Vascular Endothelial Growth Factor Receptor-2–Induced Initial Endothelial Cell Migration Depends on the Presence of the Urokinase Receptor

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Abstract—The angiogenic response of endothelial cells initiated by different growth factors is accompanied by assembly of cell surface–bound proteolytic machinery as a prerequisite for focal invasion. We have shown previously how the vascular endothelial growth factor (VEGF) initiates proteolysis by activation of pro-urokinase (pro-PA) via the VEGF receptor-2 (VEGFR-2). We now show that the cell surface receptor of the uPA-system, the urokinase receptor (uPAR), is redistributed to focal adhesions at the leading edge of endothelial cells in response to VEGF. VEGF<sub>165</sub> and VEGF-E, both interacting with VEGFR-2, but not PIGF exclusively stimulating VEGFR-1, induce within minutes internalization of uPAR via an LDL receptor–like molecule, dependent on generation of active uPA and the presence of plasminogen activator inhibitor-1 (PAI-1). uPAR seems to play a pivotal role in VEGFR-2–induced endothelial cell migration because cleavage of surface uPAR impaired the migratory response of endothelial cells toward VEGF-E, but not toward PIGF. (Circ Res. 2004;94:1562-1570.)

Key Words: angiogenesis ■ VEGF ■ urokinase receptor

The angiogenic process induced by growth factors such as vascular endothelial growth factor (VEGF) involves several key steps, including endothelial cell activation, migration and invasion, proliferation, and finally capillary strand and tube formation. Endothelial cell invasion requires degradation and reorganization of extracellular matrix proteins. Therefore, cells have to elaborate a repertoire of proteolytic enzymes, whereby both serine proteases of the plasminogen/plasmin system and matrix metalloproteinases (MMPs) are thought to be important. To focus the proteolytic machinery toward the leading edge of invading cells, urokinase, the initial enzyme of this machinery, is bound to the cell surface via its GPI-anchored receptor, uPAR (CD87). In fact, it was shown that uPAR is found at the leading edge in invading cells<sup>3,4</sup> and inhibition of functional activity of uPAR<sup>5,6</sup> significantly decreases the invasive potential in several types of cells. In addition, uPAR-deficient mice show significantly reduced leukocyte recruitment to inflamed areas.<sup>7</sup> In this context, it is, however, unclear how uPAR is redistributed to the focal adhesions in migrating cells.

During angiogenesis, endothelial cells have to invade the surrounding tissue and inhibition of the urokinase system leads to impaired angiogenesis. Furthermore, uPAR is found predominantly at invasive sites of capillary tubes.<sup>8</sup> We have shown previously that VEGF<sub>165</sub> induces activation of pro-uPA bound to its receptor uPAR,<sup>9</sup> but it is not known which of the stimuli operative during angiogenesis drives uPAR into the leading edge and how this process is initiated. It was the aim of this study to analyze how uPAR is rearranged in endothelial cells on VEGF stimulation, to study how this process is initiated, and to examine possible consequences of uPAR redistribution for endothelial cell migration.

Materials and Methods

Cell Culture
For experiments, human skin microvascular (HUSMEC) or umbilical vein endothelial cells (HUVECs) were used under serum free conditions. For all key experiments, results obtained with HUVECs were confirmed in HUSMECs; the specific cell type shown in the figure is indicated in the respective figure legend. Marine microvascular ECs were isolated and characterized from the uterus of PAI-1<sup>−/−</sup> mice, and from the respective wild-type controls.

For further details, see the expanded Materials and Methods section in the online data supplement available at http://circres.ahajournals.org.

Cytolfluorimetric Analysis
Monolayers of subconfluent ECs were treated as indicated, thereafter harvested and fixed, and aliquots were permeabilized. A monoclonal antibody against human or a polyclonal antibody against mouse uPAR and Alexa Fluor 488-conjugated secondary antibodies were
used. Samples were analyzed with FACSsort (Becton-Dickinson). Surface protein was calculated from geometric mean fluorescence values and expressed as percent of total fluorescence measured in permeabilized cells. At least three independent experiments were performed. Specific calculations used are given in the respective figure legends.

**Immunocytochemistry**

Human ECs were fixed and stained as described before. For double antigen staining, cells were simultaneously incubated with antibodies against uPAR and phospho-paxillin (pPax) for 2 hours at 37°C, washed and then incubated with a mixture of Alexa-488 labeled anti-rabbit and Alexa-568 labeled anti-mouse antibodies for 1 hour, 37°C. Deconvolution microscopy was used for analysis of stained cells (see expanded Materials and Methods).

