Research Article

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Biliary fibrosis drives liver repopulation and phenotype transition of transplanted hepatocytes

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Background & Aims: Current research focuses on developing alternative strategies to restore decreased liver mass prior to the onset of end-stage liver disease. Cell engraftment/repopulation requires regeneration in normal liver, but we have shown that severe liver injury stimulates repopulation without partial hepatectomy (PH). We have now investigated whether a less severe injury, secondary biliary fibrosis, would drive engraftment/repopulation of ectopically transplanted mature hepatocytes.

Methods: Ductular proliferation and progressive fibrosis in dipeptidyl-peptidase IV (DPPIV)$^-$ F344 rats was induced by common bile duct ligation (BDL). Purified DPPIV$^+$/green fluorescent protein (GFP)$^+$ hepatocytes were infused without PH into the spleen of BDL rats and compared to rats without BDL.

Results: Within one week, transplanted hepatocytes were detected in hepatic portal areas and at the periphery of expanding portal regions. DPPIV$^+$/GFP$^+$ repopulating cell clusters of different sizes were observed in BDL rats but not untreated normal recipients. Surprisingly, some engrafted hepatocytes formed CK-19/claudin-7 expressing epithelial cells resembling cholangiocytes within repopulating clusters. In addition, substantial numbers of hepatocytes engrafted at the intrasplenic injection site assembled into multicellular groups. These also showed biliary "transdifferentiation" in the majority of intrasplenic injection sites of rats that received BDL but not in untreated recipients. PCR array analysis showed upregulation of osteopontin (*SPP1*). Cell culture studies demonstrated increased *Itgβ4*, *HNF1β*, *HNF6*, *Sox-9*, and *CK-19* mRNA expression in hepatocytes incubated with osteopontin, suggesting that this secreted protein promotes dedifferentiation of hepatocytes.

Conclusions: Our studies show that biliary fibrosis stimulates liver repopulation by ectopically transplanted hepatocytes and also stimulates hepatocyte transition towards a biliary epithelial phenotype.

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Introduction

Chronic liver injury causes progressive fibrosis, characterized by accumulation of collagen and scar tissue, which leads to impaired hepatic function [1]. Cirrhosis is the advanced stage of fibrosis in patients with chronic liver diseases, one of the major causes of death in the US, and liver transplantation is the only effective therapeutic option for end-stage disease [2,3]. Because of the increasing scarcity of donor organs [4], alternate therapies are critical. We have therefore focused on cell-based therapies to generate healthy new hepatic tissue mass in the diseased liver and delay the end-stage. Successful application of these therapies would comprise an important clinical advance [5–8], but successful treatment will require strategies to promote engraftment and then expand the population of engrafted cells.

Elegant pioneering studies have shown that mature hepatocytes can repopulate more than 80% of the recipient liver [9–12]. Such repopulation levels are achieved under circumstances of massive injury or inhibition of the proliferative capacity of host hepatocytes, which provides a selective growth advantage of transplanted cells. However, these studies represent extreme experimental conditions that might not accurately reflect clinical therapeutic scenarios. Nevertheless, liver repopulation after cell transplantation into the diseased liver environment must be evaluated in models that reproduce the most important features of human fibrosis.

Repopulation of the healthy liver requires stimulation of regeneration by partial hepatectomy (PH) to expand the transplanted cells (reviewed in [6]). However, we recently demonstrated that the injury inherent in severe liver disease can also drive repopulation by transplanted cells without additional treatment. In these experiments, we studied advanced cirrhosis

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Abbreviations: PH, partial hepatectomy; TAA, thioacetamide; BDL, bile duct ligation; DPPIV, dipeptidyl-peptidase IV; HBSS, Hank's balanced salt solution; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; EGFP, enhanced green fluorescent protein; H&E, hematoxylin and eosin; GGT, γ-glutamyl transpeptidase; IHC, immunohistochemistry; α-SMA, alpha-smooth muscle actin; CK-19, cytokeratin-19; Sox-9, SRY-related high-mobility group box 9; Cldn-7, claudin-7; EpCAM, epithelial cell adhesion molecule; LCM, laser capture microdissection; PDGFRβ, platelet-derived growth factor receptor beta; Col1α2, collagen 1α2; TIMP1, tissue inhibitor metalloproteinase-1; Cx43, connexin43; FGF, fibroblast growth factor; IL, interleukin; SPP1, secreted phosphoprotein 1; HNF, hepatocyte nuclear factor; Itg4β, integrin beta-4.

induced by the hepatotoxin thioacetamide (TAA) and observed engrafted and repopulating cell clusters at 2 months after transplantation without the additional stimulus of PH [13].

