Effect of Steatosis and Cytokine Exposure on Hepatocyte-Derived Reporter Cells in 3-Dimensional (3-D) Culture Conditions

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Statement of Purpose: Initial stage of Non Alcoholic Fatty Liver Disease (NAFLD) is characterized by a fatty liver, referred to as steatosis, which may progress to liver failure [1]. The relationships amongst metabolic state (intracellular triglyceride), environmental stress (cytokine and oxidative stress), transcriptional regulation, and cell fate (survival, necrosis or apoptosis) represent important steps in understanding the progression from steatosis to steatohepatitis. Previous studies used primary hepatocytes or hepatoma cell lines in 2-dimensional (2-D) monolayer *in vitro* cultures to elucidate such mechanisms [2]. Unfortunately, the 2-D culture systems do not represent the complex architecture of hepatic tissue *in vivo*. Therefore, we set out to investigate the effect of steatosis and cytokine exposure on hepatocyte-derived cells in 3-D culture conditions. These studies are expected to facilitate the creation of 3-D *in vitro* liver tissue models.

Methods: *Cell Culture*. H35 rat hepatoma reporter cells, constructed by transfecting with plasmid DNA encoding a GFP reporter protein under the transcriptional control of the nuclear factor κB (NFκB) response element, were seeded at $5x10^4$ cells per well in 24-well plate, and were cultured in phenol red free DMEM with 10% fetal bovine serum containing no fat (control), 2 mM oleic acid (OA), or 2 mM linoleic acid (LA) for 72 h. Cells were also exposed to 10 ng/ml TNF-α, an inflammatory cytokine, for the last 24 h and were continuously observed under a fluorescence microscope for NFκB activation kinetics.

3-D Culture Conditions. When plated onto an elastin-like polypeptide-polyethyleneimine (ELP-PEI) coated surface, H35 cells formed cellular aggregates within 48 h. The aggregates subsequently formed 3-D spheroids with diameter of about 100 µm within 72 h. These results are in agreement with our previous studies showing formation of primary hepatocyte spheroids on ELP-PEI surface [3].

Intracellular Reactive Oxygen Species (ROS). 2',7'-dichlorodihydrofluorescein diacetate, H_2DCFDA, was used as a cell-permeant indicator for ROS. The H35 cells were treated with 10 µM of H_2DCFDA by incubating in PBS for 30 min at room temperature. Subsequently, the medium was changed to the medium with no fat (control), 2mM OA or 2mM LA and the cells were continuously observed under a fluorescence microscope for up to 72 h. All experiments were performed at least in triplicate. Results reported as mean ± 95% confidence intervals.

Results: Fig. 1 shows that the treatment of 2mM OA and 2mM LA activated the NFκB transcription factor initially at the earlier time points, with the maximum activation occurring at around 10 h. The level of activation was independent of the type of free fatty acid (FFA) used. The NFκB activation returned to baseline around 48 h. H35 reporter cells were then stimulated with TNF-α for 24 h. An activation of NFκB is shown by an increase in the GFP fluorescence, in a time-dependent manner [4]. This TNF-mediated NFκB activation was partially attenuated for the cells treated with 2mM OA and 2mM LA.

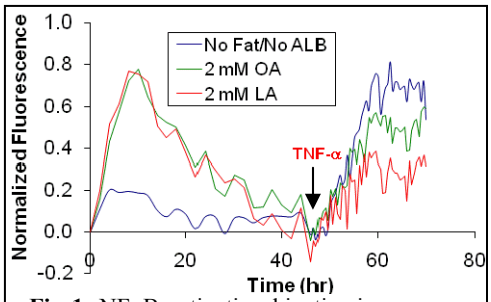

Fig 1. NFκB activation kinetics in response to TNF-α in 3-D culture of H35 cells

NFκB activation or deactivation is attributed to the intracellular ROS generated due to FFA exposure [5]. We, therefore, continuously monitored the ROS production using fluorescence microscopy. Fig. 2 shows that the ROS significantly increased during the FFA treatment at initial time points with the maximum ROS level at around 10 h, which remained constant up to 48 h and subsequently increased when the cells were stimulated with TNF-α.

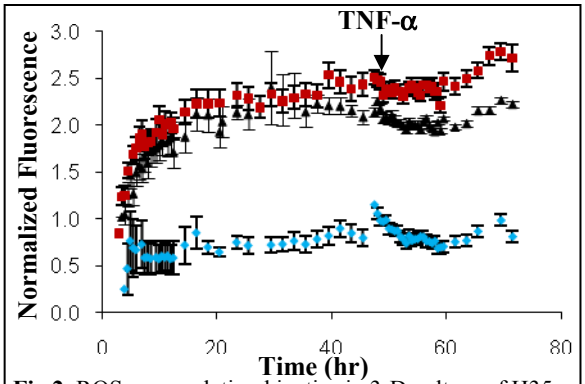

Fig 2. ROS accumulation kinetics in 3-D culture of H35 cells (♦ : Control; ▲ : 2 mM OA; ■ : 2 mM LA)

Conclusions: We showed that FFA treatment activated NFκB initially; however, partially attenuated the TNF-mediated NFκB activation at later time points. This NFκB activation/deactivation kinetics correlated with the ROS level. NFκB response to inflammatory mediators is well established. However, its role in integrating metabolic and inflammatory stimuli observed during NAFLD is unclear. These studies therefore provide a good starting point to evaluate such relationships in a 3-D *in vitro* cell culture.

References: 1. Angulo P. N Eng J Med 2002;346:1221-1231.
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