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Assessing Porcine Liver-Derived Biomatrix for Hepatic Tissue Engineering

PAUL LIN, B.S.,[1] WARREN C.W. CHAN, Ph.D.,[1,2] STEPHEN F. BADYLAK D.V.M., M.D., Ph.D.,[3] and SANGEETA N. BHATIA, M.D., Ph.D.[1]

ABSTRACT

Acellular, biologically derived matrices such as small intestinal submucosa have been extensively utilized to induce tissue regeneration and remodeling of connective tissue, vascular grafts, and urinary bladder; however, decellularized scaffolds have not been explored for their potential utility in hepatic tissue engineering. In the case of both extracorporeal hepatocyte-based devices and implantable hepatocyte–scaffold tissue-engineered constructs, maintenance of hepatocellular function is of prime importance. In this study, we specifically explored decellularized, porcine, liver-derived biomatrix (LBM) as a bioresorbable scaffold for primary hepatocytes. Primary rat hepatocytes were cultured on LBM and compared with well-characterized hepatocyte culture models—double-gel cultures that promote maintenance of liver-specific functions for many weeks, and adsorbed collagen monolayers that lead to the rapid decline of hepatocellular function and viability. Hepatocytes were maintained for up to 45 days on LBM and liver-specific functions such as albumin synthesis, urea production, and *P*-450 IA1 activity were found to be significantly improved over adsorbed collagen cultures. Our data indicate that LBM may be a favorable alternative to existing scaffolds for tissue engineering in that it is bioresorbable, can be easily manipulated, and supports long-term hepatocellular functions *in vitro*.

INTRODUCTION

THERE HAS BEEN TREMENDOUS INTEREST in the development of novel methods to treat liver diseases.[1,2] Unlike other organs that can be partially replaced by mechanical devices (heart/pump; kidney/filter), the diversity and complexity of liver functions (e.g., detoxification, metabolism, synthesis of plasma proteins, and production of bile) have thwarted attempts to support the failing liver by nonbiological strategies.[3] In contrast, purely biological approaches to liver support have included hepatocyte transplantation and xenotransplantation; however, both are still in early stages of development.[1,2] The "gold standard" for treatment of end-stage liver disease therefore remains orthotopic liver transplantation. As is the case for other organs, donor shortages remain a serious problem and the number of patients wait-listed for organs continues to rise dramatically.[4] As a result, hybrid (biological/synthetic) strategies for liver support have emerged as a means to provide partial or temporary liver support. Extracorporeal devices that house primary hepatocytes or hepatoma cells have been proposed as a means of liver support in order to "bridge" patients to transplantation. Similarly, implantable hybrid (scaffold/hepatocyte) tissue-engineered constructs are under development. Both these hybrid approaches require maintenance of viability

[1]Department of Bioengineering, University of California at San Diego, La Jolla, California.
[2]Present address: Institute of Biomaterials and Biomedical Engineering, University of Toronto, Toronto, Ontario, Canada.
[3]Department of Surgery, University of Pittsburgh, Pittsburgh, Pennsylvania.

and liver-specific function of a large mass of hepatocytes. As a result, much of the effort in hepatic tissue engineering has focused on elucidation of microenvironments that will serve to (1) maintain phenotypic stability of isolated primary hepatocytes, and (2) maximize mass transfer between the hepatocytes and the blood stream.

Isolated hepatocytes are notoriously difficult to culture *in vitro*.[5,6] In particular, they have a limited capacity to proliferate *in vitro* despite their well-documented proliferative ability *in vivo*.[7–9] Furthermore, disruption of the hepatic microarchitecture and the associated cues (cell–cell interactions, cell–matrix interactions, and blood flow) leads to a rapid loss of tissue-specific functions such as albumin synthesis, nitrogen metabolism, and cytochrome *P*-450 activity under most culture conditions. Various model systems have been reported that promote maintenance of liver-specific functions through manipulation of cell–cell interactions (e.g., cocultivation with nonparenchymal cells),[10] cell–matrix interactions (e.g., collagen sandwich, Matrigel),[11–16] and soluble factors (e.g., hormonally defined media).[7,17–19] Others have investigated hepatocyte viability and tissue-specific function on various scaffolds such as collagen microcarriers, galactose-derivatized films, encapsulation in alginate hydrogels, and seeding on degradable polyesters.[20–26]

