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Research paper

Stiffness of hyaluronic acid gels containing liver extracellular matrix supports human hepatocyte function and alters cell morphology

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ABSTRACT

Tissue engineering and cell based liver therapies have utilized primary hepatocytes with limited success due to the failure of hepatocytes to maintain their phenotype *in vitro*. In order to overcome this challenge, hyaluronic acid (HA) cell culture substrates were formulated to closely mimic the composition and stiffness of the normal liver cellular microenvironment. The stiffness of the substrate was modulated by adjusting HA hydrogel crosslinking. Additionally, the repertoire of bioactive molecules within the HA substrate was bolstered by supplementation with normal liver extracellular matrix (ECM). Primary human hepatocyte viability and phenotype were determined over a narrow physiologically relevant range of substrate stiffnesses from 600 to 4600 Pa in both the presence and absence of liver ECM. Cell attachment, viability, and organization of the actin cytoskeleton improved with increased stiffness up to 4600 Pa. These differences were not evident in earlier time points or substrates containing only HA. However, gene expression for the hepatocyte markers hepatocyte nuclear factor 4 alpha (HNF4α) and albumin significantly decreased on the 4600 Pa stiffness at day 7 indicating that cells may not have maintained their phenotype long-term at this stiffness. Function, as measured by albumin secretion, varied with both stiffness and time in culture and peaked at day 7 at the 1200 Pa stiffness, slightly below the stiffness of normal liver ECM at 3000 Pa. Overall, gel stiffness affected primary human hepatocyte cell adhesion, functional marker expression, and morphological characteristics dependent on both the presence of liver ECM in gel substrates and time in culture.

Abbreviations: HA, hyaluronic acid; ECM, extracellular matrix; HNF4α, hepatocyte nuclear factor 4; FAK, focal adhesion kinase; ILK, integrin-linked kinase; CDC42, cell division control protein 42; Rac1, Ras-related C3 botulinum toxin substrate; RhoA, Ras homolog family member A; IVC, inferior vena cava; SVC, superior vena cava; SDS, sodium dodecyl sulfate; PEG, polyethylene glycol; PEGDA, polyethylene glycol diacrylate; G′, storage modulus; HGF, hepatocyte growth factor

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1. Introduction

Rising incidence and cost of liver disease as well as a limited supply of transplantable organs have increased demand for new liver treatments. Experimental treatments currently being developed include liver cell transplant on biological scaffolds (Atala, 2011; Bajaj, 2009; Baptista et al., 2011; Ding and Shi, 2011; Joly et al., 1997). However, several limitations intrinsic to primary hepatocytes slowed development of these technologies. Challenges remain in the maintenance of viability and cell function of primary hepatocytes outside of the normal liver microenvironment (Joly et al., 1997). Therefore, developing substrates that support hepatocyte viability and function is critical to maximize the efficacy of primary hepatocytes in tissue engineering and cell therapies for liver disease.

Previous studies have identified several advantages of using tissue-specific ECM instead of generic matrix formulations such as Matrigel for supporting primary cells (Hansen et al., 2009). Cells on natural liver ECM demonstrated increased ability to proliferate and maintain function over extended time periods (Zhou et al., 2011). Hepatocytes cultured on intact or solubilized organ specific matrix also maintained normal cell morphologies as compared to cells grown on tissue culture plastic or collagen (Baptista et al., 2011; Skardal et al., 2012; Nakamura and Ijima, 2013). The underlying mechanisms for this improved phenotypic stability are still unknown but likely relate to inclusion of several components of the natural liver microenvironment, including cell and growth factor binding sites.

For this study, decellularization of normal liver was used to isolate acellular ECM for incorporation into hyaluronic acid (HA) hydrogels. The decellularization process involved perfusion of the organ with detergents to remove native cellular material, while preserving structural proteins and glycosaminoglycans (GAGs) (Shupe et al., 2010). Removal of native cells leaves a non-immunogenic ECM scaffold that can be seeded with hepatocytes that remain viable for several weeks (Mirmalek-Sani et al., 2013).

