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The interplay between hepatic stellate cells and hepatocytes in an *in vitro* model of NASH

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ABSTRACT

Background & aim: A complex interplay exists between hepatocytes and hepatic stellate cells (HSC) in hepatic fibrogenesis. The activation of HSCs after liver injury leads to production of extracellular matrix (ECM). Co-culture models could be useful to mimic the liver microenvironment. This study evaluates the effect of free fatty acids (FFA) on HSC cells and the interplay with hepatocytes *via* both soluble-mediator and cell–cell contact.
Methods: The human hepatocyte cell line (HuH7) and HSC cells (LX2) were exposed to FFA for 24 h in 3 different experimental set-ups: (A) monoculture of HSC; (B) Transwell® system (effect of soluble mediators); and (C) Simultaneous Co-Culture (SCC) (cell-to-cell connections). Intracellular FFA accumulation was assessed qualitatively (microscopy) and quantitatively (flow cytometry); the activation of HSC (alpha smooth muscle actin, α-SMA) expression of ECM components were quantified by RT-PCR.
Results: FFA exposure induces intracellular fat accumulation in all the experimental set-up but the expression of α-SMA was significantly increased only in SCC. On the contrary, the expression of ECM was substantially decreased in the transwell® system.
Conclusions: The HSC activation is independent of FFA accumulation but requires cell-to-cell interaction with hepatocyte. On the contrary, the gene regulation of ECM components seems to occur through paracrine mediators.

1. Introduction

The global obesity epidemic has dramatically increased the prevalence of fatty liver disease. At present, 14–27% of the general population in the industrialized world has non-alcoholic fatty liver disease (NAFLD) (Weiss et al., 2014). The spectrum of NAFLD ranges from liver steatosis to steatohepatitis (Chalasani et al., 2012; Rosso et al., 2014), fibrosis and in some cases, cirrhosis and hepatocellular carcinoma.

Although the mechanisms of liver injury are not completely clear, there is a complex interplay among different hepatic cell types during hepatic fibrogenesis. During liver injury, the phenotype of HSCs changes as these cells become "activated". Perpetuation of the insult induce the progressive worsening of the hepatic damage with the expression of several genes leading to an enhanced cytokine release and to the production of extracellular matrix (ECM) remodeling components, contributing to liver regeneration failure (Blaner et al., 2009) with uncontrolled ECM turnover (Mormone et al., 2011). The ECM components include matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) among others. Gelatinase A (MMP2) is expressed in culture-activated HSCs (Arthur, 2000; Takahara et al., 1997), which is involved in HSC proliferation (Arthur, 2000). MMPs are in turn regulated by TIMPs (Mormone et al., 2011), where TIMP1 has been considered an important promoter

Abbreviations: HSC, hepatic stellate cells; ECM, extracellular matrix; FFA, free fatty acids; SCC, Simultaneous Co-Culture; NAFLD, non-alcoholic fatty liver disease; MMPs, matrix metalloproteinases; TIMPs, inhibitors of metalloproteinases; MMP2, gelatinase A; COL1, collagen-1; HSP-47, heat shock protein 47; FBS, fetal bovine serum; DMSO, dimethyl sulphoxide; PBS, phosphate-buffered saline; α-SMA, alpha smooth muscle actin; GFAP, glial fibrillary acidic protein.

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of liver fibrosis (Fowell et al., 2011) and an inhibitor of activated HSC-apoptosis (Murphy et al., 2002). Regulation of MMP–TIMP ratio is crucial for an efficient ECM remodeling (Mormone et al., 2011; Park et al., 2010). Collagen-1 (COL1) represents the most abundant form in the liver and is mainly produced by activated stellate cells. Moreover, it has been reported that the collagen-specific chaperone heat shock protein 47 (HSP-47) is required for normal collagen biosynthesis and that it is involved in liver fibrosis (Brown et al., 2005).

To date, studies on fibrosis development rely mostly on *in vivo* animal models, but they are restricted to some species and might have limited predictive value for human disease due to inter-species differences (Van de Bovenkamp et al., 2007). *In vitro* models have also contributed to the understanding of liver injury mechanisms and the main advantage is the possibility to use human cells in order to minimize species differences (Van de Bovenkamp et al., 2007). The culture of one cell type (monoculture) presents some limitations since the interaction with other cell types cannot be considered. Consequently, the monoculture model does not mimic the real multicellular interaction during the progression of the disease. Co-culture, using cell inserts (transwell®), allows the growth of two cell types into different surfaces sharing only the culture media. While, simultaneous co-culture is a system where two cell types are seeded together and allows the study of cell-to-cell interactions. This approach reproduces more accurately the cell connections observed in the *in vivo* microenvironment (Green et al., 2010). The development of more complex and sophisticated liver co-culture models, will reproduce more accurately the intercellular interactions (Van de Bovenkamp et al., 2007) and the biochemical response to fat accumulation (Gomez-Lechon et al., 2007).

The present study was designed to evaluate the cellular response of HSC to FFA, and the interplay with hepatocytes both in terms of soluble mediators and in terms of cellular connections.

