

## Theme Issue Article

# uPAR – uPA – PAI-I interactions and signaling: A vascular biologist's view

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### Summary

The urokinase-type plasminogen activator (uPA), its inhibitor PAI-I and its cellular receptor (uPAR), play a pivotal role in pericellular proteolysis. In addition, through their interactions with extracellular matrix proteins as well as with transmembrane receptors and other links to the intracellular signaling machinery, they modulate cell migration, cell-matrix interactions and signaling pathways. A large body of experimental evidence from in-vitro and in-vivo data as well as from the clinics indicates an im-

portant role of the uPA-uPAR-PAI-I systems in cancer. In addition to their role in tumor cell biology, the uPA-uPAR-PAI-I systems are also important for vascular biology by modulating angiogenesis and by altering migration of smooth muscle cells and fibrin deposition in atherosclerosis and restenosis. This review will focus on the general mechanism of uPAR/uPA/PAI-I interactions and signaling and the possible relevance of this system in vascular biology.

### Keywords

Urokinase / receptor, plasminogen activator inhibitors, angiogenesis and inhibitors, atherosclerosis, restenosis

**Thromb Haemost 2007; 97: 336–342**

## uPAR – uPA – PAI-I interactome

The urokinase-type plasminogen activator receptor (uPAR) interactomes are multimolecular complexes containing molecules which are directly physically engaged with uPAR on the one hand, but also provide indirect lateral interactions for uPAR with other surface molecules on the extracellular surface or second messengers at the intracellular site. The direct contact with uPAR defines the nature of further intermolecular interactions on the (extracellular) cell surface that includes changes of activity status of the molecules involved influencing further interactions with ligands or second messengers on the inner side of the cell membrane.

### uPAR-uPA

Urokinase receptor is a GPI-anchored protein and is expected to be enriched in the glycosphingolipid rafts. However, only 20–25% of uPAR is found in detergent-resistant membranes (lipid rafts), the remainder in detergent-soluble membranes (1), and dimerization of uPAR controls its partitioning (2). uPAR binds pro-urokinase (scuPA) as well as active urokinase (uPA) to the cell surface with high affinity (1 nM) (3). uPAR consists of three domains, whereby the uPA binding site is made up of resi-

dues that are in distinct structural domains (4). The X-ray structure of uPAR has been solved recently and has revealed that uPAR binds uPA in a pocket comprised by all three domains. This keeps the entire external structure of uPAR free for interactions with other proteins, e. g. integrins, EGF-R, FPR receptors and points to a pivotal role of the domain I (DI) of uPAR indicating also a necessity for cooperation of all three receptor domains to generate high-affinity binding of ATF or uPA (5–7). Membrane anchored uPAR can be cleaved at the site of the linker region giving rise to truncated but still membrane anchored molecule consisting of domains 2 and 3 of the receptor (D2D3) (8, 9). Cleavage of the GPI anchor results in the soluble form of the receptor (suPAR) that can both be intact or truncated. All hitherto identified forms of uPAR possess functional/biological activity as reviewed by Montuori and Ragno (10). Moreover, uPAR can undergo ligand (uPA-PAI-1)-induced internalization and recycling, which requires the interaction with a specific transmembrane protein, LRP(11) as well as constitutive ligand-independent internalization via CD222 (12, 13).

All these possible structural differences of uPAR define different conformational stages exposing various ligand binding epitopes and enabling a pleiotropy of possible interactions. Ligand binding to the intact or cleaved receptor will lead again to

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Received November 26, 2006

Accepted after revision January 18, 2007

Prepublished online February 8, 2007

doi:10.1160/TH06-11-0669

conformational changes facilitating appearance of previously hidden epitopes on the surface of the molecule further broadening the spectrum of uPAR interactions. In fact, different conformations of uPAR are able to interact with different interactors, e.g. the interaction with FPR receptors appears to require cleavage of uPAR between domain 1 and domain 2 at position 84 (14). uPAR mutations still binding uPA can differentially and productively interact with different trans-membrane proteins, such as integrins and the EGF-R (15). Thus, cleavage of uPAR appears to be an important physiologic/pathologic event. Although not complete, extensive information is available on uPAR interacting proteins (the uPAR interactome). The most important of these are integrins, G-protein-coupled receptors (GPCR) of the FPR family and the EGF-R. Through these interactions uPAR directs cell adhesion, migration and proliferation (3, 16, 17).

