Cellular Signalling xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

A small molecule PAI-1 functional inhibitor attenuates neointimal hyperplasia and vascular smooth muscle cell survival by promoting PAI-1 cleavage

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ARTICLE INFO

Article history:
Received 2 December 2014
Received in revised form 15 January 2015
Accepted 15 January 2015
Available online xxxx

Keywords:
Apoptosis
Carotid stenosis
SERPIN
Vascular injury
PAI-1

ABSTRACT

Plasminogen activator inhibitor-1 (PAI-1), the primary inhibitor of urokinase-and tissue-type plasminogen activators (uPA and tPA), is an injury-response gene implicated in the development of tissue fibrosis and cardiovascular disease. PAI-1 mRNA and protein levels were elevated in the balloon catheter-injured carotid and in the vascular smooth muscle cell (VSMC)-enriched neointima of ligated arteries. PAI-1/uPA complex formation and PAI-1 antiproteolytic activity can be inhibited, via proteolytic cleavage, by the small molecule antagonist tiplaxtinin which effectively increased the VSMC apoptotic index in vitro and attenuated carotid artery neointimal formation in vivo. In contrast to the active full-length serine protease inhibitor (SERPIN), elastase-cleaved PAI-1 (similar to tiplaxtinin) also promoted VSMC apoptosis in vitro and similarly reduced neointimal formation in vivo. The mechanism through which cleaved PAI-1 (CL-PAI-1) stimulates apoptosis appears to involve the TNF-α family member TWEAK (TNF-α weak inducer of apoptosis) and it's cognate receptor, fibroblast growth factor (FGF)-inducible 14 (FN14). CL-PAI-1 sensitizes cells to TWEAK-stimulated apoptosis while full-length PAI-1 did not, presumably due to its ability to down-regulate FN14 in a low density lipoprotein receptor-related protein 1 (LRP1)-dependent mechanism. It appears that prolonged exposure of VSMCs to CL-PAI-1 induces apoptosis by augmenting TWEAK/FN14 pro-apoptotic signaling. This work identifies a critical, anti-stenotic, role for a functionally-inactive (at least with regard to its protease inhibitory function) cleaved SERPIN. Therapies that promote the conversion of full-length to cleaved PAI-1 may have translational implications.

1. Introduction

Vascular restenosis, or blood vessel re-narrowing after percutaneous coronary intervention, is a combinatorial pathological consequence of increased vascular smooth muscle cell (VSMC) migration and proliferation combined with decreased apoptosis [1,2]. Few treatment options for vascular restenosis exist aside from additional catheterization. Among several factors implicated in the vascular response to injury, plasminogen activator inhibitor-1 (PAI-1), a member of the serine protease inhibitor (SERPIN) superfamily and the major physiologic regulator of the plasmin-based pericellular proteolytic cascade, is perhaps the most prominent.

PAI-1 exists in three distinct conformations [3,4]. While initially synthesized as an active SERPIN, PAI-1 spontaneously converts to a latent form (active half-life ~2 h at 37 °C, pH 7.4) which is unable to inhibit uPA or tPA catalysis. This rather short half-life can be extended 2-to-10 fold, however, upon binding of PAI-1 to the somatomedin B domain of vitronectin where it also impacts integrin–matrix interactions and downstream signaling pathways [5–8]. During engagement of PAI-1 with its target proteases, the sissile bond in the reactive center loop (RCL) is cleaved to form a covalent ester bond between a serine hydroxyl group of the enzyme and a PAI-1 carboxyl group. Upon cleavage, the RCL N-terminus inserts into β-sheet A, while the C-terminus of the RCL forms strand s1C in β-sheet C producing a 70 Å separation of the P1 and P1′ residues, thereby deforming the complexed protease

Abbreviations: CL-PAI-1, cleaved PAI-1; FL-PAI-1, full-length PAI-1; HNE, human neutrophil elastase; HuCASMCs, human carotid artery smooth muscle cells; LRP1, low density lipoprotein receptor-related protein 1; pAkt, phospho-Akt; PAI-1, plasminogen activator inhibitor-1; PARP, poly (ADP-ribose) polymerase; Plg, plasminogen; RASMCs, rat arterial smooth muscle cells; RCL, reactive center loop; SERPIN, serine protease inhibitor; SERPINE1, serine protease inhibitor clade E member 1; TPX, tiplaxtinin; uPA, urokinase; VN, vitronectin; VSMCs, vascular smooth muscle cells.

