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Tissue specific synthetic ECM hydrogels for 3-D *in vitro* maintenance of hepatocyte function

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ABSTRACT

Despite recent advances in biomaterial science, there is yet no culture system that supports long-term culture expansion of human adult hepatocytes, while preserving continued function. Previous studies suggested that acellular liver extracellular matrix (ECM), employed as a substrate, improved proliferation and function of liver cells. Here we investigated whether extracts prepared from acellular liver ECM (liver ECM extract, LEE), or from whole (fresh) liver tissue (liver tissue extract, LTE), could be combined with collagen Type I, hyaluronic acid (HA), or heparin-conjugated HA (HP) hydrogels to enhance survival and functional output of primary human hepatocytes. The liver-specific semi-synthetic ECMs (sECMs) were prepared by incorporating LEE or LTE into the gel matrices. Subsequently, primary human hepatocytes were maintained in sandwich-style hydrogel cultures for 4 weeks. Progressive increase in hepatocyte metabolism was observed in all HA and HP groups. Hepatocytes cultured in HA and HP hydrogels containing LEE or LTE synthesized and secreted steady levels of albumin and urea and sustained cytochrome p450-dependent drug metabolism of ethoxycoumarin. Collectively, these results indicate that customized HA hydrogels with liver-specific ECM components may be an efficient method for expansion human hepatocytes *in vitro* for cell therapy and drug and toxicology screening purposes.

1. Introduction

There is a critical need for improved model systems to predict efficacy, bioavailability, and toxicology outcomes for candidate drugs. Currently, *in vivo* animal models serve as gold standards for testing prior to clinical trials, but the drawbacks associated with such models are major contributors to the costs and uncertainties in therapy development. In vitro systems that use human tissues would be preferable [1]; however, for these systems to serve as tools that reflect human biology, methods to expand human cells in culture, while preserving their functions, are key for future use in pharmacokinetic and toxicity testing. To that end, we have developed a 3-D culture system for preparing and maintaining human hepatocyte tissue constructs with potential to serve as effective drug and toxicology screening tools.

In vitro cultured primary hepatocytes are increasingly being used for screening in the pharmaceutical industry [2]. However, there is still a need for an optimal culture system that improves the long-term maintenance of liver cells with retention of liver function for *in vitro* drug screening. In recent years numerous biomaterials have been employed for *in vitro* hepatocyte culture [3]. They can be divided into two broad types: simpler non-surface modified biomaterials and more complex surface modified biomaterials. The non-surface modified scaffolds, for example, adding galactosylated hyaluronic acid to chitosan scaffolds [4] and the galactosylation of nanofibrous chitosan scaffolds [5] improved albumin and urea production and cytochrome p450 activity in primary rat hepatocytes in 15 and 7 day cultures, respectively. The surface modified biomaterials incorporate amendment of the primary scaffold components with bioactive molecules that support control over growth factor release, such as heparin or cell attachment, such as fibronectin or RGD peptides. For instance, polyethylene glycol (PEG)-heparin hydrogels supported consistent albumin and urea levels in rat hepatocytes for 3 weeks [6]. Similarly, micropatterned PEG-fibrinogen gels supported albumin and urea levels for 10 day, but only with crucial supplements such as fibronectin, epidermal growth factor (EGF), transforming growth factor- α (TGF-α), and hepatocyte growth factor (HGF) [7]. Poly(methylmethacrylate) chips containing microwells coated with RGD peptides were used to culture rat hepatocyte spheroids for 12 days. Multiple Cytochrome p450 assays were performed in which activity levels were greater in the chip wells than on collagen-coated dishes [8]. Rat

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hepatocytes were also maintained for 7 days on collagen-coated nanofibrous poly(L-lactic acid) scaffolds. Viability remained high, cells displayed good glycogen storage capability, and consistent albumin production, as well as several other markers. Cytochrome activity was inducible, but not necessarily greater than monolayer controls [9].