#### **uPAR interactions with the matrix: Vitronectin and integrins**

uPAR interacts with the matrix protein vitronectin via the somatomedin B domain (SMB) of vitronectin (18–20); this interaction might also involve uPA binding (21). In addition to such a direct interaction with matrix proteins, uPAR interacts with integrin adhesion molecules, among them it seems to have the highest affinity to the fibronectin receptors  $\alpha_3\beta_1$  and  $\alpha_5\beta_1$  integrins; functionally uPAR domain 3 seems to be important for that interaction (22). Furthermore, uPAR has been described to interact with  $\alpha_v\beta_5$  and  $\alpha_v\beta_3$  (23) and the macrophage antigen 1, Mac1 (CD11b/CD18) (24). Interactions of the soluble receptor (suPAR) with several integrins such as  $\alpha_4\beta_1$ ,  $\alpha_6\beta_1$ ,  $\alpha_9\beta_1$ ,  $\alpha_v\beta_3$  had also been shown. Although uPAR/integrin interactions occur mainly in a cis-form, a trans-form has also been described, thereby inducing cell-cell interaction (25). The interactions of uPAR with integrins have been studied extensively. Mapping the binding site of uPAR on integrin subunits, a surface loop within the  $\beta$ -propeller (W4 BC loop) of the  $\alpha_3$  integrin chain has been identified, which is outside the laminin-5 binding region. uPAR – integrin interaction regulates  $\alpha_5\beta_1$ -mediated cell migration on fibronectin (26, 27),  $\alpha_5\beta_1$  signaling (22, 28, 29) and fibronectin matrix assembly (30). Structural analysis revealed a conformational change in  $\alpha_5\beta_1$  integrin upon uPAR binding, which subsequently forms an additional binding site for fibronectin that is RGD independent and enhances cell binding to fibronectin (31). Interestingly, cells with or without uPAR adhere to the fibronectin fragment Fn III 9–11 in a RGD-dependent manner, while only uPAR expressing cell lines adhere to Fn III 12–15 independent of RGD-peptides. The notion that uPAR activates and stabilizes  $\alpha_5\beta_1$  integrin has been proposed also by Ossowski et al. based on their studies of human epidermoid carcinoma cell lines (82). Enhanced adhesion to fibronectin of tumorigenic over dormant cell lines was directly related to uPAR levels. As a consequence increased ERK phosphorylation levels and fibronectin matrix assembly were correlated with uPAR expression and could be blocked by the uPAR peptide P25, which inhibits uPAR – integrin interactions (29, 32, 33).

#### **uPAR interactions with signaling receptors: EGFR and GPCR**

Several functional interactions of uPAR have been described where the receptor is engaged in multimolecular complexes;

