

Research Article

Open-Porous Hydroxyapatite Scaffolds for Three-Dimensional Culture of Human Adult Liver Cells

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Received 24 February 2016; Accepted 25 May 2016

Academic Editor: Subhas C. Kundu

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Liver cell culture within three-dimensional structures provides an improved culture system for various applications in basic research, pharmacological screening, and implantable or extracorporeal liver support. Biodegradable calcium-based scaffolds in such systems could enhance liver cell functionality by providing endothelial and hepatic cell support through locally elevated calcium levels, increased surface area for cell attachment, and allowing three-dimensional tissue restructuring. Open-porous hydroxyapatite scaffolds were fabricated and seeded with primary adult human liver cells, which were embedded within or without gels of extracellular matrix protein collagen-1 or hyaluronan. Metabolic functions were assessed after 5, 15, and 28 days. Longer-term cultures exhibited highest cell numbers and liver specific gene expression when cultured on hydroxyapatite scaffolds in collagen-1. Endothelial gene expression was induced in cells cultured on scaffolds without extracellular matrix proteins. Hydroxyapatite induced gene expression for cytokeratin-19 when cells were cultured in collagen-1 gel while culture in hyaluronan increased cytokeratin-19 gene expression independent of the use of scaffold in long-term culture. The implementation of hydroxyapatite composites with extracellular matrices affected liver cell cultures and cell differentiation depending on the type of matrix protein and the presence of a scaffold. The hydroxyapatite scaffolds enable scale-up of hepatic three-dimensional culture models for regenerative medicine applications.

1. Introduction

Culture of liver cells within three-dimensional (3D) structures provides improved *in vitro* systems for studying hepatic cell differentiation and proliferation. The unfulfilled demand for donor organs for transplantation in chronic liver disease suggests that the development of engineered tissue transplants is necessary to provide additional metabolic capacity. Regenerative medicine techniques are currently being investigated to supplement the need for transplantable tissue. Though the developments are promising, many aspects of such technologies and procedures must be examined. Chief among the unsolved problems is the need for procedures addressing the supply of human liver tissue and cells [1], most likely expanded by proliferation *in vitro* [2]. Once such a cell source has been identified, further applications could be of interest, such as extracorporeal liver support in acute liver

failure [3] and pharmacologic *in vitro* screening of hepatic drug candidates [4, 5].

Early mouse liver cell cultures were introduced in monolayer by Evans et al. in the 1950s [6]. Later on, liver cell cultures in suspensions were developed [7]. With the introduction of extracellular matrices as dish coatings for monolayer cultures, the early loss of hepatic functions and viability in culture was somewhat improved. Liver relevant extracellular matrices such as laminin, various collagen types, hyaluronic acid hydrogel, and matrigel were implemented [8–13]. The use of sandwich culture [14–16], that is, the embedding of liver cells between two layers of collagen, was an important step in the enhancement of liver cell culture by mimicking the *in vivo* liver plate architecture. To realistically grow liver tissue from adult liver cells at higher densities and thus approach a more natural tissue situation, the use of a three-dimensional scaffold is advisable and a scale-up

of such techniques may support the cells in cultures at a size suitable for clinical use. Such a scaffold structure must have a high porosity to facilitate cell seeding and fluid flow for mass exchange around the cells but also allow sufficient space for neoendothelialized structures, while being rigid enough to structurally support the mass of the growing tissue.

Ceramic foaming techniques have been developed to create such highly porous, permeable structures [17–19]. Biocompatible calcium phosphates, specifically hydroxyapatite, have successfully been used as scaffolds for culturing several different cell types including liver cells from both human and rat sources [20, 21]. Calcium phosphate ceramics can also be manufactured to be bioresorbable by using tricalcium phosphate or taking advantage of a high temperature decomposition of hydroxyapatite to resorbable calcium phosphate phases. In the present study, we have analyzed two different extracellular matrix proteins, collagen-1 and hyaluronic acid, in combination with previously developed porous hydroxyapatite scaffolds [19]. We investigated the influence of these conditions on longer-term cultures of human adult liver cells to create scaled-up tissue structures.

