® Postepy Hig Med Dosw (online), 2019; 73: 803–808
e-ISSN 1732-2693

www.phmd.pl
Review

Received: 14.03.2019
Accepted: 10.09.2019
Published: 31.12.2019

Targeting uPAR in diabetic vascular pathologies*

uPAR jako cel terapeutyczny w powikłaniach naczyniowych cukrzycy

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Summary

The uPAR protein is one of the most important elements in fibrinolysis. uPAR is associated with many biological processes, such as cell invasion, angiogenesis and cell proliferation. Because of its multifunctional character, it is difficult to produce an effective inhibitor of uPA-uPAR interactions. The present paper shows the current state of knowledge about the contribution of uPA-uPAR complex in many biological processes and the application of uPAR inhibitors (antibodies, small-molecules, peptides), which might be potentially useful in the treatment of vascular pathologies.

Keywords: **urokinase plasminogen activator receptor • urokinase • vasculature • endothelial dysfunction**

GICID 01.3001.0013.6909
DOI: 10.5604/01.3001.0013.6909
Word count: 3684
Tables: –
Figures: 1
References: 52

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Abbreviations: **Ang-2** – angiopoietin 2, **AMD** – age-related macular degeneration, **ATF** – N-terminal fragment, **CRP** – C-Reactive Protein, **CVD** – cardiovascular diseases,**GPI** – glycosylphosphatidylinositol, **bFGF** – basic fibroblast growth factor, **LGI** – low-grade inflammation, **LRP-1** – LDL receptor- related protein-1, **MVs** – microvesicles, **PAI-1** – plasminogen activator inhibitor 1, **SDT** – spontaneously diabetic Torri rats, **suPAR** – soluble form of uPAR, **TGF-α** – transforming growth factor type α, **TGF-β** – transforming growth factor type β, **TF** – tissue factor, **tPA** – tissue-type plasminogen activator, **uPA** – urokinase-type plasminogen activator, **uPAR** – urokinase-type plasminogen activator receptor, **VEGF** – vascular endothelial growth factor.

*This work was founded by Polish National Science Center, grant PRELUDIUM 8 number UMO-2014/15/N/NZ5/01606 to M. D.-K.

INTRODUCTION

Fibrinolysis is a fundamental physiological process leading to lyse a blood clot by dissolving fibrin fibers. The equilibrium between fibrinolysis and clotting processes is needed to retain blood fluidity by preventing hemostatic clots from becoming too large and occluding the vessel. Fibrinolysis is a tightly controlled series of events involving a number of cofactors, inhibitors, and receptors and their effectiveness is greatly influenced by environmental factors (clot structure and density) [47] and also by fibrinogen isoforms and polymorphisms [45, 47], the rate of thrombin generation [30], the reactivity of thrombus-associated cells such as platelets, and the overall biochemical environment [11]. Components of the fibrinolytic system and fibrin degradation products are involved in a number of biological processes, including platelet aggregation, angiogenesis, cell migration, ovulation and tissue remodeling. The physiological role of the components of the fibrinolytic system depends on their derivation (fibrin degradation products), localization (cell membrane) and involvement in the degradation of the basal membrane and extracellular matrix proteins [41].

PLASMINOGEN ACTIVATION SYSTEM

The key protein in fibrinolysis is plasminogen, which is a proenzyme which is converted to the active plasmin as an end-product of the plasminogen activation system. This system is composed by a series of serine proteases, inhibitors and several binding proteins, which are very universal and their ancestral orthologues were identified in the number of mammalian counterpart precursors [10]. Plasmin exhibits a broad range of proteolytic activities; it targets fibrinogen and fibrin, but also extracellular matrix components (fibronectin, thrombospondin, vitronectin), growth factors, such as TGF-β (transforming growth factor type β) and bFGF (basic fibroblast growth factor), which have an important function as a mediator of inflammation and the innate immune system [20]. In addition, plasmin activates several proenzymes, like pro-tPA (pro-tissue-type plasminogen activator), pro-uPA (pro-urokinase-type plasminogen activator), plasminogen (autoactivation) and metalloproteinases zymogenes (proMMPs) [29, 33, 36].

Intravascular activation of fibrinolysis is carried out principally by tPA and occurs on the surface of fibrin. Another plasminogen activator is urokinase (uPA), which is produced by monocytes, macrophages or urinary epithelium and is involved mainly in the extravascular proteolysis [11]. The pool of the active enzyme is also controlled by plasminogen activator inhibitors, such as PAI-1 and PAI-2 [36]. Plasmin is solely inhibited by α2-antyplasmine and α2-macroglobuline [15] (Fig. 1A).

