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d-LIVER

ICT-enabled, cellular artificial liver system incorporating personalized patient management and support

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D8.6: Report on the performance of hepatocytes during a "proof of concept" clinical trial

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1. Executive Summary

A major objective of WP8 is to generate a cost-effective cell line capable of being readily expanded in number in a simple culture system and converted into functional hepatocytes using a simple regulatory switch. The function of hepatocytes generated also needs to be stably maintained in a bioreactor for long periods. Such a cell source is likely essential for any cell-based bioreactor and for it to be a realistic option in the treatment of patients with decompensated liver disease. Critically, the cells also need to be able to tolerate exposure to sera from liver disease patients, which contain higher levels of toxins than sera from healthy donors.

To examine the ability of cells to tolerate and potentially reduce the levels of toxins present, the effect of exposure to patient sera on cells over a 24 hour period was examined. In addition, a 3 day exposure was used to examine any changes in toxin levels in the sera as a consequence of exposure to the cells.

Rat B-13 cells were compared to the human pancreatic cell line and an engineered variant of this human pancreatic cell line over-expressing a transcription factor in response to the hepatic differentiating signal (H-13 cells). Their derived (through glucocorticoid exposure) hepatic variants were also examined. Compared to the 50% FCS control, control human serum (5 separate donors) was toxic to some of the human cells suggesting that healthy human serum contains factors that may adversely affect growth and/or viability. In contrast, serum from diseased liver patients (3 separate donors) was less toxic. In all cases, whether the cells were in progenitor or hepatocyte phenotype, there was clear evidence of viability over 3 days in diseased serum in that there was a removal of glucose and generation of lactate in the medium, suggestive of active glycolysis.

Given the near depletion of glucose in many cases, these data suggest that a regular replenishment of glucose and removal of lactate (which acidifies the medium) may be essential for cells in any future bioreactor. In most cases, the cells increased the levels of urea in the medium when exposed to diseased sera, suggestive of functional nitrogen detoxification. The activities were somewhat similar in both progenitor and hepatic phenotypes. In H-13/H cells there was a consistent low level reduction in bilirubin concentration when exposed to diseased sera, with some evidence of bilirubin conjugation. These data were dependent on a limited number of separate diseased sera donations available but suggest that an engineered human pancreatic progenitor cell line is able to tolerate exposure to human sera from liver disease patients and is able to mediate a low level functional detoxification in 2D culture. These data support their continued development and assessment in 3D culture, since this mode of culture may increase functionality and serum detoxification.

2. Introduction

Hepatocytes are the primary defining cell of the liver, performing the vast majority of its functions. Isolation and/or culture of hepatocytes is therefore a potential source of functional cells that could be used in a bioreactor. However, several factors limit hepatocyte utility. Hepatocytes do not proliferate *in vitro* and therefore cannot be expanded *in vitro*. Furthermore, culture results in de-differentiation and loss of function. Complex culture modifications (e.g. matrigel) can ameliorate this loss, but would introduce an array of often uncharacterised factors which likely will complicate use of primary hepatocytes in any bioreactor (e.g. immune response to xenogenic proteins), in addition to markedly increasing costs.

Human liver is in short supply and is often of poor quality. In the absence of sufficient human hepatocytes from donor livers, the main alternative for generating human hepatocytes is through differentiation of human embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). Despite significant progress over the last 15 years, it has not been possible to generate hepatocytes with function quantitatively similar to adult human hepatocytes. Stem cell-derived hepatocytes remain in a foetal state and cannot progress further unless transplanted in-vivo. Since normal hepatocytes de-differentiate into a foetal state in-vitro (even when present within culture tissue slices), a barrier to mature hepatocyte formation likely exists in stem cell-derived hepatocyte cultures. In addition, a major hurdle to the use of ESC/iPSC-derived hepatocytes is their high cost of generation (see – d-LIVER consortium (2012) Developing an "ICT-enabled, cellular artificial liver system incorporating personalized patient management and support". http://d-liver.eu/wp-content/uploads/relative-cost-of-hepatocytes-d-LIVER_TR_2012_WP8-for-web-v2a.pdf)

The backup option of using a derivative of a human pancreatic cell line was therefore developed. A piggybac vector encoding a transcription factor under control of glucocorticoid exposure was constructed and several stably transfected cell lines generated. One stable cell line (see deliverable D8.5), termed H-13, was taken forward for evaluation.

