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In vitro evidences of epithelial to mesenchymal transition in low cell-density cultured human fetal hepatocytes

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ABSTRACT

Culturing fetal hepatocytes in high cell-density allowed stabilization of the hepatocyte phenotype up to 8 weeks, including the maintenance of liver-specific functions. On the other hand, when cultured at low cell-density, fetal hepatocytes underwent morphological modifications and acquired fibroblastic morphology. Since a switch from E-cadherin to vimentin expression accompanied these changes, we hypothesized the occurrence of epithelial-to-mesenchymal transition when fetal hepatocytes were cultured at low cell-density. Changes in gene expressionsuch as up-regulation of fibrosis-related geneswere also observed, suggesting that the low cell-density culture system promoted the acquisition of a profibrotic phenotype in cultured hepatocytes. The origin of fibrogenic cells in the liver is not well known, and the role of hepatocytes as a source of fibrogenic cells is controversial. Therefore, we hypothesized that hepatocytes undergoing epithelial-to-mesenchymal transition could have a central role in liver fibrosis as a source of fibrogenic cells.

To conclude, the high cell-density culture system could be a useful model for *in vitro* studies requiring long-term cultures of hepatocytes, such as the development of pharmaceutical drugs and mechanisms of viral infections. The low cell-density culture system may provide additional insights into the origin of fibrogenic cells in the liver, thus contributing to the development of novel therapeutic approaches.

1. Introduction

Primary hepatocytes are generally considered the gold standard for establishing *in vitro* liver tissue models to reveal mechanisms underlying liver development, regeneration, physiology and pathogenesis [1,2]. As these cells show a rapid decline in viability and phenotypic functions upon isolation [3,4], various strategies for extending the life span and preserving functions of hepatocytes in primary cultures have been employed. These strategies include the use of different substrates, such as extracellular matrix (ECM) components [5], and co-cultures with epithelial liver cells [6] or stromal cells [7]. One aim of the study was providing an *in vitro* culture system allowing long-term culturing of primary fetal hepatocytes. First, we established primary cultures of fetal hepatocytes, and evaluated the effect of two different culture media on hepatocyte morphology and function. Then, we used two different culture systems, high cell-density and low cell-density cultures, and compared the effect of these two conditions in determining retention of the hepatocyte phenotype.

Next, we hypothesized that type 2 epithelial to mesenchymal transition (EMT) events may occur *in vitro* during culturing of fetal hepatocytes in low cell-density, thus explaining the rapid loss of the hepatocyte phenotype *in vitro*. EMT is the process through which immotile epithelial cells lose their polarity and cell-cell contact, and undergo biochemical changes to acquire mesenchymal features, including a migratory phenotype [8]. During the EMT, markers of epithelial cells, such as E-cadherin and CK18, are down-regulated and then lost, while markers of mesenchymal cells, such as vimentin and N-cadherin, are acquired. Nevertheless, with the development of EMT a number of cells show concomitant expression of both epithelial and mesenchymal markers. When this

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occurs, such cells arepossibly in the intermediate stage of EMT [9].

Although the ability to repair damaged tissues without scarring would be ideal, EMT may persistduring wound healing in response to continuous inflammatory stimuli, and contribute to the pathogenesis of fibrosis through the formation of activated myofibroblasts up-regulating α-SMA, and synthesizing an excess of type I collagen [10]. Activated myofibroblasts are generated from a variety of sources including resident mesenchymal cells and fibroblasts, as well as epithelial and endothelial cells undergoing EMT [11]. Evidence for a pathologic role of EMT was obtained in patients with idiopathic pulmonary fibrosis [11], as well as fibrotic renal disease [12].

Liver damage, triggered by chronic inflammation or alcohol consumption, is crucial in activating the fibrotic process [13–17]. During liver fibrosis, the excess of ECM deposition in turn can cause cirrhosis, liver failure, and portal hypertension [13,18]. Fibrogenic cells in the liver are a heterogeneous group including activated hepatic stellate cells (HSCs), myofibroblasts, and portal fibroblasts [19]. However, the role of intra-hepatic cells in the liver fibrotic process is controversial and has yet to be elucidated. In a study, the authors suggested that collagen-producing cells derive from hepatocytes undergoing EMT [20], while in another study reported no identification of hepatocyte-derived fibroblasts [21]. We hypothesized a role of hepatocytes as a source of activated fibroblasts via EMT in liver fibrosis.

The idea that hepatocyte differentiation is a definitive process has been challenged by the observation that hepatocytes are responsive to stimuli resulting in cell dedifferentiation by EMT [22]. Since E-cadherin to vimentin switch is a hallmark of EMT [23], we analyzed the expression of these two proteins in cultured fetal hepatocytes. Then, we analyzed the expression of hepatocyte-related genes, EMT-related genes, andfibrosis-related genes at different time points of the low cell-density cultures (day 1, 7, and 14). To date, no standard therapeutic treatment to reverse liver fibrosis has been developed [15,24,25]. Therefore, understanding cellular and molecular mechanisms underlying the fibrotic process may help to develop innovativetherapeutic strategies.

