was observed with uPAR upon VEGF stimulation. (C&D) Micropatterning analysis on glass coverslips patterned with either monoclonal antibody against VCAM1 (C) or PECAM1 (D). Following stimulation of endothelial cells seeded on the patterns with VEGF$_{165}$ (50ng/ml) for 60 min, samples were fixed, immunostained for uPAR and TIRF images taken as above. Scale bar 6 µm

3.4 uPAR and β1 integrin co-internalize in endocytic vesicles upon VEGF stimulation.

Based on the above observations, we surmised that uPAR and integrins co-internalize upon VEGF-stimulation. We employed confocal immunostaining to verify it was so. In unstimulated resting cells, uPAR is distributed homogenously. Upon VEGF$_{165}$ stimulation uPAR is redistributed and concentrated to newly formed focal adhesions (Figure 7A). VEGF stimulated endothelial cells were next immunostained for uPAR, integrin subunit β1 and β3.

In unstimulated endothelial cells β1 integrin was mainly found at the cell-matrix border, β3 in the perinuclear compartment and uPAR was distributed in a distinct, but diffuse pattern (Figure 7A, control). Treatment with VEGF$_{165}$ led to the redistribution of uPAR to the newly formed focal adhesions where it co-localized with β3 integrins (Figure 7B, VEGF, pink arrows). Interestingly, uPAR was also found together with β1 integrins in vesicle-like structures within the cytoplasmic compartment (Figure 7B, yellow arrows). This observation confirmed their co-internalization into the same endocytotic vesicles. Integrin and uPAR redistribution was not observed, if endothelial cells were incubated with RAP prior to stimulation with VEGF (Figure 7B, VEGF/RAP). This indicated that an LDL-receptor like molecule mediated the co-internalization of uPAR and β1 integrin. We also confirmed the above observations in polarized cells. A scratch wound was made on a confluent resting monolayer of endothelial cells. Cells were allowed to migrate into the scratch overnight thus
becoming polarized. Following VEGF stimulation samples were immuno-stained for uPAR, β1 and β3. The VEGF effect seen in polarized cells were reminiscent of resting endothelial cells. Focal adhesions positive for either uPAR or β3 integrin was observed in the unstimulated polarized cells. uPAR and β1 integrin colocalized in vesicle like structures (Figure 7C, white arrows) upon VEGF stimulation. Pretreatment with RAP inhibits the uPAR - β1 integrin colocalization. Integrins in the active state convey intracellular signals through the adaptor proteins like FAK. The phosphorylation state of the associated FAK can convey the activation state of the integrin. Therefore we immunostained endothelial cells stimulated with or without VEGF for uPAR, pFAK and integrin β1 subunit. In unstimulated cells pFAK colocalised with β1 integrin in point contacts (Figure 7D, blue arrows) and in occasional focal adhesions (Figure 7D, blue arrow head). Thus β1 integrins are the principal integrins which forms focal contacts in the resting state. Upon VEGF-stimulation colocalization of β1 integrins and phosphorylated FAK was reduced. VEGF promoted co-localization of uPAR and β1 integrins in vesicular structures (Figure 7D, orange arrows) and uPAR-pFAK colocalization in focal adhesions (Figure 7D, pink arrows). The reduction of phosphorylated FAK associated with β1 integrin verifies our hypothesis that VEGF stimulation inactivates β1 integrin; which leads to its internalization coupled with uPAR.

Figure 7A
Figure 7B

Figure 7C

Figure 7D
Figure 7. (A) uPAR is redistributed to focal adhesions upon VEGF stimulation. Serum starved endothelial cells were stimulated with VEGF$_{165}$ (50ng/ml) for 60 min. Samples were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton-X for 10 min. Following blocking with 5% goat serum for 60 min, samples were immunostained for uPAR (red) and confocal images taken using LSM-510 microscope at 100x magnification. Scale Bar 10 µm (B) Serum starved endothelial cells were stimulated with VEGF$_{165}$ (50ng/ml) for 60 min fixed and permeabilized. Sample were immunostained for uPAR (red) and β1 integrin (green) and β3 integrin (blue). Scale Bar 5 µm. Upon VEGF stimulation uPAR and β1 integrin colocalize in vesicle like structures (yellow arrows), while β3 integrin and uPAR colocalize in focal adhesions (pink arrows). Pretreatment with RAP prevents the VEGF$_{165}$ induced colocalization of uPAR and integrins. Scale Bar 5µm (C) Polarized endothelial cells were stimulated with VEGF$_{165}$ (50ng/ml) for 60 min and treated as in (B). uPAR and β1 integrin are colocalized upon VEGF stimulation (white arrows). Scale Bars 10 and 2,5 µm (D) Endothelial cells were stimulated with VEGF$_{165}$ (50ng/ml) for 60 min and immunostained for uPAR (red) and β1 integrin (green) and pFAK (blue). Unstimulated cells show colocalization of β1 integrin subunit and pFAK in focal adhesions and contacts (blue arrows). After VEGF stimulation, β1 integrin and uPAR co-localize in vesicles (yellow arrow), while uPAR was also found in focal adhesions colocalizing with pFAK (pink arrow). scale bar 10 µm