**Antisense Oligonucleotides**

Rhodamine-labeled phosphorothioate oligodeoxyribonucleotides (10 μmol/L) were freshly dissolved in PBS and added to HUSMECs. After two days of treatment, cells were stimulated with VEGF or left untreated. Cell surface uPAR was quantified by FACS-analysis as described earlier, gating rhodamine-red fluorescent events to analyze oligonucleotide-positive ECs. Efficiency of oligonucleotide uptake was verified by immunocytochemistry.

**PI-PLC Sensitivity of uPAR Redistribution**

10^5 ECs (incubated or treated as indicated) were washed and surface labeled with sulfo-NHS-SS-Biotin as described. Thereafter, they were treated either with 5 U/mL PI-PLC at 37°C for 15 minutes or with buffer as control, followed by gently washing. PI-PLC efficiency was assessed by FACS-analysis of cell surface uPAR.

**Video-Based Migration Assay and In Vivo Matrigel Angiogenesis Assay**

HUSMECs seeded on vitronectin-coated glass coverslips were stimulated as indicated for 4 hours. Therefore, the cover slips were mounted onto cell culture chambers on a heated stage insert on an Olympus AX-70 microscope for time-lapse-recording of cell migration for 4 hours, whereby 20 images per hour were recorded (F-View digital camera, Soft Imaging System). Recorded time-lapse sequences were analyzed by assessing the length of the track of every individual cell.

The effect of RAP on rm-VEGF165-induced in vivo angiogenesis was analyzed as described previously.

**Statistics**

Statistical significance was determined by paired or unpaired t test when one group was compared with the control group. To compare 2 or more groups with the control group 1-way ANOVA and Dunnett tests as posttests were used. Significance was assigned to a value of P < 0.05.

**Results**

**VEGF by Interacting With VEGFR-2, but Not by Interacting With VEGFR-1 Leads to Redistribution of uPAR**

In an initial experiment, we analyzed the possible effects of VEGF on the overall distribution of uPAR in endothelial cells. After stimulation of endothelial cells with VEGF, uPAR was rearranged to structures in the cell periphery and to the leading edges. There uPAR colocalized with phosphopaxillin (Figure 1A) and the focal adhesion kinase (not shown), indicating increased presence of uPAR after VEGF stimulation in focal adhesions. The number of focal adhesions increased on VEGF stimulation by ~2.5 times, whereas the number of uPAR-positive focal adhesions increased ~3.5 times (Figure 1B). Ninety out of 97 analyzed endothelial cells showed such an increase in uPAR-positive focal adhesions.

To analyze which VEGF receptor is responsible for the observed VEGF effect on uPAR, we used PIGF (Figure 1C) and VEGF-E (Figure 1D), specific ligands for VEGFR-1 (flt-1) and VEGFR-2 (flk-1), respectively. VEGF-E caused uPAR redistribution, whereas PIGF did not. However, PIGF-treated cells exhibited focal adhesions comparable to VEGF or VEGF-E treatment. From these data, we conclude that VEGF, when bound to its VEGFR-2, causes rapid uPAR redistribution to focal adhesions at the leading edges of endothelial cells.

**VEGF-Induced uPAR Redistribution Involves an Internalization Mechanism**

In order to investigate whether uPAR redistribution on VEGF stimulation involves internalization, we quantified the effects of VEGF on the distribution of uPAR by FACS analysis on permeabilized and nonpermeabilized cells. As shown in Figure 2, in unstimulated endothelial cells, ~96% of total cell uPAR was found on the cell surface, whereas VEGF induced a decrease in cell surface uPAR already after 30 minutes leading to a maximal decrease of surface uPAR after two hours (70.3 ± 5.2%, P < 0.01 of total uPAR).

The total amount of uPAR did not change during this initial time, consistent with previously published data; uPAR protein expression started to increase after 4 hours with a maximum after 20 hours of VEGF stimulation (Figure 2C). This indicates that during the first four hours of VEGF stimulation, synthesis of uPAR protein does not affect uPAR surface distribution. In addition, uPAR internalization in response to VEGF stimulation was independent of the protein synthesis inhibitor cycloheximide (not shown). To further analyze if uPAR internalization is also restricted to VEGFR-2, we again used specific ligands for VEGFR-1 and VEGFR-2. Only VEGF-E induced a decrease in surface uPAR comparable to VEGF, whereas PIGF was ineffective (Figure 2D). Therefore, we conclude that VEGF induces internalization and redistribution of uPAR via VEGFR-2.