Although synthetic polymers such as poly (lactic acid) (PLA) and poly(glycolic acid) (PGA) do offer the advantage of being formed into architectures that maximize mass transfer through molding or additive free-form fabrication techniques, these surfaces have not generally been shown to support high levels of liver-specific function.[27,28] In contrast, one class of biologically derived biomaterials has gained widespread use for tissue-engineering applications because of such characteristics as rapid resorption, support of angiogenesis, lack of immunogenicity, and ability to serve as a template for tissue remodeling. Acellular scaffolds are derived from the small intestinal submucosa (SIS) by mechanical removal of select portions of the mucosa and external muscle and then lysis of resident cells by peracetic acid and hypotonic washes. SIS has been used extensively to promote remodeling of musculoskeletal structures, skin, dura mater, urinary bladder, and blood vessels in animal and human studies.[29–34] The unique properties of this degradable biomaterial are thought to arise from a combination of its component extracellular matrix molecules (including collagen I, collagen IV, laminin, and fibronectin) as well as immobilized growth factors.[35]

One group has reported the use of SIS in a hepatic tissue-engineering application.[36] In this study, SIS was used to form a portal vascular graft that could, in turn, house a hepatocyte-seeded PGA mesh and sustain hepatocyte viability for 2 days; however, acellular biomaterials such as SIS have not been explored for their ability to directly sustain hepatocyte function. Badylak and co-workers modified their approach for fabrication of SIS to generate acellular matrices from other tissues including the urinary bladder and the liver.[37,38] We hypothesized that acellular liver-derived biomatrix (LBM) may provide appropriate cues to sustain liver-specific functions of hepatocytes. Therefore, in this study, we explored the ability of porcine-derived LBM to directly support the viability and function of isolated hepatocytes with the goal of exploring its utility as a biomaterial for hepatic tissue-engineering applications. We specifically examined the morphology, viability, and liver-specific functions of primary rat hepatocytes seeded on porcine-derived LBM *in vitro* in comparison with two well-characterized hepatocyte culture models: monolayer culture on adsorbed collagen (AC) and culture between collagen gels (double gel, DG). Our findings indicate that LBM supported hepatocyte adhesion, viability, and long-term (>30 days) liver-specific functions and may therefore prove to be useful for hepatic tissue-engineering applications.

MATERIALS AND METHODS

Hepatocyte isolation

Rat hepatocytes were isolated from 2- to 3-month-old adult female Lewis rats (180–200 g; Charles River Laboratories, Wilmington, MA) by collagenase perfusion and purified by filtration and Percoll centrifugation as previously described.[6] Normally, 200 to 300 million cells are isolated with 85 to 95% viability as determined by exclusion of trypan blue dye. Culture medium was Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO) and insulin, glucagon, and hydrocortisone (University of California, San Diego, pharmacy).

Preparation of LBM

Five-millimeter-thick sheets were prepared from whole porcine liver. The tissue was immersed in distilled water for 24 h at 4°C to lyse resident cells. After 24 h, the distilled water was replaced by 0.05% ammonium hydroxide solution containing 0.5% Triton X-100 for 72 h. The decellularized tissue was subsequently equilibrated with phosphate-buffered saline (PBS) at 4°C. The material was then lyophilized for 24 h and subsequently sterilized with 2.0 Mrad of γ radiation.