In the present study, we investigated whether a less severe injury, biliary fibrosis following bile duct ligation (BDL), would drive repopulation by transplanted mature hepatocytes. As in our previous studies [6,13], the experiments utilized transplantation of dipeptidyl-peptidase IV-positive ($DPPIV^+$) donor cells into allogeneic $DPPIV^-$ host rats, but because BDL made the portal vein inaccessible, cell transplantation was carried out via intrasplenic injection. The experiments indeed demonstrated that biliary fibrosis induces hepatocyte repopulation, but surprisingly, they also showed striking and rapid transition of hepatocytes to cholangiocyte-like cells in both liver and spleen. This led us to explore how BDL directs two processes: hepatocyte repopulation and reprogramming of hepatocytes to induce phenotype conversion.

Material and methods

Animals and bile duct ligation

$DPPIV^+$ F344 rats were purchased from Charles River. F344-Tg (enhanced green fluorescent protein (EGFP)) F455/Rrrc rats and $DPPIV^-$ F344 rats were originally obtained from the Rat Resource and Research Center of the University of Missouri-Columbia and used to derive male $DPPIV^+$/green fluorescent protein $(GFP)^+$ and $DPPIV^-$ F344 rats. For BDL, the common bile duct of $DPPIV^-$ F344 rats (2–3 months of age) was exposed and two ligatures were placed at the proximal and distal duct ends and tightened, followed by excision of the central part. All animal studies were conducted under protocols approved by the Institutional Animal Care and Use Committees of the University of Pittsburgh in accordance with NIH guidelines.

Isolation and purification of mature hepatocytes

Livers from $DPPIV^+$ F344 or F344-Tg (EGFP) F455/Rrrc rats were perfused with 5000 U/100 ml collagenase (Sigma), excised, minced and suspended in Hank's balanced salt solution (HBSS). Cell suspension was filtered through a 70 μm nylon mesh and centrifuged for 2 min at 50 g at 4 °C. The pellet was washed 3 times with Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), re-suspended in DMEM/10% FBS, mixed with an equal volume of Percoll (GE Healthcare Bio-Sciences) solution (containing Percoll/10 × HBSS, 9:1), and centrifuged for 10 min at 50 g at 4 °C. Cell viability of purified hepatocytes was >95%.

Cell culture

Purified hepatocytes (2×10^5) were plated on collagen-coated 6-well plates and incubated in serum-free Minimum Essential medium containing 500 ng/ml insulin (Sigma). After ~2 h, cells were incubated with or without osteopontin (R&D Systems), chenodeoxycholate, ursodeoxycholate, or taurocholate (Sigma) at various concentrations for 4 days. Triplicate cultures were pooled after cell lysis using Trizol reagent (Life Technologies), followed by total RNA isolation and subsequent qRT-PCR analysis.

Cell transplantation and liver repopulation

Purified hepatocytes ($\sim 1 \times 10^7$) were transplanted without PH into the spleen of $DPPIV^-$ F344 rats at 2 or 4 weeks after BDL or age-matched non-treated normal recipients. Rats were sacrificed at different times following cell transplantation and liver replacement was determined by enzyme histochemistry for DPPIV, as described previously [14]. For engraftment studies, transplanted hepatocytes were detected by immunohistochemistry for EGFP.

Histochemistry

Sirius Red, Masson's trichrome, and hematoxylin and eosin (H&E) staining of paraffin-embedded liver sections was performed using standard techniques. Enzyme histochemistry for γ–glutamyl transpeptidase (GGT) was determined as described previously [15].