3.5 VEGF induced PI3-kinase pathway modulates β1 integrin internalization.

We had reported that uPAR internalization upon VEGF stimulation was induced specifically through VEGF Receptor-2 (140). We verified if β1 integrin also responded similarly to VEGF receptor-2 stimulation. Serum starved endothelial cells were stimulated with VEGF-E, a ligand specific for VEGF receptor-2 or VEGF$_{165}$ a ligand which binds to all three VEGF Receptors. Analysis for cell surface expression by flow cytometry shows that following stimulation for 60 min, β1 integrin showed reduced surface expression compared to the unstimulated cells, for VEGF-E (Figure 8A). This implies that β1 integrin internalization responds to the same internalization signals as uPAR. Stimulation of VEGF receptor-2 triggers the coordinated activation of several signaling pathways (e.g., RAS-dependent signaling, phospholipase C-γ mediated protein kinase C activation, stimulation of SRC, etc) (22-26). These support the concerted reprogramming of endothelial cells that gives rise to angiogenesis. Our previous experiments showed that VEGF induced uPAR internalization via a signaling pathway comprising VEGF receptor-2, PI3-kinase and matrix metalloproteinase (MMP-2) (140). Accordingly, we assessed the effects of the PI3-kinase inhibitor wortmannin and of the MMP-2/9 inhibitor (2R)-2-[(4-biphenylsulfonyl)amino]-3-phenylpropionic acid
on integrin internalization. Serum starved endothelial cells were pretreated with these inhibitors and stimulated with VEGF-E, the specific ligand for VEGFR-2. FACS analysis showed that VEGF-E induced β1 internalization was also blunted upon inhibition of PI3-kinase and MMP-2/9 inhibitor (Figure 8Bb,c). We interrogated the signaling network by using additional inhibitors, i.e., PD98050 for blocking activation of ERK1/2, U73122 as an inhibitor of PLC-γ and PP1 to suppress the non-receptor tyrosine kinase SRC. The rationale for examining the RAS-RAF-MEK1-ERK cascade was our recent observations that activation of a receptor tyrosine kinase and of LRP-1 in the presence of uPAR resulted in sustained ERK activation and increased cellular adhesion (119). It was therefore conceivable that the VEGF-R2 and uPAR cooperated to control integrin internalization via the ERK cascade. This was not the case: VEGF-stimulated internalization of endothelial uPAR or integrins was not inhibited by the MEK1-inhibitor PD98059 (Figure 8Bd). We also examined, whether input via PLC-γ or SRC affected recycling of VEGF-induced uPAR or integrin recycling, because PLC-γ consumes the substrate of PI3-kinase and because SRC plays a prominent role in signals arising from focal adhesions. However, VEGF-induced internalization of uPAR or of integrins was neither inhibited by the SRC inhibitor PP1 nor by the PLC inhibitor U73122. Taken together, our results show that β1 integrin internalization is controlled by the same pathway (i.e., PI3-kinase-dependent activation of MMP-2) as uPAR.

![Figure 8A](image-url)
Figure 8. VEGF induced β1 internalization requires VEGFR-2 dependent PI3-kinase/MMP-2 activation. (A) Serum starved endothelial cells were stimulated with either VEGF₁₆₅ (50ng/ml) or VEGF-E (50ng/ml) for 60 min, fixed and analyzed for integrin subunit β1 surface expression using flow cytometry as in Figure 5A. VEGF stimulated sample - red histogram, Control sample - black histogram. (B) VEGF-E induced β1 internalization is inhibited by MMP and PI3-kinase inhibitor. Serum starved endothelial cells were stimulated with VEGF-E (50ng/ml) for 60 min. Prior to stimulation, cells were treated with either MMP-2/9 inhibitor {2(R)-2-[(4-Biphenyl)sulfonyl]amino-3-phenylpropionic acid} (1µmol/L), PI3-kinase inhibitor – wortmannin (100 nmol/L) or MEK1 inhibitor - PD98059 (30µmol/L) for 30 min. Cells were fixed and analyzed for integrin subunit β1 surface expression using flow cytometry. The experiment was repeated thrice, and representative histograms are shown.

3.6 Domain 3 of uPAR is necessary for β1 integrin endocytosis.

The three domains of uPAR fold to form a urokinase binding pocket with a large external surface accessible to other ligands (40). Different amino acid residues in domain-3 (DIII) have been implicated to interact with the integrin β1 subunit; particularly S245 (108) and H249 (109). Inspection of the uPAR 3D structure shows that these residues are located side by side on the external surface opposite the urokinase binding pocket.

