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Genomic and functional characterization of stellate cells isolated from human cirrhotic livers

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***Background/Aims*: Hepatic stellate cells (HSCs) are believed to participate in liver fibrogenesis and portal hypertension. Knowledge on human HSCs is based on studies using HSCs isolated from normal livers. We investigated the phenotypic, genomic and functional characteristics of HSCs from human cirrhotic livers.**

***Methods*: HSC were obtained from normal and cirrhotic human livers. Cells were characterized by immunocytochemistry and gene microarray analysis. Cell proliferation, Ca^{2+} changes and cell contraction were assessed by 3H-thymidine incorporation and by using an epifluorescence microscope.**

***Results*: HSCs freshly isolated from human cirrhotic livers showed phenotypical features of myofibroblasts. These features were absent in HSCs freshly isolated from normal human livers and become prominent after prolonged culture. HSCs from cirrhotic human livers markedly express genes involved in fibrogensis, inflammation and apoptosis. HSCs from normal livers after prolonged culture preferntially expressed genes related to fibrogenesis and contractility. Agonists induced proliferation, Ca^{2+} increase and cell contraction in HSCs isolated from human cirrhotic livers. Response to agonists was more marked in culture-activated HSCs and was not observed in HSCs freshly isolated from normal livers.**

***Conclusions*: HSCs from human cirrhotic livers show fibrogenic and contractile features. However, the current model of HSCs activated in culture does not exactly reproduce the activated phenotype found in cirrhotic human livers.**

***Keywords*: Liver fibrosis; Cell activation; Collagen; Microarray; Portal hypertension**

1. Introduction

In chronic liver diseases, hepatic stellate cells (HSCs) acquire myofibroblastic properties including collagen synthesis and contractility [1], playing a role in the pathogenesis of liver fibrosis and portal hypertension [2]. This assumption is based on experimental studies, while evidence from human studies is scarce. Early studies showed that collagen-expressing activated HSCs accumulate in patients with diseased livers [3–5]. However, the pathogenic role of HSCs in chronic liver diseases is unclear. Functional characteristics of human HSC have been extensively studied using the model of activation in culture

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Abbreviations: Ang II, angiotensin II; AT1, angiotensin receptor type 1; BK_{Ca}, big potassium-dependent calcium channel; ECM, extracellular matrix; FBS, fetal bovine serum; HSCs, hepatic stellate cells; PDGF, platelet-derived growth factor; αSMA, smooth muscle alpha actin; TGF-β1, transforming growth factor β1; TIMP-1, tissue inhibitor of metalloproteinase type 1; PAI, plasminogen activator inhibitor type 1; MCAM, melanoma cell adhesion molecule.

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(i.e. HSCs isolated from normal human livers in prolonged culture on uncoated plastic dishes) [6,7]. The functional and genomic characteristics of myofibroblastic HSCs from damaged human livers have not been assessed.

Because of their high content in vitamin A, HSCs can be isolated from normal human livers using a discontinuous gradient [7,8]. In early culture, human HSCs show a quiescent phenotype that consists of a round cell shape and numerous vitamin A-rich fat droplets [9]. After prolonged culture on plastic, human HSCs acquire characteristics of myofibroblast-like cells [7,10]. Cell activation in culture involves: (1) increased cellular proliferation in response to agonists such as platelet-derived growth factor (PDGF) and angiotensin II (Ang II) [11–14]; (2) a dramatic increase in extracellular matrix (ECM) protein expression [3]; (3) expression of metalloproteinases [15]; (4) expression of cell adhesion molecules and secretion of pro-inflammatory cytokines [16–18] as well as features of antigen-presenting cells [19]; and (5) de novo expression of cytoskeletal proteins, receptors for vasoactive substances and membrane channels [7,20].

It has been proposed that activation of human HSCs in culture reproduces the phenotypical changes that occur in chronic liver diseases [21]. However, no studies have tested this hypothesis. It is conceivable that the degree of phenotypic activation of culture-activated human HSCs exceeds the actual cell activation that occurs in patients with chronic liver diseases. Here, we characterize the phenotypic, genomic and functional profile of HSCs freshly isolated from cirrhotic human livers. Moreover, we compare the features of HSCs freshly isolated from cirrhotic human livers, with those of HSCs isolated from normal livers in early culture and after prolonged culture on plastic.

