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The tyrosine phosphatase SHP-1 inhibits proliferation of activated hepatic stellate cells by impairing PDGF receptor signaling

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ABSTRACT

The dimerization and auto-transphosphorylation of platelet-derived growth factor receptor (PDGFR) upon engagement by platelet-derived growth factor (PDGF) activates signals promoting the mitogenic response of hepatic stellate cells (HSCs) due to liver injury, thus contributing to the development of hepatic fibrosis. We demonstrate that the tyrosine phosphatases Src homology 2 domain-containing phosphatase 1 and 2 (SHP-1 and SHP-2) act as crucial regulators of a complex signaling network orchestrated by PDGFR activation in a spatio-temporal manner with diverse and opposing functions in HSCs. In fact, silencing of either phosphatase shows that SHP-2 is committed to PDGFR-mediated cell proliferation, whereas SHP-1 dephosphorylates PDGFR hence abrogating the downstream signaling pathways that result in HSC activation. In this regard, SHP-1 as an off-switch of PDGFR signaling appears to emerge as a valuable molecular target to trigger as to prevent HSC proliferation and the fibrogenic effects of HSC activation. We show that boswellic acid, a multitarget compound with potent anti-inflammatory action, exerts an anti-proliferative effect on HSCs, as in other cell models, by upregulating SHP-1 with subsequent dephosphorylation of PDGFR-β and downregulation of PDGF-dependent signaling after PDGF stimulation. Moreover, the synergism resulting from the combined use of boswellic acid and imatinib, which directly inhibits PDGFR-β activity, on activated HSCs offers new perspectives for the development of therapeutic strategies that could implement molecules affecting diverse players of this molecular circuit, thus paving the way to multi-drug low-dose regimens for liver fibrosis.

1. Introduction

Liver fibrosis is a dynamic and reversible process which occurs in response to acute or chronic liver injury and is characterized by accumulation of extracellular matrix (ECM) proteins [1,2]. Persistence of liver damage with advanced liver fibrosis may lead to life-threatening conditions, such as portal hypertension, cirrhosis and ultimately hepatocellular carcinoma. Major players in the deposition of ECM proteins are hepatic stellate cells (HSCs), which upon liver damage are activated by several inflammatory cytokines, including growth factors, interleukins, chemokines and adipokines, hence developing a myofibroblast-like phenotype with proliferative, contractile, pro-inflammatory and fibrogenic properties [3]. Among these cytokines, the platelet-derived growth factor (PDGF), which is mainly produced by Küpffer cells, acts as the most potent mitogen and effective chemotactic factor for HSCs [4,5] by engaging its cognate receptor (PDGFR), a member of the receptor tyrosine kinase (RTK) family. This event causes the dimerization, the subsequent activation of the kinase domain of PDGFR and the autophosphorylation of specific tyrosine residues in its cytosolic tail, thereby forming a phosphotyrosine (pTyr)-based platform for proteins bearing Src homology 2 (SH2) domains, which ultimately contribute to the activation the downstream signaling pathways [6,7].

To date, although there exist no agents approved as antifibrotic drugs, molecules already employed in the treatment of other diseases and, albeit not exclusively, inhibiting the kinase activity of PDGFR such as imatinib, nilotinib, sorafenib, and sunitinib, have exhibited anti-fibrotic activity [8–11]. It is to be underlined that the signals downstream of PDGFR activation are not only triggered by the kinase activity of the receptor but also governed and modulated by the action of protein tyrosine phosphatases (PTPs), whose role, albeit recognized as crucial in the regulation of signal transduction of various biological models, is still elusive in HSCs.

