Experimental and Molecular Pathology 98 (2015) 85–92

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

The importance of the interaction between hepatocyte and hepatic stellate cells in fibrogenesis induced by fatty accumulation

Pablo J. Giraudi [a], Varenka J. Barbero Becerra [a,b], Veronica Marin [a], Norberto C. Chavez-Tapia [a,b], Claudio Tiribelli [a,c], Natalia Rosso [a,*]

[a] Fondazione Italiana Fegato, Centro Studi Fegato, Area Science Park Basovizza Bldg. Q SS14 Km 163.5, 34149 Trieste, Italy
[b] Liver Research Unit, Médica Sur Clinic & Foundation, Puente de Piedra 150, Col. Toriello Guerra, Tlalpan, C.P. 14050 Mexico City, Mexico
[c] Department of Medical Sciences, University of Trieste, 34100 Trieste, Italy

ARTICLE INFO

Article history:
Received 27 November 2014
Accepted 18 December 2014
Available online 20 December 2014

Keywords:
Hepatocytes
Hepatic stellate cells
Steatosis
Extracellular matrix turnover
Fibrogenesis

ABSTRACT

Background & aims: Non-alcoholic fatty liver disease is characterized by an initial accumulation of triglycerides that can progress to non-alcoholic steatohepatitis, which can ultimately evolve to cirrhosis and hepatocellular carcinoma. Hepatic stellate cells play a key role in liver fibrogenesis by an increased activation and an altered profile of genes involved in the turnover of extracellular matrix components. To reproduce *in-vitro* the functional cell connections observed *in vivo* it is essential to consider cell-to-cell proximity and interaction. The aim of this study was to determine the response to free fatty acids in a simultaneous co-culture model of hepatocytes and hepatic stellate cells.
Methods: Simultaneous co-culture model and monoculture of each cell type (control) were exposed to FFA for 24 up to 144 h. Quantification of steatosis; stellate cell activation; assessment of fibrogenic response; expression and activity of metalloproteinases as well as collagen biosynthesis were evaluated.
Results: Free fatty acids induced comparable steatosis in simultaneous co-culture and monoculture. However, the activation of the stellate cells assessed by alpha-smooth muscle actin expression is greater when cells were in close contact. Furthermore, a time-dependent increment of tissue inhibitor metalloproteinase-2 protein was observed, which was inversely correlated with protein expression and activity of matrix-metalloproteinases, suggesting enhanced collagen biosynthesis. This behavior was absent in cell monoculture.
Conclusions: These data indicate that cell-to-cell proximity between hepatocytes and stellate cells is necessary for the initiation of the fibrotic process.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is considered as the hepatic manifestation of the metabolic syndrome, and is one of the most common liver malignancy worldwide (Adams et al., 2005; Wong, 2013). The disease begins with an aberrant accumulation of triglycerides (TG) in the liver. It is still unclear if the accumulation of neutral cholesterol esters and TG represents a real threat, but instead intermediate products such as diacylglycerol and phospholipids would be responsible for the toxic effect on liver cells. Histologically, NASH is characterized by hepatocellular ballooning, lobular inflammation and collagen deposition (fibrosis) (Kleiner et al., 2005). Current evidence indicates that the activation of hepatic stellate cells (HSCs) plays a key role in the initiation of liver fibrosis (Reeves et al., 1996; Friedman, 2008). Under normal conditions, HSCs present a quiescent phenotype, storing vitamin A in lipid droplets. Following liver injury, and in response to damage stimuli, these cells become active and acquire a myofibroblast-like morphology. Intracellular vitamin A storage is lost and expression of smooth muscle alpha-actin (alpha-SMA) increased.

Recently our group (Chavez-Tapia et al., 2012) demonstrated that fat-laden hepatocytes have a clue role in the initiation of liver fibrosis. It has been reported that injured hepatocytes release pro-inflammatory and pro-fibrotic cytokines such as interleukin-8 (IL-8), tumor necrosis alpha (TNF-α) (Chávez-Tapia et al., 2013), transforming growth factor beta 1 (TGF-beta1), connective tissue growth factor (CTGF) and platelet-derived growth factor (PDGF). Furthermore, the increased of oxygen reactive species and apoptotic hepatocytes enhance the process of HSC activation (Nieto et al., 2002; Canbay et al., 2003; Gressner and Gressner, 2008; Borkham-Kamphorst et al., 2008; Bissell

