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Inhibition of pericellular plasminogen activation by apolipoprotein(a): Roles of urokinase plasminogen activator receptor and integrins $\alpha_M\beta_2$ and $\alpha_V\beta_3$

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ABSTRACT

Background and aims: Lipoprotein(a) (Lp(a)) is a causal risk factor for cardiovascular disorders including coronary heart disease and calcific aortic valve stenosis. Apolipoprotein(a) (apo(a)), the unique glycoprotein component of Lp(a), contains sequences homologous to plasminogen. Plasminogen activation is markedly accelerated in the presence of cell surface receptors and can be inhibited in this context by apo(a).

Methods: We evaluated the role of potential receptors in regulating plasminogen activation and the ability of apo(a) to mediate inhibition of plasminogen activation on vascular and monocytic/macrophage cells through knockdown (siRNA or blocking antibodies) or overexpression of various candidate receptors. Binding assays were conducted to determine apo(a) and plasminogen receptor interactions.

Results: The urokinase-type plasminogen activator receptor (uPAR) modulates plasminogen activation as well as plasminogen and apo(a) binding on human umbilical vein endothelial cells (HUVECs), human acute monocytic leukemia (THP-1) cells, and THP-1 macrophages as determined through uPAR knockdown and overexpression. Apo(a) variants lacking either the kringle V or the strong lysine binding site in kringle IV type 10 are not able to bind to uPAR to the same extent as wild-type apo(a). Plasminogen activation is also modulated, albeit to a lower extent, through the Mac-1 ($\alpha_M\beta_2$) integrin on HUVECs and THP-1 monocytes. Integrin $\alpha_V\beta_3$ can regulate plasminogen activation on THP-1 monocytes and to a lesser extent on HUVECs.

Conclusions: These results indicate cell type-specific roles for uPAR, $\alpha_M\beta_2$, and $\alpha_V\beta_3$ in promoting plasminogen activation and mediate the inhibitory effects of apo(a) in this process.

1. Introduction

Elevated levels of plasma lipoprotein(a) (Lp(a)) have been identified as a causal risk factor for coronary heart disease (CHD) and calcific aortic valve disease (CAVD), as well as an independent risk factor for ischemic stroke, myocardial infarction, and peripheral vascular disease [1–4]. The structure of Lp(a) is similar to that of LDL, in that it contains apolipoprotein B-100 (apoB-100) with a comparable lipid content [5], but differs from LDL by virtue of the unique glycoprotein apolipoprotein(a) (apo(a)). Cloning of the apo(a) cDNA revealed a high degree of sequence similarity to that of the plasma zymogen plasminogen [6]. Apo(a) contains repeated copies of a sequence similar to that of plasminogen kringle IV (KIV) followed by sequences similar to the kringle V (KV) and protease domain of plasminogen. Unlike that of plasminogen, the protease domain in apo(a) is catalytically inactive [7]. Apo(a) KIV can also be further classified into 10 subtypes (KIV_1 to KIV_{10}) based on amino acid sequence. Each KIV subtype is present in a single copy except for KIV_2 which is present in identical repeated copies numbering from 3 to >40, giving rise to the isoform size heterogeneity present in the population [8,9]. Apo(a) is covalently linked to apoB-100 via a single disulfide bond whose formation is preceded by interactions between apoB-100 and the weak lysine binding sites of apo(a) kringle IV types 6–8 [10,11].

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The homology between plasminogen and apo(a) has been suggested to underlie the pathogenic effects of Lp(a) whereby the apo(a) component interferes with the plasminogen activation system. Plasmin, generated through plasminogen activation, is a key component not only of fibrinolysis but also of cell migration and proliferation, angiogenesis, inflammation, wound healing, and tumor cell invasion and metastasis [12–19]. Apo(a) and plasminogen both contain lysine binding sites which mediate their respective interactions with fibrin and various cell surface receptors [20]. Plasminogen activation is inherently inefficient, and biologically meaningful rates of plasmin formation require a cofactor in the form of fibrin or a cellular surface [21,22]. Apo(a) is able to inhibit plasminogen activation within the fibrin clot, leading to impaired fibrinolysis [23]. Moreover, Lp(a) and apo(a) have been recently shown to be capable of inhibiting pericellular plasminogen activation on monocytes, macrophages, and vascular endothelial cells (ECs) [24]. To date, however, the identity of lysine-containing receptors that bind apo(a) and mediate its inhibition of plasminogen activation remain obscure. Apo(a) has been shown to stimulate cell growth and migration in ECs through integrin $\alpha_V\beta_3$ and Lp(a) can recruit inflammatory cells through Mac-1 ($\alpha_M\beta_2$) integrin; direct binding of apo(a) to the latter integrin has been demonstrated [25,26].

The uPA receptor (uPAR) has been shown to be a key regulator in mediating plasminogen activation and extracellular matrix (ECM) degradation [27]. The glycosyl phosphatidylinositol (GPI)-anchored uPAR can bind to uPA with high affinity (1 nM) as well as the ECM protein vitronectin, a ligand of $\alpha_V\beta_3$ [18,27]. Activation of pro-uPA to its active form is mediated through the binding of pro-uPA to uPAR [27–29].

