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Expression and function of fibroblast growth factor (FGF) 9 in hepatic stellate cells and its role in toxic liver injury

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Abstract

Hepatic injury and regeneration of the liver are associated with activation of hepatic stellate cells (HSC). Fibroblast growth factors (FGFs) and their receptors are important regulators of repair in various tissues. HSC express FGFR3IIIc as well as FGFGR4 and different spliced FGFR1IIIc and FGFR2IIIc isoforms which differ in the presence or absence of the acid box and of the first Ig-like domain. Expression of FGF9, known to be capable to activate the HSC FGFR2/3-isoforms, was increased in HSC in liver slice cultures after exposition to carbon tetrachloride, as an acute liver injury model. FGF9 significantly stimulated 3-H thymidine incorporation of hepatocytes, but failed to induce DNA synthesis in HSC despite the fact that FGF9 induced a sustained activation of extracellular signal-related kinases (ERK) 1/2. FGF9 induced an increased phosphorylation of Tyr436 of the fibroblast growth factor receptor substrate (FRS) 2, while phosphorylation of Tyr196 which is required for efficient Grb2 recruitment remained unchanged. Our findings suggest that HSC FGF9 provide a paracrine mitogenic signal to hepatocytes during acute liver injury, while the autocrine FGF9 signaling appears to be not sufficient to induce cell proliferation.

Keywords: Fibroblast growth factor 9; Hepatic stellate cells (HSC); Mitogen-activated protein kinase (MAPK); Mesenchymal–epithelial cell signaling

The fibroblast growth factor (FGF) ligand family currently comprises at least 23 structurally related polypeptides. FGFs are important modulators of cellular proliferation, migration, and differentiation depending on cell type and tissue context. FGF stimulation leads to tyrosine auto-phosphorylation of four high-affinity tyrosine kinases FGF receptors (FGFR1–FGFR4). Alternative RNA splicing of the exon 8 and 9 encoding the second half of the third Ig-loop in the extracellular domain of FGFR1, FGFR2, and FGFR3 generates receptor isoforms with different ligand-binding specificities. The external FGF signal is transduced by recruitment of signaling proteins to the tyrosine auto-phosphorylation sites on the activated FGF receptor and to linked docking proteins creating additional binding sites for further signaling molecules. The docking protein SNT-1/FRS2 (fibroblast growth factor receptor substrate 2) plays a central role in linking FGFR activation to the RAS/MAPK and PI-3 kinase signaling pathway and is crucial for chemotactic response, differentiation, and cell proliferation [1]. Hepatic stellate cells (HSC) are quiescent vitamin A storing pericytes located between sinusoidal endothelial cells and parenchymal cells in the hepatic lobules. In the process of injury to the liver, HSC are activated and may support the wound healing by providing trophic factors for the parenchymal cells [2]. Particularly, the important role of HSC in liver regeneration is widely accepted. During regeneration after partial hepatectomy HSC are in close association with hepatocytes and activation of HSC may be necessary for HSC–hepatocyte

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interaction [3]. Expression of FGF7/KGF by activated human HSC may enhance liver regeneration and/or hepatocyte survival via activation of the de novo expressed FGF receptor 2 IIIb isoform in primed hepatocytes [4].

Here, we performed a systematic survey of the mRNA expression of FGF receptors in primary rat HSC. In addition, HSC secreted FGF9, and this ligand is up-regulated in HSC when liver slice cultures were treated with a single dose of carbon tetrachloride (CCl_4) as a model of acute toxic liver injury. FGF9 was recently recognized as important growth factor for epithelial and mesenchymal cell signaling in embryonic lung development, for epithelial to stromal signaling in prostate tumors and for the proliferation of neointimal smooth muscle cells after arterial injury [5]. Although HSC express the cognate receptors for FGF9 and FGF9 induced a sustained activation of ERK1/2, FGF9 failed to stimulate HSC proliferation. These findings were in contrast to hepatocytes which responded with high-thymidine incorporation after treatment with FGF9. Our data indicate that HSC provide a mitogenic signal to hepatocytes by secreting FGF9 during acute liver injury.

Materials and methods

Preparation of cells and cell culture. Rat HSC were prepared from male Sprague–Dawley rats (about 500 g body weight) by the pronase/collagenase method as described before [6]. Hepatocytes were isolated following the collagenase method of Seglen as described [6]. Hepatocytes were seeded at a density of $5 \times 10^4/cm^2$ on collagen coated dishes in Hepatozyme-SFM (Invitrogen, Heidelberg, Germany). Rat HSC, COS-1 cells, and the immortalized human hepatic stellate cell line LX-2 (a kind gift from Dr. S.L. Friedman, Mount Sinai Medical School, New York, NY) were cultured in DMEM, 4 mmol/L L-glutamine, supplemented with 10% FCS for HSC and COS-1 or 2% FCS for LX-2, respectively, and antibiotics (100 IU/mL penicillin, 100 μg/mL streptomycin).

