Plasmin reduces fibronectin deposition by mesangial cells in a protease-activated receptor-1 independent manner

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ABSTRACT

Background: Protease-activated receptor-1 (PAR-1) potentiates diabetic nephropathy (DN) as evident from reduced kidney injury in diabetic PAR-1 deficient mice. Although thrombin is the prototypical PAR-1 agonist, anticoagulant treatment does not limit DN in experimental animal models suggesting that thrombin is not the endogenous PAR-1 agonist driving DN.

Objectives: To identify the endogenous PAR-1 agonist potentiating diabetes-induced nephropathy.

Methods: Unbiased protease expression profiling in glomeruli from human kidneys with DN was performed using publically available microarray data. The identified prime candidate PAR-1 agonist was subsequently analysed for PAR-1 dependent induction of fibrosis in vitro.

Results: Of the 553 proteases expressed in the human genome, 247 qualified as potential PAR-1 agonists of which 71 were significantly expressed above background in diabetic glomeruli. The recently identified PAR-1 agonist plasminogen, together with its physiological activator tissue plasminogen activator, were among the highest expressed proteases. Plasmin did however not induce mesangial proliferation and/or fibronectin deposition in vitro. In a PAR-1 independent manner, plasmin even reduced fibronectin deposition.

Conclusion: Expression profiling identified plasmin as potential endogenous PAR-1 agonist driving DN. Instead of inducing fibronectin expression, plasmin however reduced mesangial fibronectin deposition in vitro. Therefore we conclude that plasmin may not be the endogenous PAR-1 agonist potentiating DN.

1. Introduction

The World Health Organization approximates that over 300 million people will suffer from diabetes in 2025 [1]. The health implications of this endemic disease are expected to be larger as diabetic patients frequently develop complications like (among others) diabetic nephropathy leading to end-stage renal disease (ESRD) [2]. Diabetic nephropathy actually emerged as the major causative pathology in patients entering ESRD worldwide and it is responsible for 30–40% of all ESRD cases. In individuals with diabetes, the presence and severity of nephropathy adversely affects their well-being, significantly contributes to disease morbidity and increases their risk of a premature death [3,4]. Although the progression of diabetic nephropathy can be delayed by strict control of plasma glucose levels and/or by lowering blood pressure, the majority of patients eventually need renal replacement therapy. The large impact of this latter therapy, both on the social and economic level [5,6], urges the need for alternative treatment options.

In the search for alternative targets to pursue in combatting diabetic nephropathy, we recently identified protease-activated receptor (PAR) − 1 as an attractive candidate. Indeed, PAR-1 deficient mice showed reduced diabetes-induced albuminuria, plasma cystatin C levels, mesangial expansion and tubular atrophy as compared to wild type diabetic controls [7]. Subsequent mechanistic experiments showed that PAR-1 activation induces proliferation and fibronectin production by MES13 mesangial cells in vitro.

PAR-1 is a seven transmembrane domain receptor that is activated by proteolytic cleavage rather than by ligand binding [8–10]. Generally, PAR-1 is recognized as a blood coagulation factor receptor and thrombin is considered the prototypical PAR-1 agonist. Importantly however, anticoagulation with low-molecular-weight heparin did not protect against diabetic nephropathy in diabetic wild type mice despite the fact that it normalized markers of coagulation (i.e. thrombin-antithrombin, dimer and renal fibrin deposition). Indeed, albuminuria, kidney weight, histological PAS scores and glomerular size were

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similar in saline and low-molecular-weight heparin treated diabetic mice [11]. Similarly, the direct thrombin inhibitor hirudin had also no significant effect on key parameters of nephropathy in diabetic wild type mice [12]. In the setting of diabetic nephropathy, thrombin may therefore not be the endogenous PAR-1 agonist potentiating kidney injury.

Next to thrombin, activated protein C (APC) is a well-recognized PAR-1 agonist [13]. APC-dependent PAR-1 activation, however, prevents hyperglycemia-induced apoptosis of endothelial cells and podocytes in vitro, whereas APC overexpression reduces diabetes-induced kidney injury in vivo [11]. These data indicate that APC is not the endogenous PAR-1 agonist potentiating diabetic nephropathy but also imply that targeting the PAR-1 agonist driving diabetic nephropathy might be a more effective treatment strategy than targeting the receptor itself. Indeed, preventing PAR-1-dependent mesangial expansion and tubular atrophy without blocking the beneficial PAR-1 effects on endothelial cells and podocytes may even further decrease diabetes-induced nephropathy.