We synthesized peptide 243-251 which contains both candidate residues to define the uPAR interacting region required for integrin internalization upon VEGF induction. We treated serum starved endothelial cells with Peptide 243-251 and with its scrambled version prior to stimulation with VEGF₁₆₅ and determined surface levels of β1 integrin and of uPAR by FACS. As positive control we used peptide 240-248, used by (Chaurasia et al, 2006) to
demonstrate uPAR/integrin β1 interaction. Peptide 243-251 and the positive control peptide inhibited VEGF_{165}-induced internalization of β1 integrin (Figure 9A). In contrast, the scrambled peptide did not have any inhibitory effect. The inhibitory peptide did not affect the VEGF-induced internalization of uPAR per se (Figure 9B). This indicates that the requirements for co-internalization were asymmetrical: internalization of β1 integrin was contingent on complex formation with uPAR, but VEGF-driven endocytosis of uPAR did not require an interaction with β1 integrin.

**Figure 9A**

**Figure 9B**
Figure 9. Peptides derived from Domain-3 of uPAR block VEGF-induced integrin internalization. (A) Representative histograms show cell surface integrin β1 expression of endothelial cells pretreated with 25 µM of the inhibitory peptide (P243-252) or it’s scrambled version for 10 min and stimulated with VEGF₁₆₅ (50ng/ml) for 60 min. P240-247 was used as a positive control. Right hand panel shows the quantification of percentage surface expression compared to control; calculated from the geometric mean fluorescence values of three independent experiments. Mean ± SD, **p < 0.01. (B) Quantification of uPAR surface expression after treatment as above **p < 0.01.

3.7 Integrin α5β1 associates with uPAR for VEGF induced endocytosis.

Six different members of the β1 family have been described on endothelial cells (76,78). Hence, we sought to determine which β1 integrin heterodimers specifically undergo VEGF induced internalization. We analyzed VEGF-induced change in surface expression of different α integrin subunits by flow cytofluorometric analysis. These experiments revealed that α3β1 and α5β1 integrins were internalized upon VEGF₁₆₅ stimulation while the surface expression of αv integrin subunit was increased. The latter can be presumed to reflect increased cell-surface translocation from the peri-nuclear vesicles (Figure 10A).

We next checked whether both α3β1 and α5β1 integrin interact with uPAR upon VEGF simulation. We verified this by resorting again to the micro-patterning approach. Micro-biochip surfaces were functionalized with an antibody to either integrin subunit α3 or α5. This forced the integrins α3β1 or α5β1 on adhering cells to accumulate according to the pattern of the immobilized antibody. We then analyzed the localization of uPAR before and after VEGF₁₆₅-stimulation of the cells. UPAR accumulated in the spots of immobilized α5β1 integrin upon VEGF stimulation (Figure 10 B). This can only be accounted for by an interaction between the two receptors. In stimulated cells, a high mean contrast of 0.21 was found between the areas of antibody-immobilized integrins versus BSA-treated areas. In contrast, the mean contrast (0.04) was low in unstimulated cells. This confirms the interaction of uPAR and α5β1 integrin. The patterned integrin α3β1 antibody did not cause any patterning of uPAR upon VEGF stimulation (Figure 10C). This indicates that these two proteins do not interact to an appreciable extent in living endothelial cells.

Immunocytochemistry shows that stimulation of quiescent endothelial cells by VEGF results in a marked redistribution of α5β1 integrins. In resting endothelial cells integrin subunit α5β1 was found mainly at the cell periphery. In contrast, in VEGF₁₆₅-stimulated endothelial cells, immunostaining at the cell borders declined and increased in the vicinity of the nuclei as
punctate structures (Figure 10D). This shift also confirms VEGF induced internalization of \( \alpha_5\beta_1 \) integrins.
The α5β1 integrin heterodimer interacts with uPAR upon VEGF stimulation and undergoes internalization. (A) Alpha integrin subunit screening for VEGF induced internalization in endothelial cells. Serum starved endothelial cells were stimulated with or without VEGF$_{165}$ (50ng/ml) for 60 min, fixed and analyzed for surface expression level of different α-integrin subunits by flow cytometry. Representative bar diagram shows percentage cell surface α-integrin expression compared to it’s respective control taken as 100%; calculated from the geometric mean fluorescence values of four independent experiments. Mean ± SD, *$p$* < 0.05 (B&C) VEGF$_{165}$ stimulation of endothelial cells induces co-distribution of α5β1 and uPAR on a micro-patterned surface. Endothelial cells were grown on microbiochips patterned with an antibody to the α5 (B) or α3 (C) integrin subunit. After stimulation with VEGF$_{165}$ for 60 min, samples were fixed and immuno-stained using a pre-labeled uPAR monoclonal antibody. Representative TIRF images show uPAR redistribution to α5β1 integrin but not α3β1 antibody patterns upon stimulation. Statistical analysis of multiple cells was done as in Figure 7A and depicted in color density plots. 100-120 cells were evaluated per condition. Scale bar 6µm. (D) VEGF$_{165}$ stimulation reduces α5-integrin (green) at the cell periphery. Resting endothelial cells were stimulated with VEGF$_{165}$ (50ng/ml) for 30 min, fixed and immuno-stained for integrin α5 subunit. Images were taken using LSM-50 microscope with a 40x objective.
3.8 uPAR is essential for VEGF induced internalization of α5β1 but not α3β1.