Hepatocyte culture

Hepatocytes were cultured under three different conditions: on LBM membranes, between two layers of collagen I gel (double gel, DG), or on adsorbed collagen I (AC) on tissue culture polystyrene. LBM membranes

were rehydrated in DMEM for 20 min before cell seeding and the membranes were held stationary in 60-mm Petri dishes by stainless steel inserts. The membrane covered ~95% of the petri dish surface. For double-gel cultures, concentrated DMEM (10×) was rapidly mixed with rat tail collagen I (1 mg/mL)[6] at a concentration of 9:1 (v/v) and kept on ice. The solution formed a gel on incubation at 37°C for 45 min. Adsorbed collagen surfaces were prepared by incubation of the polystyrene surface with collagen I (110 μg/mL) in double-distilled H_2O for 45 min. Cultures were seeded with 1.5×10^6 primary hepatocytes in 3 mL of medium. The following day, unattached cells were removed by washing with 3 mL of medium. Double-gel cultures were overlaid with a second layer of gel followed by the addition of 3 mL of medium. Medium was replaced daily and spent medium was stored at 4°C for further analysis.

Analytical assays

Metabolic and synthetic functions of hepatocytes were determined by measuring the production of albumin and urea as representative liver-specific markers. Albumin was measured by enzyme-linked immunosorbent assay (ELISA) as described previously.[10,39] Rat albumin and anti-rat albumin antibodies were purchased from MP Biomedicals (Aurora, OH). Urea synthesis was measured with a commercially available kit (Sigma). Cytochrome *P*-450 IA1 activity was measured as the rate of conversion of ethoxyresorufin (Molecular Probes, Eugene, OR) to the fluorescent product, resorufin, at excitation/emission wavelengths of 530/590 nm after induction with 5 μM naphthoflavone for 24 h.[40,41] Viability of hepatocytes was determined by dimethylthiazol-diphenyltetrazolium bromide cleavage to an insoluble purple product (MTT; Sigma-Aldrich, St. Louis, MO), extraction in 50% isoproponalanal–50% DMSO, and measurement of absorbance at 570 nm.

Microscopy

Measurements of projected surface area were performed by phase-contrast microscopy (Diaphot microscope, Nikon, Melville, NY), captured with a SPOT camera, and analyzed with MetaMorph Image Analysis software (Universal Imaging, Downingtown, PA). Twenty cells were measured for each condition. For fluorescence imaging, cultures were washed with DMEM and incubated with 5 (and 6)-{[(4-chloromethyl)benzoyl]amino}tetramethylrhodamine (CMTMR) in DMEM for 30 min. Afterward, cultures were washed three times in 10 mM PBS, pH 7.4, and fixed with 4% paraformaldehyde in PBS for 20 min. Hepatocytes were observed at excitation/emission wavelengths of 541/565 nm. For scanning electron microscopy (SEM), cultures were fixed with 4% paraformaldehye, dehydrated, and sputtered with a 100-nm layer of gold–palladium (50 mTorr; Anatech, Battle Creek, MI) and imaged with an SEM (Cambridge SEM 360; Nano Technology Systems Division of Carl Zeiss SMT, Oberkochen, Germany) at a voltage of 20.0 kV.

Statistics and data analysis

Eight separate experimental runs were performed, with typically $n = 3$ for each condition. Error bars represent the standard error. In rare cases of contamination or obvious outliers, $n = 2$. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Tukey multiple comparison test. Figures 3 and 4 depict representative trends observed in one of eight trials; however, absolute values of albumin and urea synthesis did vary significantly between trials, presumably because of cell sourcing from individual animals and different LBM batches.

RESULTS

Scaffolds and hepatocyte morphology

In this study, we explored the viability and function of primary rat hepatocytes on acellular ECM derived from porcine liver. When dehydrated and processed for use, these membranes are in a sheetlike form (Fig. 1A). At high magnification, the surface is highly porous and nonhomogeneous (Fig. 1B). LBM stayed grossly intact after 35 days in culture, although significant remodeling of the membrane was apparent by macroscopic contraction after 35 days of culture (Fig. 1C). Similarly, LBM did not exhibit significant degradation over 2 weeks in culture with human microvascular endothelial cells, keratinocytes, 3T3 fibroblasts, rat osteosarcoma (ROS) cells, and human umbilical vein endothelial cells (data not shown). Degradation of the scaffold *in vitro* can be achieved with a cocktail of collagenases and peptidases.