2. Materials and Methods

2.1. Cell Culture. Hydroxyapatite foam scaffolds were prepared from a 1 mm thick section of an emulsion foam as described in previous work [19]. Scaffolds were placed in polystyrene tissue culture plates (Becton Dickinson Biosciences) and were sterilized by autoclaving. Human total fresh liver cell suspensions from male donors were obtained from discarded grafts (Becton Dickinson Biosciences, Woburn, MA) and cell viability was determined by trypan blue exclusion. Cell number was counted in a Neubauer chamber. The cells were applied to the scaffolds either directly without extracellular matrix, or embedded in extracellular matrix protein. Two different gels of extracellular matrix proteins were prepared for the study. Hyaluronan gel (Glycosan, Alameda, CA) was prepared by dissolving freeze-dried hyaluronan in 1 mL of sterile water at 37°C. To decrease gelation time, a cross-linker (extralink) was added, and the prepared cell fraction was suspended in the mixture at a concentration of 1E6 cells/mL of gel. Collagen-1 gel was prepared by mixing rat-tail collagen type 1 (Becton Dickinson Biosciences) with sterile water, sterile 10x PBS, and sterile 1 M NaOH according to manufacturer's protocol. Cells were suspended at a concentration of 1E6 cells/mL of gel. To each well of a culture plate containing either scaffold or no scaffold, 250 µL of the cell mixtures was added and set at 37°C within 30 minutes. Controls included also cells suspended in 250 µL culture medium without addition of extracellular matrix protein. Again the concentration was 1E6 cells/mL per well. Additionally, 250 µL of supplemented Williams E medium was added to each well. Williams E medium (Life Technologies, Carlsbad, CA) was supplemented with 10% fetal bovine serum, antibiotic/antimycotic mix, 2 mM glutamax (all Life Technologies), 5 µg/L insulin, 10 µg/L transferrin, 30 nM selenium, and 100 nM hydrocortisone (all Sigma-Aldrich, St. Louis, MO). The medium was replaced every 2 to 3 days during culture and the aspirate was saved

for future protein analysis. For all experiments, cultures were kept for 5, 15, and 28 days and there were 6 biological repeats, each from a different donor.

2.2. Albumin Enzyme-Linked Immunosorbent Assay. A standard albumin sandwich enzyme-linked immunosorbent assay was used to measure secreted albumin in medium samples. MaxiSorp Immunoplates (Nalgene Nunc International, Penfield, NY) were absorbed with anti-human albumin antibody (Bethyl Laboratories), incubated with samples or standards (Bethyl Laboratories, Montgomery, TX), and conjugated with a goat anti-human albumin horseradish-peroxidase-conjugated antibody (Bethyl Laboratories). Tetramethylbenzidine substrate solution was incubated for 5 min, the enzymatic reaction was stopped with 2 M sulfuric acid (Fisher Scientific, Pittsburgh, PA), and absorbance was read at 450 nm with a Synergy H1 hybrid reader equipped with Gen5 software version 2.00 (Bio-Tek, Winooski, VT). To quantify albumin secretion of cells in culture at longer time points (days 18–28), a linear regression was completed on the data for each sample and for each condition; ANOVA was calculated with the null hypothesis being a slope of 0 (no secretion). A *p* value of 0.05 was considered statistically significant.

2.3. DNA Quantification and Gene Expression Analysis. DNA was quantified to determine cell numbers of cultures, and RNA was extracted for gene expression analyses. Total DNA and RNA were extracted from cells using the AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA). Cells were disrupted in lysis buffer of the kit on QIAshredder columns (Qiagen). Isolated DNA was quantified using the Quant-iT dsDNA BR Assay Kit on a Qubit fluorometer and compared to a sample containing 1E6 human adult liver cells from the donor. RNA was reverse transcribed to cDNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time polymerase chain reaction (PCR) was carried out using the StepOnePlus Real-Time PCR-System equipped with StepOne Software version 2.0 (Applied Biosystems). Predesigned TaqMan probes and primer sets were obtained from Applied Biosystems and used to quantify gene expression for vWF, CK19, CYP3A4, albumin, ASMA, and beta-actin using the ddCt method. Data were normalized against beta-actin expression. Negative PCR controls included no template (water).

