Biochimica et Biophysica Acta 1842 (2014) 318–325

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

# Tissue-type plasminogen activator is not necessary for platelet-derived growth factor-c activation

Kimberly J. Riehle [a,b,1], Melissa M. Johnson [a,1,2], Fredrik Johansson [a], Renay L. Bauer [a], Brian J. Hayes [a], Debra G. Gilbertson [c], Aaron C. Haran [c,3], Nelson Fausto [a], Jean S. Campbell [a,*]

[a] Department of Pathology, University of Washington School of Medicine, Seattle, WA 98195, USA
[b] Department of Surgery, University of Washington School of Medicine, Seattle, WA 98195, USA
[c] Bristol Meyers Squibb, 1201 Eastlake Avenue East, Seattle, WA 98102, USA

ARTICLE INFO

*Article history:*
Received 2 September 2013
Received in revised form 12 November 2013
Accepted 13 November 2013
Available online 19 November 2013

*Keywords:*
Platelet-derived growth factor
Plasminogen activator
Liver fibrosis

ABSTRACT

Platelet-derived growth factors (PDGFs) are critical for development; their over-expression is associated with fibrogenesis. Full-length PDGF-C is secreted as an inactive dimer, requiring cleavage to allow receptor binding. Previous studies indicate that tissue-type plasminogen activator (tPA) is the specific protease that performs this cleavage; *in vivo* confirmation is lacking. We demonstrate that primary hepatocytes from *tpa* KO mice produce less cleaved active PDGF-CC than do wild type hepatocytes, suggesting that tPA is critical for *in vitro* activation of this growth factor. We developed mice that over-express full-length human *PDGF-C* in the liver; these mice develop progressive liver fibrosis. To test whether tPA is important for cleavage and activation of PDGF-C *in vivo*, we intercrossed *PDGF-C* transgenic (Tg) and *tpa* knock-out (KO) mice, anticipating that lack of tPA would result in decreased fibrosis due to lack of hPDGF-C cleavage. To measure levels of cleaved, dimerized PDGF-CC in sera, we developed an ELISA that specifically detects cleaved PDGF-CC. We report that the absence of *tpa* does not affect the phenotype of `*PDGF-C* Tg mice. *PDGF-C* Tg mice lacking tPA have high serum levels of cleaved growth factor, significant liver fibrosis, and gene expression alterations similar to those of *PDGF-C* Tg mice with intact tPA. Furthermore, urokinase plasminogen activator and plasminogen activator inhibitor-1 expression are increased in *PDGF-C* Tg; *tpa* KO mice. Our ELISA data suggest a difference between *in vitro* and *in vivo* activation of this growth factor, and our mouse model confirms that multiple proteases cleave and activate PDGF-C *in vivo*.



## 1. Introduction

Tissue fibrosis results from an imbalance in the metabolism of extracellular matrix (ECM) proteins, and the plasminogen activation system is a critical regulator of this balance [1–3]. Tissue plasminogen activator (tPA) is a serine protease first discovered via its ability to cleave plasminogen to its active form, plasmin [1–4]. Given that tPA has a specific affinity for fibrin, its ability to activate plasminogen is more restricted to areas of thrombosis than those of other plasminogen activators. Mice deficient in tPA develop and reproduce normally and have a normal life span, though they display decreased thrombolytic potential and increased endotoxin-induced thrombosis [5]. In the liver, tPA appears to contribute to repair after injury [6], though uninjured *tpa* knock-out (KO) mice have histologically normal livers [5]. tPA, along with urokinase plasminogen activator (uPA) and plasmin, is additionally important in the degradation of collagen [4]. The balance of creation and degradation of collagen determines the composition of the ECM of multiple organs, including the liver, and imbalance of these systems can lead to organ fibrosis. Increased activity of plasminogen activator inhibitor-1 (PAI-1), which inhibits tPA and uPA function, has been shown to lead to fibrosis, for example [1]. The ECM of the liver contains latent growth factors, which upon cleavage after injury can lead to cell proliferation or inflammation, suggesting that the fibrinolytic pathway could have additional functions in the ECM of the liver, beyond collagen homeostasis.