In this respect, PTPs not only turn off pTyr-dependent signaling, but can also contribute to the fine tuning, and consequently to the specificity of signaling cascades [12], these two different functions being well exemplified by the two members of the ubiquitous Src homology 2 domain-containing phosphatase (SHP) family SHP-1 and

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SHP-2 [13,14]. Although these share high sequence homology, they often exert opposing biologic functions. In fact, whilst SHP-1 acts as a negative signal transducer involved in the downregulation of several RTKs, including PDGFR, the insulin receptor, the EGF receptor and VEGF receptor type 2, SHP-2 is a positive mediator of the Ras-extracellular signal-regulated kinase 1/2 (Erk 1/2) and Akt signaling pathways [15–18], thereby the former PTP impairing and the latter promoting cell growth and proliferation [8–10,19]. The regulation of these phosphatases is strictly dependent upon its multi-modular structure consisting of a central catalytic domain flanked by two tandem SH2 domains at the N-terminus and a C-terminal regulatory tail as well as phosphorylation at critical amino acid residues. Whilst the more N-terminal SH2 domain maintains both phosphatases in a closed inactive conformation by occupying the catalytic cleft and preventing the substrate access, engagement of the SH2 domains by tyrosine phosphorylated partner proteins disrupts the inhibitory intramolecular interactions with subsequent phosphorylation at C-terminal tyrosine residues, both events leading to full activation [20–24]. Importantly, the catalytic activity of SHP-1 can also be suppressed by phosphorylation at Ser591 by PKC or MAPK, which mechanism of inhibition has not been described for SHP-2 [25].

The aim of this work was to attempt to give a contribution to the unexplored role of these two PTPs and their relation to PDGFR activation in HSCs, and to assess whether they may represent valuable targets for drug discovery to establish new therapeutic strategies against liver fibrosis.

Here, we demonstrate that, although SHP-2 and SHP-1 are both involved in the regulation of PDGFR-dependent proliferative signals in HSCs, SHP-1 acts as a brake on HSC proliferation by dephosphorylating PDGFR-β and this effect can be enhanced by molecules that stimulate its expression (there are no known stimulators of SHP-1 activity), such as boswellic acid (BA), a compound present in the resin of *Boswellia serrata*, a plant used in Ayurvedic medicine in the treatment of a number of inflammatory diseases [26]. Notably, imatinib, the prototypical tyrosine kinase inhibitor that directly inhibits PDGFR activity and administered in certain types of cancer, and BA exhibit a striking synergism when used in association, opening new possibilities of designing more potent anti-proliferative drug combinations to effectively block HSC proliferation and prevent fibrogenesis.

2. Materials and methods

2.1. Materials and chemicals

Unless otherwise specified, reagents were obtained from Sigma (St. Louis, MO). Stealth™ RNAi for SHP-1 and SHP-2, Stealth™ RNAi Negative Control Duplexes, and Lipofectamine RNAiMAX Reagent were purchased from Invitrogen (Carlsbad, CA). FTY720 was purchased from Alexis (CH-4415 Lausen, Switzerland). Enhanced chemiluminescent detection system (ECL) was purchased from Pierce (Thermo Fisher Scientific, Rockford, IL USA). Protease inhibitor cocktail (cOmplete tablets) and Cell Proliferation ELISA, BrdU (chemiluminescent) were purchased from Roche Diagnostics (Manheim, Germany). 8-Hydroxy-7-(6-sulfonaphthalen-2-yl)diazenyl-quinoline-5-sulfonic acid (NSC-87877) was purchased from Millipore Corporation (Billerica, USA). Sodium stibogluconate and boswellic acid (cod. A9855) were from Sigma (St. Louis, MO). Antibodies were obtained as follows: anti-pPDGFR-β, anti-PDGFR-β, anti-pAkt, anti-Akt, and anti-SHP-2 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); anti-pERK1/2, anti-ERK, and anti-pY580-SHP-2, were from Cell Signaling Technology, Inc. (Danvers, MA); anti-phosphotyrosine (PY-20) monoclonal antibody, anti-SHP-1 was from Millipore Corporation (Billerica, USA); anti-pS591-SHP-1, and anti-pY536 SHP-1 were from ECM Biosciences (Versailles, KY); and anti-GAPDH, anti-α-SMA, anti-GFAP, and anti-β-actin antibodies were from Sigma-Aldrich Inc. (St. Louis, MO).

