Research Article

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Characterization of hepatic stellate cells, portal fibroblasts, and mesothelial cells in normal and fibrotic livers

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Background & Aims: Contribution of hepatic stellate cells (HSCs), portal fibroblasts (PFs), and mesothelial cells (MCs) to myofibroblasts is not fully understood due to insufficient availability of markers and isolation methods. The present study aimed to isolate these cells, characterize their phenotypes, and examine their contribution to myofibroblasts in liver fibrosis.

Methods: Liver fibrosis was induced in Collagen1a1-green fluorescent protein (*Col1a1*^{GFP}) mice by bile duct ligation (BDL), 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet, or CCl_4 injections. Combining vitamin A (VitA) lipid autofluorescence and expression of GFP and glycoprotein M6a (GPM6A), we separated HSCs, PFs, and MCs from normal and fibrotic livers by fluorescence-activated cell sorting (FACS).

Results: Normal *Col1a1*^{GFP} livers broadly expressed GFP in HSCs, PFs, and MCs. Isolated VitA+ HSCs expressed reelin, whereas VitA−GFP+GPM6A− PFs expressed ectonucleoside triphosphate diphosphohydrolase-2 and elastin. VitA−GFP+GPM6A+ MCs expressed keratin 19, mesothelin, and uroplakin 1b. Transforming growth factor (TGF)-β1 treatment induced the transformation of HSCs, PFs, and MCs into myofibroblasts in culture. TGF-β1 suppressed cyclin D1 mRNA expression in PFs but not in HSCs and MCs. In biliary fibrosis, PFs adjacent to the bile duct expressed α-smooth muscle actin. FACS analysis revealed that HSCs are the major source of GFP+ myofibroblasts in the injured *Col1a1*^{GFP} mice after DDC or CCl_4 treatment. Although PFs partly contributed to GFP+ myofibroblasts in the BDL model, HSCs were still dominant source of myofibroblasts.

Conclusion: HSCs, PFs, and MCs have distinct phenotypes, and PFs partly contribute to myofibroblasts in the portal triad in biliary fibrosis.

Keywords: Entpd2; Fibrosis; Gpm6a; Mesothelin; Myofibroblasts; Reelin; Uroplakin.

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Abbreviations: MCs, mesothelial cells; HSCs, hepatic stellate cells; PFs, portal fibroblasts; VitA, vitamin A; GPM6A, glycoprotein M6a; Col1a1, collagen1a1; GFP, green fluorescence protein; TGF-β, transforming growth factor-β; PDGF, platelet-derived growth factor; ACTA2, α-smooth muscle actin; ELN, elastin; ENTPD2, ectonucleoside triphosphate diphosphohydrolase-2; SLCs, second-layer cells; CFs, capsular fibroblasts; Krt, keratin; MSLN, mesothelin; PDPN, podoplanin; FACS, fluorescence-activated cell sorting; UPK, uroplakin; RFP, red fluorescent protein; BDL, bile duct ligation; NPCs, nonparenchymal cells; PI, propidium iodide; qPCR, quantitative-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DES, desmin; SMCs, smooth muscle cells; RELN, reelin; Ccnd1, Cyclin D1; P-SMAD3, phosphorylated-SMAD3.

Introduction

Hepatic stellate cells (HSCs) reside in the space of Disse in the liver and store vitamin A (VitA) lipids as retinyl ester [1,2]. Upon liver injury, HSCs transform into myofibroblasts that express α-smooth muscle actin (ACTA2) [1,2]. Myofibroblasts form fibrous scars, actively synthesize extracellular matrices and proinflammatory cytokines, and participate in the progression from an injured liver to fibrosis and cirrhosis. Cell lineage tracing indicated that HSCs are mesodermal in origin and are the major source of myofibroblasts [3–5]. Clinical cases and animal studies suggest that fibrosis is reversible [6–9]. During fibrosis regression, activated HSCs undergo apoptosis or revert to quiescent HSCs [6,8,9]. Thus, the suppression of HSC activation has been considered to be a therapeutic target for treating liver fibrosis.