The platelet-derived growth factor (PDGF) family has been extensively studied over the past 40 years, and these ligands are required

*Abbreviations:* PDGF, platelet-derived growth factor; tPA, tissue-type plasminogen activator; Tg, transgenic; KO, knock-out; uPA, urokinase plasminogen activator; ECM, extracellular matrix; PAI-1, plasminogen activator inhibitor-1; IP, intraperitoneally; CUB, complement subcomponents Clr/Cls, Uegf, Bmp1; BrdU, bromodeoxyuridine; H&E, hematoxylin & eosin; NPC, non-parenchymal cell; HCC, hepatocellular carcinoma

* Corresponding author at: Department of Pathology, University of Washington, Box 357705, Seattle, WA 98195, USA. Tel.: +1 206 221 5244; fax: +1 206 543 3967.
*E-mail address:* campjs@u.washington.edu (J.S. Campbell).
[1] These authors contributed equally.
[2] Current address: Division of Public Health Sciences, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue North, P.O. Box 19024, Seattle, WA 98109, USA.
[3] Current address: Gilead Sciences, Inc., 199 East Blaine Street, Seattle, WA 98102, USA.

0925-4439/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbadis.2013.11.013

for normal development [7]. PDGFs appear to be critical for adult physiologic functions, as their overexpression results in a number of pathological responses via mesenchymal cells, including liver fibrosis and cirrhosis [8–10]. PDGF-C and PDGF-D specifically are secreted as latent factors, from which the complement subcomponents Clr/Cls, Uegf, Bmp1 (CUB) domain must be cleaved to allow the active growth factor dimers to bind their receptors [11,12]. Activated PDGF-CC binds to either PDGFRα homodimers or PDGFRα/β heterodimers to initiate intracellular signaling cascades, leading to diverse cellular processes such as proliferation and migration. Upon the initial discovery of PDGF-C, it was suggested that plasmin was the primary protease responsible for cleavage of the CUB domain from the growth factor domain, thus allowing downstream receptor activation [12]; a second group of investigators demonstrated that this cleavage is serum-dependent [11]. Later studies in fibroblasts demonstrated that tPA has the ability to cleave full-length PDGF-C to the active form [13–15]. Further, it has been suggested that activation of latent PDGF-C by tPA in ischemic stroke is responsible for the neurotoxic side effects of tPA in this setting; use of imatinib, a kinase inhibitor whose targets include PDGFRs, has been suggested for use in stroke patients to prevent this toxicity [15,16]. Direct *in vivo* studies confirming the relationship between tPA and PDGF-C activation are lacking, however.

We have recently developed a mouse model in which liver fibrosis, angiogenesis, and tumor development are driven by hepatocyte-specific over-expression of human *PDGF-C* [17]. Elevated serum levels of cleaved, activated growth factor in these mice are associated with up-regulation of pro-fibrotic genes and liver fibrosis that progresses with age [17]. To maintain high levels of PDGF-CC, ongoing cleavage of full length PDGF-C by an *in situ* protease or proteases is required. The purpose of the current study was to determine whether tPA is indeed the protease responsible for activation of PDGF-C in the liver in an *in vivo* system. To this end, we intercrossed *PDGF-C* transgenic (Tg) mice with those deficient for tPA. Here we report that tPA appears important for PDGF-C cleavage *in vitro*, including in primary hepatocytes, but in the more complex *in vivo* environment cleavage and activation of the growth factor occurs normally in the absence of tPA. Moreover, *PDGF-C* Tg; *tpa KO* mice display a histologic phenotype identical to that of *PDGF-C* Tg mice with intact tPA. Our findings suggest that tPA is not the sole protease that is important for *in vivo* cleavage and activation of PDGF-C, and that there is significant redundancy amongst PDGF-C-activating proteases.

## 2. Materials and methods

### 2.1. Enzyme-linked immunosorbent assay (ELISA) for determination of PDGF-CC in serum

We use the following nomenclature for PDGF-C protein: PDGF-C = non-cleaved, full-length protein including the growth factor domain and the CUB domain; PDGF-CC = cleaved, dimerized, activated growth factor domain. Levels of PDGF-CC in media or mouse sera were quantified using an antibody capture ELISA with two monoclonal antibodies specific for human PDGF-CC, as described previously [11]. Other ligands tested to validate the specificity of the ELISA included PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD (all from R&D), as well as full-length (FL) PDGF-C and PDGF-CC isolated from conditioned media derived from BHK cells overexpressing these two constructs (indicated as 'z' since they were developed at Zymogenetics) [11].