Hepatocytes on LBM were relatively rounded as seen by electron microscopy in Fig. 2A and B. These data were corroborated by fluorescent live cell visualization as seen in Fig. 2C. Hepatocytes are clearly visualized (orange) attached to the autofluorescent membrane (green). The average surface area of hepatocytes on the LBM scaffolds was 517 ± 35.5 μm^2. In comparison, hepatocytes cultured in a collagen sandwich double-gel system (DG) are cuboidal in shape and had an average surface area of 1111.3 ± 77.3 μm^2, whereas hepatocytes cultured on polystyrene surfaces with adsorbed collagen (AC) were highly spread with an average surface area of 3147.2 ± 214.8 μm^2. In addition, lamellopodia as long as 100 μm were observed on AC, whereas no visible lamellopodia formation was observed on LBM.

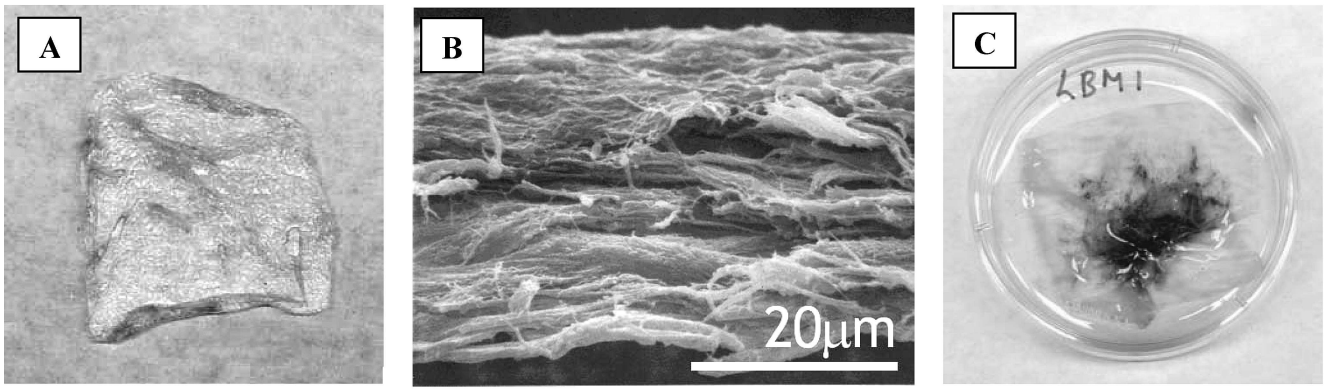

FIG. 1. Macroscopic and microscopic features of LBM. (**A**) Bright-field photograph of rehydrated LBM. (**B**) SEM image of LBM. (**C**) Bright-field photograph of remodeled LBM after 35 days of culture. Viable hepatocytes are stained with MTT (purple precipitate). Original magnification: (**A**) ×1; (**B**) ×2200.

Liver-specific functions of primary hepatocytes in vitro

The synthetic and metabolic functions of hepatocytes were assessed on the basis of albumin secretion and urea synthesis as representative markers of liver-specific function. Figures 3 and 4 compare hepatocyte function on LBM with that in two conventional hepatocyte culture models; collagen sandwich double-gel (DG) culture and adsorbed collagen (AC) culture over 33 days. DG culture is a well-characterized, robust model system that preserves a variety of liver-specific functions for weeks[14] whereas AC culture does not support the maintenance of liver-specific functions. Indeed, DG cultures produced ~27 μg of albumin per day on day 3 and continued to increase albumin production throughout the 33 days of culture. In contrast, cells on the adsorbed collagen surface rapidly declined and had almost no albumin production after day 5. In comparison, hepatocytes on LBM functioned at an intermediate, but sustained level, producing ~17 μg/day on day 3 and plateauing at ~7 μg/day. The dynamics of albumin secretion observed in Fig. 3 (a decline followed by a slow increase), were not consistently observed for other liver-specific functions such as urea synthesis, nor were results as dramatic in all trials as in this case. On the basis of our experience with other culture models (double gel, coculture, adsorbed collagen), we hypothesize that hepatocyte products (growth factors, matrix) may secondarily modulate the microenvironment and may collaborate with LBM to cause partial secondary induction of hepatic functions. Future experiments could be performed to decellularize hepatocyte-conditioned LBM and compare the induction kinetics of naive hepatocytes; however, these studies were beyond the scope of the current report.