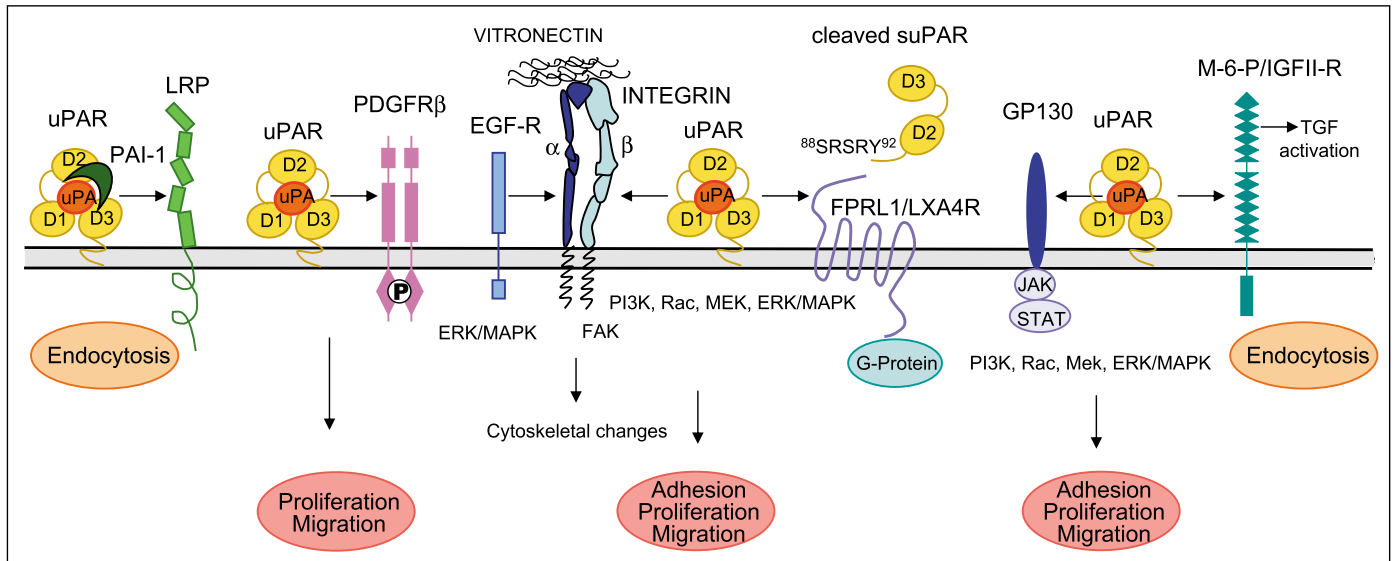
however, the details of physical interactions within these complexes are still to be determined. Reciprocal modulation of uPAR dependent biological responses can simultaneously involve EGFR and integrins, GPCR, members of the LDLR-family and integrins or matrix metalloproteinases (34–37). Interaction of uPAR with the FPR family (FPRL-1 and FPR) of GPCR require an uPAR chemotactic epitope located in domain 1 at position 84–92 (14). Activity of FPRL-1 is also modulated by the soluble D2/3 of the uPAR (16, 38).

#### **uPAR interactions with “modulators”**

uPAR can be functionally modulated by the tetraspanin CD82/KAI1 (with tumor suppressor activity) is influencing uPAR –  $\alpha_5\beta_1$  interaction (39). Availability of uPAR on the cell surface seems to be modulated by the mannose-6-phosphate/insulin like growth factor-II receptor (M6P/IGF-IIR; CD222) which interacts directly with uPAR via the N-terminal region of CD222 (40). High-molecular-weight kininogen (HK) interacts with the soluble receptor suPAR at several sites (41) thereby interfering with uPA binding. Involved are domains 2/3 (D2/3) of uPAR (42) and domain 5 (kininostatin) of kininogen (43) and this results in anti-adhesion the cell (44). Cytokeratin-1 (CK-1) is suspected to be part of a multiprotein receptor complex for HK binding (45). Gangliosides interfere with integrin  $\alpha_5\beta_1$  and uPAR or integrin and EGFR interactions modulating thereby uPAR – integrin-dependent functions (37, 46).

#### **uPAR – uPA – PAI-I in extra- (peri-) cellular proteolysis**

Urokinase (uPA) is a plasminogen activator; however, in contrast to tissue type plasminogen activator (tPA) neither uPA nor its receptor uPAR participate predominantly in regulating vascular fibrinolysis, as the absence of uPA does not result in the accumulation of fibrin as shown by studies in uPA and uPAR deficient mice (3, 47). Single-chain urokinase (scuPA) is the proenzyme form of uPA that is generated mainly in a positive feedback loop by plasmin converted from plasminogen by uPA. In addition to plasmin, also some metalloproteinases (MMPs [48]) as well as matrilysin (49), hepsin (50) and serase 1B (51) are capable to generate active uPA. scuPA is converted to uPA bound to its cell surface receptor uPAR, and it seems that under certain conditions binding of scuPA/uPA to its receptor modulates activation of scuPA to uPA or inactivation of uPA by its specific inhibitor plasminogen activator inhibitor 1 (PAI-1) (52–54). Active uPA then initiates a series of different cascades influencing cell adhesion, migration and survival. Firstly, pericellular proteolysis of the matrix is initiated by generation of plasmin from its zymogen plasminogen. Plasminogen is not merely contained in the extracellular fluid, but locally bound to its cellular receptor annexin I. Plasmin in turn is at the top of a cascade of matrix metalloproteinases, and their activation is initiated by plasmin providing the cellular armor to digest the extracellular matrix for invasion (53, 55). Another pathway is initiated by plasminogen bound to another cellular receptor M-6-P/IGF receptor CD222 which also interacts with uPAR and binds the latent TGF $\beta$  complex. Plasmin generated at the CD222 side then releases active TGF $\beta$  (13, 40,