Many previous studies utilized ECM coated tissue culture plastic for growing primary hepatocytes. However, this method is only suitable for two-dimensional culture and is not easily translated into a three-dimensional form for use in cell based therapies (Zhang et al., 2009; DeQuach et al., 2010). The current study uses a hydrogel that may be formed into three-dimensional units suitable for transplantation. Preliminary findings indicated that solubilized ECM combined with hyaluronic acid gel (HA) maintained a normal epithelial morphology and strong tight junction formation with little evidence of transition to a fibroblast-like morphology, commonly seen in traditional collagen cultures. Solubilization of the ECM also prevented hepatocyte apoptosis induced by phagocytosis of cryomilled ECM powders. Other studies published by our group demonstrated that HA based hydrogels avoided an immunogenic response measured by human lymphocyte proliferation, while other gel types such as rat tail collagen 1 induced a low level immune response (Mirmalek-Sani et al., 2013; Murphy et al., 2013). Better phenotype maintenace and biocompatibility upon transplantation signaled that HA gel was the best substrate for incorporating decellularized liver in future assays.

In the development of a liver ECM substrate, the physical characteristic of stiffness is important to cell physiology and mechanics. The general conclusions of previous publications indicate that stiff substrates promote hepatocyte spreading, proliferation, and dedifferentiation; while soft substrates promote maintenance of the functional hepatocyte phenotype (Wells, 2008; Hansen et al., 2006). Substrate stiffness also regulates cell aggregation, growth factor responsiveness, and cell motility with the highest cell migration occurring on intermediate stiffness levels (Semler et al., 2000; Zaman et al., 2005). However, several of these previous studies were done on stiffness ranges well above physiological liver stiffness, which has been reported anywhere from 1.5 to 8.5 kPa (Fung et al., 2013; Mueller and Sandrin, 2010; Arena et al., 2008; Klatt et al., 2010; Millonig et al., 2008; Schrader et al., 2011).

For the current study, the crosslinker concentration was the sole variable for adjusting gel stiffness. Crosslinking eliminated potential confounding effects found in more complex systems where concentrations of structural compounds are altered. Gel stiffnesses were also limited to a narrow, physiologically relevant range of 600–4600 Pa that was near the stiffness of normal liver ECM at 3000 Pa. Assays were conducted to measure overall hepatocyte function as well as the mechanisms by which substrate stiffness affected cell phenotype. Specific tests performed in this study were designed to explore the mechanisms by which substrate stiffness affected substrate/cell adhesion, primary hepatocyte function, and morphology. Cell junction formation is critical for the maintenance of epithelial cell phenotype; regulating a broad range of cellular properties through cell-cell communication, adhesion, and diffusion of water and solutes (Vinken et al., 2006; Lee and Luk, 2010). Tight junctions act as semi-permeable barriers that establish cell polarity, which is crucial for the maintenance of bile canaliculi (Balda and Matter, 2008; Kojima and Sawada, 2011). The tight junction protein, claudin-1, is required for maintenance of the selective barrier properties between adjacent hepatocytes (Gunzel and Yu, 2013). Occludin is not essential for maintaining tight junction structures, but plays a role in regulating cytoskeletal organization, cell polarity, and cell migration (Du et al., 2010; Rao, 2009; Van Itallie et al., 2010).

The effects of substrate stiffness on intracellular signaling were also studied through measurement of two cytoplasmic kinases: focal adhesion kinase (FAK) and integrin-linked kinase (ILK). Cells adapt their cytoskeletal structure to specific microenvironmental conditions through interactions between focal adhesion molecules and intracellular kinases. Appropriate expression of these kinases in hepatocytes is required for normal growth factor responsiveness, prevention of apoptosis, and normal metabolic function (Ilic et al., 1998; Dedhar et al., 1999; Su et al., 2005). Silencing or overexpression of these factors can lead to cellular dysfunction and, in the case of neoplasia, tumor progression (Persad et al., 2000). Increased expression also mediates cytoskeletal reorganization and cell protrusion formation which drives cell migration (Wu and Dedhar, 2001; Fabry et al., 2011). In other experimental systems, FAK and ILK expression have been shown to correlate with cell junction remodeling, specifically through