Urea production, a marker of nitrogen metabolism, displayed a similar response (Fig. 4). In DG, hepatocytes produced urea at 134 μg/day on day 1 and progressively

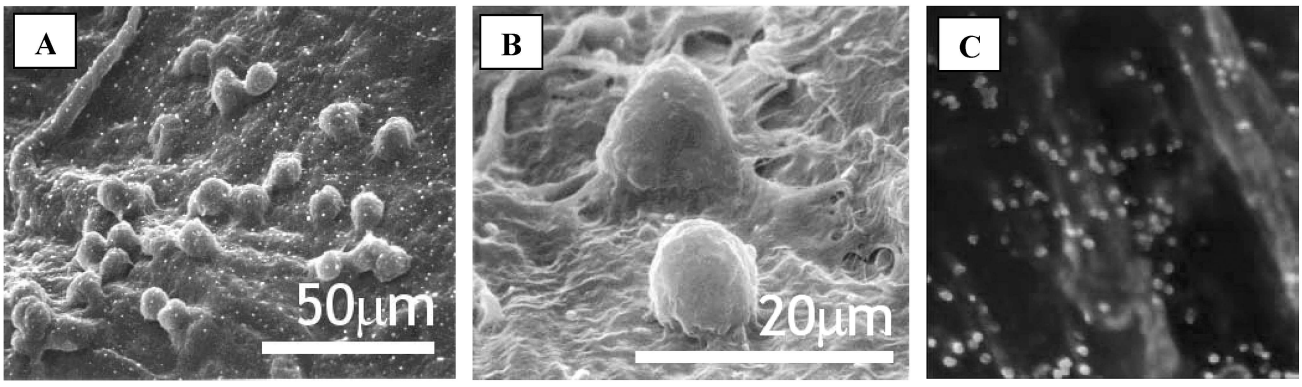

FIG. 2. Primary rat hepatocyte morphology on LBM after 7 days of culture. SEM images of cells at (**A**) ×850 and (**B**) ×3200 (original magnification). (**C**) Fluorescently labeled hepatocytes (orange) and autofluorescent LBM (green). Original magnification: ×20.

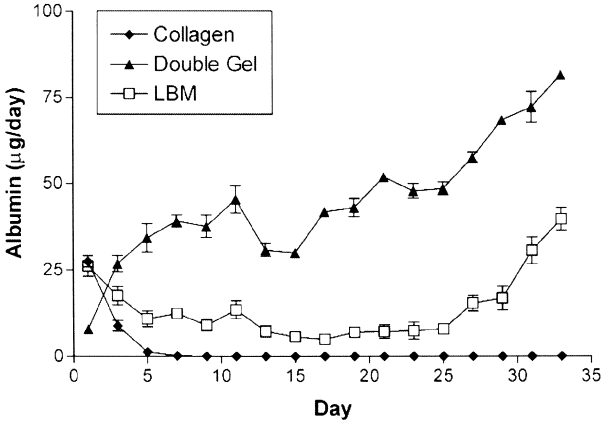

FIG. 3. Albumin secretion under different culture conditions. LBM exhibited an intermediate response between the stable culture model (DG) and the unstable culture model (AC).

increased their daily production over time. Hepatocytes on AC displayed a dramatic decrease in urea production after initial seeding. Finally, hepatocytes on LBM displayed an intermediate response, whereby the decline in urea synthesis was less dramatic than noted for AC. After 17 days, hepatocytes on LBM still produced urea at 54 μg/day whereas hepatocytes seeded on adsorbed collagen surfaces produced close to 1 μg/day.