To fully appreciate the importance and potential clinical relevance of the PAR-1 pathway in diabetic nephropathy, it is important to identify the endogenous PAR-1 agonist that potentiates kidney injury during diabetes. In the current manuscript, we therefore aimed to pinpoint candidate proteases as endogenous PAR-1 agonists potentiating diabetic nephropathy. To this end, we employed an unbiased approach in which expression levels of all proteases expressed in the human genome were assessed in diabetic glomeruli.

2. Materials and methods

2.1. Mining of publicly available RNA microarray dataset

The GSE10009 [14] dataset was downloaded from Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo). This dataset reports whole-genome gene expression of glomeruli isolated from patients with diabetes mellitus. Background expression was determined for individual chips using Affymetrix negative control probes and protease expression levels above the mean + 2SD were considered significantly expressed and were used for further analysis. Subcellular localisation of proteases was assessed using the UniProtKB/Swiss-Prot and COMPARTMENTS databases [15].

2.2. Cell culture and stimulation

Mouse mesangial cells (SV40 MES13; CRL-1927 ATCC) were cultured according to the recommended protocol (https://www.lcststandards-attc.org/Products/Cells_and_Microorganisms/By_Tissue/Kidney/CRL-1927.aspx?geo_country=nl#culturemethod) using a 3:1 mixture of Dulbecco's Modified Eagle's Medium containing 1 g/L glucose with Ham's-F12 medium, supplemented with 5% heat inactivated fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine. Cells were cultured at 37 °C in an atmosphere of 5% CO2. Cells were serum starved overnight in low glucose (1 g/L) medium and separated on 10% SDS-PAGE gel and transferred onto Immobilon-PL membranes (Millipore) as described before [20]. Membranes were blocked for 1 h at room temperature in 5% bovine serum albumin (BSA) in TBS + 0.1% Tween-20 (TBS-T) and incubated on TBS-T membrane at 4 °C. The immunodetection was performed using an ImageQuant western blot machine and corresponding software (Software release 1.5.0 (1.5.0.39), Roche, Almere, the Netherlands) on an ImageQuant LAS 4000 biomolecular imager (GE Healthcare, Zeist, the Netherlands).

2.3. MTT assay

Cells were seeded at a density of 5000 cells/well in 96 well plates. After stimulation with plasmin or PAR-1 agonist peptide for 24 h, MTT (St. Louis, MO, USA) was added to the culture medium. After 2 h incubation at 37 °C, cells were lysed with DMSO and OD570 was measured using a microplate reader (Synergy HT, BioTek).

2.4. Western Blot

Cells were seeded at a density of 50000 cells/well in 24 well plates. After stimulation for 24 h, cells were lysed with RIPA lysis buffer (50 mM Tris HCl (pH 7.4), 150 mM NaCl, 0.5% deoxycholate, 1.0% Triton X-100, 0.1% SDS and 1 mM EDTA) supplemented with 1x Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, #78445). Cell lysates were subsequently diluted (1:1) in Laemmli buffer and separated on 10% SDS-PAGE gel and transferred onto Immobilon-PL membranes (Millipore) as described before [20]. Membranes were blocked for 1 h at room temperature in 5% bovine serum albumin (BSA) in TBS + 0.1% Tween-20 (TBS-T) and subsequently incubated with the following primary antibodies, diluted in TBS-T: mouse-anti-tubulin 1:2500 (Santa Cruz; sc-23948) or goat-anti-fibronectin 1:1000 (Santa Cruz; sc-6953). After overnight incubation, the membranes were washed 3 times with TBS-T and incubated 1 h at room temperature with horseradish peroxidase (HRP)-conjugated (1:1000, DakoCytomation, Glostrup, Denmark) secondary antibodies diluted 1:5000 in TBS-T. Membranes were washed 3 times with TBS-T and imaged using Lumina Forte western blot substrate (Merck Millipore, Billerica, Massachusetts, USA) on an ImageQuant LAS 4000 biomolecular imager (GE Healthcare, Zeist, the Netherlands).