**VEGF165 Induces uPAR Internalization Dependent on Active Urokinase and a Member of the LDL Receptor Family**

Two well-known processes are described for internalization of uPAR. One is dependent on formation of a ternary complex of uPAR with its specific ligand urokinase (uPA) and the specific plasminogen activator inhibitor-1 (PAI-1) that leads to internalization of the uPAR/uPA/PAI-1 complex to the endosomal compartment via a member of the LDL receptor family. This internalization is followed by recycling of unoccupied uPAR to the cell surface. The second mechanism is internalization of uPAR via binding to the cation-independent mannose 6-phosphate/insulin-like growth factor-II receptor (CIMPR). Endocytosis via the former mechanism is inhibited by the 39-kD receptor-associated protein (RAP), whereas the latter one is inhibited by a peptide derived from the interaction site of uPAR and CIMPR as described by us previously (Peptide B).
was blocked by the addition of 200 nmol/L RAP (P<0.01), whereas Peptide B (15 μmol/L) was ineffective, indicating an LDL-receptor family member–dependent mechanism.

Receptor-bound activated uPA is a prerequisite for complex formation with its inhibitor PAI-1 triggering binding of the uPAR complex to a member of the LDL-receptor family. When complex formation between uPA and PAI-1 was inhibited by the low molecular weight competitive uPA inhibitor benzamidine, no uptake of uPAR complexes occurred (Figure 3C). Because of the high PAI-1 synthesis in endothelial cells, the most effective way to assess participation of PAI-1 in such complex formation was to analyze endothelial cells derived from PAI-1/−/− mice. Indeed, rm-VEGF165 did not induce uPAR internalization in these cells as compared with the respective wild-type cells (Figure 3D). These data indicate that VEGF-induced uPAR internalization in endothelial cells is dependent on formation of a uPAR/uPA/PAI-1 complex and its binding to a member of the LDL-receptor family.

**Figure 1.** VEGF165 and VEGF-E, but not PIGF induce redistribution of uPAR to focal adhesions. A, Immunofluorescence detection of uPAR and phosphopaxillin in HUVECs: under control conditions (top), little uPAR (green) is located in focal adhesions highlighted by pPax (red; middle), whereas after VEGF165 stimulation (50 ng/mL, 2 hours), the number of focal adhesions positive for uPAR is increased. Right, Images of uPAR and pPax double-staining obtained by deconvolution microscopy; nuclear counter stain, DAPI (blue). Size bar=10 μmol/L. B, Statistical analysis of focal adhesions colocalizing with uPAR before and after VEGF165 stimulation. C, Double immunolabeling of PIGF-stimulated (10 ng/mL, 2 hours) human microvascular ECs: uPAR (green) is rarely observed in PIGF-induced focal adhesions (phospho-FAK, red); nuclei stained with DAPI (blue). Size bar=10 μmol/L. D, VEGF-E (30 ng/mL, 2 hours) stimulated human microvascular ECs stained as in C. VEGF-E induced relocalization of uPAR to focal adhesions to a similar extent as VEGF165 (A and B). Size bar=10 μmol/L.

**uPAR Internalization in Response to VEGF165 Is MMP-2 and MT1-MMP, PI3-Kinase, and Integrin Dependent**

Urokinase is synthesized in its inactive precursor form pro-uPA, which binds to its receptor uPAR. To form complexes with its inhibitor PAI-1, pro-uPA has to become activated. Previously, we could demonstrate that VEGF165 stimulation of endothelial cells activates pro-uPA when bound to its receptor uPAR via a mechanism, which requires a PI3-kinase–dependent change in conformation of integrin β, as well as active MMP-2. In order to analyze if uPAR internalization is a consequence of VEGF165-induced pro-uPA activation, we used a set of experiments that interfered with the step of pro-uPA activation and analyzed uPAR internalization.

First, we used 1,10-phenanthroline, a broad inhibitor of MMPs, and the specific MMP-2 inhibitor [(2R)-2-[4-Biphenylylsulfonyl]amino]-3-phenylpropionic acid], both shown by us to prevent VEGF165-induced pro-uPA activa-
tion. In fact, these inhibitors were also capable to inhibit VEGF165-induced uPAR internalization, whereas specific inhibitors of other matrix-metalloproteinases were not (Figure 4A). Activation of pro-MMP-2 is readily achieved on the cell surface by membrane-type 1 matrix metalloproteinase (MT1-MMP), an MMP with a transmembrane domain. To test the involvement of MT1-MMP in our system, we used human MT1-MMP antisense oligonucleotides as they were used before by others. In addition, MMP-2 antisense oligonucleotides and respective scrambled oligonucleotides were used. In cells treated with MT1-MMP antisense as well as in those treated with MMP-2 antisense, VEGF165 had no effect on uPAR redistribution, whereas in cells treated with scrambled oligonucleotides VEGF165 was still effective with respect to uPAR internalization (Figure 4B).