We verified if for the observed internalization of α5β1 integrins, the concomitant internalization of uPAR is co-incidental or required. We employed 3 different strategies: a) phosphatidylinositol-specific phospholipase C (PI-PLC) treatment b) Pretreatment with uPAR inhibitory peptide and c) transient knock down of uPAR. Endothelial cells were pretreated with PI-PLC to cleave off all GPI-anchored proteins including uPAR from the cell surface. Surface expression of α5β1 and α3β1 integrins was subsequently assessed by FACS analysis after VEGF_{165} stimulation. Addition of VEGF_{165} led to internalization of ~25% and ~35% of α3β1 and α5β1 integrin, respectively. However, the lack of uPAR impaired VEGF induced α5β1 integrin but not α3β1 integrin internalization (Figure 11A versus Figure 11B). Pretreatment of endothelial cells with the uPAR inhibitory peptides also yielded similar results. The inhibitory peptide P243-251 and the control peptide P240-248 inhibited the internalization of α5β1 but not that of α3β1 integrin (Figure 11C,D). The scrambled peptide did not have any inhibitory effect. A dose response curve for the inhibitory effect of P243-251 and the scrambled peptide was done. P243-251 could inhibit α5β1 integrin internalization starting from a concentration of 25μM with no obvious effect of the scrambled peptide (Figure 11E). We also knocked down uPAR in endothelial cells using siRNA strategy. Transfection of endothelial cells with uPAR siRNA strongly inhibited the expression of uPAR compared to the scrambled siRNA (Figure 11F – western blot). Upon stimulation with VEGF uPAR silenced endothelial cells did not show α5β1 integrin internalization (figure 11F). These observations indicate that the complex formation between uPAR and α5β1 integrin is necessary to drive efficient endocytosis of α5β1 integrin.

Figure 11A
Figure 11B

Figure 11C
Figure 11D

Figure 11E
Figure 11F

Figure 11 uPAR is necessary for VEGF induced internalization of α5 integrin subunit but not α3. (A) Cleavage of uPAR from endothelial cell surface diminishes VEGF induced α5-integrin internalization. Serum starved endothelial cells were treated with 5U ml⁻¹ of PI-PLC for 15 min, stimulated with VEGF₁₆₅ (50ng/ml) for 60 min and prepared for flow cytometry as described in Figure 5A. Quantitative analysis of cell surface α5 integrin subunit calculated from the geometric mean fluorescence values of four independent experiments is shown in the right panel. Mean ± SD, **p < 0.01, *p < 0.05 (B) The experiment was repeated as above but analyzed for α3 integrin surface expression. (n=3) (C) Dose response of P243-251 inhibitory effect of VEGF induced α5β₁ integrin internalization. Endothelial cells were pre-treated with P243-251 or scrambled peptide with concentrations ranging from (0.25µM-50µM). Samples were stimulated with VEGF₁₆₅ (50ng/ml) for 60 min and assessed for α5β₁ integrin internalization as above. Complete inhibition of α5β₁ integrin internalization was observed at a concentration of 25µM. n=4 Mean ± SEM, **p< 0.01 (D) Representative histograms show cell surface expression of α5-integrin on endothelial cells pretreated with the uPAR-derived (P243-251, P240-247; 25 µM) or the scrambled control peptide (PScramble, 25 µM) for 10 min and stimulated with VEGF₁₆₅ (50 ng/ml) for 60 min. Right panel shows bar diagram summarizing 4 experiments (means±S.D.) with immuno-staining in control cells representing 100%. (E) Conditions were as in panel A, but surface levels of α3-integrin were quantified. (F) Knock-down of uPAR by siRNA impairs VEGF-induced internalization of integrin α5β1. Endothelial cells were transfected with either uPAR or scrambled siRNA (100nM) for 36 h. Knock-down of uPAR was assessed by immunoblotting for uPAR and actin (loading control) in lysates of siRNA-traesfected cells. Samples were stimulated with VEGF₁₆₅ (50ng/ml) for 60 min and assessed for α5β₁ integrin internalization as above. n=3 Mean ± SD, **p< 0.01
3.9 The LDL-Receptor is necessary for VEGF induced endocytosis of α5β1 integrin.