Laser capture microscopy

Cryosections from liver tissues at 1 month after BDL and non-treated normal livers (n = 3/3 rats) were stained by Cresyl Violet (Ambion) to visualize the fibrotic septa and surrounding parenchymal tissue regions, which was followed by laser capture microdissection (LCM) using the LMD6500 Laser Microdissection System (Leica Microsystems). By collecting laser-captured liver tissue samples from 5–10 fibrotic septa regions and equally sized regions from surrounding parenchyma of each rat, we isolated 50–150 ng RNA/sample using the PicoPure RNA isolation kit (Life Technology). LCM-derived RNA had a very high integrity without DNA contamination (RNA integrity numbers were between 8.0 and 9.3), as determined using the 2100 Bioanalyzer system (Agilent Technologies). RNA was amplified using the Ovation PicoSL WTA System V2 (NuGEN Technologies). After one round of amplification, at least 7 μg complementary DNA/sample was obtained, subsequently pooled for each group and used for RT-PCR, qRT-PCR analysis for selected genes or PCR array analysis.

RT-PCR, qRT-PCR, and qRT-PCR array analysis

Total RNA was extracted from cell isolates or snap-frozen liver tissue using Trizol reagent (Life Technologies) and treated with DNAse I (NEB), followed by a cleanup step using RNeasy Plus Mini/Micro Kit (Qiagen).

For RT-PCR analyses, RNA was reverse transcribed using Verso cDNA Synthesis Kit (Thermo Scientific). A complete list of primers with the number of cycles is shown in Supplementary Table 1. cDNA was amplified by Choice-Taq DNA Polymerase (Denville Scientific) for 10 min at 95 °C, followed by 25 to 35 cycles at 94 °C for 30 s, 60 °C for 20 s, 72 °C for 60 s and a final cycle at 72 °C for 7 min.

qRT-PCR was performed with at least two independent experiments, each with duplicate assays, using the StepOnePlus Real Time PCR System (Applied Biosystems). All samples were analyzed using Power SYBR Green Master Mix (Applied Biosystems). mRNA abundance was determined by normalization of the data to the expression levels of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA. RT-PCR data were analyzed using the $2^{-\Delta\Delta Ct}$ (ddCt) method. A complete list of primers is shown in Supplementary Table 1.

Rat Growth Factors RT^2 Profiler™ PCR arrays (SA Biosciences) containing 84 genes encoding specific growth factors (SA Biosciences; www.sabiosciences.com) were used to determine mRNA expression levels in laser-captured liver tissue samples. A complete list of the genes, as well as housekeeping genes/controls can be found at www.sabiosciences.com. cDNA synthesis, qRT-PCR, and data analysis were performed according to the manufacture's instructions. For data analysis, the SA Bioscience web-based PCR Array Data Analysis tool was used (www.sabiosciences.com/pcrarraydataanalysis.php).

Quantification of dedifferentiated hepatocytes

To determine the % of bile ductules derived from transplanted $DPPIV^+$ hepatocytes, 3 liver sections (representing 3 different lobes) from each rat (n = 3) were analyzed for the presence of bile duct-like structures in $DPPIV^+$ cell clusters.

Additional information can be found in the Supplementary materials and methods.

Results

Characterization of hepatic fibrosis after bile duct ligation

To directly address the influence of a mild injury on the engraftment and repopulation of transplanted hepatocytes in a diseased liver environment, we induced progressive portal tract fibrosis by common bile duct ligation in $DPPIV^-$ F344 rats. Changes were assessed at 2 and 4 weeks after BDL, using Sirius Red (Fig. 1A), Masson's trichrome, and H&E staining (Fig. 1B). Immunohistochemistry for CK-19 showed strong cholangiocyte proliferation after BDL (Fig. 1B). Compared to normal liver tissue, increased numbers of α-SMA^+ (Fig. 1A) and $laminin^+$ cells were detected at 4 weeks after BDL (Fig. 1A), indicating activation of fibroblasts and stellate cells (additional immunohistochemical analyses can be found in Supplementary Fig. 1).

To determine the expression levels of fibrosis-related genes, qRT-PCR analyses were performed on laser-captured liver tissue samples at 1 month after BDL (Fig. 1C). We observed elevated

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