Abbreviations: NAFLD, non-alcoholic fatty liver disease; TG, triglycerides; NASH, non-alcoholic steatohepatitis; HSCs, hepatic stellate cells; alpha-SMA, smooth muscle alpha-actin; IL-8, interleukin-8; TNF-alpha, tumor necrosis alpha; TGF-beta1, transforming growth factor beta 1; CTGF, connective tissue growth factor; PDGF, platelet-derived growth factor; ECM, extracellular matrix; TIMPs, tissue inhibitors of metalloproteinases; MMPs, matrix metalloproteinases; Col1A1, collagen type I, alpha I; HSP47, heat shock protein 47; FFAs, free fatty acids; SCC, simultaneous co-culture; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum.

* Corresponding author at: Fondazione Italiana Fegato — ONLUS, Area Science Park, Basovizza, SS14 Km 163.5, 34149 Trieste, Italy.
E-mail address: natalia.rosso@csf.units.it (N. Rosso).

http://dx.doi.org/10.1016/j.yexmp.2014.12.006
0014-4800/

et al., 2001). Once activated, HSCs increase the proliferative rate and present a dysregulation of gene expression profile particularly those involved in extracellular matrix (ECM) turnover, such as tissue inhibitors of metalloproteinases (TIMPs), matrix metalloproteinases (MMPs), collagen type I, alpha I (Col1A1) and heat shock protein 47 (HSP47) (Tsukada et al., 2006).

Data obtained in *in vitro* models have contributed to understanding the molecular mechanisms and signaling cascades involved in many liver diseases; however only few data has been obtained in NAFLD (Chavez-Tapia et al., 2011). The main limitation in the use of *in vitro* systems is the high heterogeneity among the experimental conditions used among the research groups. Cell models include the use of primary cultures of hepatocytes, non-parenchymal cells (Gómez-Lechón et al., 2004) or established hepatic cell lines. More complex approaches widely used are also the co-cultures (Wang et al., 2006; Krause et al., 2009), liver slices (Rius et al., 2014) and/or perfused liver models (Janorkar et al., 2011). It has been reported that cell-to-cell vicinity and interaction are essential to retain *in-vitro* the physical and functional cell connections observed *in vivo* (Rojkind et al., 1995; Fontana et al., 1997; Thomas et al., 2005). This point is generally disregarded. To our knowledge, there is no evidence about the response to exposure to free fatty acids (FFAs) of hepatocytes and HSCs.

In the present study, we explored the cellular response to FFAs in terms of HSC activation and ECM remodeling in the initiation of the fibrotic process by using a simultaneous co-culture (SCC) model of hepatocytes and HSCs where both cell types are in close contact.

2. Materials and methods

2.1. Chemicals

Cell culture medium Dulbecco's modified Eagle's high glucose medium (DMEM) (ECB7501L-50), L-glutamine (ECB3000D), penicillin/streptomycin (ECB3001D), fetal bovine serum (ECS0180L) and EuroGOLD RNAPure were purchased from Euro-clone (Milan, Italy). Bicinchoninic acid solution-kid (BCA) (B9643); bovine albumin Cohn V fraction (A4503); dimethyl sulfoxide (DMSO) (D2438); gelatin type A (G1890); Nile Red (N3013); oleic acid (C18:1) (O1008), palmitic acid (C16:0) (P0500); paraformaldehyde (P6148), phosphate-buffered saline (PBS) (D5652); picric acid (197378) and Direct Red 80 (365548) were obtained from Sigma Chemical (St. Louis, MO, USA). Reagents for cDNA synthesis and iQ SYBR Green Supermix were from Bio-Rad Laboratories (Hercules, CA, USA). Cell lysis buffer #9803 was purchased from Cell Signaling Technology.

