Research Article

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β-PDGF receptor expressed by hepatic stellate cells regulates fibrosis in murine liver injury, but not carcinogenesis

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Background & Aims: Rapid induction of β-PDGF receptor (β-PDGFR) is a core feature of hepatic stellate cell activation, but its cellular impact *in vivo* is not well characterized. We explored the contribution of β-PDGFR-mediated pathway activation to hepatic stellate cell responses in liver injury, fibrogenesis, and carcinogenesis *in vivo* using genetic models with divergent β-PDGFR activity, and assessed its prognostic implications in human cirrhosis.

Methods: The impact of either loss or constitutive activation of β-PDGFR in stellate cells on fibrosis was assessed following carbon tetrachloride (CCl_4) or bile duct ligation. Hepatocarcinogenesis in fibrotic liver was tracked after a single dose of diethylnitrosamine (DEN) followed by repeated injections of CCl_4. Genome-wide expression profiling was performed from isolated stellate cells that expressed or lacked β-PDGFR to determine deregulated pathways and evaluate their association with prognostic gene signatures in human cirrhosis.

Results: Depletion of β-PDGFR in hepatic stellate cells decreased injury and fibrosis *in vivo*, while its auto-activation accelerated fibrosis. However, there was no difference in development of DEN-induced pre-neoplastic foci. Genomic profiling revealed ERK, AKT, and NF-κB pathways and a subset of a previously identified 186-gene prognostic signature in hepatitis C virus (HCV)-related cirrhosis as downstream of β-PDGFR in stellate cells. In the human cohort, the β-PDGFR signature was not associated with HCC development, but was significantly associated with a poorer outcome in HCV cirrhosis.

Conclusions: β-PDGFR is a key mediator of hepatic injury and fibrogenesis *in vivo* and contributes to the poor prognosis of human cirrhosis, but not by increasing HCC development.

Keywords: HCC; Cirrhosis; Receptor tyrosine kinase; Gene expression signatures; Pathway analysis.

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Abbreviations: β-PDGFR, beta platelet-derived growth factor receptor; HCV, hepatitis C virus; CCl_4, carbon tetrachloride; DEN, diethyl nitrosamine; GFAP, glial fibrillary acidic protein; BDL, bile duct ligation; bw, body weight; AST, aspartate aminotransferase; ALT, alanine aminotransferase; mRNA, messenger ribonucleic acid; PCR, polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; αSMA, alpha smooth muscle actin; H&E, hematoxylin and eosin; BrdU, 5-bromo-2′-deoxyuridine; CD45, cluster of differentiation 45; UV, ultraviolet; PBS, phosphate buffered saline; HCC, hepatocellular carcinoma; IL1R, interleukin 1 receptor; GSEA, gene set enrichment analysis; FACS, fluorescence-activated cell sorting; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; RTK, receptor tyrosine kinase; EGFR, epidermal growth factor receptor; NES, normalized enrichment score; FDR, false discovery rate.

Introduction

Among mitogenic pathways in stellate cells, signaling by the beta platelet-derived growth factor receptor (β-PDGFR) is the most potent [1,2]. The expression of PDGF receptors is low in healthy liver, but dramatically increases in stellate cells during injury [2,3]. In both mice and humans, the PDGF signaling network is comprised of four ligands, PDGF A-D, which transduce their signals through dimeric transmembrane receptors α- and β-PDGFR, which can form hetero- and homodimers [4]. Upon ligand binding, receptor dimerization provokes phosphorylation of the tyrosine residues within the intracellular domain, leading to activation of the Fas-MAPK pathway, signaling through the PI3K-AKT/PKB pathway and activation of PKC family members [5].

Antagonism of β-PDGFR has been an appealing target to treat hepatic fibrosis. Indeed, our previous study and those of others [6–8] have demonstrated that the RTK inhibitor imatinib mesylate (Gleevec®) whose targets include β-PDGFR, inhibits stellate cell activation and reduces fibrosis.

Recent evidence links the behavior of stromal cells, that can be driven by the ligand PDGF-B, not only to the pathogenesis of fibrosis, but also to inflammation, regeneration and cancer [9]. Sorafenib (Nexavar®), a multi-receptor tyrosine kinase inhibitor

whose targets include β-PDGFR, remains the only drug approved for treatment of advanced, non-resectable HCC [10].

Despite the suggestion that promoting fibrosis by β-PDGFR can accelerate HCC development, this important question has yet to be addressed experimentally. Here we have specifically explored the contribution of β-PDGFR signaling by activated hepatic stellate cells (HSCs) to injury, fibrosis and cancer by exploiting complementary genetic models using a Cre-Lox strategy: one model exhibited the deletion of the receptor in stellate cells, and in the other the receptor was auto-activated. Importantly, we have also addressed whether the overall prognosis for human cirrhosis can be linked to β-PDGFR signaling in HSCs.

Methods

For detailed description of methods see Supplementary Methods.

Animals

β-PDGFR$^{fl/fl}$ mice, as previously described [11], (on the 129S4/SvJaeSor background) were crossed with a transgenic FVB line expressing Cre-recombinase under control of the human glial fibrillary acidic protein (GFAP) promoter to generate β-PDGFR$^{fl/fl}$ GFAP-Cre mice with a deletion of β-PDGFR in stellate cells – this GFAP promoter has been successfully validated in prior studies to be active in HSCs [12,13]. To create animals with constitutively activated β-PDGFR in stellate cells, β-PDGFR$^{beta1/+}$ mice, as previously described [14], (on the 129S4/B6 background) were also crossed with a transgenic GFAP-Cre line to generate β-PDGFR$^{beta1/+}$ GFAP-Cre mice. These animals harbor HSCs with auto-activation of β-PDGFR, owing to an activating mutation knocked into the β-PDGFR locus, plus addition of a lox-stop-lox cassette between the splice acceptor and the initiating codon of the cDNA [14].