VEGF165, when bound to its receptor VEGFR-2, activates among other signaling cascades, including MAP kinase p38 and ERK-1/2 pathways, and also the PI3-kinase pathway, which leads to a change in integrin affinity, causing pro-uPA activation. Therefore, we studied the effects of respective inhibitors of signaling pathways originating from the VEGFR-2 on uPAR internalization and found that the PI3-kinase inhibitor wortmannin (100 nmol/L) and the PKA-inhibitor H-89 (20 μmol/L) prevented VEGF165-induced uPAR internalization (Figure 4C). When a possible effect of these inhibitors on VEGF165-induced pro-uPA activation was bypassed by the addition of active uPA in the presence or absence (not shown) of VEGF165, wortmannin was ineffective, whereas the PKA inhibitor H-89, known to inhibit the endocytic process itself, prevented both VEGF165 and uPA-induced uPAR internalization. This indicates that H-89 acts on the endocytotic process, whereas wortmannin is effective upstream. Other pathway inhibitors (PD098059 and SB203580), which were already ineffective at the level of pro-uPA activation, were also ineffective at the level of uPAR internalization.

As further indication that VEGF165-induced pro-uPA activation is operative also in VEGF165-induced rapid uPAR redistribution, we determined the effects of VEGF165-induced changes in integrin affinity, shown previously by us to induce pro-uPA activation, on uPAR redistribution. Indeed, the divalent cation manganese that induces a strong conformation change of integrins abolishes VEGF165-induced uPAR internalization (Figure 4D).

Therefore, in addition to the requirement of active uPA (Figure 3C) all mechanisms that prevented pro-uPA activation in response to VEGF165 also prevented uPAR internalization, and we conclude that pro-uPA activation by VEGF165 is a prerequisite for uPAR internalization.
VEGF165-Induced uPAR Internalization Is Followed by Its Redistribution to Focal Adhesions

In order to analyze whether interference with uPAR internalization would also block uPAR rearrangement to focal adhesions, the presence of uPAR in focal adhesions on VEGF165 stimulation was followed in the presence of the MMP-2/9 inhibitor or RAP both shown to inhibit uPAR internalization (Figures 3A, 3B, and 4A). The MMP-2/9 inhibitor as well as RAP largely impaired uPAR redistribution to focal adhesions (Figure 5A). In the presence of RAP or the MMP-2/9 inhibitor, no significant change of surface uPAR levels of HUVECs was observed in VEGF165 (50 ng/mL)-treated human endothelial cells. In non-VEGF165–stimulated endothelial cells derived from PAI-1+/− mice or respective wild-type mice. Stimulation with 50 ng/mL rm-VEGF165 for 2 hours did not induce a change in cell surface uPAR of PAI-deficient ECs, whereas cell surface uPAR of wild-type ECs decreased by 33%. Note that unspecific fluorescence measured by nonimmune control IgG was set to zero (shadowed curve). Geometric means of fluorescence intensity: wild type: 11.34, unstimulated; 7.6, rm-VEGF165–treated ECs; PAI-1+/−: 11.65, unstimulated; 11.13, rm-VEGF165–treated ECs.

VEGF165-Induced uPAR Internalization and Redistribution Contributes to the Migratory Response Toward VEGFR-2

Having shown that VEGF165 induces uPAR internalization and redistribution dependent on pro-uPA activation, uPAR/uPA/PAI-1 complex formation and the interaction with an LRP-like molecule, we were interested if this sequence of

