Research Article

JOURNAL OF HEPATOLOGY

# Gas6/Axl pathway is activated in chronic liver disease and its targeting reduces fibrosis via hepatic stellate cell inactivation

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**Background & Aims**: Liver fibrosis, an important health concern associated to chronic liver injury that provides a permissive environment for cancer development, is characterized by accumulation of extracellular matrix components mainly derived from activated hepatic stellate cells (HSCs). Axl, a receptor tyrosine kinase and its ligand Gas6, are involved in cell differentiation, immune response and carcinogenesis.

**Methods**: HSCs were obtained from WT and *Axl*$^{-/-}$ mice, treated with recombinant Gas6 protein (rGas6), Axl siRNAs or the Axl inhibitor BGB324, and analyzed by western blot and real-time PCR. Experimental fibrosis was studied in $CCl_4$-treated WT and *Axl*$^{-/-}$ mice, and in combination with Axl inhibitor. Gas6 and Axl serum levels were measured in alcoholic liver disease (ALD) and hepatitis C virus (HCV) patients.

**Results**: In primary mouse HSCs, Gas6 and Axl levels paralleled HSC activation. rGas6 phosphorylated Axl and AKT prior to HSC phenotypic changes, while Axl siRNA silencing reduced HSC activation. Moreover, BGB324 blocked Axl/AKT phosphorylation and diminished HSC activation. In addition, *Axl*$^{-/-}$ mice displayed decreased HSC activation *in vitro* and liver fibrogenesis after chronic damage by $CCl_4$ administration. Similarly, BGB324 reduced collagen deposition and $CCl_4$-induced liver fibrosis in mice. Importantly, Gas6 and Axl serum levels increased in ALD and HCV patients, inversely correlating with liver functionality.

**Conclusions**: The Gas6/Axl axis is required for full HSC activation. Gas6 and Axl serum levels increase in parallel to chronic liver disease progression. Axl targeting may be a therapeutic strategy for liver fibrosis management.



Keywords: Experimental fibrosis; TAM receptors; HSC activation; Chronic liver patients; Gas6/Axl serum levels.

*Received 28 October 2014; received in revised form 7 April 2015; accepted 10 April 2015; available online 20 April 2015*

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*Abbreviations:* ALD, alcoholic liver disease; COL1A1, Collagen 1A1; ECM, extracellular matrix; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HSCs, hepatic stellate cells; MMP, matrix metalloproteinase; PCNA, proliferating cell nuclear antigen; ProS, Protein S; rGas6, recombinant Gas6; sAxl, soluble Axl; α-SMA, α-smooth muscle actin; TAM receptor, Tyro3/Axl/MERTK receptor; TGF-β1, transforming growth factor-β1; WT, wild type.

## Introduction

Activation of hepatic stellate cells (HSCs) is responsible for the liver fibrosis associated to chronic liver injury of any etiology, being HSCs the main collagen-producing cells in the damaged liver [1,2]. Liver fibrosis, critical pre-stage in the development of liver cirrhosis, may lead to hepatic transplantation or promote a favorable microenvironment for cancer development [3]. HSCs transform during chronic liver injury from a quiescent state into a myofibroblast-like phenotype, which proliferate and migrate towards areas of necrosis and regeneration [4,5]. Activated HSCs alter extracellular matrix (ECM) composition due to the upregulation of proteins such as α-smooth muscle actin (α-SMA), interstitial collagens such as Collagen 1A1 (COL1A1), and matrix metalloproteinases (MMPs) such as MMP9, as well as tissue inhibitor of metalloproteinases (TIMPs), and proteoglycans. Activated HSCs also generate hepatic cytokines such as TGF-β, PDGF, CTGF, FGF, HGF, and VEGF, and recruit inflammatory cells, mono- and polymorphonuclear leukocytes that produce chemokines, including MCP-1, RANTES, CCL21, CCR5. Although HSC critical role in liver fibrosis was proposed a decade ago [6], recent data demonstrates that irrespective of the underlying etiology of liver disease, the majority of myofibroblasts come from the liver-resident HSC population [7]. Moreover, after cessation of the fibrotic triggering insult, around 50% of the activated HSCs survive in an apparently quiescent state, being primed to quickly reactivate into myofibroblasts in response to fibrogenic stimuli [8,9]. Therefore, effective antifibrotic therapies aimed to inhibit activated HSCs, although positive to prevent extracellular matrix deposition, may be insufficient to definitely revert fibrosis, probably requiring the elimination of activated

HSCs for fibrosis resolution in the treatment of chronic liver disease.

Growth arrest-specific gene 6 (*Gas6*) product is a vitamin K-dependent protein that activates a family of receptor tyrosine kinases including Axl, MERTK and Tyro3, known as TAM receptors, whose immunologic and oncogenic properties have been described in detail [10,11]. Among them, Axl receptor signaling has been related to processes leading to cell differentiation and carcinogenesis. Gas6 possesses a high structural homology and sequence identity to the natural anticoagulant protein S (ProS). However, Gas6 and ProS have clearly different biological roles [12,13].

In liver pathologies, a hepatoprotective role for Gas6 has been reported in ischemia/reperfusion-induced damage [14], and in the wound healing response to liver injury [15,16]. In normal liver, Gas6 is mainly expressed in Kupffer cells, while Axl is found in macrophages and in quiescent HSC [17]. Moreover, after acute $CCl_4$ administration increased Gas6 expression was observed in activated HSCs and macrophages, while Gas6 *in vitro* protection to HSCs was mediated by the Axl/PI3-kinase/AKT pathway [17]. However, the role of Gas6/Axl signaling in chronic liver disease, the potential use of related proteins as serological markers of disease progression, and Gas6/Axl targeting in future liver therapies are aspects that merit further investigation.