2.4. Immunocytochemistry. At each of the three time points, cell culture samples were fixed with 4% para-formaldehyde (Sigma-Aldrich). Portions of these cultures were blocked with 10% goat serum (Sigma-Aldrich) and 1% FCR block (Miltenyi Biotec, Auburn, CA) in phosphate-buffered saline and stained with diamidino-phenylindole dihydrochloride (Sigma-Aldrich) for cell nuclei and AF568-conjugated phalloidin for intracellular actin filaments (Life Technologies). To evaluate CK19 expression cells were stained with mouse anti-CK19 primary antibody (Fisher Scientific) and AF488-conjugated goat anti-mouse secondary antibody (Life Technologies). To examine endothelial cells in culture separate portions were stained with rabbit anti-vWF primary antibody

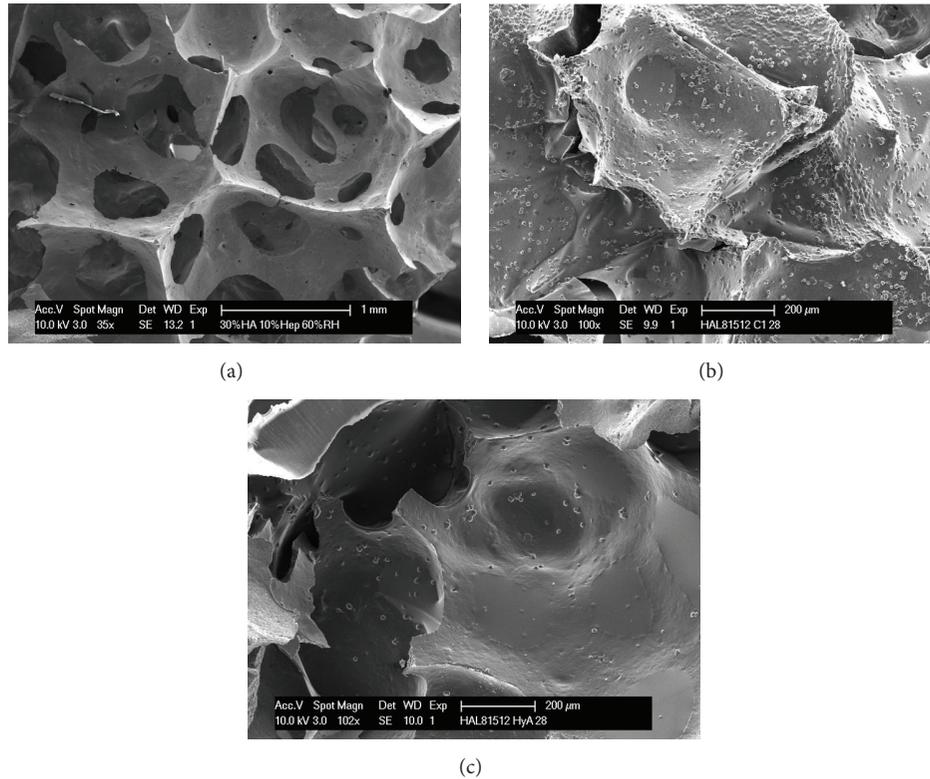


FIGURE 1: Scanning electron microscopy. Porous foamed hydroxyapatite scaffolds (a) were used for cell culture. After 28 days of culture, numerous cells attached to the scaffold could be observed in cultures with collagen-1 (b), whereas considerably less cells were attached to scaffolds in culture with hyaluronan (c).

(Abcam, Cambridge, MA) and AF488-conjugated goat anti-rabbit secondary antibody (Life Technologies). All stainings were analyzed by confocal microscopy using a Fluoview 1000 system (Olympus, Center Valley, PA).