Of note was the significant variability we observed in hepatocellular responses to distinct LBM membranes. Two of three membranes typically displayed responses similar to those shown in Figs. 3 and 4 whereas one membrane would commonly exhibit a diminished hepatocellular response. We speculated that this variability was due to differences in hepatocyte adhesion due to uncharacterized variations in LBM—perhaps related to location in the porcine liver, "sidedness," or animal-to-animal variability. To quantitatively compare liver-specific functions on the various substrates, viable cell number was measured in one trial on day 35 by MTT assay (Fig. 5A). These data were used to normalize and compare albumin and urea secretion rates on day 35 (Fig. 5B and C). Normalized hepatocellular function was comparable for LBM or DG for albumin secretion whereas both conditions displayed improve function relative to AC. MTT was used as an estimate of cell number in lieu of total DNA or cell counting because of residual DNA on decellularized matrices and difficulty in obtaining single-cell suspensions from mature cultures, respectively.

DISCUSSION

Acellular, biologically derived scaffolds have been successfully utilized in many areas of tissue engineering. In this study, we explored the utility of a porcine liver-derived biomatrix as a scaffold to support the liver-specific function of isolated primary hepatocytes for applications in implantable tissue constructs or extracorporeal devices.[21–26,42] LBM was evaluated for its ability to support hepatocyte adhesion and viability, and synthetic (albumin) and metabolic (urea synthesis) functions, by comparison with two well-characterized culture models: double-gel culture, known to support a variety liver-specific functions for weeks, and adsorbed collagen culture, known to precipitate the rapid decline of liver-specific functions.

In general, our findings over eight replicate trials indicate that LBM was capable of supporting liver-specific functions for 1 month in culture and performed at an intermediate level between double-gel and adsorbed collagen cultures. Synthetic (albumin), metabolic (urea), and detoxification (*P*-450 IA1, data not shown) functions were maintained to some extent for up to 36 days in culture. In our longest culture, hepatocellular functions were detected on LBM after 45 days of culture. Cell attachment experiments showed that cells attach better to the double-gel and adsorbed collagen configuration than to LBM (data not shown). This suggests that on a per-cell basis, the hepatocytes on LBM might be functioning better than Figs. 3 and 4 would suggest. This observation is consistent with hepatocellular functions when normalized to viable cell number as seen in Fig. 5B (i.e., hepatocytes on LBM were comparable to those on DG and significantly more functional than those on AC). Indeed, significant variability in seeding efficiency and performance of individual membranes was consistently observed—perhaps related to location in the porcine liver, "sidedness," biomatrix topography, or animal-to-animal variability. Future molecular analysis of individual LBM

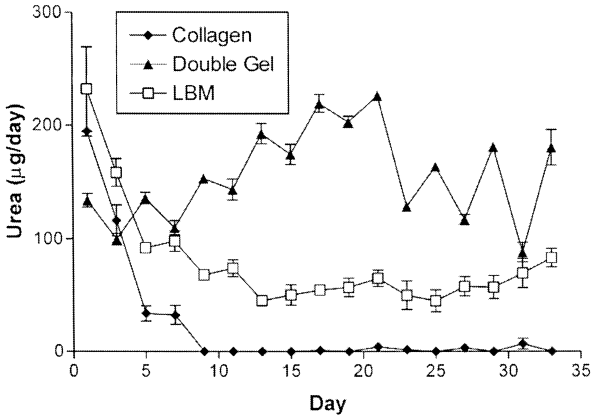

FIG. 4. Urea secretion under different culture conditions. LBM exhibited an intermediate response between the stable culture model (DG) and the unstable culture model (AC).