To do so, we used both a genetic model of Axl deficiency ($Axl^{-/-}$), and a pharmacologic approach, the Axl inhibitor BGB324 [18]. Our results revealed that Axl receptor is an interesting target to block HSC transformation *in vitro* and demonstrated the efficacy of both strategies, genetic and pharmacologic, to diminish experimental liver fibrosis after chronic administration of $CCl_4$. Moreover, we analyzed data from patients at different stages of ALD and HCV infection providing evidence of the involvement of the Gas6/Axl axis in human liver fibrosis, and showing the correlation between Gas6/Axl serum levels and liver dysfunction.

In conclusion, our results underscore a critical role of the Gas6/Axl in fibrogenesis and in the progression of chronic liver diseases, suggesting that therapies aimed to inhibit Axl signaling deserve to be undertaken for the treatment of liver fibrosis, particularly now that small molecule inhibitors of Axl have been tested in clinical trials for cancer treatment [19].

## Materials and methods

*Animal procedures*

All procedures were performed according to protocols approved by the Animal Experimentation Ethics Committee from the University of Barcelona. *In vivo* liver fibrogenesis was analyzed after chronic carbon tetrachloride ($CCl_4$) administration. To this aim, WT or $Axl^{-/-}$ mice were treated with $CCl_4$ at a dose of 5 μl (10% $CCl_4$ in corn oil)/g of body weight, by intraperitoneal injection twice a week for five-six weeks. Control animals received corn oil alone. Treatment with Axl inhibitor (BGB324) or vehicle (saline solution) was performed daily for the last ten days of the study via oral gavage at a dose of 80 μg/g body weight. In previous experiments with rodents at similar doses, BGB324 reached serum concentration in the low micromolar range [18], being safe for animal treatment. Control animals received vehicle alone.

*HSCs isolation and culture*

Wild type and Axl knockout mice livers (male, 8–10 week-old littermates) (C57BL/6 strain) were perfused with collagenase and HSCs cultured as previously described [20,21]. Culture purity, assessed routinely by retinoid autofluorescence at 350 nm, was >95%. Lack of staining for F4/80 confirmed the absence of Kupffer cells. HSCs and LX2 human activated stellate cells [20,22] were cultured in DMEM supplemented with 10% FBS and antibiotics at 37 °C in a humidified atmosphere of 95% air and 5% $CO_2$. Experiments to compare protein or mRNA content were always performed with cells extracted at the same time of culture, previously treated with recombinant Gas6 (R&D), Axl inhibitor (BGB324, BerGenBio), or siRNA silencing (Santa Cruz) after Lipofectamine 2000 exposure for the indicated periods of time.

*SDS-PAGE and immunoblot analysis; RNA isolation and real-time RT-PCR; In Vitro Small Interfering RNA Transfection; Nuclear extract isolation; Immunohistochemical staining; and liver collagen determination*

These methods were performed as previously indicated [20,21,23] with modifications as specified in Supplemental methods.

*Determination of Gas6, and soluble Axl (sAxl) levels*

Measurements of Gas6 and sAxl human levels were carried out using commercial antibodies (R&D Systems) to develop specific ELISAs that use the sandwich technique as described [24]. Serum Gas6 mouse levels were determined using a commercial kit (DuoSet mGas6 ELISA, R&D). Serum sAxl mouse levels were determined by western blot.

*Human samples*

a) The ALD study group comprised serum samples from 40 individuals: ten healthy normal adult controls (C) and 30 alcoholic patients with different degrees of liver disease as diagnosed after hepatic biopsy and Fibroscan measurement: ten patients with initial fibrosis (Fibroscan score $\leqslant$7 KPa, mean = 5.2 ± 0.4) (F), ten patients with compensated cirrhosis (CH) and, 10 patients with decompensated cirrhosis (DCH), five of them due to ascitis, three due to spontaneous bacterial peritonitis (SBP) and two due to gastrointestinal bleeding by esophageal varices and portal hypertension. Relevant biochemical data are shown in Table 1. b) The HCV study group comprised serum samples from 51 individuals at different stages of liver fibrosis (8 F0, 15 F1, 17 F2, and 11 F3/F4), as stated by liver biopsies, before initiation of treatments. None of the HCV patients exhibited signs of decompensation. Relevant biochemical data are shown in Table 2. All subjects gave written informed consent in accordance with the Declaration of Helsinki, and the protocol, approved by ethical committees from the Hospital Clinic, followed ethical guidelines on handling human samples.

*Statistical analyses*

Results are expressed as mean ± standard deviation, unless indicated, with the number of individual experiments detailed in Figure legends. Statistical comparisons were performed using unpaired two-tailed Student's *t* test or One-way ANOVA followed by Newman-Keuls Multiple Comparison Test (GraphPad Prism). A *p* value less than 0.05 was considered significant.

## Results

*TAM receptors and ligands levels during HSC activation*

Gas6 and ProS are the ligands of the tyrosine kinase family of receptors named TAM (Tyro3, Axl, and MERTK), which have been involved in numerous processes related to cell transformation and cancer. Since TAM receptor participation in HSC activation has not been explored, we analyzed the presence of transcriptional changes during HSC transdifferentiation in mouse-derived primary cultures of HSCs. A significant increase in the mRNA levels of *Gas6*, but not of *ProS*, was detected (Fig. 1A). In parallel, strong upregulation of Axl was observed, with no significant changes in MERTK levels (Fig. 1B). Tyro3 mRNA levels were not detectable in these samples. Of note, increased secretion of Gas6 protein expression was confirmed in HSCs after different days in culture, as determined by ELISA in 24 h cell conditioned