Figure 3. Receptor associated protein (RAP) inhibits VEGF165-induced redistribution of uPAR. A, Representative immunocytofluorimetric histograms of cell surface uPAR on HUVECs stimulated with 50 ng/mL VEGF165 for 2 hours (top). Treatment of cells with 200 nmol/L GST-RAP for 30 minutes before growth factor stimulation (bottom) prevented VEGF165-induced decrease of cell surface uPAR. B, Changes of cell surface uPAR measured with FACS analysis (n=3) in HUVECs presented as percent of total receptor. RAP prevented VEGF165-induced decrease of cell surface uPAR indicating the involvement of a member of the LDL-R family. Peptide B, inhibiting uPAR/CIMPR interaction, had no effect on VEGF165-induced internalization. Mean±SD; **P<0.01 (n=4). C, Cytofluorimetric analysis of surface uPAR changes. In the presence of the uPA inhibitor benzamidine (10 μmol/L), no significant change of surface uPAR levels of HUVECs was observed in VEGF165 (50 ng/mL)-treated human endothelial cells. Mean±SD; **P<0.01 (n=3). D, Representative immunocytofluorimetric histograms of cell surface uPAR on microvascular ECs derived from PAI-1+/− mice or respective wild-type mice. Stimulation with 50 ng/mL rm-VEGF165 for 2 hours did not induce a change in cell surface uPAR of PAI-deficient ECs, whereas cell surface uPAR of wild-type ECs decreased by 33%. Note that unspecific fluorescence measured by nonimmune control IgG was set to zero (shadowed curve). Geometric means of fluorescence intensity: wild type: 11.34, unstimulated; 7.6, rm-VEGF165–treated ECs; PAI-1+/−: 11.65, unstimulated; 11.13, rm-VEGF165–treated ECs.
events has a biological significance. One major cellular phenomenon, in which uPAR-dependent proteolytic activity as well as the continuous formation of focal adhesions is required, is cell migration. VEGF initiates endothelial cell migration already within 1 hour after stimulation. We therefore analyzed whether depletion of uPAR itself would alter VEGF-induced endothelial cell migration. When we stimulated endothelial cells with VEGF-E or PlGF for 4 hours, we observed an increase in the migratory response for both growth factors (141 ± 11% for VEGF-E and 125 ± 3% for PlGF) (Figure 6A). After cleavage of the GPI-anchor of uPAR using PI-PLC, the migratory response toward VEGF-E was diminished (107 ± 18%), whereas the migratory response toward PlGF was unaffected, consistent with the observation shown in Figure 1C that PlGF induces focal adhesion formation without any participation of uPAR. When internalization of the uPAR/uPA/PAI-1 complex was inhibited by RAP, the migratory response of endothelial cells toward VEGF165 was reduced by ≈70% (Figure 6B), indicating a biological significance for VEGF-induced uPAR internalization. Also in an in vivo endothelial cell migration assay using matrigel plugs,15 the number of invading cells on rm-VEGF164 stimulation was reduced by ≈68% when RAP was incorporated into such an assay (Figure 6C).

Discussion

Growth factors and chemokines activate cells to migrate and invade surrounding tissues. For invasion, the coordinated formation of a localized proteolytic machinery is necessary. Focusing uPAR toward the leading edge of migrating cells provides such armor8 and inhibition of uPA binding to its receptor inhibits invasion of endothelial cells.5 It is not known, however, at which step of the invasive process redistribution of uPAR occurs and how it is achieved. We could show previously that VEGF165 inducing pro-uPA activation in endothelial cells is responsive for VEGF-dependent fibrinolytic activity.9 In this study, we show that VEGF165 interacts with its receptor VEGFR-2 rapidly induces pro-uPA activation that is dependent on a change in integrin affinity, on activation of MMP-2, and on pro-uPA being bound to its surface receptor uPAR.9 This VEGF-induced pro-uPA activation in endothelial cells is responsible for VEGF-dependent fibrinolytic activity.9 In this study, we show that VEGF165 induces via the same mechanism also internalization of uPAR. This suggests that the initial step in this process is VEGF-induced pro-uPA internalization required, is cell migration. VEGF initiates endothelial cell migration already within 1 hour after stimulation. We therefore analyzed whether depletion of uPAR itself would alter VEGF-induced endothelial cell migration. When we stimulated endothelial cells with VEGF-E or PlGF for 4 hours, we observed an increase in the migratory response for both growth factors (141 ± 11% for VEGF-E and 125 ± 3% for PlGF) (Figure 6A). After cleavage of the GPI-anchor of uPAR using PI-PLC, the migratory response toward VEGF-E was diminished (107 ± 18%), whereas the migratory response toward PlGF was unaffected, consistent with the observation shown in Figure 1C that PlGF induces focal adhesion formation without any participation of uPAR. When internalization of the uPAR/uPA/PAI-1 complex was inhibited by RAP, the migratory response of endothelial cells toward VEGF165 was reduced by ≈70% (Figure 6B), indicating a biological significance for VEGF-induced uPAR internalization. Also in an in vivo endothelial cell migration assay using matrigel plugs,15 the number of invading cells on rm-VEGF164 stimulation was reduced by ≈68% when RAP was incorporated into such an assay (Figure 6C).
activation followed by uptake of the uPAR/uPA/PAI-1 complex by an LDL-receptor like molecule. Although we have no direct evidence that formation of the trimolecular complex is a prerequisite for uPAR internalization, data obtained from PAI-1−/− cells indicate that uPAR internalization in response to VEGF is PAI-1 dependent. Thus, initially receptor-bound pro-uPA needs to become activated in order to form a complex with PAI-1 that is taken up in an LDL receptor–like molecule dependent fashion. uPAR internalization itself was originally described by others,16,30 but VEGF-induced uPAR internalization is shown for the first time in this study. Consistent with this model, we found that in case of VEGF-induced uPAR internalization a uPA inhibitor could inhibit VEGF-induced uPAR internalization. An alternate mechanism of internalization of uPAR via the mannose 6-phosphate/insulin-like growth factor-II receptor (CIMPR) was also described by others18 and us,19,20 but because respective inhibitory peptides derived from CIMPR were ineffective in our system, we can exclude participation of CIMPR in VEGF-induced uPAR internalization. Taken together our data indicate that VEGF induces uPAR internalization by initially activating pro-uPA followed by complex formation with PAI-1 and interaction of the ternary complex uPAR/uPA/PAI-1 with a member of the LDL receptor–like family.