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http://dx.doi.org/10.1016/j.cellsig.2015.01.009
0898-6568/

and rendering it inactive. A substrate form of PAI-1 exists as well in which PAI-1 is cleaved by its target proteases without formation of a covalent PAI-1:protease complex [3,9–11].

Due to the complexity of the PAI-1 structure/function, several low-molecular weight antagonists of PAI-1 were developed to evaluate specific contributions of this SERPIN to disease pathology [12]. Tiplaxtinin (PAI-039), a well-studied PAI-1 small-molecule inhibitor, attenuates asthmatic episodes, reduces hyperlipidemia and hyperglycemia and suppresses tumor angiogenesis [12–19]. The mechanism by which tiplaxtinin antagonizes the anti-fibrinolytic activity of PAI-1 appears to involve promotion of a substrate-like conformation resulting in PAI-1 cleavage and impaired uPA and tPA inhibition [20,21].

This paper reports that PAI-1 levels are elevated in injured VSMCs in vivo. Tiplaxtinin, which promotes PAI-1 cleavage, and elastase-cleaved PAI-1 (CL-PAI-1) both attenuate neointima formation in response to carotid artery ligation and stimulate plasmin-dependent, terminal VSMC apoptosis. CL-PAI-1, but not the functionally-stable full-length recombinant PAI-1 mutant 14-1b (FL-PAI-1), sensitizes VSMCs to TWEAK (TNF-α weak inducer of apoptosis)-induced apoptosis, a likely consequence of full-length PAI-1/LRP1-mediated down-regulation of FN14 receptor expression. This work identifies a novel, anti-stenotic, role for cleaved PAI-1 in the tissue response to arterial injury. Therapies that promote the conversion of full-length to cleaved PAI-1 may have translational implications in the therapy of cardiovascular pathologies.

2. Experimental procedures

2.1. Cell culture

Newborn rat arterial smooth muscle cells (RASMCs; a gift from Dr. Peter A. Jones) were cultured in DMEM with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a humidified 5% CO_2 atmosphere at 37 °C. Human carotid artery SMCs (HuCASMCs; Cell Applications Inc., San Diego, CA) were grown in a Smooth Muscle Cell Growth Medium containing 5% FBS, 1% penicillin/streptomycin and 0.5% each of hEGF, insulin and bFGF/heparin, conditions which maintain VSMC in the de-differentiated, synthetic phenotype.

2.2. Recombinant proteins

Endotoxin levels in full-length, stabilized recombinant 14-1b PAI-1 (FL-PAI-1), R76E-PAI-1 (LRP1-binding deficient mutant; Molecular Innovations, Novi, MI), human neutrophil elastase (Abcam, Cambridge, UK) and high-molecular weight two chain uPA (Seiksui Diagnostics, LLC, Lexington, MA) preparations were measured using the Limulus Amebocyte Lysate Kit QCL-1000 (Lonza, Basel, Switzerland) and all were found to be <0.14 EU/ml, the acceptable threshold [22]. Recombinant Human TWEAK/TNFSF12 was obtained from R&D Systems (Minneapolis, MN).

2.3. RNA interference

siRNA designed against human PAI-1 mRNA (5′-AAGGATGAGATC AGCACCACA-3′) and a scrambled control (sc) siRNA (5′-AATTCTCCGA ACGTGTCACGT-3′) [23] were obtained from QIAGEN Inc. (Valencia, CA). HuCASMCs were transfected with 20 nM of PAI-1 siRNA or sc siRNA using Lipofectamine 2000 (Invitrogen).