Models of murine liver injury and fibrosis

Liver fibrosis was induced either by ligation of the common bile duct (BDL) [15] or by intraperitoneal (i.p.) injections of carbon tetrachloride (CCl_4, Sigma, St. Louis, MO) [16]. For acute CCl_4 injury studies, mice received a total of 3 i.p. injections (alternating days) of either corn oil or 10% CCl_4 (diluted in corn oil) at a dose of 0.5 μl/g body weight (bw). For the chronic injury model, mice received i.p. injections of CCl_4 3 times per week for a total of 6 weeks.

Induction of carcinogenesis

Mice received a single dose of diethylnitrosamine (DEN, Sigma, St. Louis, MO) (25 μg/g bw i.p.) at day 15 post-partum. Starting two weeks after DEN, mice received a total of 22 injections of CCl_4 (0.5 μl/g bw i.p., 1 injection/week) [17]. Mice were sacrificed 48 h following the last CCl_4 injection. Nodule number and size were documented as described by counting and measuring the diameter of each lesion using a caliper.

Primary hepatic stellate cell isolation and cell culture

Mouse HSCs were isolated from β-PDGFR$^{fl/fl}$ GFAP-Cre negative and β-PDGFR$^{fl/fl}$ GFAP-Cre positive mice by enzymatic pronase and collagenase digestion and density gradient centrifugation as previously described [18]. Cells were cultured with Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum. Cells were either treated with or without PDGF-B (10 ng/ml; Peprotech, Princeton, NJ) diluted in albumin (vehicle) containing serum-free media (DMEM).

Histologic and immunohistochemical studies

Liver samples were formalin-fixed, paraffin-embedded, sectioned at 4 μm, and processed routinely for H&E staining. Sirius Red, combined with morphometry, was used to quantify collagen using Bioquant image analysis software (Bioquant Image Analysis Corporation, Nashville, TN). Immunohistochemical staining of αSMA and desmin was performed on formalin-fixed, paraffin-embedded liver sections with a rabbit polyclonal antibody (Abcam, Cambridge, England). A pathologist blindly to the experimental protocol scored 5 random areas per slide for necrosis, inflammation and dysplasia.

Genome-wide expression profiling

Genome-wide gene expression profiling of mouse primary HSCs was performed, in triplicate, by using MouseWG-6 v2.0 Expression BeadChip (Illumina) according to the manufacturer's protocol. Raw scanned data were normalized by using cubic spine algorithm implemented in the GenePattern genomic analysis toolkit (www.broadinstitute.org/genepattern) [19]. Probe-level expression data were collapsed into gene-level by calculating the median of multiple probes, and converted to human genes based on an orthologous mapping table provided by the Jackson laboratory (www.informatics.jax.org). The dataset (GSE#52253) is available at NCBI Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo).

Bioinformatics and statistical analysis

Enrichment of molecular pathways was evaluated by Gene Set Enrichment Analysis (GSEA) [20] on a comprehensive gene set collection in Molecular Signatures Database (see Supplementary Methods).

Results

β-PDGFR expression is induced upon liver injury in vivo and in vitro

We generated a mouse line in which the expression of β-PDGFR was deleted in HSCs by crossing β-PDGFR$^{fl/fl}$ mice with animals expressing Cre-recombinase under the human GFAP promoter (GFAP-Cre) (Supplementary Fig. 1A) [13,21].

To first confirm the induction of β-PDGFR following acute injury, β-PDGFR$^{fl/fl}$ GFAP-Cre negative animals were treated with three doses of CCl_4 during one week. Whole liver lysates contained increased β-PDGFR expression and phosphorylation, as well as upregulation of αSMA (Fig. 1A).

We next analyzed isolated HSCs from β-PDGFR$^{fl/fl}$ Cre negative mice (β-PDGFR) as well as their β-PDGFR$^{fl/fl}$ Cre expressing littermates (Δβ-PDGFR) (Fig. 1B and C). Isolated stellate cells were maintained in primary culture for six days following isolation. To first validate the knockdown and diminished activation of β-PDGFR in the Cre expressing population, cell lysates were analyzed via immunoblot after incubation with either vehicle or PDGF-BB (Fig. 1B). Stellate cells from Δβ-PDGFR mice displayed attenuated receptor expression and lack of activation at baseline and after 30 min of ligand exposure. Next, the expression of collagen I and αSMA were compared in primary stellate cells of the two groups. Stellate cells isolated and cultured for 6 days displayed significant knockdown of the receptor in the Δβ-PDGFR group, with substantially reduced expression levels of collagen I and αSMA compared to stellate cells from β-PDGFR animals (Fig. 1C). β-PDGFR expression was upregulated during hepatic injury and correlated with stellate cell activation *in vitro*.

Deletion of β-PDGFR in stellate cells attenuates liver fibrosis in vivo

Since the upregulation of β-PDGFR on stellate cells associated with fibrogenic markers in response to acute liver injury, we further analyzed control and Δβ-PDGFR animals (Supplementary Fig. 1A) following acute (1 week) and chronic (6 weeks) liver injury. To do so, we injected both groups with either oil or CCl_4 (3 i.p. injections weekly). Macroscopically, there were no differences in the livers between control and Δβ-PDGFR littermates after treatment (Supplementary Fig. 1B and C), but less necrosis