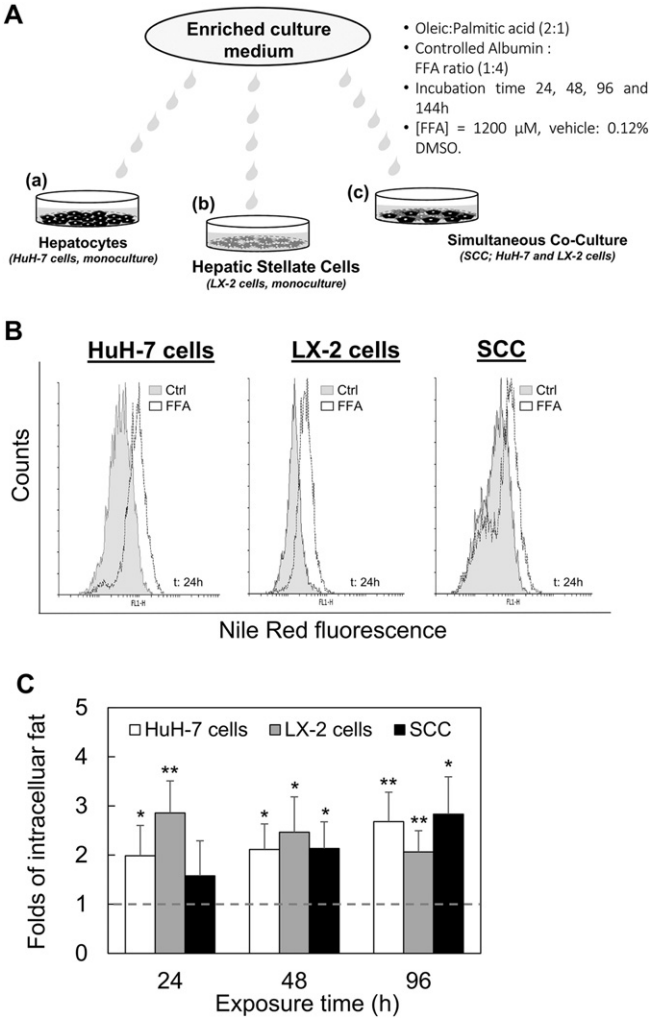

Fig. 1. Intracellular lipid accumulation. (A) Experimental set-up used for the different *in vitro* systems in the study. (B) Representative flow cytometry fluorescence histograms of lipid droplets stained with Nile Red. (C) Kinetics of intracellular lipid content quantified by flow cytometry of Nile Red staining. Data represent mean ± SD of three independent experiments and expressed as folds of relative fluorescence *vs.* control. *$p < 0.05$; **$p < 0.01$ *vs.* vehicle-treated control (—).

2.2. Cell lines and co-culture experiments

The human hepatoma cell line HuH-7 (JHSRRB, Cat#JCRB0403) was obtained from the Health Science Research Resources Bank (Osaka, Japan). HSC cell line LX-2 cells were kindly provided by S.L. Friedman (Mount Sinai School of Medicine, New York, NY). HuH-7 cells were grown as previously described (Chavez-Tapia et al., 2012). LX-2 cells were cultured under the same conditions but using 1% v/v of fetal bovine serum as described (Xu et al., 2005).

2.3. FFA treatment

The effect induced by exposure to 1200 µM FFA (oleic:palmitic molar ratio 2:1) was analyzed at 24, 48, 96 and 144 h on hepatocytes (HuH-7), HSC (LX-2) and in SCC (Fig. 1A). The FFA concentration (1200 µM) was selected based on our previously published study (Chavez-Tapia et al., 2012), since this dose is able to reproduce in hepatocytes the events involved in the progression of NAFLD without compromising the cell viability. The solution of medium containing FFA was prepared as described previously (Chavez-Tapia et al., 2012) and was sonicated briefly in order to facilitate the FFA solubility. The maximal concentration of vehicle (DMSO) was 0.12% v/v. All treatments were carried out using DMEM/high glucose media supplemented with 1% of FBS. For SCC, HuH-7 cells were adapted to grow in 1% FBS medium, by reducing gradually the medium serum concentration during the two weeks before each experiment. Cell density in monocultures was 30,000 cells/cm^2, and in SCC the ratio between hepatocytes:HSC was 5:1 (25,000 cells/cm^2 and 5000 cells/cm^2, respectively) (Bhatia et al., 1999).

2.4. Determination of intracellular fat content using Nile Red staining

Intracellular lipid droplets were determined fluorometrically based on Nile Red staining as described previously (Chávez-Tapia et al., 2013). Briefly, cells were detached by trypsinization and washed with PBS. After centrifugation, cells were resuspended in PBS and stained with Nile Red dye (1 µg/mL). Nile Red intracellular fluorescence was determined by flow cytometry using a Becton Dickinson FACSCalibur System on the FL2 emission channel. Fluorescence was measured in 10,000 events and analyzed using Flowing Software 2.5.1 (University of Turku, Finland).