During endothelial cell migration, uPAR is redistributed to focal adhesions on the leading edge. Such redistribution can occur either by lateral movement or by internalization and recycling of the receptor. In this study, we provide evidence that in endothelial cells on VEGF stimulation, redistribution of uPAR occurs mainly via recycling of the endocytosed receptor. This is shown by focal adhesions largely devoid of uPAR when they are formed on VEGF stimulation under conditions where uPAR internalization is inhibited. We clearly could show this phenomenon for MMP-2/9 inhibitor–treated cells, in which receptor-bound pro-uPA activation9 as well as uPAR internalization were inhibited. We also found a significant percentage of focal adhesions lacking uPAR despite VEGF stimulation in cells where uPAR internalization was inhibited by RAP. Because under these experimental conditions few focal adhesions still exhibit uPAR staining, contribution of other cellular events to the reduction of uPAR in focal adhesions cannot be excluded. Therefore, in another set of experiments, we labeled surface proteins of endothelial cells with glutathione cleavable biotin. When internalization of uPAR by VEGF was induced in these cells and remaining surface label was removed by treatment with glutathione, surface-labeled proteins appeared in focal adhesions and colocalized with uPAR. When GPI-anchored proteins, which include uPAR, were removed from the surface after labeling, significantly less surface label appeared in focal adhesions after VEGF stimulation, indicating that GPI-linked proteins are recycled to the leading edges. These data suggest that on VEGF stimulation of endothelial cells pro-uPA activation leads not only to uPAR internalization, but also to a controlled recycling of uPAR to focal adhesions at the leading edge.

uPAR redistribution seems to participate in endothelial cell migration induced by VEGFR-2 because cleavage of the GPI-anchor diminished the migratory response significantly, whereas PIGF-induced endothelial cell migration was not affected by uPAR cleavage. This indicates that different growth factors induce different migratory pathways. The migratory response and the respective involved pathways might depend also on the matrix that supports migration. The effects shown in this study were observed using a vitronectin matrix. Our data, showing that RAP that inhibits internalization of uPAR/uPA/PAI-1 complex also inhibits endothelial cell migration ex vivo on a matrigel matrix and in a matrigel plug assay in vivo,
Figure 6. uPAR contributes to the VEGF-E-induced, but not to the PIGF-induced endothelial cell migration in a RAP dependent manner. A, Video-based migration assay. Both VEGF-E (30 ng/mL) and PIGF (10 ng/mL) significantly increased the migratory activity of HUSMECs plated on vitronectin, although to a different extend. In PI-PLC–treated cells, the migratory response toward VEGF-E was decreased significantly, whereas the migratory response toward PIGF was unaffected. Mean±SEM; **P<0.01 (n=3). B, Video-based migration assay. VEGF-E increased migratory activity of HUSMECs plated on matrigel was significantly blocked by addition of RAP (200 nmol/L); **P<0.01. C, In vivo matrigel angiogenesis assay. In vivo invasion of cells into the matrigel plug in response to rm-VEGF-E, VEGF-induced increase in invasion was significantly inhibited by RAP. Invasion is given in cell number per cross sectional area (175 000 μm²); endothelial cells were stained with a rat monoclonal antibody against mouse CD31. **P<0.01.

Further indicate that uPAR internalization and in turn relocation to focal adhesions is a general important part of the VEGF-R–induced endothelial cell response. From these data, it is also obvious that the mechanism described not only provides the necessary proteolytic potential at the leading edge for invasion, but it is also important for migration itself. This pathway is clearly not the only one operative in endothelial cells, because PlGF interacting with VEGFR-1 does neither cause pro-uPA activation nor uPAR internalization and redistribution, but still induces migration independent of the presence of uPAR.