Endocytosis of the uPAR tri-molecular complex is mediated by the LDL-Receptor (134-137). We have shown that VEGF induced endocytosis of uPAR can be inhibited by RAP - the high affinity ligand of LDL-Receptor (140). As α5β1 integrin requires uPAR interaction for its internalization, it would be predictable that VEGF induced endocytosis of α5β1 integrin can be inhibited by RAP. This was the case as pre-treatment of endothelial cells with RAP inhibited the internalization of α5β1 integrin (Figure 12A). As expected, this was not the case for α3β1 integrin (Figure 12B). Thus hindrance of the LDL-Receptor like protein inhibits both uPAR and α5β1 integrin internalization. The uPAR inhibitory peptides inhibited the internalization α5β1 but not uPAR (Figure 9A). This suggests that the complex for internalization assembles in hierarchical order and that the internalization of α5β1 is contingent on uPAR. Taken together, these data indicate that it is specifically α5β1 integrin that interacts with uPAR upon VEGF stimulation. α3β1 integrin internalization occurs independently of uPAR mediated by a separate VEGF dependent pathway.

Figure 12A
VEGF-induced α5β1-integrin internalization requires LDLR-protein dependent uPAR interaction. Endothelial cells were treated with RAP (200 nM) for 30 min prior to stimulation with VEGF for 60 min; surface expression of α5 integrin (A) and α3 integrin (B) expression by flow cytofluorometry. Bar diagrams summarize data from 3 experiments (means ± S.D.). Statistical significance was assessed by ANOVA (**p < 0.01).

3.10 Inhibition of uPAR-α5β1 interaction impairs VEGF induced migration of endothelial cells.

Our results till now indicate that uPAR specifically interacts with α5β1 integrin in response to VEGF stimulation. α5β1 integrin is a fibronectin receptor and fibronectin is an essential component of the provisional extracellular matrix generated during wound healing and angiogenesis, (77,78) but is not a ligand for the urokinase receptor. If co-internalization of integrin and uPAR is functionally relevant, its disruption is predicted to have an effect on VEGF-induced endothelial cell migration. This was examined in a modified boyden chamber assay where the filter inserts were coated with fibronectin. Serum starved endothelial cells treated with the uPAR-derived inhibitory peptide were allowed to migrate towards a VEGF gradient. The inhibitory peptide significantly reduced migration towards VEGF (Figure 13A). This observation is consistent with the interpretation that uPAR-mediated integrin internalization is a necessary step in VEGF induced cell migration.

We also examined whether RAP (which precluded LDL-receptor like protein mediated uPAR-integrin internalization) suppressed VEGF induced migration of endothelial cells on fibronectin and collagen. We tested on collagen because collagen degradation exposes RGD binding sites conducive for α5β1 integrin ligation (74). In a modified boyden chamber

Figure 12B
chemotaxis assay RAP (Figure 13B,C) significantly reduced migration towards VEGF in both fibronectin as well as a collagen matrix. Taken together our results demonstrate that efficient integrin recycling is contingent on the co-internalization of uPAR, integrins and LDL-receptor like protein. Blockage of uPAR or LDLR-like protein impairs integrin recycling and in turn cell migration.

Figure 13A

![Graph showing cell migration](image)

Figure 13B

![Graph showing cell migration](image)

Figure 13C

![Graph showing cell migration](image)
Figure 13 VEGF-induced endothelial migration in vitro requires the interaction of the fibronectin receptor α5β1 integrin with uPAR. Serum-starved endothelial cells were treated with the uPAR-derived (P243-251, 25 µM) or the control peptide (PScramble, 25 µM) (A) or with RAP (200 nM) (B&C) for 10 min and then allowed to migrate through transwell membranes towards VEGF$_{165}$ for 4 h at 37°C. The transwell inserts were coated with fibronectin (5µg/ml) (A,B) or collagen (50µg/ml) (C) overnight at 4°C prior to seeding of the cells. Cells that had migrated to the underside of the membrane were fixed and counted (AnalySiS® software, Olympus). The results from three (A) and four (B,C) independent experiments are shown; statistically significant differences were verified by ANOVA (**p < 0.01).