### 2.2. Culture of primary hepatocytes and adenovirus infection

Primary hepatocytes were isolated by collagenase perfusion and Percoll purification from wild type and *tpa* KO mice and cultured as previously described [18]. Primary hepatocytes were infected with adenoviruses over-expressing *Lac-Z* or full-length human *PDGF-C* [11] at an MOI of 2000; uninfected cells served as an additional control. Twenty-four hours after infection, infectious media were washed off and replaced with feeding media containing EGF (10 ng/ml). Three days after plating, media were collected for detection of PDGF-CC using the PDGF-CC specific ELISA; and hepatocytes were harvested for RNA analysis as described below.

### 2.3. Animal studies

The generation and characterization of *PDGF-C* Tg mice have been previously described [17]. *PDGF-C* Tg mice were crossed with mice deficient for tPA (*Plat*$^{tm1Mlg/J}$, Jackson Laboratories stock number 002508, referred to as *tpa* KO) to generate mice with the four genotypes used in study: wild type, *tpa* KO (lacking both *tpa* alleles), *PDGF-C* Tg, and PDGF-C Tg; *tpa KO* (expressing the *PDGF-C* transgene and lacking both *tpa* alleles). All mice were on the C57BL/6 background; mice heterozygous for *tpa* were initially kept and examined, but as we did not see any evidence of a gene dosage effect, *tpa*$^{+/-}$ mice were not used in our final analyses. Bromodeoxyuridine (BrdU, 50 μg/kg body weight) was injected intraperitoneally (IP) 2 h prior to necropsy as described [17,19]. At the indicated ages, mice were sacrificed by $CO_2$ inhalation, sera collected by cardiac puncture, and livers and spleens harvested, weighed, and fixed or snap frozen for analysis. All animal studies were carried out under approved Institutional Animal Care and Use Committee protocols at the University of Washington.

### 2.4. Histology

Mouse livers were fixed in 10% neutral buffered formalin or Methacarn (60% methanol, 30% chloroform and 10% acetic acid: v/v/v) overnight, processed to paraffin blocks, sectioned and stained with hematoxylin and eosin (H&E), picrosirius red, or trichrome using standard techniques. Immunostaining was performed on methacarn fixed sections as described [17,19,20] using a primary antibody specific for BrdU (Dako, Carpinteria, CA) and the mouse on mouse kit (Vector Labs). Nuclear incorporation of BrdU into both hepatocytes and non-parenchymal cells (NPCs) was used to measure cell proliferation; data are presented as the number of BrdU positive hepatocyte nuclei or NPCs present in thirty 400× fields (1.3 $mm^2$; approximately 3000 hepatocytes). Morphometric analyses of liver fibrosis were performed on picrosirius red and trichrome stained sections magnified to 40× using a method similarly described [21], using NIH Image J software.

### 2.5. RNA isolation and real-time PCR

Mouse livers or primary hepatocytes were harvested with TRIzol Reagent (Invitrogen) per the manufacturer's protocol. 1 μg of RNA template was used for reverse transcription by MMLV-RT (Applied Biosystems, Carlsbad, CA) per the manufacturer's protocol. 50 ng of the resultant cDNA was subjected to real-time PCR analysis using the following FAM labeled TaqMan primers (Applied Biosystems): *18s*, *Pdgfrα*, *Pdgfrβ*, *Col1a1*, *Col3*, *Col4a*, *Plat*, *Plau*, and *Serpine1*; SYBR Green-labeled primers were used to detect expression of human *18S* (Ambion, Life Technologies, Grand Island, NY) or *PDGF-C* (5′ ACAGAGGA GGTAAGATTA TACAGC 3′, 5′ AATTGTGGAGACAACAGGCACAGT 3′). A RotorGene 3000 thermocycler (Corbett Research, San Francisco, CA) was used for detection, and the ΔΔCt method was used to calculate fold change normalized to *18s* or *18S* [22].

### 2.6. Statistical analyses

Statistical analysis was done by non-parametric analysis using the Mann-Whitney U t-test using GraphPad Prism software (GraphPad for Science Inc., San Diego CA). Data are presented as average +/− S.E.M., with $p < 0.05$ (*) considered statistically significant.