3. Results

3.1. Cell Viability, Attachment, and Number. Human adult liver cell suspensions had viabilities of 65–83%.

After 28 days in culture, cells were analyzed for their attachment on hydroxyapatite scaffolds (Figure 1). In culture with collagen-1, numerous cells could be observed being attached on the scaffold (Figure 1(b)), whereas in culture with hyaluronan considerably fewer cells were attached on the scaffold (Figure 1(c)).

We also investigated cell numbers (based on DNA correlation) in the various culture conditions after 5, 15, and 28 days of culture (Figure 2). Cell numbers in all conditions decreased after 5 days of culture when compared to initial seeding numbers. After 28 days of culture, very few cells could be detected in hyaluronan cultures, both with and without hydroxyapatite scaffold, and were significantly lower than any other condition at day 15 and day 28 ($p < 0.05$). Conversely, cells cultured in collagen-1, both with and without scaffold,

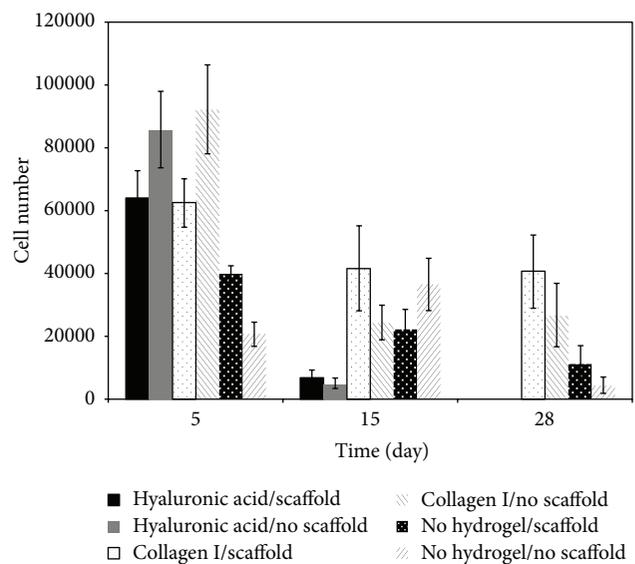


FIGURE 2: Numbers of liver cells in culture. Cells were cultured for 5, 15, and 28 days with (w) or without (wo) hydroxyapatite scaffolds embedded in hyaluronan gel (HyA), collagen-1 gel (C1), or no gel (NG), and cell numbers were determined by correlation with DNA concentration. Data are given as means from 6 biological repeats \pm standard deviation.

TABLE 1: Gene expression of liver cell cultures.

Gene	Culture time (days)	Hyaluronic acid		Collagen I		No hydrogel	
		Scaffold	No scaffold	Scaffold	No scaffold	Scaffold	No scaffold
CK19	5	288.16	95.97	263.85	192.42	164.23	148.33
	15	453.34	202.45*	2063.79*	278.88	391.36	145.73
	28	873.87	791.60	1064.61	309.64	309.73	274.06
vWF	5	1.39	0.84	0.50	0.58	1.18	0.28
	15	0.078 ^{<}	0.06 ^{<}	0.93	1.65	4.27*	2.64*
	28	0.12	0.03	0.19 ^{<}	0.34 ^{<}	3.62	0.80 ^{<}
CYP450 3A4	5	63.15	40.56	0.76	0.81	0.26	0.03
	15	5.35 ^{<}	32.39	4.11	2.14	0.11	0.02
	28	41.87	39.76	0.04	0.04	0.02	0.02
Albumin	5	0.46	0.43	0.56	0.58	0.10	0.20
	15	2.03*	0.34	0.35	0.43	0.15	0.21
	28	0.27 ^{<}	0.42	0.16	0.12	0.08	0.12
ASMA	5	0.00	0.55	24.62	36.62	17.57	14.69
	15	0.00	10.21*	4.06 ^{<}	18.34	6.88	3.19 ^{<}
	28	4.64*	0.00 ^{<}	2.50	0.99 ^{<}	1.50	1.37

Cells were cultured for 5, 15, and 28 days with (w) or without (wo) hydroxyapatite scaffolds embedded in hyaluronic acid gel (HyA), collagen-1 gel (C1), or no gel (NG), and gene expression was measured by PCR for cytokeratin-19 (CK19), Von Willebrand Factor (vWF), cytochrome P450 3A4 (CYP3A4), albumin, and Alpha-Smooth Muscle Actin (ASMA). Data are given as means from 6 biological repeats, normalized against beta-actin expression, and significance ($p < 0.05$) is marked by * to indicate a value significantly greater than the previous time point and < to indicate a value significantly less than the previous time point.