While undoubtedly useful, the studies discussed above only employ rat hepatocytes. Primary human hepatocytes are desirable, but are difficult to obtain and maintain in culture. Hepatoma cell lines, such as HEPG2, are often used as alternatives to primary hepatocytes due to their low cost and ease of culture. Unfortunately, the key drug metabolism enzymes necessary for drug screening are often absent or expressed only in low levels in HEPG2 cells [10]. Hepatocytes, on the other hand, are fragile when removed from liver tissue and cultured on plastic *in vitro*. The cells quickly lose liver-specific functions and tend to apoptosis due to lack of appropriate environmental support. However, our previous study demonstrated that decellularized liver ECM used as a substrate improved expansion and function of human primary hepatocytes over a 3 week time course [11]. Additionally, in recent years use of tissue-derived ECMs from different organs has been widely explored for culture of other cell types, including the concept of solubilizing ECM and reconstituting it at a later time. ECMs derived from porcine urinary bladder matrix were solubilized in pepsin, and subsequently self-assemble into a gel at physiological conditions, supporting expansion of rat aortic arch smooth muscle cells [12]. Moreover, alginate microspheres containing urinary bladder matrix and Sertoli cells that were implanted into nonobese diabetic mice resulted in diabetes prevention and reversion [13]. The beneficial effects of ECM-based supplements are likely a result of the ECM being a source of many specific factors including, but not limited to, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), EGF, transforming growth factor- β (TGF-β), keratinocyte growth factor (KGF), HGF, and platelet-derived growth factor (PDGF) [14]. The structural components in the ECM, such as collagens, glycosaminoglycans, fibronectins, laminins, and elastin, might improve bioactivity of materials through mechanical and biochemical cues. By incorporating liver-specific materials into hydrogels, we aimed to improve the *in vitro* viability, and support the long-term function of primary human hepatocytes. Our hypothesis in this study is that HA-heparin biomaterials can combine with growth factors and ECM components existing in liver tissue or liver ECM extracts so that these factors be slowly slow released, enhancing maintenance of functional liver cells. For the structural foundation of our constructs we chose Type I rat tail collagen as a control, as it has been used as a standard for cell culture, and an HA-based hydrogel, since HA-derivatives have been extensively implemented as a cell carrier for regenerative medicine applications in recent years [15]. Examples include wound healing of corneal lacerations [16], embryonic stem cell expansion [17], soft tissue engineering *in vitro* and *in vivo* [18–21], tumor xenograft models [22–24], and bioprinting of cellularized tubular structures [25,26]. The HA variety of semi-synthetic ECMs (sECMs) are commercially available as Extracel™ and HyStem™ for research use, and are in clinical use for veterinary medicine by Sentrx Surgical. Products for clinical use in humans are undergoing FDA review. Herein, we fabricated sECMs containing extracts from fresh or acellular liver tissues and investigated their support for survival, expansion, and function of primary human hepatocytes.

2. Materials and methods

2.1. Decellularization of liver tissues

Fresh porcine livers (Disher Packing, Inc., Yadkinville, NC) were pre-rinsed through the hepatic portal vein with chilled Dulbecco's phosphate buffered saline (DPBS). The liver was cut into 7.6 cm by 10.2 cm blocks and flash frozen at −80C. Frozen liver blocks were sectioned into 3 mm slices. Slices (6–10) were transferred to 500 ml distilled water and shook on a rotary shaker at 200 rpm for 3 days at 4 °C, during which water was changed three times per day. The liver slices were treated with 2% Triton X-100 for 4 days followed by 2% TX-100 + 0.1% NH_4OH for 24 h. During the TX-100 rinses, solutions were changed twice daily. The decellularized liver tissues were washed for 2 additional days in distilled water to remove any traces of TX-100, after which they were stored at 4 °C until further use.

2.2. Preparation of tissue and ECM digest

Fresh liver tissues and decellularized liver ECMs were lyophilized for 48 h. Following lyopholization, samples were ground into a powder with a freezer mill. One gram of liver tissue or liver ECM powder was mixed with 100 mg Pepsin (Porcine gastric mucosa, 3400 units of protein, Fisher Scientific, Fair Lawn, NJ) and sterilized by gamma irradiation (1 Mrad). All subsequent procedures following sterilization were carried out under sterile conditions. Hydrochloric acid (0.1 N, 100 μL) was added to the sterilized materials and incubated for 48 h at room temperature. The resulting mixture was transferred to a 50 ml conical tube and centrifuged at 3000 rpm for 15 min. The supernatant was removed and the pellet was discarded. This was repeated 3 times until the supernatant was clear. To ensure there was no more particulate matter remaining, the suspension was filtered through a 0.2 μm syringe filter (Fisher Scientific). The resulting liver decellularized ECM extract (LEE) and liver tissue extracts (LTE) were stored at −80 °C until further use.