**Figure 1: Compilation of important interactions within the uPAR/uPA/PAI-1 systems leading to signaling events.**

56). Active TGF $\beta$  then could upregulate synthesis of PAI-1 that would act in a negative feedback loop to terminate TGF $\beta$  activation.

A further substrate for uPA is uPAR; uPA cleaves uPAR, initiating a cell migration cascade through an interaction between cleaved uPAR, integrins and the GPCRs FPRL-1 and FPR (9, 14).

### uPAR in cell signaling

Figure 1 provides a summary of important interactions of the uPAR/uPA/PAI-1 systems involved in cellular signaling.

#### Signaling via integrins

uPAR – integrin interaction not only mechanically influences cell functions, but also adhesion-dependent signal transduction. Disruption of this interaction by interfering peptides blocks the association of src family kinases with beta integrins, thereby markedly impairing integrin functions. This can be explained by the fact that uPAR stabilizes caveolin – integrin complexes, whereby Src family members might be tracked to the integrin  $\beta$ -chain via caveolin-1. A src – integrin interaction is essential for the initiation of adhesion-dependent cell signaling (57). Another group has described a mechanism by which overexpression of uPAR disrupts tumor dormancy by inducing FAK and src activation leading to ERK phosphorylation via integrin  $\alpha_5\beta_1$ , which is necessary for tumor growth *in vivo* (33). Consistently, disrupting uPAR expression or blocking FAK leads to tumor dormancy in human carcinoma cells T-Hep3 (29).

uPA binding to its receptor uPAR induces high affinity binding of uPAR-uPA to  $\alpha_3\beta_1$  (Kd < 20nM), thereby enhancing cell spreading and FAK phosphorylation on fibronectin and collagen type I in a pertussis-toxin sensitive manner (58). Also fibronectin matrix assembly is mediated through a uPAR-dependent sequential activation of src kinase, EGFR, and  $\beta_1$  integrins (30).

#### Signaling via GPCRs

uPAR also transduces signals via GPCRs. Thereby a motif between domain 1 and 2 consisting of <sup>88</sup>Ser-Arg-Ser-Arg-Tyr<sup>92</sup> has to be unmasked either by uPA binding to uPAR or by cleavage of domain 1 (D1) of uPAR, revealing a chemotactic epitope to induce cytoskeletal changes and intracellular signal transduction (59–61). The involvement of a GPCR was found by a pertussis toxin-sensitive signal transduction upon the addition of soluble D2D3 to uPAR deficient cells (62). At least one GPCR has been identified – FPRL1 (a homologue of the fMLP receptor) – to transduce chemotactic activity of uPA or D2D3. Cells deficient in FPRL1 have been shown to be resistant against soluble D2D3 as well as against uPA-induced chemotaxis as were FPRL1 positive cells, which were blocked by specific anti-FPRL1 antibodies. In uPAR-deficient cells, expression of FPRL1 exhibited responsiveness against exogenous D2D3, but resistance against uPA, indicating that uPA acts indirectly via uPAR (63).