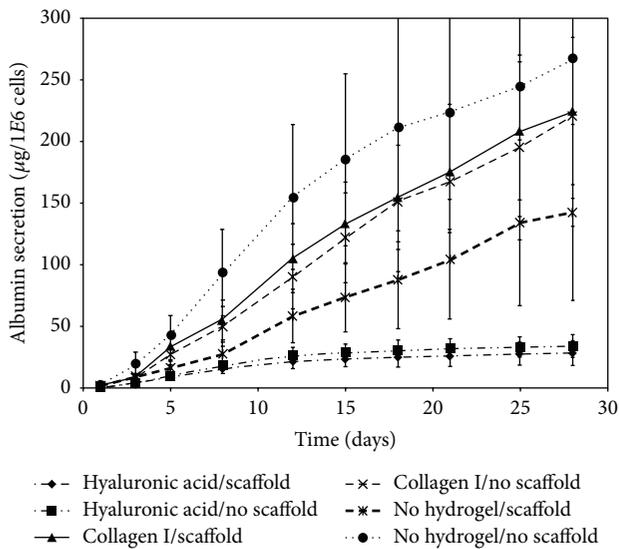


FIGURE 3: Albumin secretion of liver cell cultures. Cells were cultured for 5, 15, and 28 days with (w) or without (wo) hydroxyapatite scaffolds embedded in hyaluronic acid gel (HyA), collagen-1 gel (C1), or no gel (NG), and albumin secretion was measured by ELISA. Data are given as means from 6 biological repeats \pm standard deviation.

showed constant cell numbers between 15 and 28 days. Cultures with the scaffolds were showing slightly higher cell numbers at 28 days than those without (40,643/well on average compared to 26,793/well).

3.2. *Albumin ELISA.* Albumin secretion of the cultures (Figure 3) varied between samples; however, certain trends

developed that were consistent between donors. Of hydrogel conditions, culture in collagen-1 showed consistently higher overall secretion than in hyaluronan. In addition, when hydroxyapatite scaffold was not present the cells ceased significantly albumin secretion after day 15. During the last ten days of culture (days 18–28) only the cells cultured on hydroxyapatite in collagen-1 showed a significant increase in albumin secretion. ANOVA linear regressions of the data demonstrated a nonzero slope in cumulative albumin secretion ($p < 0.01$) in this condition showing significant secretion in the late stages of culture (78.5 ± 40.1 ($\mu\text{g}/1\text{E}6$ cells)/day). No other condition had a slope in cumulative albumin secretion significantly different from zero over the last 10 days of culture.

3.3. *Gene Expression.* Changes in expressions of genes specific for the various cell types of the liver were analyzed after different time points in culture; data are given relative to freshly isolated cells, which were set as 1. The expression of CK19, a biliary epithelium specific gene, was highly expressed in all samples (Table 1) throughout the 28-day experiment when compared to original cell suspensions. Cells cultured in collagen-1 on scaffolds had the highest expression; after 28 days cells cultured in collagen-1 with scaffold had an expression approximately 1000 times more than that of day 0 donor samples. For the other three conditions (cultures on collagen-1 without scaffold and both no-gel conditions), this expression was around 500 times that of day 0 donor cells and significantly less than cells cultured on collagen-1 with scaffold ($p < 0.05$). The expression of vWF, a mature endothelium specific marker, showed differences between samples (Table 1), with the highest expression after 15 and 28 days in cells cultured