In addition to HSCs, different types of liver mesenchymal cells also differentiate into myofibroblasts in fibrosis. Portal fibroblasts (PFs) around the bile duct in the portal tract express COL15A1, elastin (ELN), ectonucleoside triphosphate diphosphohydrolase-2 (ENTPD2/NTPDase2/CD39L1), and THY1 and do not store VitA lipids in the rat liver [10–14]. In biliary fibrosis, PFs are believed to be the source of myofibroblasts in the portal area. In addition to PFs, second-layer cells (SLCs) in the central vein and capsular fibroblasts (CFs) beneath the mesothelium were characterized based on their morphology and location in the liver [15]. However, little is known about how these cells contribute to fibrosis

because the availability of markers and isolation methods for each cell type is limited.

Mesothelial cells (MCs) form a single epithelial cell sheet and cover the liver surface [16]. MCs express glycoprotein M6A (GPM6A), mesothelin (MSLN), and podoplanin (PDPN) [16–18]. During liver development, mesodermal MCs migrate inward from the liver surface and give rise to both HSCs and PFs [19]. Upon liver injury, MCs give rise to HSCs or myofibroblasts near the liver surface, depending on the etiology [16,18]. Similar to HSC activation, MCs change their phenotype to myofibroblasts in response to transforming growth factor-β (TGF-β) [16].

In the present study, we separated HSCs, PFs, and MCs by fluorescence-activated cell sorting (FACS) from collagen1a1 promoter-green fluorescent protein (*Col1a1*^{GFP}) transgenic mouse livers and quantified contribution of these cells to myofibroblasts in liver fibrosis.

Materials and methods

Mouse models

Col1a1^{GFP} mice were obtained from Dr. David Brenner [20]. Uroplakin 1b-red fluorescent protein (*Upk1b*^{RFP}) knock-in mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Fibrosis was induced by bile duct ligation (BDL) for 3 weeks, 0.1% 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet for 1 month, or CCl_4 injection 12 times as previously described [4,16]. All animal experiments were performed in accordance with the NIH guidelines under the protocol approved by the IACUC at the University of Southern California.

Immunohistochemistry

Mouse liver tissues were embedded in freezing medium without fixation or after fixation with 4% paraformaldehyde. Cryosections (7 μm) were used for immunohistochemistry. After blocking, the sections were incubated with primary antibodies for 1 h. The primary antibodies and additional treatment are listed in Supplementary Table 1. The primary antibodies were detected with secondary antibodies conjugated with AlexaFluor dyes. Nuclei were counterstained with DAPI. Signals were captured with 90i microscope (Nikon, Melville, NY). The paraffin-embedded specimens for normal human livers (n = 2) and alcohol-induced fibrosis (n = 2) at the Harbor-UCLA Medical Center or biliary atresia (n = 3) at Children's Hospital Los Angeles were used for immunohistochemistry under study protocols approved by the institutional review boards (HS-11-00476, CCI-10-00148). The primary antibodies were detected with SuperPicure HRP Polymer (Life Technologies, Grand Island, NY).

Cell isolation

Nonparenchymal cells (NPCs) were isolated by the NPC core supported by NIAAA grant (R24AA012885) [16]. Mouse livers were perfused through the superior vena cava by 0.5% pronase (Roche, Indianapolis, IN) and 0.044% collagenase (Sigma, St. Louis, MO). After agitation of the digested tissue with 10 μg/ml DNase, the cells were placed on the top of four OptiPrep gradients (1.034, 1.043, 1.058, 1.085) in Beckman ultracentrifuge tubes and were centrifuged in the SW-41Ti rotor at 20,000 rpm for 15 min. The 1.058 fraction was used as NPCs.

FACS

NPCs were incubated with the anti-GPM6A antibody (MBL, Woburn, MA) at 1500-fold dilution for 30 min. After washing, the primary antibody was detected with anti-rat IgG AlexaFluor647 (Life Technologies). After excluding propidium iodide (PI)+ dead cells, PI− live cells were analyzed with a krypton laser and a 424 nm filter to detect VitA autofluorescence with FACS Aria I (BD Bioscience, San Jose, CA) in the USC Flow Cytometry Core[4,16]. The VitA− fraction was further separated based on the signal intensities for GPM6A and GFP.

