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uPAR
A modulator of VEGF-induced angiogenesis

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Vascular endothelial growth factor (VEGF)-initiated angiogenesis requires both coordinated proteolytic degradation of extracellular matrix provided by the urokinase plasminogen activator/urokinase receptor (uPA/uPAR) system and regulation of cell-migration provided by integrin–matrix interaction. Previously we have shown that stimulation of pericellular proteolysis induced by VEGF occurs via the VEGF receptor-2 leading to redistribution of uPAR to focal adhesions at the leading edge of endothelial cells. In our recent work published in *Cardiovascular Research*, we investigated the mechanisms underlying the uPAR-dependent modulation of VEGF-induced endothelial migration. By applying a micropatterning technique we described that VEGF stimulation results in complex formation between uPAR and $\alpha_5\beta_1$-integrin on the cell surface. The subsequent internalization of this complex, important for receptor redistribution, was demonstrated by flow-cytometry and immunohistochemistry. Targeting of the interaction site between uPAR and $\alpha_5\beta_1$ impairs receptor internalization and leads to the inhibition of endothelial cell migration in vitro and in an angiogenesis model in vivo. This proof-of-principle that the interface of uPAR and $\alpha_5\beta_1$-integrin may represent a promising site to therapeutically target tumor angiogenesis raises hope for the development of an anti-angiogenic approach that is limited to only the mobilizing effect of VEGF to endothelial cells, and does not interfere with the inarguably positive effect of VEGF as survival factor.

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In pathological and physiological angiogenesis, new endothelial cells penetrate avascular zones by sprouting from existing vascular vessels. For this process, vascular endothelial growth factor (VEGF) plays a critical role, because it regulates all steps required for angiogenesis, i.e., it induces endothelial cell proliferation and migration, increases vascular permeability and allows for the expression of active proteases on the cell surface.[1] As a consequence, matrix molecules are degraded and new provisional extracellular matrix (ECM) is created that promotes invasion of the surrounding tissue by endothelial cells.

The urokinase-plasminogen activator receptor (uPAR) binds urokinase-plasminogen activator (uPA),[2,3] which in turn localizes the activation of plasminogen to the extracellular protease, plasmin.[4] Plasmin then catalyzes degradation of the ECM and also activates other proteases, which together facilitate endothelial cell invasion.

As we have shown previously,[5] the above described activation of the proteolytic activity can be induced by VEGF via the VEGF receptor-2 (VEGFR-2). This process involves activation of pro-urokinase, its inactivation by plasminogen activator inhibitor-1 (PAI-1) and leads to subsequent redistribution of uPAR to focal adhesions at the leading edge of endothelial cells. It also involves internalization of uPAR via an LDL receptor-like molecule. We showed that such redistribution of uPAR was dependent on the VEGFR-2 since the VEGF isoforms $VEGF_{165}$ and VEGF-E (both interacting with VEGFR-2), but not PlGF (binding only to VEGFR-1) induced internalization of uPAR via an LDL receptor-like molecule within minutes. We concluded that VEGF-induced uPAR redistribution to the leading edge of migrating

endothelial cells provides them with the localized proteolytic capacity to degrade the basement membrane and invade the surrounding tissue during sprout formation.

In our recent work published in *Cardiovascular Research*, we studied the uPAR-dependence of endothelial cell migration and angiogenesis upon VEGF stimulation. We had previously shown that the migratory response of endothelial cells to VEGF depends on the uPAR.[5] This we had observed on vitronectin—an ECM protein that is a ligand for uPAR.[6] Here, we addressed our observation, that also on fibronectin, which is not a ligand for uPAR, the VEGF-induced endothelial migration still depended on uPAR. We surmised that uPAR, which has been described to be able to interact with and bind to several members of the integrin receptor family, might thereby mediate integrin redistribution in response to VEGF thus fostering migration.

In our work we demonstrate that the VEGF-signal is funneled through uPAR in order to control—besides proteolysis—a second limb of the angiogenic response, namely integrin-dependent cell migration. For this we provided several lines of evidence: (1) VEGF promoted assembly of a complex comprising uPAR and integrin $\alpha_5\beta_1$; (2) The internalization of the complex into endosomes, that were also decorated with clathrin immunoreactivity, was mediated by LDLR-like protein; (3) In the absence of uPAR, VEGF failed to trigger internalization of the fibronectin receptor $\alpha_5\beta_1$ and thus initiate the redistributive cycle of integrin endocytosis and exocytosis. This translated into impaired endothelial cell migration in vitro and reduced endothelial cell invasion and vessel formation in vivo (**Fig. 1**).