2. Materials and methods

2.1. Cell cultures and media composition

Fetal hepatocytes were isolated from second trimester human fetal livers from fetuses undergoing therapeutic abortion, as previously described [26], and according to a protocol approved by ISMETT's Institutional Research Review Board (IRRB) and Ethics Committee. Informed consent was obtained from each donor. Fetal hepatocytes were plated onto collagen type I-coated 6-well plates (BD Biosciences, Franklin Lakes, NJ, USA), and cultured as previously described [27]. After attachment, the cells were cultured in two chemically and hormonally defined culture media: Kubota's Medium (KM) [28] and Lazaro's Medium (LM) [29]. Dexamethasone (10^{-7} M) (Hospira, Lake Forest, IL, USA) and epidermal growth factor (EGF) (20 ng/ml) (Sigma-Aldrich) were added to LM at each medium change.

2.2. Albumin secretion and urea synthesis in culture supernatants

The secretion of albumin was measured with the enzyme-linked immunosorbent assay (ELISA) Kit (Abnova, Heidelberg, Germany) according to manufacturer's instructions. The absorbance was measured on a microplate reader (Model 680, Bio-Rad, Hercules, CA, USA). Urea production was determined by blood urea nitrogen (BUN) test using an automated system (Dimension RxL Max, Siemens Healthcare Diagnostics, Tarrytown, NY, USA), as previously described [27]. The results of both assays were normalized to the total number of adherent cells, and expressed as ng/ml/10^6 cells/hour for albumin, and as mg/dL10^6 cells/hour for urea.

2.3. Activity of cytochrome P450 (CYP3A7 assay)

Activity of the fetal isoform of cytochrome P450, CYP3A7, was assessed with the pGlo Kit Luciferin-PFBE (6'-pemtafluoro-benzyl ether) (Promega, Madison, WI, USA) according to manufacturer's instructions for non-lytic CYP, as previously described [27]. The results were normalized to the total number of attached cells and expressed as relative light units (RLU)/10^6 cells/hour.

2.4. Flow cytometry analysis

To assess the immunophenotype of cells at different time points of the low cell-density cultures, a flow cytometric analysis was done, as previously described [30]. The primary antibodies used were anti-CK18 fluorescein isothiocyanate (FITC)-conjugated (Abcam, Cambridge, UK), and anti-vimentin phycoerythrin (PE)-conjugated (Abcam). Data were analyzed with FACS Diva software (BD Biosciences).

2.5. Immunofluorescence analysis

Cells were fixed according to the antibody manufacturer's instructions, and stained as previously described [30]. The primary antibodies used were anti-E-cadherin (Abcam) and the anti-vimentin (Abcam). The secondary antibodies used were Alexa Fluor fluorescence-conjugated (Invitrogen, Carlsbad, CA, USA). Cells were mounted with Prolong Gold Antifade (Invitrogen), which included diamidino-2-phenylindole (DAPI) for nuclear counterstaining, and stored in the dark at 4 °C until fluorescence microscope analysis. Cell imaging was done with a microscope (Nikon Eclipse 50i, Melville, NY, USA) coupled with a camera (Olympus XM10, Tokio, Japan), and CellF software for image acquisition (Olympus).

2.6. Analysis of gene expression profile by real-time polymerase chain reaction

Genes were divided into three panels related to mature hepatocyte phenotype, EMT, and fibrotic phenotype, respectively. The CK19 genewas included as a marker of immature hepatocytes. Alpha-SMA and MMP2 genes were included in both EMT and fibrosis marker panels. Total RNA was extracted from cells at 1, 7, and 14 days of the low cell-density cultures with the RNeasy Kit (Qiagen, Hilden, Germany). Then, 1 μg of RNA was retro-transcribed with the high capacity RNA-to-cDNA kit protocol (Applied Biosystems, Foster City, CA, USA). Gene expression analysis was donewith real-time PCR using SYBR® Green PCR Master Mix on StepOnePlus™ Real-Time PCR System (Applied Biosystems). Assayed gene and corresponding primers are listed in Table 1. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as housekeeping gene. Quantification of relative gene expression was done according to the comparative method. The value of $2^{-\Delta\Delta Ct}$ indicated the fold change in mRNA values for each gene relative to the control sample normalized to GAPDH. Hierarchical cluster analysis of gene expression was used to group expression patterns of similar samples. Cells with similar gene expression pattern were grouped together using the hierarchical clustering algorithm in the Gene Cluster 3.0 program. The heatmap was generated using the Java Tree View program.

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