#### Signaling via members of the LDLR family

The low-density lipoprotein receptor-related protein (LRP-1) binds and mediates endocytosis of multiple ligands, transports the uPAR and other membrane proteins into endosomes and binds intracellular adaptor proteins involved in cell signaling. It has been shown that LRP-1 functions as a major regulator of Rac1 activation in an uPAR-dependent manner. In LRP-1-deficient MEFs as well as in RAP-treated cells where LRP-ligand binding is blocked, increased Rac1 activation and cell migration was seen. However, the same parameters were unaffected by RAP in uPAR<sup>-/-</sup> cells. In addition to Rac1, LRP-1 suppressed activation of extracellular signal-regulated kinase (ERK) in MEFs (64).

#### Signaling via the Jak/Stat signaling pathway

Binding of uPA to its specific receptor uPAR induces further intracellular signaling events in some cells. By using co-immunoprecipitation techniques, association of uPAR with several

tyrosine kinases of the src family (p60fyn, p53/p56lyn, p56<sup>lck</sup>, p59fgr) has been identified upon uPA/uPAR association (65). Furthermore, it was shown that clustering of uPA/uPAR by a monoclonal antibody induces JAK1 kinase association with uPAR in the kidney epithelial cell line TCL. As a consequence STAT1 phosphorylation and dimerization and nuclear translocation was induced, leading to its binding to the specific DNA sites GAS (interferon-gamma activation site) and ISRE (interferon-stimulated response element). Thereby, most likely the transmembrane protein gp130, which also co-localizes with gp130 upon uPA binding, mediates the extracellular signaling event (66). Similar findings were reported by Dumler et al. showing that uPA binding to its receptor induces the Jak/Stat pathway, thereby regulating migration of vascular smooth muscle cells (67, 68). Thereby other kinases (69, 70) as well as the PDGF receptor (71) seem to be involved.