That integrin $\alpha_5\beta_1$ and uPAR would be able to interact had been suggested by earlier experiments: in uPAR rich cells, $\alpha_5\beta_1$-integrin had been recovered in the immuno-precipitate[7,8] and this correlated with persistent ERK1/2 activation and enhanced tumor growth in vivo.[9]

In our study we employed a micropatterning approach[10] that allowed us for direct visualization of VEGF-promoted formation of a complex between uPAR and $\alpha_5\beta_1$-integrin on the endothelial cell surface. This method 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). 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 occurred indeed at the cell surface. This new approach does not differentiate though, whether there is a direct interaction between uPAR and integrins or whether additional proteins—other than LDLR-like proteins—must be recruited to stabilize and internalize the complex. In detergent solution, in any case, purified uPAR and purified $\alpha_5\beta_1$-integrin can directly interact. Furthermore, that functional association of uPAR with integrin $\alpha_5\beta_1$ can indeed be mediated by a specific site of uPAR, was shown by the use of a 9-mer peptide derived from a sequence in domain III (residues 240–248) which was able to bind purified $\alpha_5\beta_1$-integrin. Substitution of a single amino acid (S245A) in this peptide, or in full-length soluble uPAR, impaired binding of the purified integrin.[11]

Our observations in the micropatterning experiment suggest that the interaction of uPAR and integrin $\alpha_5\beta_1$ in the cell membrane is subject to regulation that is brought about by a conformational change. This increases the mutual affinity of the partners or drives their association by recruiting an additional molecule into the complex. Our earlier results suggest that the integrin is inactivated by VEGF-induced inside-out signaling via a PI3-kinase dependent mechanism[12] before interacting with uPAR. This is underscored by the fact that in our micropatterning experiment the activating antibody 12G10 to $\alpha_5\beta_1$[13] inhibited the VEGF-induced recruitment of uPAR.

In our publication in *Cardiovascular Research*, we furthermore report that VEGF triggered in endothelial cells endocytosis of another integrin, $\alpha_3\beta_1$. Surprisingly however, we did not detect any direct interaction of integrin $\alpha_3\beta_1$ with uPAR in our micropatterning experiment, though this method is, in our opinion, among the most sensitive methods to record interactions in the native cell membrane.[10] Thus, it seems likely that, in endothelial cells, VEGF indeed does not promote complex formation between integrin $\alpha_3\beta_1$ and uPAR. This our conclusion is also supported by our observation that $VEGF_{165}$-triggered endocytosis of $\alpha_3\beta_1$-integrin was not impaired by the receptor-associated protein (RAP), which blocks LDLR like protein mediated internalization of uPAR.

While previous reports documented direct binding of purified integrin $\alpha_3\beta_1$ to uPAR,[7,14] the discrepancy between these and our findings could most likely be accounted for by cell type-dependent differences. In fact, uPAR can recruit various integrins in a cell type-dependent manner.[11,14,15] An alternative explanation would be delocalization of integrin $\alpha_3\beta_1$by the tetraspanin CD9 upon VEGF stimulation previously described.[16]

The findings presented in our recent study strongly suggest that absence of integrin $\alpha_5\beta_1$ or targeting of the interaction sites between uPAR and $\alpha_5\beta_1$ might lead to the inhibition of endothelial cell migration and angiogenesis.

In fact, blockage of integrin $\alpha_5\beta_1$ by an antibody was recently shown to inhibit angiogenesis in a murine tumor model, where human rhabdomyosarcoma cells were xenografted into immuno-deficient mice.[17] Accordingly, integrin $\alpha_5\beta_1$ is currently being targeted by using the pertinent chimeric antibody in phase II clinical trials in advanced human cancer.[18]

The binding sites for integrin $\alpha_5\beta_1$ to uPAR are thought to reside on domain-3 of uPAR and point mutation of S245 or H249 resulted in inhibition of the association of uPAR and integrin $\alpha_5\beta$.[11,19] We employed a peptide comprising the residues 243–251 of uPAR (TASMCQHAH) containing both, S245 and H249. This peptide efficiently blocked VEGF-induced internalization of the fibronectin receptor in vitro and angiogenesis in vivo.

The interaction site between uPAR and integrin $\alpha_5\beta_1$ fulfils several criteria of a candidate drug binding site: (1) it is readily accessible, because it is on the extracellular surface thus obviating the cell membrane as a permeation barrier; (2) it allows for discrimination, because it can be specifically targeted; (3) there is no major toxicity that can be a priori anticipated. Furthermore, targeting only VEGF-induced integrin redistribution—without affecting VEGF-mediated maintenance of the endothelium is expected