Under physiological conditions, uPA is synthesized mainly by epithelial, endothelial and smooth muscle cells, fibroblasts and monocytes/macrophages as a non-active zymogene (pro-uPA) in the form of a single chain urokinase-type plasminogen activator (sc-uPA), with low proteolytic activity, which after two-steps proteolytic cleavage, becomes the two-chain (tc-uPA) active form [36]. Sc-uPA activity is necessary for the generation of small amounts of plasmin, which hydrolyses the sc-uPA into tc-uPA. Both forms of uPA exhibit their enhanced proteolytic activity after binding to the specific receptor (uPAR), which is present on the cell surface [3, 13, 29]. Increased synthesis of uPA is observed in tumor cells, in particular malignant tumors of the cervix, ovaries, thyroid gland and breast, which is considered as diagnostic and prognostic biomarker [4, 13, 22, 24, 27, 36]. In contrast, the uPAR deficiency has a significant impact on fibrin accumulation in tissue, leading to decreased endothelial cell motility, impaired angiogenesis and finally tissue hypoxia and ulceration [2, 37, 42].

Immediately after uPA (free or bound to uPAR) activation, the enzyme is combined with PAI-1 (plasminogen activator inhibitor 1) to form an inactive uPA/PAI-1/uPAR complex, which is up taken by LDL receptor-related protein-1 (LRP-1) *via* clathrin dependent mechanism [14]. Inside the cell, the residual uPA/PAI-1 complex is transported to the lysosomes, where it is degraded; nevertheless, the uPAR molecule is recycled in macropinosome-like vesicles and endosomal compartments, where it can be transported to the cell surface and binds another urokinase [12].

Urokinase-type plasminogen activator receptor (uPAR), also known as CD87, is a glycoprotein, attached to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor [12, 50]. This single chain (313 a.a.) peptide consists of three domains with different biological functions: D1 – binding the N-terminal fragment of urokinase (ATF), D2 and D3 – controlling interaction of uPAR with vitronectin. The crystal structure of uPAR has been proposed by Llinas at al. and the uPA binding central cavity has been modeled [34].

The short amino acid chain, the D1/D2 linker region has properties similar to chemokines when it is cleaved by MMPs, plasmin, chymotrypsin, and uPA [36]. uPAR expression was determined in most normal cells as well as in cancer cells [23, 43]. uPAR mediates a variety of cellular processes, such as angiogenesis, metastasis, cell adhesion, invasion and inflammation [29, 36, 43].

BIOLOGICAL AND CLINICAL IMPORTANCE OF UPA/UPAR COMPLEX

It has been well proven that the uPA proteolytic system is especially involved in cancer malignancy and other diseases associated with 'low-grade inflammation' (LGI), which is an undefined subclinical chronic inflammatory state, e.g. sepsis, autoimmune disorders or cardiovascular diseases including arrhythmias [9, 25, 26, 44, 48]. The most commonly used biomarker of LGI is a pentraxin called C-Reactive Protein (CRP), which shows positive correlation with a soluble form of uPAR (suPAR) in many LGI processes [9, 44, 48]. In contrast to other LGI biomark-

