Research Article

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Fibroblast growth factor signaling regulates the expansion of A6-expressing hepatocytes in association with AKT-dependent β-catenin activation

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Background & Aims: Fibroblast Growth Factors (FGFs) promote the proliferation and survival of hepatic progenitor cells (HPCs) via AKT-dependent β-catenin activation. Moreover, the emergence of hepatocytes expressing the HPC marker A6 during 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)-induced liver injury is mediated partly by FGF and β-catenin signaling. Herein, we investigate the role of FGF signaling and AKT-mediated β-catenin activation in acute DDC liver injury.

Methods: Transgenic mice were fed DDC chow for 14 days concurrent with either *Fgf10* over-expression or inhibition of FGF signaling via expression of soluble dominant-negative FGF Receptor (R)-2IIIb.

Results: After 14 days of DDC treatment, there was an increase in periportal cells expressing FGFR1, FGFR2, and AKT-activated phospho-Serine 552 (pSer552) β-Catenin in association with up-regulation of genes encoding the FGFR2IIIb ligands, *Fgf7*, *Fgf10*, and *Fgf22*. In response to *Fgf10* over-expression, there was an increase in the number of pSer552-β-Catenin$^{(positive)+ive}$ periportal cells as well as cells co-positive for A6 and hepatocyte marker, Hepatocyte Nuclear Factor-4α (HNF4α). A similar expansion of A6^{+ive} cells was observed after *Fgf10* over-expression with regular chow and after partial hepatectomy during ethanol toxicity. Inhibition of FGF signaling increased the periportal A6^{+ive}HNF4α^{+ive} cell population while reducing centrolobular A6^{+ive} HNF4α^{+ive} cells. AKT inhibition with Wortmannin attenuated FGF10-mediated A6^{+ive}HNF4α^{+ive} cell expansion. *In vitro* analyses using FGF10 treated HepG2 cells demonstrated AKT-mediated β-Catenin activation but not enhanced cell migration.

Conclusions: During acute DDC treatment, FGF signaling promotes the expansion of A6-expressing liver cells partly via AKT-dependent activation of β-Catenin expansion of A6^{+ive} periportal cells and possibly by reprogramming of centrolobular hepatocytes.

Introduction

Human diseases such as congenital biliary atresia and primary biliary cirrhosis are marked pathologically with periportal fibrosis surrounding expanding biliary ductular reactions, the cells of which exhibit characteristics consistent with those of epithelial progenitor and stem cells [1,2]. Treatment of rodents with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) induces an analogous fibrosis with ductular reactions populated with hepatic progenitor cells (HPCs) expressing SOX9 [3], 1C3 [3], PROMININ-1 (CD133) [4], and A6 [5]. Recent lineage tracing studies suggest that the HPC transdifferentiation toward a hepatocyte cell fate during DDC injury is negligible [6–8]. Instead, hepatocytes undergo some degree of reprogramming or transdifferentiation with *de novo* expression of A6, SOX9, CK19, and OPN, which are conventionally considered HPC or biliary epithelial cell (BEC) markers [9].

Fibroblast Growth Factor (FGF) signaling regulates hepatogenesis [10–12], progenitor cell expansion, and liver regeneration [13,14]. The FGF family comprises 22 polypeptide ligands that bind to 4 promiscuous tyrosine kinase FGF receptors (FGFR), each expressed as two isoforms [15]. FGFRs are principally located in the cell membrane although nuclear localization has been described [16,17]. We previously demonstrated that during early hepatogenesis, FGF10, expressed by embryonic mesenchymal hepatic stellate cells, promotes HPC proliferation via β-catenin activation [10]. Postnatally, hepatocyte proliferation following liver injury is regulated in part by activation of FGF signaling

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Abbreviations: HPCs, hepatic progenitor cells; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; CD133, Prominin-1; FGF, Fibroblast Growth Factors; FGFR, Fibroblast Growth Factor Receptor; RTK, receptor tyrosine kinase; WT, wild type; dnFGFR, dominant negative Fibroblast Growth Factor Receptor; PCNA, Proliferating Cell Nuclear Antigen; NHF, non-hepatocyte fraction; PFA, paraformaldehyde; H&E, hematoxylin and eosin; cDNA, complementary DNA; qPCR, quantitative PCR; HGF, Hepatocyte Growth Factor; HNF4α, Hepatocyte Nuclear Factor-4α; GSK3β, glycogen synthase kinase-3 beta; PI3K, Phosphoinositide 3-kinase.

via FGFR2IIIb [14]. During DDC-induced liver injury, mesenchymal cell expression of FGF7 is known to regulate HPC expansion, and over-expression of *Fgf7* reduces hepatocyte damage and cholestatic liver injury [13].