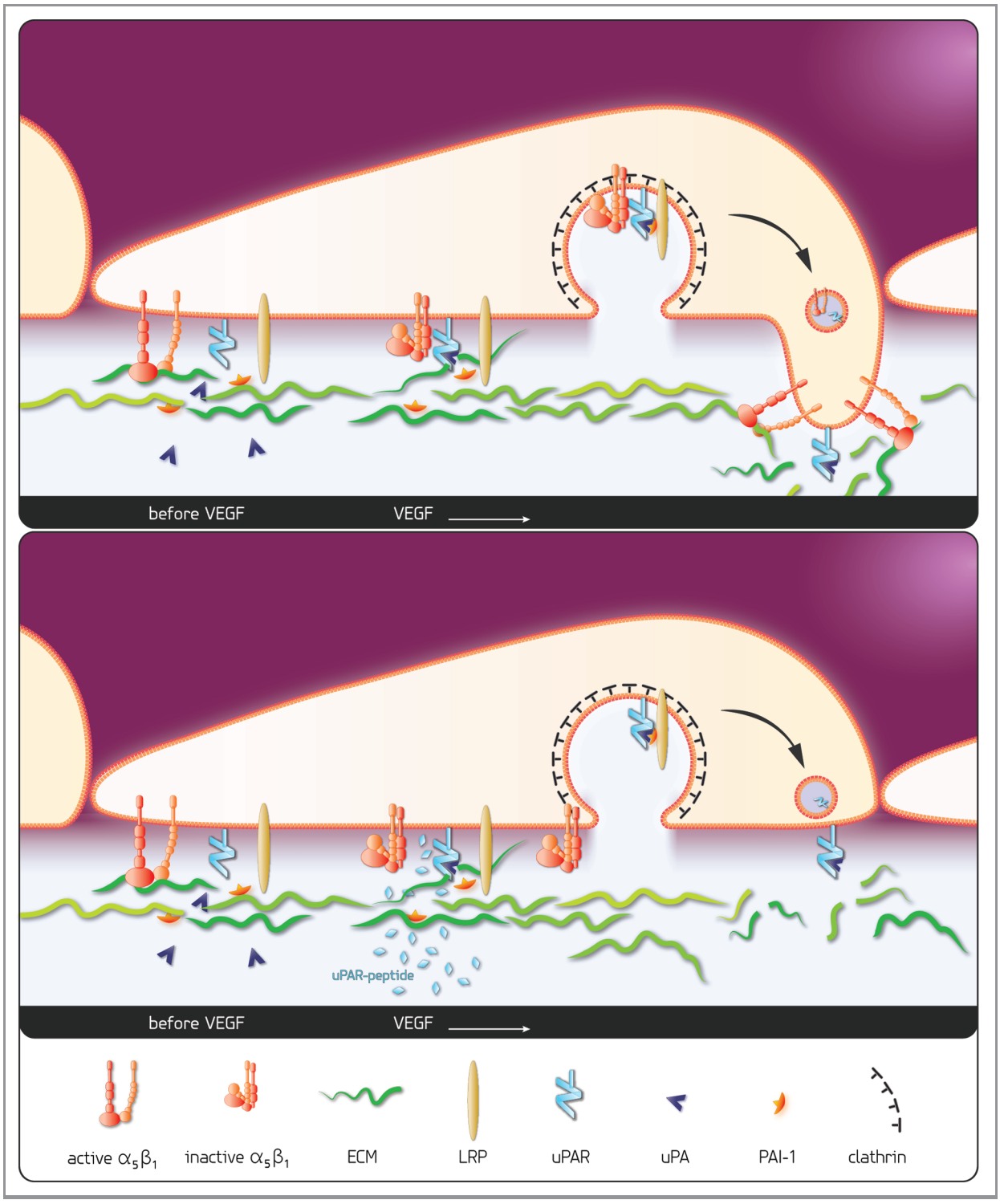

Figure 1. Scheme of proposed mechanism: VEGF leads to interaction of uPAR and the inactivated fibronectin receptor, integrin $\alpha_5\beta_1$, resulting ultimately in the cointernalization of integrin $\alpha_5\beta_1$ via "LRP/clathrin dependent endocytosis" and redistribution to the leading edge VEGF-stimulation of endothelial cells leads to inactivation of the fibronectin receptor integrin $\alpha_5\beta_1$, to activation of urokinase (uPA) bound to its receptor (uPAR) and to matrix degradation. PAI-1, released from the degraded matrix, inactivates uPA and the resulting trimolecular complex (uPAR/uPA/PAI-1) is internalized via "LRP/clathrin dependent endocytosis" dragging along the fibronectin receptor $\alpha_5\beta_1$. The redistribution of both, proteolytic as well as adhesive capacity, to the leading edge of the cell enables invasive cell migration and fosters angiogenesis. The uPAR derived peptide aa 243–251 corresponding to the proposed interaction site of uPAR and integrin $\alpha_5\beta_1$ prevents the VEGF-induced interaction of the two receptors. Thus, the co-internalization and redistribution of integrin $\alpha_5\beta_1$ is prevented impairing cell migration and invasion during VEGF-stimulated angiogenesis.

to reduce the adverse side effects of a broad anti-VEGF therapy and might even foster the normalization of the tumor vasculature. Our results therefore provide a proof-of-principle that the interface of uPAR and $\alpha_5\beta_1$-integrin may represent a site to therapeutically target tumor vasculature.

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