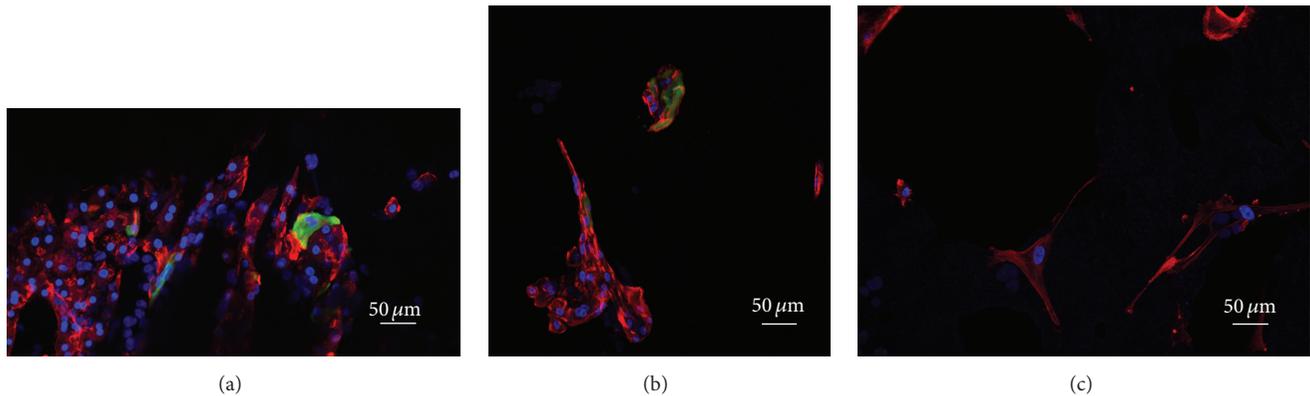


FIGURE 4: Immunocytochemistry of liver cell cultures. Liver cells were cultured for 28 days and stained, and images were taken by confocal microscopy. (a) Cells cultured in collagen-1 gel; (b) cells cultured in collagen-1 gel on hydroxyapatite scaffold; (c) cells cultured on hydroxyapatite scaffold only. Blue = DAPI, red = phalloidin, and green = CK19.

without any gel. Cells cultured without any gel on hydroxyapatite had the highest vWF expression, with expression maintained between day 15 and day 28 ($p < 0.05$). The expression of CYP3A4 (Table 1), a cytochrome P450 enzyme of mature hepatocytes, was highest in cultures with hyaluronan. Cells in hyaluronan maintained expression throughout the 28-day culture, significantly greater than all other conditions at day 28 ($p < 0.05$), and cells cultured in collagen-1 gel maintained expression through 15 days and lost expression after 28 days. Cells cultured without hydrogel downregulated expression of CYP3A4 in culture. The gene expression of albumin (Table 1), a secreted protein of mature hepatocytes, was highest in cells cultured in hyaluronan after 28 days, and always lowest in cultures without extracellular matrix addition. The expression of albumin is downregulated with the secretion of albumin, which is why cultures with high albumin secretion seen in ELISA results have lower gene expression. The downregulation of the expression of fibroblastic gene ASMA (Table 1) in most samples during culture suggests that there was no overgrowth of fibroblasts in culture.

3.4. Immunocytochemistry. Cells cultured in collagen-1 gel, regardless of the presence of hydroxyapatite, formed hepatocyte cordlike structures similar to liver plates (Figures 4(a) and 4(b)). Cells were found in small numbers in both hyaluronic acid and the no-gel conditions without the cordlike structures seen in cells cultured in collagen-1. In addition, both collagen-1 conditions also showed many cells positive for CK19 (Figures 4(a) and 4(b)). In those samples containing hydroxyapatite, ring-like structures of CK19 positive cells were found in a few instances (Figure 4(b)), similar to bile ductular structures *in vivo*. Cells cultured on scaffolds without collagen-1 exhibited a much different morphology with cells stretching out over the surface of the ceramic (Figure 4(c)).