Immunoblot analysis. For Western blotting cell extracts were resolved by SDS–PAGE (12.5% SDS–polyacrylamide gel in Tris–glycine) and transferred to nitrocellulose membranes. The washed blots were then incubated with the appropriate secondary antibody coupled to horseradish peroxidase (PIERCE, Perbio Science, Bonn, Germany).

RNA isolation and RT-PCR analysis. RNA from rat HSC was extracted with the RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. One microgram of extracted total RNA was reverse transcribed with Omniscript Reverse Transcriptase Kit (Qiagen) using oligodT as primer. cDNAs were amplified using the Taq DNA Polymerase Kit (Qiagen). PCR primers were obtained from Invitrogen. The RT-PCR conditions have been optimized regarding temperature and primer sequence. RT-PCR products were confirmed by DNA sequencing. Real-time PCR was performed using the LightCycler FastStart DNA Master SYBR Green I from Roche Diagnostics GmbH according to manufacturer's protocol.

Animal treatment, liver slices, and histological examination. All animal protocols were in full compliance with the guidelines for animal care and were approved by the local Animal Care Committee. Sirius red staining was performed by standard procedure as described [6]. Livers from adult rats were sliced into 200 mm thick slices using a Krumdieck tissue slicer (Alabama Research and Development, USA), were placed onto semi-porous membranes at a fluid–air interface as previously described [6]. In order to induce an acute toxic injury on cells of the liver slices 10 μl CCl_4 was added on filter paper in a six-well plate and allowed to evaporate and equilibrate with the medium [7]. The sections were immunostained using a rabbit polyclonal antibody against FGF9 and the avidin–biotin complex method (Vectastain Elite ABC-Kit, Vector Laboratories, Burlingame, CA) as described before [6].

Immunofluorescence analysis. Rat HSC were cultured on glass coverslips coated with polylysine at a density of 2×10^4 cells per 35 mm dish. Immunostaining was performed as described before [6]. FGF9-related immunoreactivity was detected by using a rabbit polyclonal anti-FGF9 antibody (sc-7876, 1:100) (Santa Cruz, Heidelberg, Germany).

Proliferation assay. To determine proliferation of primary rat hepatocytes, cells were starved in basal medium (Williams modified Eagle's medium, Sigma, St. Louis, MO) supplemented with glutamine (2 mM), insulin (1.67 μM), dexamethasone (3 μM), and heparin (0.5 μg/mL) for 24 h. Subsequently, the medium was changed to serum-free conditions with and without various concentrations of growth factors as indicated. Primary HSC were seeded in DMEM supplemented with 10% FCS overnight and the medium was changed to DMEM plus 0.5% FCS 24 h before stimulation with the indicated growth factors. After 16 h, cells were exposed to 3-H-thymidine (673 GBq/mmol; 2 μCi/mL medium; Hartmann Analytic GmbH, Braunschweig) for a period of 3 h as described [6]. The mean (±SD) activity was determined from triplicate wells. Hepatozyme-SFM, FCS or PDGF served as positive controls. Recombinant human PDGF and FGFs were obtained from PeproTech (London, UK).

Results

Expression of FGFR isoforms in HSC

Since FGF tyrosine kinase receptors bind FGFs with different binding affinities and alternative spliced variants exhibit unique FGF-binding specificity, we investigated the expression of the four FGF tyrosine kinase receptors in freshly isolated HSC and in HSC cultured for 3 days. To examine the expression of differentially spliced FGFR1, FGFR2, and FGFR3 isoforms by RT-PCR in detail, we first used oligonucleotide primers (set I depicted in Fig. 1A) that encompass the second half of Ig-like domain II and the alternatively spliced second half of the third Ig-like domain to distinguish between the IIIc and IIIb isoforms (Fig. 1A). All amplified products were confirmed by DNA sequencing (data not shown). Freshly isolated rat HSC expressed all four FGF tyrosine kinase receptors. The alternative spliced IIIc isoform was the dominant form of FGFR 1, 2, and 3 (Fig. 1B).

To further analyze the N-terminal spliced variants of FGFR 1, 2, and 3, amplification of the FGFR encoding transcripts was performed with a second set of primers (set II, Fig. 1A) encompassing the signal peptide and the complete Ig-like domains encoding sequence of the IIIc receptor isoforms (Fig. 1A). All products were confirmed by sequencing (data not shown). The predominant amplified FGFR1IIIc variant was FGFR1βIIIcAB, which misses the first Ig-loop (β form) and contains the acidic box (AB). Additionally, two minor FGFR1IIIc receptor cDNAs were detected, FGFR1αIIIc and FGFR1αIIIcAB (both contain the first Ig-loop (α form)). Amplification of the FGFR2 transcripts with the set II primers as depicted in Fig. 1C yielded three PCR fragments corresponding to FGFR2βIIIcAB, FGFR2αIIIcAB, and FGFR2βIIIc. The last was the predominantly expressed isoform of FGFR2. Freshly isolated and 3-day-old HSC expressed only the