Regulatory Peptides 188 (2014) 5–12

Contents lists available at ScienceDirect

Regulatory Peptides

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

Octreotide modulates the effects on fibrosis of TNF-α, TGF-β and PDGF in activated rat hepatic stellate cells ☆

Stefanos Klironomos [a], George Notas [a,b], Ourania Sfakianaki [a], Foteini Kiagiadaki [b], Costas Xidakis [a], Elias Kouroumalis [a,*]

[a] Liver Research Laboratory, Medical School, University of Crete, Voutes 71003 Crete, Greece
[b] Laboratory of Experimental Endocrinology, Medical School, University of Crete, Voutes 71003 Crete, Greece

ARTICLE INFO

Article history:
Received 15 May 2013
Received in revised form 12 November 2013
Accepted 19 November 2013
Available online 26 November 2013

Keywords:
Hepatic stellate cells
Somatostatin
Somatostatin receptors
Procollagen
Fibrosis

ABSTRACT

Background and aims: Somatostatin and its analogs may influence hepatic fibrosis interfering through several mechanisms. The aim of this study was to investigate the effect of octreotide on cytokine activated hepatic stellate cells (HSC).

Methods: Primary HSCs were isolated from rats and were cultured on plastic for activation. Expression of somatostatin receptors (SSTR) was investigated in cultured HSCs by immunofluorescence and western blot. The effect of octreotide on cellular proliferation was studied with the MTT assay and western blot for α1-procollagen (α1-PROC) production in TNFα, TGF-β1 or PDGF treated HSCs. Phosphotyrosine (PTP) and phosphoserine–phosphothreonine (STP) phosphatases inhibition was performed with sodium orthovanadate and okadaic acid respectively.

Results: Activated HSC express SSTR subtypes 1, 2A, 2B, 3 and 4 and their expression is enhanced by further HSC activation. Octreotide did not have an effect on HSC proliferation but inhibited plastic induced α1-PROC production. Interestingly, it enhanced PDGF-induced HSC proliferation but inhibited PDGF and TGFβ1 dependent expression of α1-PROC, while an opposite effect was observed in TNFα-induced cell proliferation and collagen production. PTP inhibition reversed the inhibitory effect of octreotide on α1-PROC, but potentiated its effect on PDGF and TGFβ1 dependent α1-PROC production. Finally, STP inhibition profoundly inhibited α1-PROC expression in all cases suggesting that both STP and PTP phosphatases are important regulators of pro-fibrotic mechanisms.

Conclusions: The net effect of octreotide on HSCs and therefore liver fibrosis is subject to the cytokine microenvironment of these cells. This effect is modulated by PTPs and STPs inhibition. Especially in the case of STPs their profibrotic effects could be an interesting new therapeutic target in liver fibrosis.

1. Introduction

Hepatic stellate cells (HSCs) play a central role in the pathogenesis of liver fibrosis [1]. In the normal liver, HSCs are localized within the space of Disse and function as retinoid storing cells [2]. After various forms of liver injury, HSCs undergo trans-differentiation to a myofibroblastic phenotype, a process termed activation [3,4]. Activated HSCs are characterized by expression of alpha smooth muscle actin (aSMA) [5], increased contractility [6], proliferation, migration [1,7,8], release of proinflammatory cytokines, and increased production of extracellular matrix (ECM) components [4,9,10] as well as matrix metalloproteinases (MMPs) [11] and their inhibitors (TIMPs) [12]. Among many cytokines, growth factors, ECM components and transcription factors affecting the complex biochemical process of activation, transforming growth factor β-1 (TGFβ1) and platelet-derived growth factor (PDGF) are considered the most prominent mediators of this process. TGFβ1 is widely accepted as the strongest stimulus for the transdifferentiation of HSCs [13] while PDGF is one of the most potent mitogen for activated HSC [14,15]. Tumor necrosis factor alpha (TNFα), produced mainly by Kupffer cells and Th1 lymphocytes during inflammatory responses, is another central cytokine in both acute and chronic liver disease and has been shown to affect activated HSC proliferation [16,17], MMP production [18] and collagen gene expression [19,20]. Hepatic fibrosis is the final common endpoint for almost all chronic liver diseases, irrespective of the underlying initial cause of liver injury [21–25]. Many of the changes that have been

Abbreviations: HSC, Hepatic stellate cell; SSTR, Somatostatin receptor; α1-PROC, α1-Procollagen; PTP, Phosphotyrosine phosphatase; STP, Phosphoserine–phosphothreonine phosphatase.