2. Materials and methods

2.1. Isolation and culture of human HSCs

HSCs were isolated from human cirrhotic livers obtained from patients submitted to liver transplantation ($n=10$). Eight patients had hepatitis C virus-induced liver cirrhosis and two had alcohol-induced liver cirrhosis. Twenty-five grams of liver tissue were digested by two enzymatic solutions. First digestion was performed in Gey's Balanced Salt Solution (GBSS, Sigma Chemical Co., St Louis, MI) containing 0.33% pronase, 0.053% collagenase, and 0.003% DNase (Roche Diagnostics, Mannheim, Germany) for 45 min at 37 °C. Second digestion was performed in GBSS containing 0.08% pronase, 0.04% collagenase, and 0.003% DNase for 30 min at 37 °C. The resulting cell pellet was centrifuged over a gradient of Nycodenz 13% (Sigma). Cells obtained from the upper layer were seeded for 15 min in serum free medium to allow Kupffer cell attachment. To remove contaminating leucocytes, non-attached cells were recovered and purified using magnetic anti-CD45 beads (Dynal Biotech, Compiègne, France). Average yield per isolation was 2.5×10^5 cells/g liver. HSCs were also isolated from fragments of normal human livers obtained from resections of liver metastasis ($n=5$). Briefly, liver tissue was digested by two enzymatic solutions. First digestion was performed in GBSS containing 0.33% pronase, 0.035% collagenase, and 0.001% DNase for 30 min at 37 °C. Second digestion was performed in GBSS containing 0.06% pronase, 0.035% collagenase and 0.001% DNase for 30 min at 37 °C. The resulting cell pellet was centrifugated over a gradient of 10% Nycodenz. Average yield per isolation was 5×10^5 cells/g liver. HSCs isolated from both cirrhotic livers were studied at 24 h after isolation. HSCs from normal livers were studied at 24 h after isolation (quiescent phenotype) and after the second serial passage (culture-activated phenotype). In all cultures, no staining was found for CD45, factor VIII related-antigens, and Cam 5.2 (Dako, Glostrup, Denmark), indicating the absence of mono/macrophagic, endothelial, and epithelial cells. Cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM, BioWhittaker, Verviers, Belgium) containing 15% fetal bovine serum. The protocol was approved by the Investigational Review Board of the Hospital Clínic of Barcelona.

2.2. Cell proliferation assay

DNA synthesis was estimated by methyl-3H-thymidine (Amersham Biosciences, Buckinghamshire, UK) incorporation, as described in detail previously [22].

2.3. Immunocytochemistry studies

Cells were fixed in methanol at −20 °C for 10 min, blocked in PBS containing goad 2% FCS for 30 min, and incubated with primary antibodies anti-smooth muscle α-actin (αSMA) (1:100) (Dako), vimentin (1:200), fibronectin (1:100) (Sigma), nerve growth factor (1:100) (Santacruz), Synaptophysin (1:50) (Dako), neural cell adhesion molecule (1:100) (Sigma) for 1 h. Cells were incubated with fluorescent secondary antibody for 1 h.

2.4. Gene expression analysis

Total RNA was isolated from HSCs freshly isolated from cirrhotic livers, HSCs isolated from normal livers after prolonged culture and total normal human livers with Trizol (Life Technologies, Inc., Rockville, MD). RNA integrity and concentration was assessed with a microfluidic glass chip platform (Bioanalizer 2100, Agilent, Palo Alto, CA). Six micrograms of total RNA were used for microchip hybridizations. Preparation of cRNA probes, hybridization, and scanning of arrays were performed according to manufacturer's protocol (Affimetrix, Santa Clara, CA). CELL files generated by Affymetrix Microarray Suit Software were imported into the dChip Software to calculate the perfect match/mismatched difference model expression value [23]. Arrays were normalized against the median intensity array. Approximately 8400 genes included in HG-Focus array were analyzed. Genes with a coefficient of variation of less than 0.5 across all samples, and a P call of at least 30% were filtered, yielding 2142 genes that were considered for further analysis. To identify differentially expressed genes, a combined comparison of the different group of samples was performed. A fold change exceeding 2 and a *t*-test *P*-value less than 0.01 were considered significant. Unsupervised hierarchical clustering of the 740 resulting genes was performed. Gene Ontology annotations were used to assess enriched functional clusters ($P<0.001$ was considered significant) [24].

2.5. Quantitative polymerase chain reaction (PCR)

Pre-designed Assays-on-Demand TaqMan probes and primer pairs for collagen α1(I), transforming growth factor type 1 (TGF-β1), tissue inhibitor of metalloproteinase type 1 (TIMP-1), intercellular adhesion molecule type 1 (ICAM-1), lysyl oxidase, thrombospondin type 1, plasminogen activator inhibitor type 1 (PAI), and melanoma cell adhesion molecule (MCAM) were obtained from Applied Biosystems (Foster City, CA) (Table 1). Information on these Assay-on-Demand is available in: http://myscience.appliedbiosystems.com/cdsEntry/Form/gene_expression_keyword.jsp. TaqMan reactions were carried out in duplicate on an ABI PRISM 7900 machine (Applied Biosystems).