Although clearly the primary aim of any bioreactor is to stabilize a decompensating liver patient and reverse the metabolic changes responsible for their deterioration, there remains a potential that the patient's plasma will be toxic to the cells within the bioreactor and the cells will not be capable of bridging liver function. In order to examine the potential toxicity of liver patients' plasma to cells in a bioreactor, H-13 cells were exposed to patient sera and their viability examined. In addition, the ability of the cells to modulate the levels of some metabolites in the sera was examined.

3. Generation of H-13 cell lines

3.1. Methods

Cell culture

Cells (B-13, a human pancreatic cell line and H-13 lines) were cultured in low glucose Dulbecco's minimum essential medium (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS), 80 U/ml penicillin and 80 µg/ml streptomycin. Cells were incubated at 37 °C in a humidified incubator gassed with 5% CO_2 in air. Dexamethasone (DEX) was purchased from the Sigma Chemical Co. (Poole, UK) and was added to medium from 1000-fold concentrated ethanol vehicle solvated stocks, control cells were treated with 0.1% (v/v) ethanol alone as control to convert cells into their hepatocyte phenotype (10 nM for B-13 cells, 1 µM for the human pancreatic cell line and H-13 cells).

Human sera

Blood was collected from 5 healthy controls and from 3 patients with chronic liver disease, allowed to clot and serum prepared by centrifugation. Ethical approval was granted on the 17th February 2014 through a modification of the Wright laboratory's approval for research operated under the Newcastle Hepatopancreatobiliary Research Tissue Bank.

Cell treatment with serum

Cells were exposed to 50% v/v serum for 24 hours prior to a viability determination. Cells were also exposed for 3 days to serum, prior to a repeat clinical chemistry analysis.

Viability assay

Viabilities were determined through assaying of thiazolyl blue tetrazolium bromide (MTT) reduction by cells. MTT reduction was determined by replacing culture media with media containing 0.5 mg/ml MTT and returning cells to the incubator for between 2-4 hours. The medium was then replaced with an equal volume of isopropanol and after mixing, absorbance was determined at 570 nm (with background absorbance at 690 nm also determined and reading subtracted from reading at 570 nm). The results are expressed as percentage absorbance relative to vehicle-treated cells.

Clinical chemistry

Clinical chemistry measurements were performed using validated clinical chemistry referee methods and carried out in the Department of Clinical Biochemistry at the Newcastle Hospitals NHS Foundation Trust. Sodium, potassium and ammonia were measured using ion-selective electrode methodology. Lactate concentrations were determined using blood gas analyser. Creatinine levels were determined sing a kinetic colorimetric assay based on the Jaffé reaction as modified by Bartels, bilirubin by a colorimetric assay, total bile acids by an enzyme-linked reduction assay quantified spectrophotometrically and albumin was determined colorimetrically using bromocresol green binding. Serum ALT and ALP were determined as previously described [1].

Other clinical scores

The Model for End-Stage Liver Disease, or MELD, is a scoring system for assessing the severity of chronic liver disease and is based on patient values for serum bilirubin, creatinine and the international normalized ratio for prothrombin time (INR) to predict survival. MELD is calculated according to a formula [2] and the predicted 3-month survival for a range of scores

is MELD > 40 (71.3% mortality); MELD 30–39 (52.6% mortality); MELD 20–29 (19.6% mortality); MELD 10–19 (6.0% mortality) and MELD <9 (1.9% mortality) [2].

The Child-Pugh score is used in the clinic to assess the prognosis of chronic liver disease, mainly cirrhosis. The score employs five clinical measures of liver disease. Each measure is scored 1-3, with 3 indicating most severe derangement (see Table 1).

Table 1: Criteria used in calculating Child-Pugh score for chronic liver disease patients. Chronic liver disease is classified into Child-Pugh class A to C, employing the added score from above with 5-6 points (class A: survival, 100% after 1 year, 85% after 2 years); 7-9 points (class B: survival, 81% after 1 year, 57% after 2 years) and 10-15 points (class C: survival, 45% after 1 year, 35% after 2 years). For an online calculator, see: http://www.liverpoolmedics.co.uk/clinicalcalculator/childpugh.php.