2.3. Collagen and hyaluronic acid liver sECM and liver tissue gel preparation

Type I rat tail collagen (BD Biosciences, Bedford, MA) was diluted to 1 mg/mL in PBS and adjusted to pH 7.0 by 1 M NaOH. LEE and LTE solutions (1 mg/ml) were also adjusted to pH 7.0 by 1 M NaOH. Collagen only gels (COL) were formed by mixing the prepared collagen solution with William's E Media (Invitrogen, Carlsbad, CA) 1:1 by volume. Collagen with LEE or LTE gels (COL + EG and COL + TG, respectively) were formed by mixing the collagen solution with LEE or LTE 1:1 by volume. Gels were allowed to crosslink for 1 h before use.

For HA and HP gels, Extracel and Extracel-HP (Glycosan Biosystems, Alameda, CA) components were dissolved in sterile water. Briefly, Glycosil (for HA gels), Heprasil (for HP gels), and Gelin-S were dissolved in water to make 2% w/v solutions. Extralink, the crosslinker, was dissolved in water to make a 4% w/v solution. For HA-only and HP-only gels, Glycosil (or Heprasil), Gelin-S, and Extralink were mixed 2:2:1 by volume. The resulting solution was mixed 1:1 with water and vortexed. For gels containing LEE or LTE (HA + EG, HA + TG, HP + EG, and HP + TG), LEE or LTE were substituted for the water in final mixing step. Solutions were allowed to crosslink for 30 min before use. Formulation of liver-specific collagen and hyaluronic acid gels are displayed in Table 1.

2.4. Extract extracellular matrix component and growth factor analysis

LEE and LTE solutions (1 mg/mL) were prepared as described above were analyzed for collagen, elastin and glycosaminoglycan (GAG) content. To measure each component, 25 mg of starting material was employed ($n = 3$). Following additional chemical digestion with HCL (collagen assay), papain (GAG assay) and oxalic acid (elastin assay), samples were analyzed with the Blyscan Collagen, GAG, and Elastin assay kits (Biocolor Life Sciences Assays, Carrickfergus, UK) according to the manufacturer's instructions in order to quantify the amount of each component present.

Additionally, LEE and LTE solutions were analyzed for growth factor content using a Quantibody® Human Growth Factor Array (RayBiotech, Norcross, GA). LEE and LTE solutions (1 mL aliquots) were prepared as described above for analysis.

2.5. Primary human hepatocyte culture on liver-specific sECMs

Primary human hepatocytes were extracted from the liver of a Caucasian, 50yr old male (Invitrogen). Cells were suspended in 25 ml of William's E Medium solution and counted on the hemocytometer with Trypan blue to assess viability (85%). Substrate groups (50 μL in 96-well plates) were prepared as described above and 60,000 cells were seeded on top of each substrate and incubated for 3 h (37 °C, 5% CO_2). Following incubation substrates were washed 3 times with warm DPBS to remove any dead or unattached cells. An additional layer of the corresponding gel solutions were added to each well and allowed to crosslink for 1 h to create sandwich culture tissue constructs. After the gels were sufficiently crosslinked, 100 μl of fresh William's E Medium or conditioned TS or ES media was added to the appropriate wells. The hepatocytes were maintained in culture for 4 weeks, with media changes every 2 days.

Every week, the cultures were analyzed for cellular morphology. Phase microscopy photos were taken with a Zeiss Axiovert (Carl Zeiss) at 10× and 20× magnification. Proliferation was evaluated by measuring mitochondrial metabolism, using the MTS assay at week 1, 2, 3, and 4, in the same manner as described above. On week 4, cell viability was assessed using by LIVE/DEAD© as described above,