☆ Dr. Foteini Kiagiadaki was supported by the European Union Programs Regional Potential/Translational Potential Grant 285948.

* Corresponding author at: University of Crete, Faculty of Medicine, P.O Box 1393, Heraklion 71409 Crete, Greece. Tel.: +30 2810 392356; fax: +30 2810 542085.
E-mail address: kouroum@med.uoc.gr (E. Kouroumalis).

0167-0115/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.regpep.2013.11.002

identified during HSC activation in vivo are also observed in HSCs grown in vitro on plastic [26] and therefore this model has been extensively used to study cellular events involved in the activation process.

Somatostatin exerts its effects through binding to a family of five specific receptors, named sst_1 to sst_5. Somatostatin receptor genes are intronless with the exception of sst_2, whose splice variants are named sst_{2A} and sst_{2B} [27,28]. Binding of somatostatinergic ligands on ssts leads to transactivation of one or more inhibitory G-proteins (Gi/Go), which in turn inhibit adenylyl cyclase activity and decrease the concentration of intracellular cAMP [29]. Other G-protein-mediated actions common to all SSTRs are activation of a vanadate-sensitive phosphotyrosine phosphatase (PTP) and modulation of mitogen-activated protein kinases (MAPKs) [29]. Somatostatin is secreted in two biologically active forms: a 14 amino acid peptide (somatostatin-14) and a 28 amino acid peptide (somatostatin-28). Both forms bind with high affinity to all five receptor subtypes [30]. The widely used synthetic analog, octreotide, binds with high affinity only to sst subtypes 2 and 5 and with lower affinity to sst_3.

In earlier studies we have shown that octreotide profoundly affects the production of pro-inflammatory cytokines, pro and anti-fibrotic agents by rat Kupffer cells [31,32]. Therefore, in the present study we investigated the presence of somatostatin receptors in isolated primary rat stellate cells and the effects of the synthetic somatostatin analog octreotide on plastic or cytokine activated HSC proliferation and collagen production. We also studied the effect of PTPs and phosphoserine–phosphothreonine (STP) phosphatases on the regulation of these octreotide effects.

2. Materials and methods

2.1. Cell isolation and culture

Animals were treated according to the principles stated in Greek law and EU Directive 2010/63/EU for animal experiments on animal experiments and local institutional guidelines and were approved by the University of Crete School of Medicine ethics committee. HSCs were isolated from pathogen-free male Sprague–Dawley rats (450–600 g) by collagenase–pronase digestion of the liver followed by centrifugation in a Nycodenz (Axis-Shield PoC Oslo, Norway) solution. In situ cannulation and perfusion and enzymatic/mechanical disruption of live tissue were performed as previously described [33]. Collagenase B, and pronase were purchased from Roche (Mannheim, Germany). Tissue homogenate was washed twice in HBSS containing 10 μg/ml DNase I (Roche) and centrifuged at 400 ×*g* for 7 min at 4 °C. HSCs were isolated from the homogenate by centrifugation in Nycodenz solutions in 2 steps. The cell pellet was suspended in 50 ml solution of 17% Nycodenz in HBSS supplemented with 10 μg/ml DNase I, distributed to 4 15-ml tubes and centrifuged at 1400 ×*g* for 15 min at 4 °C. The resulting floating band of sinusoidal cells was collected, washed in HBSS, resuspended in a solution of 10% Nycodenz in HBSS containing 10 μg/ml DNase and centrifuged again at 1400 ×*g* for 15 min at 4 °C. The resulting floating band consisted of highly purified HSC cells while the pellet consisted mainly of Kupffer, endothelial and other sinusoidal cell fractions. The average yield of this method was 30×10^6 cells with >98% viability as determined by trypan blue exclusion and >95% purity as evidenced by autofluorescence of vitamin A esters and desmin immunostaining. HSC cells were suspended in DMEM with high glucose, supplemented with GLUTAMAX (Invitrogen, Carlsbad, USA), 20% fetal bovine serum (FBS), 0.1 mM nonessential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin. They were seeded in tissue culture dishes or flasks (Nunc, Thermo Fisher Scientific, Langenselbold Site) at a density of 30,000 cells/cm^2 and cultured at 37 °C in a 5% CO_2 atmosphere. The culture medium was replaced every other day and FBS content was reduced to 10% after the first 48 h. At 70–80% confluency, the cells were trypsinized and subcultured in a 1:2 split ratio. After day 7 in culture, HSC characteristically stained positive for aSMA. For all experiments, cells between passages 3 and 5 were used.