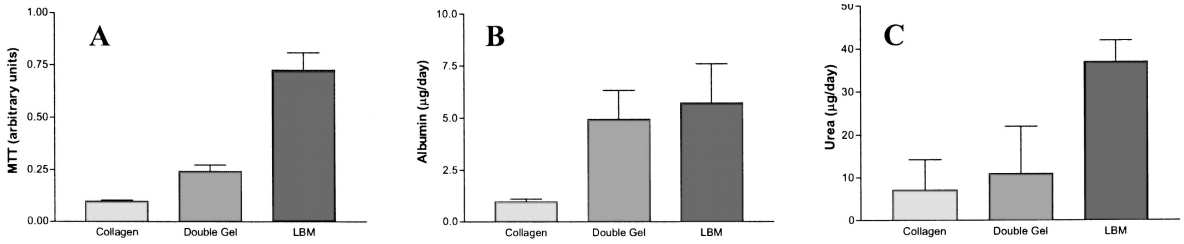

FIG. 5. Normalized hepatocellular function under different culture conditions. To compare hepatocellular function on a per-cell basis, cell viability on day 35 was measured by MTT assay (**A**) and used to normalize albumin (**B**) and urea (**C**) production. In general, DG and LBM cultures were comparable on a per-cell basis for albumin secretion whereas AC (collagen) cultures exhibited a consistently diminished response. In this experiment, AC and DG cultures were significantly less than LBM for MTT ($p < 0.01$), but not significantly different from one another.

samples will likely elucidate which biochemical constituents mediate the variability in cell adhesion.

The molecular mechanisms by which LBM supports hepatocellular function have yet to be elucidated. In other studies, a number of investigators have correlated lack of cell spreading (i.e., "cell shape") with differentiated hepatocyte function.[43–46] Indeed, adherent hepatocytes on LBM displayed a round, spherical morphology reminiscent of hepatocytes seeded on Matrigel, polyester foams, low-density fibronectin surfaces,[46] and Primaria dishes.[11,21,22,25,43,47] Nonetheless, it is not clear whether hepatocyte cell shape itself leads to differentiated gene expression as there are numerous examples of well-spread hepatocytes that exhibit high levels of liver-specific function.[6,10] Hepatocytes in double-gel culture, for example, form monolayers of well-spread cells whose phenotypic stability is thought to arise, in part, from the orientation of extracellular matrix binding and β_1-integrin ligation ("sandwich") that mimicks that found *in vivo*. The observed improvements in hepatocellular function on LBM over AC suggest that providing a matrix overlay (or sandwich) on LBM cultures might further increase liver-specific functions on this biomatrix.

Another possible mechanism for the improved function of LBM over a simple adsorbed collagen monolayer is the presence of additional matrix components such as glycosaminoglycans,[48] immobilized growth factors,[35] or even cellular remnants. To assess whether liver-specific matrix components were responsible for the effects of hepatocyte function, we compared the response of hepatocytes on LBM with that of cells seeded on small intestine submucosa (SIS) and urinary bladder matrix (UBM). In pilot studies, the average albumin secretion levels of hepatocytes on SIS and UBM were 225 and 125%, respectively, of those on LBM over 35 days of culture whereas average urea synthesis levels on SIS and UBM were 125 and 69% of those on LBM. Thus, the three matrices performed within the same order of magnitude, suggesting that liver-specific constituents were not required for hepatocellular signaling. In addition, these data suggest that many decellularized tissues may hold promise for hepatic tissue-engineering applications.

In summary, we describe the use of liver-derived biomatrix for the support of hepatocyte function for many weeks *in vitro*. Although the performance of double-gel cultures was generally superior to that of LBM cultures, transport limitations of the double-gel system limit its utility for therapeutic applications. In contrast, LBM provides a bioresorbable, biologically derived scaffold that supports hepatocyte function without the presence of an intervening gel layer (as in DG), without the need for nonparenchymal cells (as in coculture models), and without the need for hormonally defined media. In moving forward, it will be critical to isolate variables that influence membrane performance as well as to identify mechanisms by which LBM influences the differentiated function of mature hepatocytes. Regardless of the molecular mechanism by which LBM supports hepatocyte function, this biologically derived scaffold presents a favorable alternative to existing materials for hepatic tissue-engineering applications.

ADDENDUM

We respectfully acknowledge contributions made by several groups using rat liver biomatrix for the support of hepatocytes.[49–52] The material used in the current work was derived from a large animal source (porcine) that may be well-suited for clinical applications due to its size,

availability, and the precedence of employing porcine-derived small intestinal submucosa as a biomaterial in patients.

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Address reprint requests to:
Sangeeta N. Bhatia
University of California, San Diego
Department of Bioengineering
9500 Gilman Drive
La Jolla, CA 92093-0412

E-mail: sbhatia@bioeng.ucsd.edu