Measure	1 point	2 points	3 points
Total bilirubin, µmol/l (mg/dl)	< 34 (<2)	34-50 (2-3)	> 50 (>3)
Serum albumin, g/dl	> 3.5	2.8-3.5	< 2.8
Prothrombin time, prolongation (s)	< 4.0	4.0-6.0	> 6.0
Ascites	None	Mild	Moderate to Severe
Hepatic encephalopathy	None	Grade I-II (or suppressed with medication)	Grade III-IV (or refractory)

3.2. Results

Blood was collected from donors and serum prepared. An aliquot of each donor serum was examined for a variety of clinical chemistry endpoints. Table 2 demonstrates that for K^+, bilirubin, ALT, ALP and albumin, liver disease patient sera showed significant differences to healthy controls. Clinical scores also demonstrate significant chronic liver disease in donors.

Table 2: Clinical parameters for normal and liver disease sera used in detoxification studies. $^{\$}$based on n = 4 (due to one sample with high haemolysis); n/d, not determined, n/a not applicable. *Significantly different ($p < 0.05$) from control sera using the Student T test (two tailed). Note, the lactate normal range is 0.5 – 2.2 mM and urea normal range is 2.5 - 7.8 mM.

Parameter	Control sera (n=5)	Patient #1 68 years ♂ ARLD	Patient #2 60 years ♀ PBC	Patient #3 34 years ♂ ARLD	Disease patient Mean ± SD
Na^+ (mmol/l)	141 ± 1.1	139	135	142	139 ± 3.5
K^+ (mmol/l)	4.5 ± 0.37$^{\$}$	3.4	4.1	3.9	3.8 ± 0.36*
Creatinine (µmol/l)	83.4 ± 17.4	140	111	78	110 ± 31.0
NH_4 (µmol/l)	n/d	191	49	44	95 ± 83.5
Urea (mM)	n/d	9.3	8	4	2.0 ± 0.40
Total bile acids (µmol/l)	2.8 ± 0.84	92	35	336	150 ± 160
Lactate (mM)	n/d	2.4	1.6	2.1	2.0 ± 0.40
Bilirubin (µmol/l)	6.8 ± 1.30	70	32	243	120 ± 112*
ALT (units/ml)	22 ± 16.0	82	49	58	63 ± 17.1*
ALP (units/ml)	57 ± 16.0	170	172	147	160 ± 13.9*
Albumin (g/L)	49 ± 2.9	28	27	35	30 ± 4.4*
International Normalised Ratio (INR)	n/d	1.5	1.1	2.2	1.6 ± 0.56
MELD score	n/a	21	12	25	19 ± 6.7
Child Pugh score	n/a	12	9	10	10 ± 1.5
Additional comments		Child Pugh Grade C	Child Pugh Grade B	Child Pugh Grade C	
Ascites		Severe	Severe	Moderate	
Encephalopathy		Severe	none	None	

Using the MTT reduction assay, the viability of B-13, the human pancreatic cell line and H-13 cells and their hepatocyte-like derivatives (B-13/H, the human pancreatic cell line + DEX, and H-13/H) were determined. Figure 1 demonstrates that, compared with the 50% FCS control, control human serum was toxic to some of the human cells suggesting that healthy human serum contains factors that may adversely affect growth and/or viability. In contrast, serum from diseased liver patients was less toxic.