Wnt/β-catenin signaling has been implicated in HPC-mediated liver regeneration [18,19]. Binding of the Wnt ligand to Frizzled receptor leads to dephosphorylation, activation, and nuclear translocation of the transcriptional regulator β-CATENIN. Using *ex vivo* embryonic liver cultures, Sekhon *et al.* showed that FGF signaling promotes β-catenin-mediated proliferation of hepatoblasts [20]. Activation of β-CATENIN can also occur non-canonically via receptor tyrosine kinase (RTK) activation through AKT-dependent [21,22] and Protein Kinase A (PKA) mediated [23] phosphorylation of β-CATENIN at Serine-552 (pSer552-β-CATENIN). FGF signaling promotes HPC proliferation *in vitro* via AKT-dependent β-catenin activation [24]. Postnatal HPC proliferation induced by DDC treatment is mediated in part via β-catenin activation through increased expression of Wnt ligands [19]. Hyper-activation of liver-specific β-catenin during chronic DDC-induced liver injury leads to increased expansion of A6-expressing hepatocytes in association with improved hepatic repair and resolution of cholestasis [25].

In this study, we further investigate the role of FGF signaling in the emergence and expansion of A6^{+ive} cells during DDC-induced liver injury. We also demonstrate a link between FGF signaling and β-catenin activation during acute DDC liver injury, during which the initial expansion of A6^{+ive} cells is induced, analogous to what is observed in embryonic liver development.

Materials and methods

Experimental animals and procedures

Six-week old, C57BL/6J (wild-type, WT) male mice (Jackson Laboratories) were fed either a standard diet or 0.1% DDC diet (Test Diet, Richmond) up to 14 days. Inducible transgenic and littermate control mice were given water with 1% doxycycline (Clontech) 2 days prior to and throughout DDC treatment. *CMV^{cre};Rosa26$^{rtTA/-}$;tet(O)-sFGFR2-IIIb$^{+/-}$* mice (dnFGFR) exhibited induced ubiquitous expression of dominant-negative soluble FGFR2-IIIb as confirmed by qPCR (Supplementary Fig. 1B) [26]. *CMV^{cre};Rosa26$^{rtTA/-}$;tet(O)-Fgf10$^{+/-}$* mice (*Fgf10*-induced) exhibited induced *Fgf10* over-expression in uninjured, and DDC treated mice [27]. In a separate experiment, *Fgf10*-induced mice were given daily intra-peritoneal injections of the AKT inhibitor, Wortmannin (*Fgf10* + Wort, 0.7 mg/kg) or vehicle solution (*Fgf10* + vehicle) in DMSO with 1x phosphate buffered saline (PBS) (Sigma-Aldrich), two days prior to and throughout DDC treatment. *Fgf10* was also induced two days prior to and throughout 70% partial hepatectomy (PHx) combined with ethanol (EtOH) gavage (1 g/kg, every 12 h pre- and post-PHx). All procedures were done in compliance with the IACUC of Children's Hospital Los Angeles/Saban Research Institute guidelines for use of laboratory animals.

Tissue collection

After carbon dioxide euthanasia, 1x PBS was flushed through the portal vein. Portions of the right lobe were collected for histology and total RNA. Single cell suspension of whole liver and non-hepatocyte fractions were obtained by mechanical and enzymatic digestion followed by serial centrifugation [4] to enrich the non-hepatocyte fractions (NHF). qPCR for *Albumin* and *Hnf4α* was performed to confirm depletion of hepatocytes from the NHF (Supplementary Fig. 1A).