MCs were isolated from the liver surface as previously reported [16]. After whole liver digestion with 1 mg/ml pronase, the cells were incubated with the anti-GPM6A antibody. The primary antibody was detected with anti-rat IgG AlexaFluor568 antibodies and VitA−GPM6A+ MCs were sorted by FACS.

Cell culture and immunocytochemistry

After FACS, the cells (2×10^4 cells) were plated on collagen-coated 24-well plates in DMEM containing 10% FBS. The cells were treated with 10 ng/ml TGF-β1 (Sigma) or 100 pg/ml PDGF-BB (eBioscience, San Diego, CA). Immunocytochemistry was performed as previously described [16,18]. The cells cultured on a glass cover were fixed with 4% paraformaldehyde. After blocking, the sections were incubated with primary antibodies. The primary antibodies are listed in

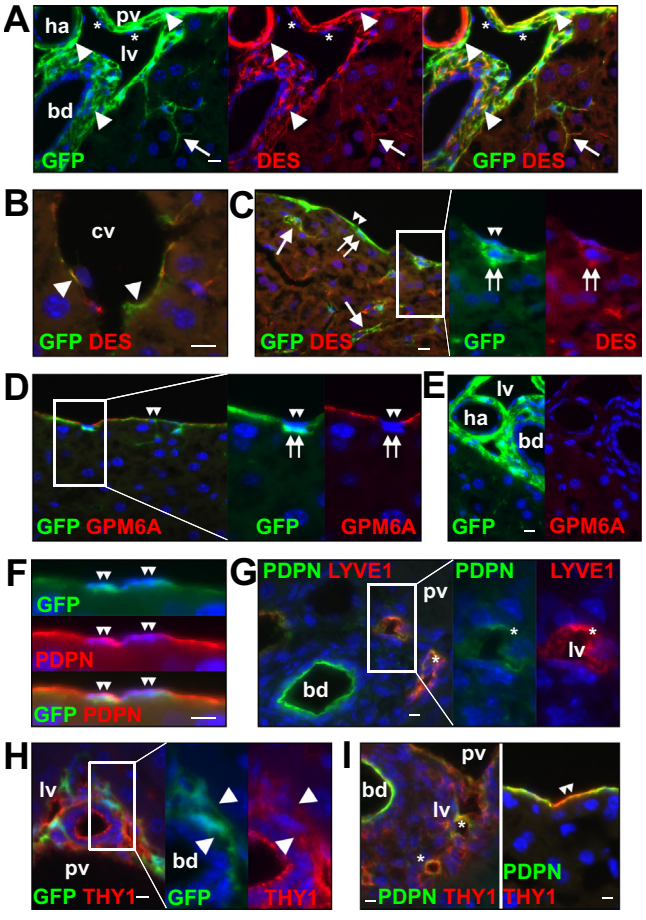

Fig. 1. Expression of GFP in HSCs, PFs, SMCs, SLCs, CFs, and MCs in *Col1a1*^{GFP} mouse livers. Expression of GFP and different cell markers was examined by immunohistochemistry in the *Col1a1*^{GFP} (A–F, H) or wild-type (G, I) mouse livers. (A) GFP expression is observed in DES+ HSCs (arrows), PFs (arrowheads) adjacent to bile duct (bd), and SMCs (arrowheads) in the hepatic artery (ha) and portal vein (pv). Asterisks indicate lymphatic vessels (lv). (B) GFP expression is observed in DES+ SLCs (arrowheads) in the central vein (cv). (C) MCs (double arrowheads) and CFs (double arrows) express GFP. Arrows indicate GFP+ HSCs. (D) Both GPM6A+ MCs (double arrowheads) and GPM6A− CFs (double arrows) express GFP. (E) No GPM6A expression in the portal triad. (F) PDPN+ MCs express GFP (double arrowheads). (G) Bile duct and lymphatic vessels (asterisks) are positive for PDPN. (H) THY1+ PFs express GFP (arrowheads). (I) Expression of THY1 in lymphatic vessels (asterisks). Some MCs express THY1 (double arrowheads). Scale bar: 10 μm.