2.5. RNA isolation and RT-qPCR

Cells were seeded at a density of 50000 cells/well in 24 well plates. After stimulation for the indicated time points, mRNA was isolated using Trizol and separated in 1x RNase-free water. All mRNA samples were quantified by spectrophotometry and stored at −80 °C until further analysis. 0.75 μg of mRNA was treated with DNase using the RQ1 DNase kit (M101, Promega, Madison, WI, USA) and subsequently converted to cDNA using M-MLV reverse transcriptase (M1705, Promega, Madison, WI, USA) and random hexamer primers (#SO142, Fisher scientific, Landsmeer, the Netherlands) according to the manufacturers recommendations. qPCR and subsequent analysis were performed using a Roche lightcycler with SYBR green PCR master mix (#04707516001; Roche Diagnostics) according to the manufacturers recommendations. cDNA samples were run in triplicates on an Mx3005P machine and corresponding software (Software release 1.5.0 (1.5.0.39), Roche, Almere, the Netherlands). Expression levels were normalized using the mean average expression levels of HPRT and TBP. The following primer sequences were used: Fibronectin forward 5′-CCATGTTAGGAAACAGTGCGCA-3′ and reverse 5′-GAAGCACTCAATGGGGCA-3′; TBP forward: 5′-GGAGAATCATGGAACAGAA-3′ and reverse: 5′-GATGGAAATTCCAGAGTCA-3′; HPRT forward: 5′-TGCCTGGTTGCTGATTCTGTTT-3′ and reverse: 5′-CCTGGTGTACATCGCTATCTC-3’. (80 °C until

2.6. Statistics

All values are expressed as mean ± SEM. Differences between groups were analysed using a Mann-Whitney U-test for non-parametric data. All analyses were performed using GraphPad Prism version 5.01.

3. Results

The proteolytic activation mechanism of PAR-1 dictates the endogenous PAR-1 agonist inducing glomerular expansion and subsequent diabetic nephropathy as a beateze. Consequently, we assessed protease mRNA expression levels in glomeruli obtained from patients with diabetic nephropathy using the publically available GSE1009 gene expression omnibus database [14]. From the total of 553 genes that have been annotated to encode proteases or protease homologues in the
human genome [21], 93 genes were excluded as candidate PAR-1 agonist as they represent catalytically inactive pseudogenes and 26 were excluded as they do not have murine counterparts (Fig. 1). Based on subsequent subcellular location analysis another 187 intracellular proteases were excluded. From the remaining 247 secreted or outer cell membrane-bound proteases, expression data were available for 166 proteases of which 70 were significantly expressed above background levels (Supplemental Table 1; Fig. 2A). Serine and metalloproteases constitute the large majority of these 70 candidate PAR-1 agonists.

Tissue plasminogen activator (tPA) is the highest expressed protease in diabetic glomeruli, whereas its physiological substrate plasminogen is also within the upper tertile of expressed proteases (Fig. 2B). This is particularly interesting as plasin is a known PAR-1 agonist [17,22–25] and thus a likely candidate to drive PAR-1 activation during diabetic nephropathy. As shown in Fig. 3A, plasin does not affect mesangial proliferation as opposed to PAR-1 agonist peptide that significantly induced proliferation of MES13 cells in vitro. PAR-1 activation on MES13 mesangial cells using PAR-1 agonist peptide also induced fibronectin expression, which was not observed after plasin stimulation (Fig. 3B). In fact, plasin even decreased fibronectin deposition by MES13 cells in a dose-dependent manner (Fig. 3C). Pretreatment of MES13 cells with the PAR-1 inhibitors P1pal12 or vorapaxar did not affect unstimulated fibronectin levels (Fig. 3D) but did also not prevent the plasin-dependent reduction in fibronectin levels showing that this response is PAR-1 independent (Fig. 3E). The reduction in fibronectin deposition after plasin stimulation was not accompanied by a decrease in fibronectin mRNA levels (Fig. 3F).