Immunohistochemistry and immunofluorescence analysis

Collected tissues were fixed in 4% paraformaldehyde (PFA) or 30% sucrose for 4 h. 5 μm sections of paraffin-embedded and OCT-embedded tissues were utilized for immunostaining (Supplementary Table 1). Hematoxylin & Eosin (H&E) was utilized for morphologic analyses. Quantification of A6^{+ive}HNF4α^{+ive} cells per lobular region was performed by separating each lobule into periportal (0–96 μm), mid (97–194 μm), and central regions (195–290 μm) (Fig. 2E). Immunohistochemistry was performed with the Dako EnVision+ Dual Link System-HRP (DAB+) kit (Dako). Immunofluorescence (IF) images were taken using a Leica DM5500B microscope (Leica). All IF quantifications were performed on at least three images per animal with three animals per treatment (n = 3).

Quantitative PCR analysis of gene expression

Total RNA was isolated with the Qiagen RNeasy Mini kit (Qiagen). Complementary DNA (cDNA) was prepared with iScript cDNA synthesis kit (Bio-Rad). Quantitative real-time PCR (qPCR) was performed using the Light-Cycler Taqman Master (Roche Applied Science) and probes from the Universal Probe Library (Roche Applied Science) against intron spanning, gene specific primers (Supplementary Table 2). *18s* was selected as the optimal internal control for all analyses.

Cell migration assays

HepG2 cells were cultured in DMEM with 1% L-Glutamine, 1% Pen/Strep, and 10% Fetal Bovine Serum (Gibco). Scratch assays in serum free conditions were used to determine the dose response of HepG2 cell migration to recombinant FGF10 (rFGF10) (n = 8). Images were taken at 0, 24, and 48 h post scratch and the width determined by three measurements per image. An optimal dose of rFGF10 (10 ng/ml) was determined from a generated standard curve for trans-well cell migration assays. 40,000 cells were cultured in 8 μm pore trans-well inserts (BD Falcon) for 24 h and stained with crystal violet to quantify cell migration by cell counting and spectrophotometer analysis (Victor3, Perkin Elmer). Western blot analysis was performed as previously described to confirm signaling pathway activation in rFGF10 treated HepG2 cells (Supplementary Table 1) [24].

Statistical analysis

ANOVA-Post hoc Fisher's PLSD test or Mann-Whitney Rank Sum was performed using Statview (SAS Institute, Inc.) to calculate statistical significance (p <0.05).

Results

Expression of FGF ligands and receptors is up-regulated during acute DDC liver injury

FGF ligands expressed by mesenchymal cells regulate HPCs and liver regeneration during chemically induced liver injury [13,16,17]. Thus, qPCR was performed on RNA extracted from a density centrifugation-enriched, non-hepatocyte cell fraction (NHF) and from whole liver tissue in order to assess FGF ligand and receptor gene expression during acute DDC injury. Partial enrichment of small non-hepatocyte cells was validated by qPCR with a marked reduction in expression of hepatocyte genes *Albumin* and *Hnf4α* relative to the hepatocyte cell fraction (Supplementary Fig. 1A) [4]. Within the first 14 days of DDC treatment, we observed 11.6-fold and 12.4-fold increases in *Fgfr1IIIb* and *Fgfr2IIIb* expression, respectively, in the NHF (Fig. 1A, p <0.05), indicating FGFR activation as previously described [24]. FGF1, FGF2, FGF3, and FGF10 possess high binding affinity for FGFR1IIIb, while FGF1, FGF3, FGF7, FGF10, and FGF22 exhibit high binding affinity for FGFR2IIIb [28]. qPCR was performed in lieu of western blot analyses given the lack of reliable antibodies against FGF ligands. *Fgf3* expression was not detected by qPCR. *Fgf10* expression was significantly up-regulated 14 days after DDC injury while *Fgf7* and *Fgf22* were upregulated throughout the entire observation period (Fig. 1B, p <0.05). *Fgf1* expression was transiently down-regulated at 3 days and significantly upregulated by 14 days of DDC treatment (Fig. 1B, p <0.05). In contrast, gene expression profiles from whole liver samples for *Fgfr2IIIb*