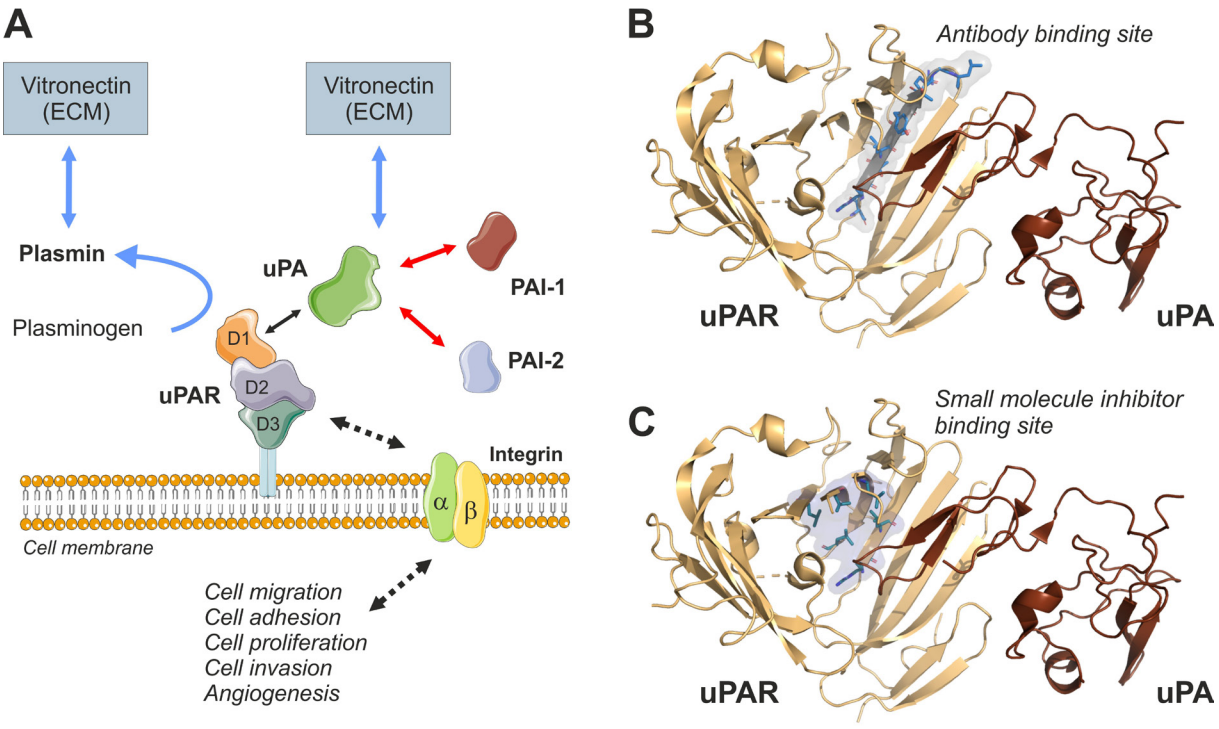

Fig. 1. Functional uPAR-uPA interactions in cell physiology and their molecularlandscape for the design of targeted inhibitors; **A** – Urokinase-type plasminogen activator (uPA) activity is controlled by two plasminogen activator inhibitors (PAI-1 and PAI-2) and its specific receptor (uPAR); uPA and uPA/uPAR complex activate plasmin and interact with extracellular matrix protein (ECM) – vitronectin, which modulates their activity; uPA/uPAR contributes in different cellular processes; **B** – Pocket in uPAR-uPA interaction targeted by inhibitory antibodies; **C** – Pocket in uPAR-uPA interaction used for the design of small molecular weight inhibitors. Depicted molecular coordinates correspond to the crystal structure of the uPAR-uPA complex determined at 1.9 A resolution (PDB code: 2FD6)

ers, suPAR is independent of age, which assigns it to be a good predictor both in young and old individuals [25]. In the large international project determining trends in cardiovascular diseases (MONICA 10), it was proven that a shorter life expectancy and the increased risk of CVD (cardiovascular diseases), type 2 diabetes mellitus or cancer are associated with elevated plasma suPAR concentrations [18]. Moreover, the level of suPAR is increased with renal dysfunction (albuminuria) and other diabetic complications [31]. suPAR is associated with glomerular focal sclerosis due to podocyte and basal membrane damage and modification of renal barrier function [50, 51]. Experiments carried out on a mouse allograft model showed that uPAR contributes to cellular apoptosis and renal ischemia [21], whereas inhibition of uPAR decreases proteinuria in mice with glomerular disease [50, 52].

ROLE OF UPAR IN ENDOTHELIAL PATHWAY REGULATIONS

What is more intriguing, uPAR stimulates the activity of TCF-responsive promoter and transactivation of uPA and cyclin D-1 genes and the overexpression of u-PAR and uPA has also been shown in atherosclerotic plaques [40]. Other data indicate that VEGF (vascular endothelial growth factor) stimulates uPA expression by inducing endothelial cells hyperpermeability due to the activation of the uPA/uPAR system through beta-catenin signaling [5]. What is interesting, uPA-induced permeability is rapid and sustained, in contract to a two-step transient/delayed VEGF-induced permeability. In the delayed phase of endothelial dysfunction, uPAR upregulation was observed in the correlation with beta-catenin nuclear translocation and downregulation of ocludin [5]. The mechanisms responsible for VEGF-induced endothelial permeability include fenestration due to VE-cadherin , β-catenin tyrosine phosphorylation and actin cytoskeleton rearrangement [17]. These results suggest that u-PA/u-PAR and Wnt pathways are tightly related with presently non-revealed manner.