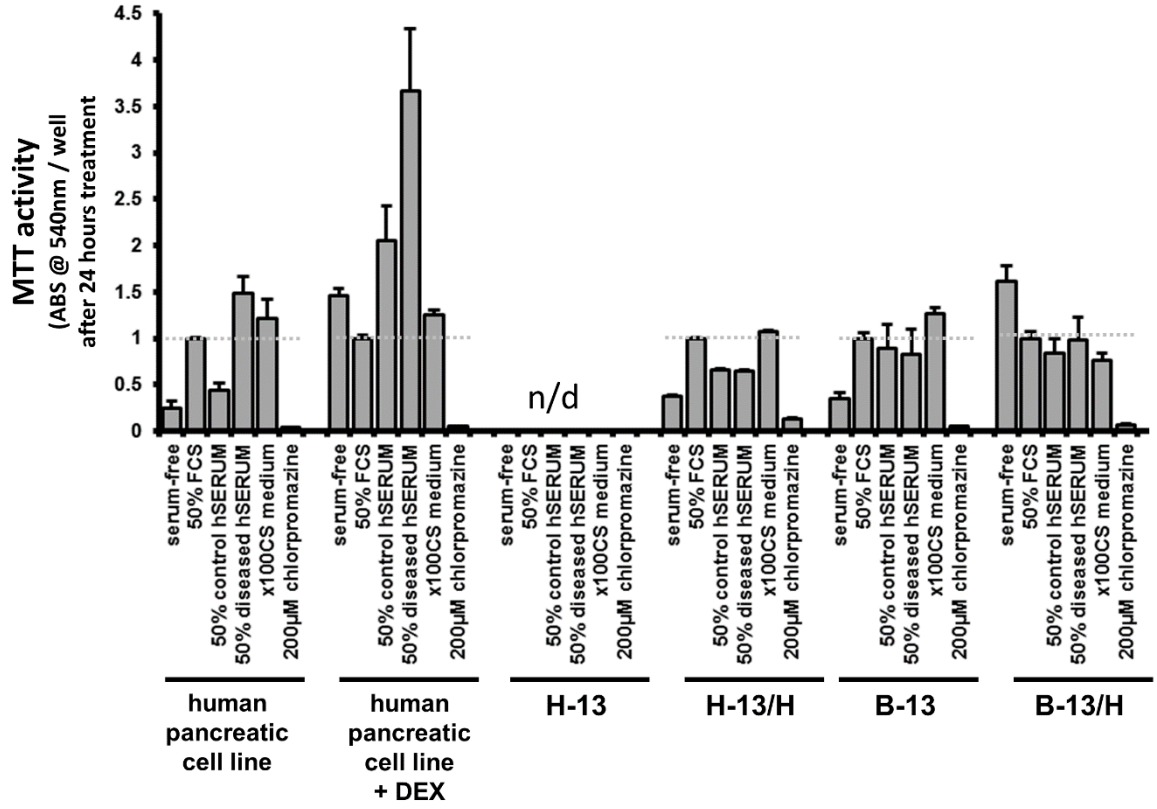

Figure 1: Effect of high concentrations of serum from healthy and liver disease patients and a synthetic cirrhotic sera on the viability of progenitor and progenitor-derived hepatocytes.

Cell lines were typically cultured with 10% foetal calf serum (FCS). However, the concentration of serum in a bioreactor being used in therapy is likely to be at least 50% (v/v). Cells were cultured serum-free (n=3) or with 50% FCS (n=3); 50% human serum (hSERUM) obtained from healthy individuals (n=5); 50% hSERUM obtained from patients with chronic liver disease (n=3); medium containing a synthetic serum containing endotoxins at levels 100-fold higher than calculated to be present in a cirrhotic patient (n=3) or 200 µM chlorpromazine, used as a positive toxic drug control (n=3). Cells were incubated for 24 hours prior to performing an MTT assay. Data are the mean and standard deviation MTT activity expressed relative to 50% FCS control for each cell type / treatment. Note, that there may have been different growth rates over 24 hours in the different sera and so therefore the "true control" MTT activity/well will likely be between the serum-free and 50% FCS values.

Note also that, due to a shortage of H-13 cells as a result of pressures to provide material for a variety of other analyses, incubation with H-13 has yet to be completed, but is expected to be similar to the human pancreatic cell line cells.

Using clinical chemistry assays, the effects of B-13, the human pancreatic cell line and H-13 cells and their hepatocyte-like derivatives (B-13/H, the human pancreatic cell line + DEX, and H-13/H) on medium toxins levels were determined (Tables 3 – 5).

In all cases, whether the cells were in progenitor or hepatocyte phenotype, there was clear evidence of viability in diseased serum in that there was a removal of glucose and generation of lactate in the medium, suggestive of active glycolysis. Given the near depletion of glucose in many cases, these data suggest that a replenishment of glucose and removal of lactate (which acidifies the medium) may be essential for cells in any future bioreactor.

In most cases, the cells generated increased levels of urea when exposed to diseased sera, suggestive of functional ammonia detoxification. However, the activities were somewhat similar in both progenitor and hepatic phenotypes.

In H-13/H cells there was a consistent low level reduction in bilirubin concentration when exposed to diseased sera, with some evidence of bilirubin conjugation.

Table 3. B-13 and B-13/H cell modulation of liver disease sera.