4. Discussion

The use of hydroxyapatite ceramic scaffolds should have several advantages for the scale-up of cultures. Previously [18]

we reported that a heat treatment process can be used to control the relative amounts of hydroxyapatite and tricalcium phosphate in calcium phosphate scaffolds. This could open new directions in the utilization of such resorbable scaffolds for implantable constructs, as tissue implants which could initially support neovascularization and subsequently disintegrate once perfused tissue is formed. The inherent local liberation of calcium may also be of interest for creation of local calcium gradients around cells immobilized near the scaffolds surface, as calcium is thought to support endothelial structure reformation. Calcium ions contribute to the maintenance of endothelial cells and also the formation of vascularized tissue [22]. Calcium ion concentration has been shown to affect proliferation of adult rat hepatocytes directly in a tight compositional range [23]; their highest proliferation rates *in vitro* were observed at physiological concentrations of 0.4 mM while lower or higher concentrations resulted in lower proliferation rates. Biodegradable ceramic scaffolds have even been seen to influence angiogenesis in bone marrow cell cultures by creating a localized calcium rich environment [24]. The three-dimensional scaffold itself can also be used to induce endothelial cells for culture of vascularized tissue constructs by creating a surface suitable for the actin filaments of the endothelial cells to attach, as well as providing a microenvironment suitable for cell proliferation [25, 26].

Few studies have been published using ceramic structures for liver cell cultures. Ceramic plate-like structures with circular cavities were developed, and rat hepatocytes were demonstrated to attach within the cavities [27]; however, cultures were maintained only for 24 h and no liver specific functions were analyzed. The applicability of cell-seeded hydroxyapatite scaffolds for potential future clinical transplantation studies has been demonstrated by rodent transplantation studies; hydroxyapatite scaffolds seeded with immortalized mouse liver cells were successfully transplanted into the omentum and kidney of mice [21]; transplantation of hydroxyapatite disks seeded with normal rat hepatocytes intraperitoneally into Nagase albuminemic rats significantly increased albumin secretion within the host [20].

The usefulness of extracellular matrix proteins in the improvement of hepatic cell cultures has been widely discussed in the context of cell structures for implantation or bioreactors for temporary extracorporeal use [28] (for review, see [29]). Extracellular matrix proteins not only provide mechanical stability for cell constructs but also interact directly with cells through receptors influencing their cell type specific function. We used hyaluronan and collagen-1 hydrogels for our studies on establishing culture models involving hydroxyapatite, because these extracellular proteins have been used successfully to culture human adult hepatocytes and other primary cell types [13–15, 30–32]. Of the culture conditions examined, the maintenance of cells in culture was clearly best in the collagen-1/hydroxyapatite composite showing almost two times as many cells after 28 days compared with any other condition. These findings were also supported by the other parameters examined. Although gene expression of albumin, a secreted protein of mature hepatocytes, was highest in cells cultured in hyaluronan after 28 days, actual secretion of albumin protein (as measured by ELISA) was higher in collagen-1 culture. This fact can be explained by the known feedback mechanism of albumin protein on gene expression, by which gene expression of albumin is downregulated with the secretion of albumin protein [33], which is why cultures with high albumin secretion seen in ELISA results have lower gene expression. Collagen-1 hydrogels are a common culture model for liver cells because of the abundance in liver tissue [34, 35]. This extracellular matrix provides an environment on the lowest level of organ structure similar to native liver tissue and might be expected to be the best hydrogel for supporting the culture of adult liver cells *in vitro*. When cells were cultured on porous hydroxyapatite in collagen-1 the composite structure also provided improved cell maintenance during the last two weeks of culture, compared to the negative control and also the collagen-1 sandwich cultures of primary human hepatocytes found in the literature [31]. We have shown previously that such a three-dimensional condition supported hepatic cell differentiation and proliferation in perfusion culture [32, 36]. However, it must be noted that in the model used here the static conditions did not involve medium perfusion, which was found to further enhance culture longevity [37, 38].

Competing Interests

No competing interests were identified for all authors.

Acknowledgments

The authors thank the Center for Biological Imaging (CBI) at the University of Pittsburgh for its support. This study was funded by the National Institutes of Health (Grant no. 1R01HL108631).

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