2.2. Culture conditions for stimulation with cytokines

HSCs at 60–70% confluency cells were washed in HBSS and placed in serum free culture medium for 24 h prior to every treatment. Following serum starvation cells were treated with one of TNF-α 70 ng/ml, TGF-β1 5 ng/ml or PDGF 32 ng/ml (all from R&D, Minneapolis, USA). Octreotide (Novartis, Basel, Switzerland) was used at concentrations 10^{-6}–10^{-10} M.

2.3. Immunofluorescence for SSTRs

HSCs were plated on Permanox chamber slides (Nunc). At a confluence of 60–70%, the cells were washed with cold PBS and fixed with 4% paraformaldehyde in PBS for 10 min. Following fixation the cells were washed with TBS twice and treated with quench buffer (20 mM glycine in TBS) for 5 min and incubated in blocking buffer (TBS with 0.2% TritonX-100, 1% Fish Skin Gelatin (Sigma Aldrich) and 2 mM $MgCl_2$) for 10 min. Subtype specific rabbit anti-rat antibodies against sst_1 (Chemicon, Chemicon International Concord Road Billerica, MA) and ssts 2A, 2B, 3, 4 and 5 (Gramsch Laboratories, Schwabhausen, Germany) were used. Following blocking, the cells were incubated in the primary antibody at a dilution of 1:1000 for ssts 1, 2A, 2B, 4, 5 and 1:500 for sst_3 at 4 °C overnight. Following incubation, the cells were blocked again for 10 min and incubated in the secondary antibody, Alexa Fluor 488 goat anti-rabbit (Invitrogen) at a dilution of 5 μg/ml, for 1 h at RT. The slides were then washed with TBS and the cells were incubated for 5 min in TO-PRO-3 iodide (Invitrogen/Molecular Probes) at a concentration of 1:1000, for 5 min. Finally, the cells were washed in TBS, and the slides were mounted with UltraCruz mounting medium (Santa Cruz, CA, USA). HepG2 cells were used as positive controls for sst2A, sst3 and sst5, HT29 colon cancer cells were used as positive controls for sst4 and rat brain was used as a positive control for sst1 and sst2B.

2.4. Western blot

The cells were washed 2 times with ice cold PBS and were lysed in a buffer containing 20 mM TrisHCl pH 7, 4% CHAPS and protease inhibitors (Complete, EDTA-free from Roche). The lysates were sonicated for 5 s on ice and centrifuged at 10,000 ×*g* for 5 min. The supernatants were collected and the protein content was measured with the BCA method [34]. Sample buffer (4×) consisting of 0.25 M TrisHCl pH 6.8, 8% SDS, 40% glycerol, 2 mM DTT and 0.008% bromophenol blue was added, and the samples were briefly heated at 98 °C. Sample volumes containing 10 μg protein were resolved on a 8% acrylamide gel (30/0.8 acrylamide/bisacrylamide ratio). The resolved proteins were transferred on Immuno-Blot PVDF membranes (Bio-Rad, Hercules, CA) overnight, at 22 mV, at 4 °C. To minimize nonspecific binding, the membranes were incubated in TBST and 5% casein (Sigma Aldrich) for 1 h in room temperature. For the sst receptor blots, casein was substituted with bovine serum albumin (BSA) (Sigma Aldrich), the buffer was supplemented with 0.5% Fish Skin Gelatin (Sigma Aldrich) and the membranes were blocked overnight at 4 °C. Following blocking, the membranes were incubated with the primary antibody, overnight at 4 °C. The antibodies used were rabbit anti rat ssts 1, 2A, 2B, 3, 4, and 5 (Gramsch Laboratories, Schwabhausen Germany), mouse anti rat β-actin (Santa-Cruz), mouse anti rat anti-α1-procollagen (Santa Cruz), mouse anti rat aSMA (Millipore/Chemicon). The membranes were then washed 3 times in TBST and incubated in the secondary goat anti-mouse or goat anti-rabbit HRP conjugated antibody (Chemicon) for 1 h in RT. Following thorough washing in TBST, positive bands were visualized using ECL substrate (Thermo Scientific/Pierce, Rockford, IL 61105 USA). Following band visualization the films were scanned and band density was measured using ImageJ software.