2.2. Cell isolation and culture

HSCs were isolated from the livers of normal male Wistar rats. All animals were purchased from Charles River Laboratories and housed in the Animal Research Facility of the Molecular Medicine Department, in accordance with the National Institute of Health guidelines for the care of laboratory animals. They were maintained under a 12-hour light–dark cycle and given rat chow and water ad libitum. According to the method described in Zhang et al. [27], livers were perfused with a calcium-free buffer solution to wash out the blood, subsequently with a wash solution (calcium-free solution with $CaCl_2$ 0.294 g/l, $MgSO_4$ 0.097 g/l) and finally incubated in a digestion buffer (collagenase 0.6 g/l in Gey's Balanced Salt Solution with Ca^{2+} and Mg^{2+}, pH 7.5) for 1 h (37 °C, CO_2 5%) to digest the extracellular matrix. The organs were cut into small pieces and subjected to homogenization to produce a single cell-suspension, which was centrifuged at 1450 *g* for 18 min in a 12% (wt/vol) Nycodenz density gradient to obtain a pure HSC fraction. Both the number and viability of HSCs were determined using the trypan blue dye exclusion test. HSCs were collected and washed with Hank's balanced salt solution and resuspended at a concentration of 1×10^5 cells/ml, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin and cultured on collagen type I coated well-plates (10 μg/ml) in a 5% CO_2-humidified atmosphere. After passage 2, FBS was diluted to 10%. All experiments were performed using HSCs between passages 3 and 8.

2.3. Cell proliferation assay and calculation of drug synergism

Proliferation assays were performed by using Roche Cell Proliferation ELISA, BrdU (chemiluminescent). Briefly, 7.5×10^3 cells were seeded on 96-well flat bottom microplates and incubated in DMEM medium alone for 24 h to stop cell growth. Cells were preincubated at time points as indicated in the Results in the absence or presence of NSC-87877, SSG, BA and imatinib, and then treated with PDGF-BB (10 ng/ml) at different incubation times. HSCs were labeled with 10 μM BrdU 10 h before each single incubation time point. The following treatment was carried out according to the manufacturer's guidelines. The microplates were then washed three times with washing solution and 100 μl substrate solution was added and incubated for 5 min. Absorbance of the samples was measured using a luminometer. To evaluate the effects of BA and imatinib in combination, we calculated the combination index (CI), which turns out to be less than 1, equal to 1 and more than 1 in the case of synergy, additive effect and antagonism, respectively, by using the CompuSyn software [28].

2.4. Western blot analysis

Cells were seeded on 24-well plates, cultured in DMEM medium supplemented with 10% FBS to confluence and then incubated for 24 h in DMEM medium alone. Cultured cells were subjected to various stimuli, as described in the figure legends, detached by scraping and centrifuged at 1200 rpm for 5 min. Cells were resuspended and quickly lysed in a buffer containing 62 mM Tris/HCl solution (pH 6.8), 5% glycerol, 0.5% SDS, and 0.5% β-mercaptoethanol. Samples were run in 10% SDS-PAGE and transferred to nitrocellulose membranes. After treatment with 3% bovine serum albumin for 1 h, membranes were incubated with the appropriate antibodies overnight and, after washing, with secondary horseradish peroxidase-conjugated polyclonal antibody for 30 min. Bound antibodies were detected by the ECL detection system (Pierce, on a Kodak Image Station 440CF and visualized with Kodak 1D Image software (Rochester, NY). Membranes, when required, were reprobed with other primary antibodies after stripping with 0.1 M glycine (pH 2.5), 0.5 M NaCl, 0.1% Tween 20, 1% β-mercaptoethanol and 0.1% NaN_3 for 2×10 min.