Three-Dimensional Tissue Constructs to Facilitate the Study of Liver Diseases

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Statement of Purpose: Non Alcoholic Fatty Liver Disease (NAFLD) is a family of liver diseases associated with obesity. Initial stage of NAFLD is characterized by a fatty liver, referred to as steatosis, which progresses in some individuals to nonalcoholic steatohepatitis (NASH) and liver failure [1]. Understanding the relationships amongst metabolic state (intracellular triglyceride), environmental stress (cytokine and oxidative stress), transcriptional regulation, and state-dependent stress responses (survival, necrosis or apoptosis) represent important steps in understanding the progression from steatosis to steatohepatitis. Several studies in the past have attempted to elucidate these mechanisms using primary hepatocytes or relevant hepatoma cell lines in 2-dimensional (2-D) monolayer *in vitro* cultures [2]. These 2-D planar culture systems, unfortunately, do not represent the complex architecture of hepatic tissue *in vivo*. Therefore, we set out to design new biopolymer conjugates that will facilitate the creation of 3-D *in vitro* liver tissue models. To this end, we have engineered elastin-like polypeptide (ELP)-polyelectrolyte (PE) polymers and investigated their utility as suitable substrates for representative H35 rat hepatoma cells. ELPs are a family of polypeptides derived from a portion of the primary sequence of mammalian elastin, VPGXG, where V = valine, P = proline, G = glycine, and X = any amino acid except proline [3]. ELPs and their derivatives have been used for a number of applications, including drug delivery, protein purification, and tissue engineering.

Methods: *Expression and Purification of ELP.* The synthetic gene for $(VPGVG)_{40}$ was synthesized via recursive directional ligation using standard recombinant techniques. The ELP was produced in shaker flask culture using *E. coli* BLR(DE3) at 37°C for 24 h. The cells were subsequently lysed by sonic disruption and cell debris were separated by centrifugation. The ELP was purified by an inverse transition temperature purification method and had purity of >95% by SDS-PAGE.

Chemical Conjugation of ELP to PE. ELP was conjugated to polyethylene imine (PEI) using carbodiimide chemistry. The ELP-PEI was adsorbed to 24-well TCPS plate by placing 200 µl of a 5 mg/ml solution in PBS in each well. The plate was incubated at 37°C for 48 h and the remaining solution was aspirated.

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 $1x10^5$ cells per well in 24-well plate, and were maintained in phenol red free DMEM with 10% fetal bovine serum. Cells were exposed to 10 ng/ml TNF-α, a prototypic inflammatory cytokine, for 24 h and were continuously observed under an optical and fluorescence microscope for NFκB activation kinetics.

Results: H35 rat hepatoma cells seeded on these surfaces exhibited dramatically different morphologies (Fig. 1). On TCPS and ELP surfaces, H35 cells spread and formed a confluent monolayer within 48 h. The morphology observed on these surfaces indicates dominant adhesive cell-surface interactions, consistent with previous work examining adherence of liver cell lines to neutral surfaces.

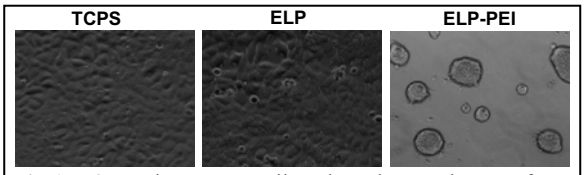

Fig 1. H35 rat hepatoma cells cultured on various surfaces

By contrast, when plated onto a 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 [4].

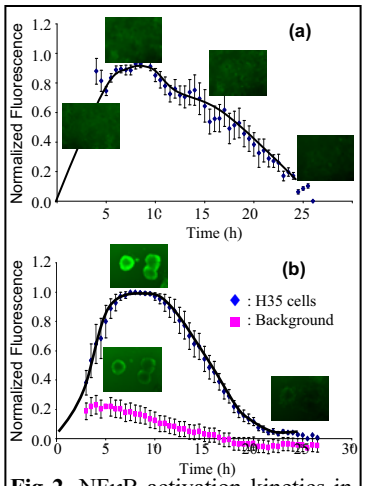

Fig 2. NFκB activation kinetics in response to TNF-α in (a) 2-D and (b) 3-D culture of H35 cells.

H35 reporter cells were then stimulated with TNF-α for 24 h. Fluorescence microscopy was used to observe the cellular GFP fluorescence. An activation of NFκB is demonstrated by an increase in the GFP fluorescence, in time-dependent manner [5]. Fig. 2 shows that the TNF-mediated NFκB activation followed a similar kinetics in both 2-D and 3-D H35 cell cultures.

Conclusions: We have shown that ELP is a suitable for H35 cell culture and its chemical modification with PEI profoundly influenced cellular morphology. Further, TNF-α activated NFκB similarly in both 2-D and 3-D culture settings. NFκB is a transcription factor with well established role in response to inflammatory mediators. However, its role in integrating metabolic and inflammatory stimuli is unclear. These studies therefore provide a good starting point to evaluate such relationships observed during NAFLD in a 3-D *in vitro* cell culture.

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