ROLE OF UPAR IN VASCULAR PATHOLOGIES

Endothelial extracellular vesicles (EVs) can be considered as a hallmark of vascular pathology [46]. Especially the population of the bigger EVs called microvesicles (MVs) or ectosomes is very active in the conveyance of both procoagulant and fibrinolytic factors including tissue factor (TF), phosphatidylserine or uPAR [1, 6, 49]. The uPAR activity in endothelial dysfunction has been previously described and its role in the modulation of angiogenetic response of endothelial MVs has been documented [32]. Endothelial cells provide a catalytic surface for the conversion of plasminogen into plasmin by expressing the uPA/uPAR complex, thus decreasing uPAR activity on dysfunctional endothelial cells by releasing uPAR-bearing MVs turns vascular lining into pathological prothrom-

botic state [6]. By contrast, the increase of soluble uPAR-bearing endothelial MVs contributes to the fibrinolytic and proangiogenic activity of MVs [32]. This MVs-uPAR interplay might explain a molecular mechanism associated with an increased risk of vascular complications in patients with diabetes mellitus.

TARGETING UPAR IN DIABETIC VASCULAR COMPLICATIONS

The spontaneously diabetic Torri (SDT) rats can develop diabetic retinopathy (DR) in a similar way to human, which is why they are one of the most popular models for studying the pathologic signs of type 2 diabetes. UPARANT is a new designed tetrapeptide which mimics the amino acid sequence responsible for uPAR agonists binding in the cell membrane [8]. In the laser-induced choroidal neovascularization (CNV) mice model, UPARANT disturbs inflammation and angiogenesis through transcription factors encoding inflammatory and angiogenesis genes [7]. These results suggest that UPARANT might be potentially useful in the treatment of age-related macular degeneration (AMD) and diabetic retinopathy [7, 8]. Additionally, this tetrapeptide down-regulates the expression of uPAR and its membrane partners in SDT rats and diabetic mice, which resulted in the reduction in levels of inflammatory (IL-1β, IL-6, TNF-α) and pro-angiogenic factors (FGF-2, VEGF, IGF-1, Ang-2) [7, 8]. Another peptide, derived from the non-receptor-binding region of urokinase (Å6), blocks the retinal vascular permeability and reduces the loss of VE-cadherin in diabetes, which suggests its potential role as a therapeutic agent in the treatment of diabetic macular edema [39].

Also, antibody-blocking strategy is very promising in targeting uPAR function. Bacteriophage display libraries were used to produce short amino acids fragments of monoclonal antibody against uPAR. The mAb IIIF10 significantly reduce the binding of uPA to uPAR, which suggest that this sFv fragment is located within or close to the uPA-binding site in uPAR [35]. Experiments carried out on human ovarian cancer cell line OV-MZ-6 indicated that the IIIF10 antibody decreased uPA-induced cell proliferation by disrupting uPA/uPAR interactions [19]. In clinic, antibodies against uPAR might be useful not only for therapeutic purposes but also for imaging in tumors with high expression of uPAR [16].

Here, we propose the conformational analysis for the uPA/uPAR complex and docking antibody and small molecule inhibitors (Fig. 1B and C).

There are a few small molecule inhibitors of the uPA/uPAR protein-protein interaction; the biological role and functional properties of two of them were thoroughly analyzed: IPR-456 and IPR-803. They were discovered by a bioinformatical analysis against various conformations of uPAR derived from explicit-solvent molecular dynamics simulations [28]. *In vitro*, the IPR-456 compound blocks uPA/uPAR and inhibits MDA-MB-231 cell invasion [28]. However, there was no impact on cell adhesion and the effect on cells migration was weak, which suggests that other ligands (no uPA) are associated with the mediation of these processes. Similarly, the IPR-803 compound impairs MDA-MB-231 cell adhesion and migration and, what is most important, cell invasion by blocking MMP activity [38].

CONCLUSIONS

Several antibodies, small molecules and peptides have been developed to target uPAR in vascular pathologies by the blocking uPAR/uPA pathway. The main challenge in developing the efficient uPAR inhibitor is the pleiotropic nature of uPAR interactions and its multifunctional properties in coagulation, inflammatory and vascular systems. Moreover, the uPAR gene is under control of the Wnt pathway (beta-catenin). All these data suggest that the uPA/uPAR system contributes in many cellular processes, including endothelial cell proliferation or dysfunction and targeting uPAR may imbalance these processes unknowingly.

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The authors have no potential conflicts of interest to declare.