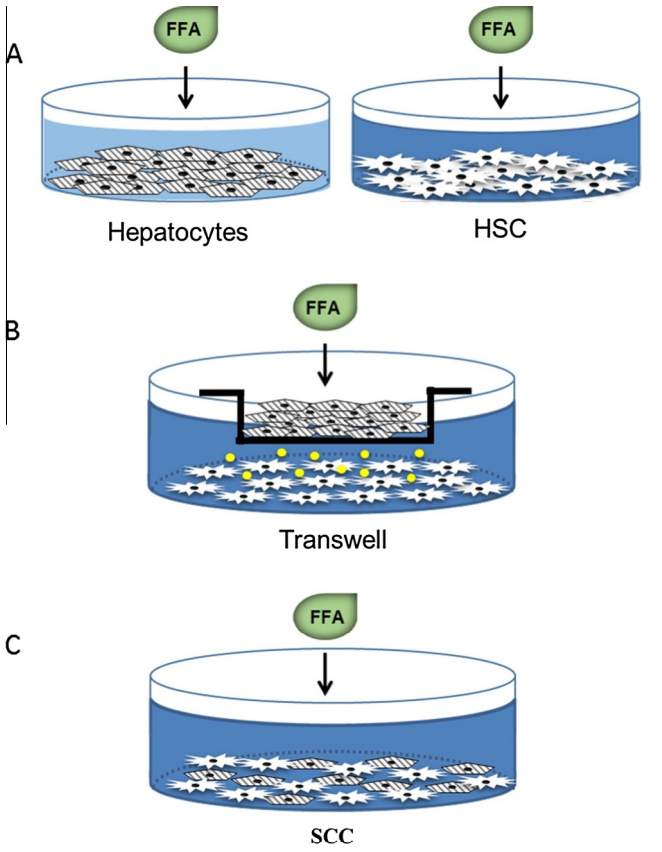

Fig. 1. Experimental set-ups designed for the *in vitro* culture systems. (A) Monoculture, HSC or HuH7. (B) Transwell® system. (C) Simultaneous Co-culture System (SCC).

2. Materials and methods

2.1. Human cell lines & culture media

The human hepatoma cell line HuH7 (JHSRRB, Cat#JCRB0403) was obtained from the Health Science Research Resources Bank (Osaka, Japan). HSC cell line (LX-2 cells) was kindly provided by S.L. Friedman (Mount Sinai School of Medicine, New York, NY). Cells were grown in DMEM high glucose medium (ECB7501L-50, Euroclon, Italy) supplemented with 10% v/v (HuH7) (Chavez-Tapia et al., 2012) and 1% v/v (LX-2) (Xu et al., 2005) of fetal bovine serum (FBS) (ECS0180L, Euroclon, Italy), 2 mM L-Glutamine (ECB3000D, Euroclon, Italy) and 10,000 U/mL penicillin and 10 mg/mL streptomycine (ECB3001D, Euroclon, Italy). Simultaneous co-culture experiments were performed in 1% v/v FBS medium, in order to avoid LX-2 auto activation. HuH7 cells were adapted to grow in low FBS media 1 week before the experiments (Giraudi et al., 2014).

2.2. Cell culture & FFA treatment

For the FFA treatment, 25×10^3 cells/cm^2 were seeded into 6 wells plates, and were allowed to attach overnight. The following day, cells were exposed for 24 h to a mixture of free fatty acids, oleic acid (C18:1) (O1008, Sigma Chemical USA) and palmitic acid (C16:0) (P0500, Sigma Chemical USA), at 1200 μM in molar ratio 1:2 palmitic:oleic respectively (Fig. 1A). The solution of medium containing FFA was prepared as described previously (Chavez-Tapia et al., 2012) but using DMEM/high glucose media supplemented with 1% of FBS. Control cells were treated with the equivalent concentration v/v of the vehicle (DMSO) (D2438 Sigma Chemical USA).

2.2.1. Transwell® system

Cells were cultured using hanging cell culture inserts (3 μm pore size, Millipore) to separate both cell populations. Wells (Multiwell 6 well, Becton Dickinson) and inserts with growth media were let to stabilized for 24 h at 37 °C as manufacturer's indications. HuH7 cells were seeded in the insert (75×10^3 - cells/cm^2) and let to attach overnight. The following day, the insert was placed in a 9.6 cm^2 well with 15×10^3 cells/cm^2 of LX-2 cells and exposed HuH7 cells to 1200 μM FFA. In this system, HuH7 were placed on the top to make a gravity gradient of the released mediators (Fig. 1B). HuH7 and LX-2 were seeding in a ratio of 5:1 respectively; since it is representative of the ratio of parenchymal/non-parenchymal cells in liver (Nieto and Cederbaum, 2003). Cells and supernatants were harvested and recovered for further analysis.

2.2.2. Simultaneous co-culture system

A pool of HuH7 and LX-2 were seeded in the same plate considering a ratio of 5:1 (25 and 5×10^3 cells/cm^2) respectively. After 24 h, cells were washed with PBS and exposed to FFA for 24 h, as described previously (Fig. 1C). Cells and supernatants were harvested and recovered for further analysis.

2.3. Qualitative analysis of intracellular FFA – Nile Red staining

Intracellular lipid droplets were determined by fluorescence microscopy based on Nile Red (N3013, Sigma Chemical USA)