3.11 UPAR-integrin interaction is necessary for in vivo angiogenesis.

Based on our observations inhibition of uPAR-mediated integrin recycling must have an effect on endothelial cell migration in vivo. We investigated the anti-angiogenic effect of the mouse uPAR-derived peptide m.P243-251 in a directed in vivo angiogenesis assay (DIVAA). The peptide m.P243-251 “TASWCQGSH” corresponds to the domain 3 peptide of human uPAR. Angioreactors filled with basement membrane extract containing either mVEGF or the combination of mVEGF and m.P243-251 were subcutaneously implanted for 11 days in the dorsal flanks of wild type BL6 mice. As predicted, capillary tubes formed in reactors containing mVEGF. In contrast, angiogenesis was significantly inhibited in reactors filled with VEGF and the inhibitory peptide m.P243-251 (Figure 14A). The presence of endothelial cells was verified by immuno-staining sections from the reactors for CD31 (Figure 14B,C). Sections from mVEGF containing angioreactors exhibited well developed blood vessels confirming the macroscopic examination of the reactors. Compared to the mVEGF, reactors containing mVEGF and the inhibitory peptide showed little or no CD31 positive staining. These results prove that the uPAR mediated integrin recycling is a necessary prerequisite for in vivo angiogenesis. In the absence of efficient recycling of integrins endothelial cells cannot invade and reorganize to form functional blood vessels.
Figure 14 uPAR mediated integrin recycling is necessary for in vivo angiogenesis. Angioreactors of size 1.0 cm length and 0.15 cm diameter were filled with 25 µl basement membrane extract either alone or containing 2 µg/ml VEGF_{164} and 1mg/ml heparin with or without 250µM m.P243-251. A reactor each was implanted on either side of the dorsal flank of 6-8 week BL6 mice for 11 days. Eight reactors were used for each group. (A) Representative angioreactor from each group retrieved after 11 days. (B) The liquid N\textsubscript{2} frozen angioreactors were sectioned and stained for rat anti mouse CD31 (green) and Hoescht (blue). Immunofluorescence pictures were taken using the AX-70 Olympus microscope. scale bar 100 µm (C) Sections were analyzed and quantified for invading endothelial cells (CD31 positive area) using the CellP\textregistered imaging software (Olympus). n=8 p<0.05; ANOVA
4. DISCUSSION

VEGF-directed reprogramming shifts endothelial cells from quiescence to an activated state that enables invasion of the surrounding tissue. Invasion is supported by the redistribution of uPAR to the leading edge of endothelial cells, which results in focused proteolysis of the extracellular matrix (52, 140). Invading endothelial cells sample the environment for guiding cues by dynamically changing membrane protrusion. Because these advancing cellular filopodia are continuously remodeled, uPAR must also be subject to continuous redistribution. This is achieved by complex formation between the GPI-anchored uPAR and a member of the LDL receptor family and their subsequent internalization (133-136). VEGF drives this endosomal recycling of uPAR by a signaling cascade that emanates from the VEGF-receptor-2/Flk1 (140). Our current observations document that the VEGF-signal is funneled through uPAR to control a second, integrin-dependent limb of the angiogenic response. VEGF-induced recycling of α5β1 integrin requires uPAR and is contingent on the internalization of a complex that also contains LDL-receptor related protein (Illustrated in Figure 15). This conclusion is based on the following findings: (i) VEGF promoted assembly of a complex comprising uPAR and α5β1 integrin. (ii) This complex was internalized upon VEGF stimulation; accordingly β1 integrins and uPAR were visualized in the same intracellular compartment. (iii) The internalization of uPAR and α5β1 integrin is dependent on the LDL-receptor like protein. (iv) In the absence of uPAR, VEGF failed to trigger internalization of α5β1 integrin and thus to initiate the redistributive cycle of integrin endocytosis and exocytosis. This translated into impaired endothelial cell migration in vitro and in vivo.

Figure 15. Schematic representation of uPAR mediated integrin internalization for VEGF stimulated endothelial cells.
The urokinase receptor can associate with integrin heterodimers in a cell-type dependent manner. The original observation was made with β2 integrins in complex with αL and αM; these associate with uPAR on monocytes (67). Formation of complexes between uPAR, integrins and an LDL-related receptor was first documented in HT1080 fibrosarcoma cells (128). Ligation of the LDL-related receptor protein-1 with PAI-1 drives the internalization of integrins αvβ3 and αvβ5 in conjunction with uPAR. Earlier experiments also suggested a functional interaction of α5β1 integrin and uPAR: upon increasing expression of uPAR, integrin α5β1 was recovered in the immunoprecipitate and this correlated with persistent ERK1/2 activation and enhanced tumor growth in vivo (97,98). We employed a micropatterning approach that allowed for direct visualization of VEGF-promoted formation of a complex between uPAR and α5β1 integrin on the endothelial cell surface. The salient techniques that are used to probe for protein interactions, such as co-immunoprecipitation or tandem affinity purification have several disadvantages. Protein interactions are probed in detergent lysed cell lysates where the spatio-temporal distribution of cell membrane proteins as well as their native confirmation is lost. Such settings are prone to false positives as well as false negatives where weak interactions can be lost. Endothelial cells seeded on the integrin antibody functionalized micropattern surfaces provide a platform for analyzing interactions occurring on the surface of live cells. The fact that integrin internalization is precluded in this experimental setup did not only facilitate quantitative assessment of uPAR recruitment but also afforded the unequivocal demonstration that the interaction happened indeed at the cell surface. This new approach does not allow to differentiate whether there is a direct interaction between uPAR and integrins or whether additional proteins - other than LDL receptor-like proteins - must be recruited to stabilize and internalize the complex. It is worth noting that purified uPAR and purified α5β1 integrin can directly interact in detergent solution (108). Dynamin and clathrin-mediated endocytosis of various integrins has been documented in several instances (68-70). LDL-receptor like proteins internalize uPAR into clathrin coated vesicles (136). Experiments from our lab show that VEGF stimulation of endothelial cells stimulates internalization of β1 integrins into clathrin-coated vesicles. Therefore the hypothesis that uPAR and β1 integrins are internalized by LDL-related proteins via clathrin coated vesicles is concurring with the observation that uPAR and β1 integrins were found in the same endocytic vesicles following VEGF stimulation. Growth factors involved in wound healing trigger the redistributive cycle of integrin trafficking to control cell migration (88,89). The downstream signalling pathways are not well understood. However, in endothelial cells, VEGF-A-triggered αvβ3 integrin recycling is contingent on activation of PKD1 (143).
observations in signaling pathway studies are in line with our findings: integrin internalization was triggered by the receptor specific for VEGF-E (FLK1/VEGFR-2) and blunted by wortmannin. This compound inhibits PI3-kinase, which is also the upstream activator of PKD1. Our earlier observation showed that VEGF induced a PI3-kinase dependent activation of MMP-2 (139). VEGF induced recycling of uPAR was contingent on this activation of MMP-2 and prevented by an inhibitor of MMP2/9 (140). Accordingly, we used this inhibitor interrogate the VEGF-controlled signaling network. These experiments further supported our conclusion that the VEGFR-2 orchestrates both, the accumulation of uPAR and a repertoire of proteolytic enzymes at the leading edge of an invading endothelial cell and regulated retrieval of β1 integrins via the same signaling pathway. Accordingly, uPAR is an essential bottleneck through which the VEGF-generated signal must be funnelled: in the absence of uPAR, neither focused proteolysis nor integrin-dependent migration can be elicited by VEGF.