4. Discussion

PAR-1 activation potentiates diabetic nephropathy [7], but the endogenous agonist responsible for PAR-1 activation during diabetes remains elusive. In the current manuscript, we performed an unbiased protease profiling approach to identify candidate PAR-1 agonists driving diabetic nephropathy. This approach identified the tPA/plasmin axis as prime candidate but subsequent in vitro experiments seem to exclude plasin as endogenous PAR-1 agonist aggravating diabetes-induced kidney injury.

In the upper tertile of expressed proteases, plasinogen is the only protease that has been implicated as potential PAR-1 agonist. Already in 1999, it was shown that plasin cleaved a soluble N-terminal exodomain of PAR-1 as effectively as thrombin [23]. Plasin-PAR-1 signaling was subsequently shown to, amongst others, induce Cbfα1 expression -a growth factor-like gene involved in cell proliferation, adhesion, and migration [23]- and transforming growth factor beta production [24]. Moreover, plasin stimulation of murine tubular epithelial cells induced their phenotypic transition to fibronectin-producing fibroblast-like cells which was inhibited by PAR-1 siRNA and by a specific PAR-1 antagonist [25]. Despite these data pinpointing plasin-PAR-1 signaling as a key pathway in profibrotic responses, here we show that plasin does not induce proliferation of mesangial cells whereas it even inhibits fibronectin deposition by these cells.
Although plasmin is probably best known as a key protease of the fibrinolytic pathway involved in clot lysis, it can also cleave/degrade extracellular matrix proteins like laminin and fibronectin [26]. Already in the early 1980’s it was shown that plasmin efficiently cleaved purified fibronectin \textit{in vitro} [27] whereas the proteolytic activity of plasmin towards fibronectin \textit{in vivo} was confirmed more recently [28,29]. As plasmin stimulation of MES13 cells did not affect fibronectin mRNA levels, reduced fibronectin deposition by MES13 cells after plasmin stimulation is most likely not the result of reduced fibrinogen synthesis but is actually caused by enhanced degradation.

Plasmin does not mimic the previously reported effect of thrombin-PAR-1 signaling on mesangial cells. Indeed, thrombin induced proliferation and extracellular matrix deposition in a PAR-1 dependent manner [7], whereas plasmin-PAR-1 signaling does not affect these processes. Such divergent responses of different PAR-1 agonists are well described, and the capacity of agonists to trigger distinct signaling pathways is referred to as biased signaling (excellently reviewed in [30]). The contrasting effects of thrombin and activated protein C (APC)-induced PAR-1 signaling on endothelial barrier function is a classic example hereof [31]. Interestingly however, PAR-1-induced
biased signaling has also been described for podocytes as APC-PAR-1 signaling inhibits whereas thrombin-PAR-1 signaling induces apoptosis of podocytes [11]. Next to biased signaling, plasmin-cleaved PAR-1 may not induce profibrotic responses of mesangial cells because plasmin cleavage is not only described to activated PAR-1 signaling [17,22–25] but may actually also desensitize PAR-1 [32]. Irrespective the actual explanation, our data show that plasmin does not induce fibroblast production by mesangial cells.

The PAR-1 agonists described to date are either serine (i.e. APC [13], Factor X [33], thrombin [8–10], plasmin [22–25], Granzyme K [34], kalikirein-1 [35] and –6 [36], kalikirein-related peptide 4 [37], neutrophil elastase [38], PRSS3 [39], proteinase 3 [38]) or metalloproteases (MMP-1 [40] and MMP-13 [41]). Highly expressed proteases of these types may thus be prime PAR-1 agonist candidates and actually all serine and metalloproteases in the upper tertile of expressed proteases in diabetic glomeruli (Fig. 2B) theoretically qualify as candidates. Except tPA/plasminogen, none of the proteases from the upper tertile have however been implicated in diabetic nephropathy and/or to modify PAR-1 signaling. Moreover, the endogenous PAR-1 agonist driving diabetic nephropathy may not be diabetic nephropathy and/or to modify PAR-1 signaling. Moreover, the endogenous PAR-1 agonist driving diabetic nephropathy may not be synthesized locally in the glomeruli at all, but may actually be produced in cardiac cells [11]. The endogenous PAR-1 agonist in the setting of diabetic nephropathy therefore remains elusive.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbrrep.2017.03.009.

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