Parameter	**Patient #1** 68 years ♂ ARLD	**Patient #2** 60 years ♀ PBC	**Patient #3** 34 years ♂ ARLD
B-13 cells			
ALT (Units/ml)	-10	0	-4
Glucose depletion (mM)	5.6	3.3	5.4
Lactate production (mM)	8.8	5.6	9.2
Creatinine (µmol/l)	+2	0	0
Urea (mM)	+1.2	+1.1	+0.9
Free bilirubin (µmol/l)	-1	-3	-6
Conjugated bilirubin (µmol/l)	n/det	n/det	n/det
B-13/H cells			
ALT (Units/ml)	-38	0	+22
Glucose depletion (mM)	5.6	5.6	5.7
Lactate production (mM)	6.5	8.0	10
Creatinine (µmol/l)	-40	+14	+1
Urea (mM)	-1.3	+0.3	+1.7
Free blirubin (µmol/l)	-7	+22	+15
Conjugated bilirubin- (µmol/l)	n/det	27	-10

DMEM culture media was mixed 1:1 (v/v) with serum isolated from 3 separate liver disease patients (see Table 2 for details) and incubated with cells for 3 days. n/det, not detectable. Lactate, normal range in serum 0.5 - 2.2 mM; urea, normal range in serum 2.5 - 7.8 mM.

Table 4: The human pancreatic cell line and human pancreatic cell line + DEX treatment cell modulation of liver disease sera.

Parameter	**Patient #1** 68 years ♂ ARLD	**Patient #2** 60 years ♀ PBC	**Patient #3** 34 years ♂ ARLD
Human pancreatic cell line			
ALT (Units/ml)	-25	+2	-3
Glucose depletion (mM)	5.6	5.6	5.8
Lactate production (mM)	10.3	10	9.0
Creatinine (µmol/l)	+2	+10	+9
Urea (mM)	+0.9	+1.1	+0.7
Free bilirubin (µmol/l)	+1	0	-1
Conjugated bilirubin (µmol/l)	n/det	n/det	-17
Human pancreatic cell line + DEX cells			
ALT (Units/ml)	-16	+1	-5
Glucose depletion (mM)	5.3	5.6	5.6
Lactate production (mM)	9.9	10.2	9.8
Creatinine (µmol/l)	+1	-2	+1
Urea (mM)	+0.8	+0.8	+0.8
Free bilirubin (µmol/l)	-14	0	-3
Conjugated bilirubin (µmol/l)	n/det	n/det	-18

DMEM culture media was mixed 1:1 (v/v) with serum isolated from 3 separate liver disease patients (see Table 2 for details) and incubated with cells for 3 days. n/det, not detectable. Lactate, normal range in serum 0.5 - 2.2 mM; urea, normal range in serum 2.5 - 7.8 mM.

Table 5: H-13 and H-13/H cell modulation of liver disease sera.

Parameter	Patient #1 68 years ♂ ARLD	Patient #2 60 years ♀ PBC	Patient #3 34 years ♂ ARLD
H-13 cells			
ALT (Units/ml)	-14	-2	-4
Glucose depletion (mM)	4.4	4.5	3.3
Lactate production (mM)	8.9	8.7	7.1
Creatinine (µmol/l)	0	-2	0
Urea (mM)	+0.7	+0.9	+0.8
Free bilirubin (µmol/l)	-1	-2	+1
Conjugated bilirubin (µmol/l)	n/det	n/det	+15
H-13/H cells			
ALT (Units/ml)	-28	-2	-4
Glucose depletion (mM)	1.7	2.7	2.0
Lactate production (mM)	3.6	5.7	4.3
Creatinine (µmol/l)	-2.3	0	+5
Urea (mM)	-1.2	+0.8	+1.1
Free bilirubin (µmol/l)	-5	-2	-3
Conjugated bilirubin (µmol/l)	n/det	n/det	+19

DMEM culture media was mixed 1:1 (v/v) with serum isolated from 3 separate liver disease patients (see Table 2 for details) and incubated with cells for 3 days. n/det, not detectable. Lactate, normal range in serum 0.5 - 2.2 mM; urea, normal range in serum 2.5 - 7.8 mM.

4. Conclusions

These data indicate that an engineered human pancreatic progenitor cell line is able to tolerate exposure to sera from liver disease patients and can mediate a low level functional detoxification in 2D culture. These data support their continued development and assessment in 3D culture, since this may increase functionality and serum detoxification.

5. References

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