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References


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Material and Methods (extended)

Cell Culture, Reagents, Antibodies

Human endothelial cells (skin microvascular or umbilical vein origin) were cultured in M199 (Sigma) supplemented with 20% serum, and bovine endothelial growth supplement (Technoclone; Vienna, Austria). Experiments were performed using subconfluent cultures up to passage 5, silenced under serum reduced conditions for 24 hours, followed by serum deprivation for additional 4 hours in M199 containing 1% BSA. Murine microvascular endothelial cells isolated from uterus of PAI-1\(^{-/-}\) mice, and from the respective wild-type controls were cultured in DMEM supplemented with 10% serum. Wild type (wt), uPAR\(^{-/-}\) and PAI\(^{-/-}\) mice, were of the same background (75% C57BL/6; 25% 129S/V).

The proteins and reagents were purchased as follows: recombinant human VEGF\(_{165}\) (Promocell GmbH; Heidelberg, Germany); VEGF-E and PlGF-1 (RELIATech; Braunschweig, Germany); recombinant mouse VEGF\(_{164}\) (Calbiochem; La Jolla, CA); active uPA (Ebewe Pharma; Unterach, Austria); RGD-S peptides (Sigma Chemicals; St Louis, MO). The inhibitors PD098059, LY294002 and the MMP-inhibitors (MMP-2/MMP-9 Inhibitor I, MMP-8 Inhibitor I, MMP-3 Inhibitor II), Calbiochem (La Jolla, CA); wortmannin and benzamidine (Sigma Chemicals; St Louis, MO); matrigel solution (Becton-Dickinson, San Jose, CA) were obtained as indicated.

Peptide B was a kind gift from Dr. Stockinger (Department of Immunology, University of Vienna, Austria). The rat receptor-associated protein (RAP, 39 kD Protein) was made as a recombinant fusion protein with glutathione S-transferase (GST) as previously described\(^1\).

Antibodies: Monoclonal antibody against the domain 2 of uPAR (American Diagnostica; Greenwich, CT); goat polyclonal antibodies against (a peptide mapping near the carboxyterminal region of) uPAR of mouse origin (Santa Cruz Biotechnology, Inc.; Santa
Cruz, CA); monoclonal antibodies against phospho-paxillin (Becton Dickinson; Franklin Lakes, NJ); anti-phospho-FAK (Upstate, VA). Secondary antibodies: Alexa Fluor 488 conjugated anti-mouse IgG antibody, biotinylated goat anti rabbit antibody, Alexa 568 labeled goat anti-mouse and Zenon\textsuperscript{TM} One Mouse IgG1 Labeling Kit (Molecular Probes; Leiden, Netherlands); DAPI (Vector Laboratories; Burlingame, CA) were obtained as indicated. To account for unspecific binding in all immunoassays purified non-immune mouse IgG or rabbit IgG (Sigma, St Louis, MO) were used.

The effectiveness of the growth factors and of the respective signal transduction pathway inhibitors used in endothelial cells was analyzed in replicate experiments determining the induced phosphorylation of specific target molecules by Western blots\textsuperscript{2;3}. The used growth factors induced activation of the respective pathways that was inhibited by the respective pathway inhibitors\textsuperscript{2;3}.

**Cytofluorimetric analysis**

Monolayers of subconfluent endothelial cells, serum deprived, were treated as indicated. After the respective times, cells were transferred to 4\(^\circ\)C, washed twice with PBS, harvested with EDTA 1\% and fixed with 2\% paraformaldehyde in PBS for 10 min. Each sample was divided into two aliquots, whereby one was permeabilized with 0.2\% Tween-20. For surface and total uPAR detection cells were labeled with monoclonal antibodies against human uPAR or polyclonal antibodies against mice uPAR and with Alexa Fluor 488 conjugated secondary antibodies. Samples were analyzed with FACSort (Becton-Dickinson, San Jose, CA).