Our observations indicate that the uPAR tri-molecular complex removes inactivated β1 integrins from the cell surface and in effect permitting migratory αvβ3 integrin to be exported to the cell surface. We have previously shown that VEGF stimulation leads to β1 integrin inactivation through a PI3-kinase pathway (140). Our hypothesis is supported by two facts. UPAR did not show any interaction with the activating β1 integrin antibody patterned microbiochip. pFAK associated with the β1 integrin reduced following VEGF stimulation. The absence of pFAK with uPAR and β1 integrin complexes implies that the integrin is in the inactive state.

In endothelial cells, VEGF triggered endocytosis of α3β1 integrin. Previous experiments documented that the binding of α3β1 integrin to uPAR occurred in a uPA-dependent manner (147). The direct binding of purified uPAR to purified α3β1 integrin and the recovery of uPAR in complex with α3β1 integrin is contingent on the presence of uPA (147,101). Surprisingly, micropatterning did not detect any direct interaction of α3β1 integrin with uPAR. In our opinion, micropatterning is among the most sensitive methods to record interactions in the native cell membrane, because it does not require any modification of the interacting molecules (e.g., by attaching fluorescent moieties) or any change in their stoichiometry (e.g., by heterologous expression). Thus, we conclude that, in endothelial cells, VEGF does not promote complex formation between α3β1 integrin und uPAR. This conclusion is also supported by the observation that VEGF-triggered endocytosis of α3β1 integrin was not impaired by RAP. The discrepancy between our findings and results from earlier studies (147,101) is most likely accounted for by cell type-dependent differences. In fact, uPAR can recruit various integrins (96-104) in a cell type-dependent manner. This also
supports the conjecture that these complexes are stabilized by the recruitment of additional proteins.

In our model the VEGF-induced signal is propagated via uPAR to regulate the fibronectin receptor - α5β1 integrin. In the absence of uPAR (PI-PLC treatment or uPAR inhibitory peptides) VEGF induced internalization of α5β1 integrin is inhibited. Preventing the internalization of uPAR with RAP inhibits α5β1 integrin internalization convincing that it’s internalization is contingent on uPAR. This subsequently also affects VEGF induced migration on collagen and fibronectin. The significance of our observations was proved with the inhibition of in vivo angiogenesis using the murine equivalent of the uPAR inhibitory peptide. In the presence of the inhibitory peptide VEGF induced endothelial invasion was significantly reduced with no visible blood vessel formation. This predicts and proves that the absence of α5β1 integrin ought to suppress tumor angiogenesis. Mice rendered genetically deficient in integrin α5 integrin die during embryonic development (86,87). As uPAR knock out animals are also viable the phenotype of mice with double knockout of α5 integrin and uPAR in endothelial cells would be interesting. However, blockage of α5β1 integrin by an antibody suppressed angiogenesis in a murine tumor model, where human rhabdomyosarcoma cells were xenografted into immunodeficient mice (148). Accordingly, α5β1 integrin is being explored as a potential target in advanced human cancer; the pertinent chimeric antibody, is currently in phase II clinical trials (149).