## uPAR – uPA – PAI-1: Biologic role in the vasculature

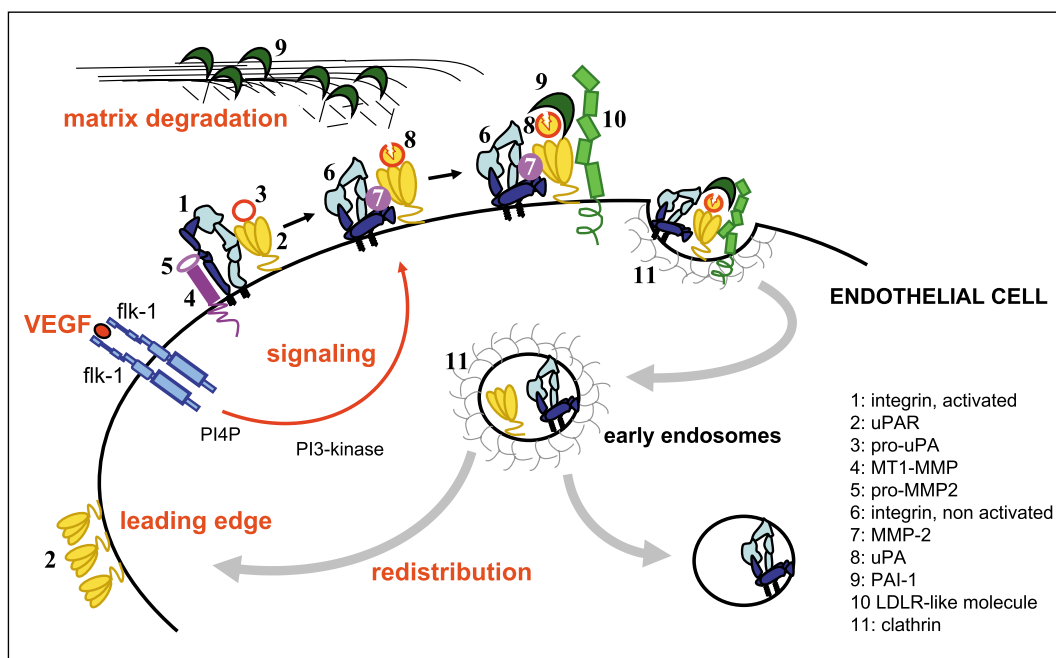
### uPAR – uPA – PAI-1 in angiogenesis

Blood vessels form the largest network in the body, thereby providing oxygen and nutrients to every cell in the organism. In contrast, dysregulation of blood vessel formation contributes to a number of diseases of malignant, infectious, ischemic and inflammatory origin. Vessels can grow in different ways: Vasculogenesis is the formation of new blood vessels by endothelial progenitor cells, whereby angiogenesis refers to sprouting of pre-existing vessels (72–78). Angiogenesis in tumors was already described 100 years ago (79), and in 1971 Folkman proposed that tumor growth and metastasis are angiogenesis-dependent; blocking angiogenesis could be a strategy to block progression of the disease (80). Meanwhile it is widely accepted that pre-cancerous tissues acquire angiogenic capacities on their way to becoming cancerous. Thereby the net balance between pro-angiogenic and anti-angiogenic molecules is tipped in favor of angiogenesis. In fact, tumors produce pro-angiogenic growth factors. Among them the vascular endothelial growth factor, VEGF, is thought to be the most important (76). Also under physiological conditions VEGF is upregulated, e.g. by hypoxia, thereby inducing angiogenesis. VEGF acts pro-angiogenic by augmenting all steps of angiogenesis: vascular permeability, which allows exudation of plasma proteins that lay down a provisional scaffold for detached endothelial cells, endothelial cell proliferation, endothelial cell migration and capillary-like tube formation. For endothelial cells to emigrate from their residential site they need to be set free by loosing cell-cell contacts and to be polarized to focus the newly formed proteolytic machinery at the leading edge, which is essential for matrix degradation. Thereby, proteinases of the plasminogen system, matrix-metalloproteinase system, chymase and heparanase families are thought to be important. So, inhibition of functional activity of the receptor of urokinase-type plasminogen activator, uPAR, significantly decreases the invasive potential of endothelial cells (81, 82), whereas the absence of the host plasminogen activator inhibitor-1 (PAI-1) prevents cancer invasion and metastasis (83). Inhibition of MMP-2 binding to integrin  $\alpha_3\beta_3$  by the non-catalytic MMP-2

fragment PEX inhibits tumor angiogenesis (84). Paradoxically, the inhibitor of plasminogen activation, PAI-1, as well as the tissue inhibitor of MMPs 1 (TIMP-1) correlates with poor prognosis in cancer patients, probably by preventing excessive proteolysis or other not yet defined mechanism.

When surrounding matrix proteins become degraded at the leading edge, cryptic binding sites for adhesion molecules like integrins are revealed. Especially migrating cells continuously form focal contacts at the leading edge by new integrin-matrix interactions. The cell matrix contacts persist until they reach the trailing end, where integrins have to release their ligands in order to allow cell locomotion (85, 86). Endocytosis and recycling of integrins during cell migration is the favored hypothesis for integrin redistribution (87). It is still unclear how integrins are internalized, but the involvement of clathrin-coated vesicles has been suggested (88). The NPXY internalization/signaling (89) motive present in receptors undergoing regulated endocytosis via clathrin-coated vesicles is actually found also in the cytoplasmatic domain of most integrin beta-subunits. However, the internalization process of integrins was not affected by point mutations of this motive (90), suggesting an additional mechanism for integrin internalization.

When endothelial cells are activated by VEGF, several proteolytic enzymes are transcriptionally upregulated. Although most of these enzymes are secreted in their latent precursor form, we previously were able to identify a mechanism via which VEGF<sub>165</sub> interacting with its receptor VEGFR-2 rapidly induces pro-urokinase (pro-uPA) activation on the surface of endothelial cells (91). This involves a phosphatidylinositol 3-kinase (PI3-kinase)-dependent change in integrin affinity, leading to activation of proMMP-2 and pro-uPA, when pro-uPA is bound to its surface receptor uPAR. As a consequence, this VEGF-induced pro-uPA activation on endothelial cells is responsible for VEGF-dependent local fibrinolytic activity and might be one of the initial steps in matrix degradation during the angiogenic process. Furthermore, active uPA forms complexes with its inhibitor PAI-1, which – when bound to uPAR – can be internalized and degraded. Internalization is performed via a member of the LDL receptor family (92), involving clathrin-coated vesicles formation. Thereafter, uPAR itself can recycle back from the endocytotic compartment to the cell surface (93). In VEGF-stimulated endothelial cells we were able to show that pro-uPA activation not only lead to extracellular matrix degradation, but – as a consequence – led to a coordinated internalization of uPAR by an LDL-receptor like molecule. Data obtained from PAI-1<sup>-/-</sup> cells indicate that uPAR internalization in response to VEGF is PAI-1-dependent, which is consistent with the prerequisite of a uPAR/uPA/PAI-1 complex formation. As a consequence we were able to show that uPAR recycles back to the cell surface not by random distribution, but focused on newly formed focal adhesions at the leading edge (94). Internalization and target-oriented recycling of uPAR to the leading edge plays a role in VEGF-induced endothelial cell migration, because cleavage of the GPI-anchor of uPAR, via which uPAR is fixed to the cell surface, diminished the migratory response significantly. In contrast, placental-like growth factor PlGF, which does not induce pro-uPA activation on the endothelial cell surface and consequently no uPAR internalization and recycling to the leading