Statistical analysis of data obtained with cytofluorimetric assays: Cell surface protein expression was calculated from median or geometric mean fluorescence values of surface levels detected with cytofluorimetric methods and expressed as percent of total amount measured in the permeabilized cells. Data showing surface level changes of different molecules were obtained from at least three independent experiments and calculated as
Immunocytochemistry

Human microvascular endothelial cells seeded on gelatin-coated cover slips, stimulated as described, were fixed with 4% paraformaldehyde, then permeabilized with 0.1% Triton X-100 for 5 minutes and blocked with 5% normal goat serum in Primary Antibody Dilution Buffer (DAKO) for 1 hour. For single antigen detection primary antibodies were applied for 1 hour at 37 °C, for double antigen staining cells were simultaneously incubated with antibodies against uPAR and phospho-paxillin or phospho-FAK for 2 hours at 37 °C, washed and then incubated with a mixture of Alexa 488 labeled anti rabbit and Alexa 568 labeled anti mouse antibodies for 1 hour at 37 °C. Samples were mounted in Vectashield (Vector; Burlingame, CA), visualized on an Olympus AX70 microscope and digital images were recorded using an F-View camera and the software package AnalySiS Pro (both: Soft Imaging System; Münster, Germany). Stained cells were also analyzed by deconvolution microscopy using the 3D-Deconvolution Module from Soft Imaging System.

Antisense Oligonucleotides

Human MT1-MMP antisense, MMP-2 antisense and a scrambled control rhodamine-labeled phosphorothioate oligodeoxyribonucleotides were synthesized by VBC-Genomics, Vienna, Austria. The sequences of human MT1-MMP antisense oligonucleotides and the scrambled control were used as described before \(^4\): antisense oligonucleotids for human MT1-MMP, 5’-GCCGTTAAACTTCTG-3’ and control, scrambled oligonucleotide, 5’-ATCTCGGATCAGACT-3’. The sequence of the MMP-2 specific antisense oligonucleotide was 5’-CGATGGCAAGGTGTG-3’. These oligonucleotides were freshly dissolved in PBS and added to human microvascular endothelial cells at 10 µM. After two days of treatment,
cells were stimulated with VEGF or left untreated before uPAR on the cell surface was quantified by FACS analysis as described above. Cells had been washed carefully before they were cytofluorimetric analyzed. To analyze only oligonucleotide positive endothelial cells, rhodamine red fluorescent events were gated to quantify uPAR levels at the cell surface. In addition, the efficiency of oligonucleotide uptake was verified by immunocytochemistry.

**PI-PLC sensitivity of uPAR redistribution**

$10^5$ endothelial cells - incubated or treated as indicated - were washed and surface labeled with sulfo-NHS-SS-Biotin as described by Weaver-AM et al.\(^5\). Thereafter they were treated either with 5 U ml\(^{-1}\) PI-PLC at 37 °C for 15 min or with buffer as control. After incubation the supernatants were removed and cells were gently washed with M199 containing 10 mg ml\(^{-1}\) BSA. As control for the efficiency of PI-PLC, the presence of uPAR on the cell surface was analyzed by FACS in parallel experiments. Approximately 85% of uPAR were removed from the cell surface by this protocol.

**Video-based migration assay**

Human microvascular endothelial cells were seeded on vitronectin coated glass coverslips in serum-free medium and stimulated for 4 hours with 30 ng ml\(^{-1}\) VEGF-E or 10 ng ml\(^{-1}\) PlGF after cells were treated with PI-PLC as described above or left untreated as control. Additionally, the effect of RAP on VEGF\(_{165}\)-induced endothelial cell migration was evaluated in the same system. The cover slips were mounted onto cell culture chambers on a heated stage insert on an Olympus AX-70 microscope for time-lapse-recording of cell migration for 4 hours, whereby 20 images per hour were recorded (F-View digital camera, Soft Imaging System; Münster Germany). Recorded time-laps sequences were then analyzed manually by assessing the length of the track of every recorded individual cell.
In migration assays for endothelial cells derived from either wild type or uPAR deficient mice matrigel was used as matrix; mice endothelial cells have been stimulates with or without mouse recombinant VEGF$_{164}$ in a comparable set of experiments as described above.

**In vivo Matrigel angiogenesis assay**

Three hundred microliters of matrigel solution supplemented with vehicle or 300ng/ml VEGF$_{164}$ with and without 200nM RAP were injected subcutaneously into the flank of C57BL/6 mice. On day 6 post injection the mice were sacrificed, the matrigel plug removed and snap frozen. Sections of the frozen plug were also prepared and stained with hematoxylin (Merck, Darmstadt, Germany), DAPI (Vector Laboratories, Burlingame, CA) or rat anti-mouse CD31 antibodies (Oxford, UK). Tissue samples were visualized with AX-70 Olympus microscope (Olympus Optical Co., Japan) and photographed using an Optronics DEI-750D CCD camera (Optronics, Muskogee, OK).

**Statistics**

Statistical significance was analyzed by paired or unpaired t-Test when one group was compared with the control group. To compare two or more groups with the control group “one way analysis of variance” and Dunnett’s tests as post tests were used. Significance was assessed to p-values of less 0.05.