A variety of receptors have been identified on different cell types which can mediate both plasminogen activation as well as plasmin binding [19,30]. Most plasminogen receptors are synthesized with a carboxyl terminal lysine or expose a lysine on the cell surface which can mimic a carboxyl-terminal lysine. The receptors that bind plasminogen are redundant and broadly distributed on various cells with binding sites ranging from 10^5 to 10^7 per cell [30]. Known receptors for plasminogen on vascular cells include annexin A2/p11, actin, $\alpha_V\beta_3$ integrin, histone H2B, $\alpha_M\beta_2$ integrin, Plg-R_{KT}, α-enolase, $\alpha IIb\beta_3$, and the TAT-binding protein-interacting protein [31–37]. Collectively, these receptors serve to accelerate plasminogen activation as well as to protect plasmin from its natural physiological inhibitor, α_2-antiplasmin.

In the present study, we describe the role of various specific receptors – namely uPAR, $\alpha_M\beta_2$, and $\alpha_V\beta_3$ – in regulating plasminogen activation as well as plasminogen and apo(a) binding.

2. Materials and methods

2.1. Construction, expression, and purification of recombinant apo(a)

The construction and expression of the various r-apo(a) variants have been previously described. The pRK5 expression vectors encoding 10 K, 12 K, 12ΔV, 17 K, 17 KΔLBS_{10} and 17 KΔV were stably transfected into human embryonic kidney (HEK) 293 cells and purified from conditioned media by lysine-Sepharose affinity chromatography as previously described [7,38–40]. All r-apo(a) protein concentrations were determined spectrophotometrically and assessed for purity by SDS-PAGE under non-reduced and reduced conditions followed by silver staining.

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) and human acute monocytic leukemia (THP-1) cells were cultured, and the latter cell line differentiated into macrophage-like cells, as described previously [24].

2.3. Plasmid mutagenesis

A plasmid containing the cDNA encoding carboxyl-terminally GFP tagged human uPAR, in the pCMV-AC-GFP vector, was purchased from Origene (catalog number RG201222). The plasmid was mutagenized to introduce a stop codon immediately preceding the GFP tag via site directed mutagenesis, thereby restoring the natural carboxyl-terminal Thr residue in uPAR. Site directed mutagenesis of the plasmid was conducted using the QuikChange II-E kit (Stratagene) as per the manufacturer's recommendations with the following primers: forward 5′-TCT CCT CTG GAC CTA GCG TAC GCG GCC G-3′ and reverse 5′-CGG CCG CGT ACG CTA GGT CCA GAG GAG A-3'. Base pairs AC were mutated to TA as indicated to introduce the stop codon. The resultant plasmid was used in overexpression experiments.

2.4. Transient transfection

HUVECs, THP-1 monocytes, and THP-1 macrophages were transfected with uPAR siRNA or control siRNA (Santa Cruz Biotechnology) at a concentration of 40–80 nM with transfection reagent and medium from Santa Cruz Biotechnology following the manufacturer's protocols. Transfection mixture was removed following 5–6 h incubation and replaced with complete medium. Cells were assayed 72 h following transfection (determined to have the most robust effect on KD). Percent knockdown was determined using qRT-PCR. HUVECs, THP-1 monocytes, and THP-1 macrophages were transiently transfected with uPAR expression using Lipofectamine LTX and Plus Reagent (Invitrogen) as per the manufacturer's protocol. HUVECs were transfected with 0.3 μg/mL of DNA and incubated with transfection mixture for 4 h, after which the cells were incubated in fresh medium for 48 h prior to assay. THP-1 monocytes and macrophages were transfected with 0.75 μg/mL of DNA and incubated with transfection mixture for 24 h, after which the cells were incubated in fresh medium for 48 h prior to assay. 72 h post-transfection was determined to correspond with the peak overexpression of uPAR, as determined through Western blot analysis.

2.5. Plasminogen activation and cell binding assays

Plasminogen activation experiments were performed as previously described [25], and utilized the fluorogenic plasmin substrate H-D-Val-Leu-Lys-7-amido-4-methylcoumarin (Bachem). The rate of plasminogen activation was taken between 10 and 40 min from the slope of relative fluorescent units (RFU) against min^2. Rates obtained for each individual experiment performed in triplicate were normalized to the rate of plasminogen activation in the absence of apo(a). For experiments using blocking monoclonal antibodies, prior to the assay of plasminogen activation, cells were washed three times with blocking solution (10 mM Tris-HCl, pH 7.4, 0.14 mM NaCl, 0.1% (w/v) BSA, 1 mM $CaCl_2$, and 1 mM $MgCl_2$) followed by incubation with mouse anti-human integrin $\alpha_V\beta_3$ (Millipore), mouse-anti human CD18 clone IB4 (Labs Inc Biotechnology), and/or mouse-anti human CD11b clone 44aacb (Imgenex) for 1 h at 37 °C. Cells were then washed three times with blocking solution followed by plasminogen activation assay as described above.

Cell binding assays used purified recombinant apo(a), fluorescently labeled using Alexa Fluor 488 protein labeling kit (Invitrogen), or fluorescein isothiocyanate-labeled human plasminogen (purchased from Molecular Innovations) as previously described