2.4. Balloon catheter denudation and carotid artery ligation

All animal protocols were approved by the Institutional Animal Care and Use Committee of Albany Medical College (IACUC approved protocol #13-09001) and conducted in accord with the EU Directive 2010/63/EU for animal experimentation. Rodents were housed in the College Animal Resource Facility, licensed by the USDA and New York State Department of Health, Division of Laboratories and Research. Male Sprague–Dawley rats (400–450 g, Taconic Farms, Germantown, NY) were anesthetized by i.p. injection of ketamine (0.1 mg/g) and xylazine (0.01 mg/g), the left common carotid artery exposed by a midline cervical incision and blunt dissection performed alongside the artery with dull forceps to expose the carotid bifurcation into the internal/external branches. After blood flow cessation by arterial clamping, a 2F Fogarty balloon catheter (Edwards) was introduced via a small arteriotomy in the external carotid and advanced to the common carotid artery [24]. The balloon was inflated by 1.6 atm pressure, inserted and withdrawn three times. For carotid ligation, FVB/NJ mice (Jackson Labs) were anesthetized by i.p. injection of ketamine (0.1 mg/g) and xylazine (0.01 mg/g). Following site preparation, the left carotid artery was exposed with a small 8–10 mm midline incision in the neck and blunt dissected to free the left common carotid and branches from surrounding tissue. The common carotid artery was ligated just proximal to the internal and external carotid bifurcation with a 6-0 sterile silk suture. Animals were treated with tiplaxtinin (3 mg/kg; oral gavage), full-length (FL-PAI-1; 3 mg/kg; i.p. injection), and cleaved PAI-1 (CL-PAI-1; 3 mg/kg; i.p. injection), or vehicle control once daily for 14 days following ligation.

2.5. Northern blotting

Total RNA (10 μg) from the left (denuded) and (right) uninjured carotid arteries was separated on 1.2% agarose/formaldehyde gels, transferred to Nytran membranes by capillary action in 10× SSC (3 M NaCl, 0.3 M Na citrate, pH 7.0) and immobilized by UV crosslinking prior to incubation for 2 h at 42 °C in a prehybridization buffer (50% formamide, 5× Denhardt's reagent, 1% SDS, 100 μg/ml sheared/heat-denatured salmon sperm DNA [ssDNA], 5× SSC). RNA blots were incubated with random-primed ^{32}P-dCTP-labeled cDNA probes (5×10^6 cpm) to rat PAI-1 or A50 (loading control) for 16 h at 42 °C in a hybridization buffer (50% formamide, 2.5× Denhardt's reagent, 1% SDS, 100 μg/ml ssDNA, 5× SSC, and 10% dextran sulfate). Membranes were washed in 0.1× SSC/0.1% SDS at 42 °C, followed by three 15 min washes in 0.1× SSC/0.1% SDS at 55 °C and exposed to an X-OMAT AR-5 film using intensifying screens. Probe was removed from blots by washing in 55% formamide, 2× SSC, 1% SDS for 1 h at 65 °C and rinsed in 0.1× SSC/0.1% SDS prior to rehybridization with A50.

2.6. Immunohistochemistry

Paraffin-embedded tissue sections (5 μm) were stained with hematoxylin and eosin (H&E). For immunohistochemistry, sections were incubated with primary antibodies to smooth muscle α-actin (Sigma-Aldrich, St. Louis, MO; 1:6400 dilution) and PAI-1 (Affinity Bioreagents, Waltham, MA; 1:1000 dilution), washed, incubated with biotinylated anti-rabbit IgG and avidin D horseradish peroxidase (1:1000 dilution) and color reactions developed using an ABC kit prior to mounting coverslips in a Vectashield medium (Vector Labs, Burlingame, CA). Isotype IgG controls were included. Sections were analyzed using a Nanozoomer 2.0RS Digital Microscope equipped with NDP 2.2.1 software (Hamamatsu Photonics, Hamamatsu, Japan).

2.7. Flow cytometry

Fluorescence intensity was measured using a FACS LSRII (BD Biosciences, San Jose, CA). Ten thousand events were counted for each sample and experiments were analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

2.7.1. Cell cycle analysis

After treatment, RASMCs and HuCASMCs were harvested by trypsinization, centrifugation, washed twice with ice-cold phosphate-buffered saline (PBS) and fixed in 70% ethanol for 1 h. Fixed cells were washed twice with ice-cold PBS and incubated for 2 h in the dark in

Please cite this article as: T.M. Simone, et al., Cell. Signal. (2015), http://dx.doi.org/10.1016/j.cellsig.2015.01.009