**Figure 2: Initial events in endothelial cells following VEGF stimulation:** pro-uPA is activated forms complexes with PAI-1 that in turn are internalized through a LRP pathway. uPAR is then redistributed to focal adhesions at the leading edge.

edge, also induces endothelial cell migration, but independently of the presence of uPAR (94).

Therefore, we conclude that for endothelial cell migration the generation of proteolytic machinery is necessary, whereby proteases which participate in extracellular matrix degradation have to be focused on the leading edge. Degradation of the extracellular matrix is thought to set free matrix bound PAI-1, which consequently forms a complex with uPA/uPAR to become internalized. Although there might be different mechanisms by which generation of a localized proteolytic machinery is performed, at least in VEGF-induced endothelial cell migration the presence of uPAR seems to be essential. This notion is consistent with data obtained initially by Bajou et al. in PAI-1<sup>-/-</sup> mice showing that tumor angiogenesis is critically dependent on host PAI-1 in tumor transplantation models in mice (83, 95–97).

Taken together, for an integrated response of endothelial cells to growth factor stimulation, it is mandatory to provide a suitable mechanism for a coordinated activation of local proteolytic cascades to enable endothelial cells to invade the matrix. In addition uPAR-uPA-PAI-1 complex formation and internalization seems to be decisive for the initial migratory response of endothelial cells, whereby likely signaling events initiated by that process and induced integrin redistribution might be involved (Fig. 2). This indicates that the uPAR-uPA-PAI-1 system – in addition to a possible effect of the plasminogen cleavage product angiostatin (98–101) – is critically involved in the angiogenic part of vascular biology.

#### uPAR – uPA – PAI-1 in macrovascular diseases

In contrast to angiogenesis, where the uPAR-uPA-PAI-1 system seems to be decisive, in thrombotic macrovascular diseases rather the tPA-PAI-1 pathway of fibrinolysis is important. This is exemplified – perhaps with the exemption of aneurysm (102) and neointima formation (103) – by knock out (47, 104, 105) and transgenic (106) mouse models and by a large body of patient studies correlating tPA and mainly PAI-1 levels with disease (107–116). The mechanism involved seems to be predominantly PAI-1 dependent, because PAI-1 enhances neointima formation in several models (117–119). This effect on neointima formation might be due to PAI-1-induced endothelial cell proliferation (120) or an effect on cell migration (121) and/or indirectly via the prothrombotic effect of high PAI-1 levels. It also can not be excluded that interference with the uPA-induced activation of the STAT pathway in smooth muscle cells that inhibits cell proliferation (122) might contribute to that PAI-1 effect.

Taken together, while the correlation between elevated PAI-1 levels and thrombus formation seems to be plausible and is supported by in-vivo studies in mice as well as by data from patients, an additional direct effect of the uPAR – uPA – PAI-1 system as well as of the tPA – PAI-1 system on macrovascular diseases is less supported.

#### Acknowledgements

This work was performed within the Network of Excellence EVGN of the EU 6th framework program (contract number LSHM-CT-2003–503254) and the Integrated Project Cancerdegradome (LSHC-CT-2003–503297).

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