The binding sites for α5β1 integrin are thought to reside on domain-3 of uPAR. Specifically, point mutation of S245 or H249 resulted in inhibition of the association of uPAR and α5β1 integrin (99,100). This allows for selective disruption of the complex, a concept that was verified by using a peptide comprising the residues 243-251 of uPAR (TASMCQHAH) that contains both S245 and H249. The peptide efficiently blocked VEGF-induced internalization of the fibronectin receptor. The interaction site between uPAR and α5β1 integrin fulfils several criteria of a candidate drug binding site: (i) it is readily accessible, because it is on the extracellular surface thus obviating the cell membrane as a permeation barrier. (ii) It allows for discrimination, because it can be specifically targeted. (iii) There is no major toxicity that can be a priori anticipated. The absence of uPAR allows for the development of a viable animal (64) but it interferes with VEGF-induced angiogenesis. Our results therefore provide a proof-of-principle that the interface of uPAR and α5β1 integrin may represent a site to be targeted for anti-angiogenic therapy.
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Figure 1. The VEGF-Receptor signalling pathway.

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Figure 3. The integrin family.
CURRICULUM VITAE

Revu Ann Alexander
Institute of Vascular Biology and Thrombosis,
Center for Biomolecular Medicine and Pharmacology,
Schwarzpanierstrasse 17, Vienna, 1090 Austria
Ph: +43 069910298997
revu.alexander@meduniwien.ac.at

PERSONAL DETAILS

Date of Birth 07.06.1983
Nationality INDIAN
Gender Female

EDUCATION

Oct’ 2007 - Present
PhD (Vascular Biology)
Group: Late Univ. Prof. Bernd Binder
Univ. Prof. Dr. Michael Freissmuth
Medical University of Vienna, Vienna, Austria.

Jun’ 2004 – Aug’ 2006
Master of Science
Specialization: Microbial Gene Technology, Molecular Biology
Madurai Kamaraj University, Madurai, India.
Graduated with 83.3% marks

Jun’ 2001 – Apr’ 2004
Bachelor of Science
Specialization: Biotechnology, Zoology, Chemistry
University of Kerala, Thiruvananthapuram, India.
Graduated with 80.7% marks

Apr’1999 – Mar’ 2001
Pre-Degree/ Higher Secondary
Biology/Physics/Chemistry
H.H. The Maharaja’s College for Women, Thiruvananthapuram.
Graduated with 87.1% marks

RESEARCH TRAINING


This work brought in a new dimension into how the proteolytic enzyme uPAR; by mediating the adhesion receptors affects endothelial cell migration and angiogenesis. We showed that on VEGF stimulation of endothelial cells uPAR interacts and mediates the internalization of α5β1 integrins, which is necessary for endothelial migration.
Techniques: Primary endothelial cultures, Fluorescence Activated Cell Sorting (FACS), Immunocytochemistry, Confocal microscopy, Cell migration assay, Western Blots.

2. Visiting Student, Group: Dr. Gerhard Schütz (Jul’ 2010 – Dec’ 2010)
Johannes Kepler University, Linz, Austria.
Discerning uPAR – integrin interaction using Micropatterning.
The novel “micropatterning“ technique created by the group was modified to suit primary cell lines and investigate protein interactions on single molecule level. Alternatively also developed a methodology to assess multiple protein interactions in the same cell.
Techniques: TIRF Microscopy.

3. Junior Research Fellow, Group: Prof. Satyajit Mayor (Sep’ 2006 – Aug’ 2007)
National Center for Biological Sciences, Bangalore, India.
Whole Drosophila genome screening using shRNA for novel genes in fluid phase and clathrin- mediated endocytosis.
Techniques: Designing and generation of shRNA, Endocytosis assays, Fluorescence microscopy.

4. Msc Dissertation, Project Supervisor: Prof. S. Shanmuga Sundaram
Aptamer C1 MGT-20, a bacterial collagenase interacting peptide (Aug’ 2005- April 2006)
Using a random peptide display library, inhibitory peptides were identified which could inhibit bacterial collagenases. The interacting region was identified using bioinformatics and enzyme inhibitory activity assessed.
Techniques: Culturing, isolation and identification of microorganisms, Construction of genome libraries.

SCIENTIFIC PRESENTATIONS

Feb’ 2010 (Talk): Gordon Research Conference (Plasminogen Activation And Extracellular Proteolysis) Ventura, California

Sep’ 2009 (Poster): 5th European Meeting On Vascular Biology And Medicine, Marseille, France.

Jun’ 2009 (Poster): 3rd Young Scientist Association Meeting, Vienna, Austria.


ACHIEVEMENTS

○ (2008-2010): Organizing Committee Member, 1st - 3rd International workshop on “Cell Communication in Health & Disease”, Vienna, Austria.

○ (2004): Seventh rank in Kerala University for Bachelor’s Degree.

○ (2003) Organizing Committee Member for State Level Seminar – BIOQUEST.
PUBLICATIONS

VEGF induced endothelial cell migration requires urokinase receptor (uPAR)- dependant integrin redistribution. Alexander R.A., Prager G.W., Mihaly-Bison J et al. (Submitted).

INTERESTS

Avid Reader on contemporary writers.
Enjoys travelling.