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ORIGINAL ARTICLE

Co-transplantation of encapsulated HepG2 and rat Sertoli cells improves outcome in a thioacetamide induced rat model of acute hepatic failure

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Summary

Hepatocyte transplantation offers therapeutic opportunities in liver disease. Xenogeneic hepatocytes are a potential resource, but rejection presents a major problem. We combined cell encapsulation with modulation by local generation of an immunosuppressant by co-encapsulating Sertoli cells with HepG2 cells. We assessed *in vitro* rat leukocyte proliferative responses and HepG2 cell survival after intraperitoneal injection in rats. Empty beads, and beads containing HepG2 cells or HepG2/Sertoli cells were injected intra-peritoneally into rats and survival of implanted cells followed over 4 weeks; in some animals acute hepatic failure (AHF) using thioacetamide (TAA) was also induced. The marked proliferative response of rat leukocytes to HepG2 cells and HepG2-containing beads was reduced by Sertoli cell-conditioned medium and HepG2/Sertoli encapsulates. After intra-peritoneal transplantation, Sertoli cells co-encapsulation protected the HepG2 cells in normal and AHF animals. Combined encapsulation and locally generated immuno-suppression may be a valuable strategy in hepatocyte transplantation.

Introduction

Hepatocyte transplantation offers therapeutic opportunity in acute and chronic liver disease and many inborn errors of metabolism. Problems with the availability of human hepatocytes, and technical issues concerning the expansion of primary hepatocytes in culture and cryopreservation have prompted consideration of the use of xenogeneic hepatocytes [1]. Despite intrinsic immunosuppressive actions inherent in isolated hepatocytes [2] prevention of rejection constitutes a major challenge in the development of xenogeneic liver cell transplantation.

Cell encapsulation inside a membrane, which prevents direct access of host cells to the transplant, and impedes transfer of large immunologically active molecules, provides one strategy, which has prolonged survival of grafted allogeneic and xenogeneic cells [3,4]. Transplant survival has been prolonged by encapsulation within microporous polypropylene [5], acetonitrile methallyl sulphonate co-polymers [6,7], and alginate with poly-L-lysine (PLL) [4]. This approach does not prevent the development of immune responses to transplant-derived peptides, which can cross the physical barrier. Conventional systemic immunosuppression can be utilized to prolong graft survival, but modulation of rejection by the generation of an immunosuppressant locally is an attractive alternative approach. One such approach is co-transplantation of Sertoli cells within cell grafts [8,9].

Sertoli cells are central to the immune privilege demonstrated in the testis, and their actions have been utilized to protect cell transplants [10]. Co-transplantation of Sertoli cells prolonged survival of allogeneic dopaminergic neurones in rat brain [11], and of both allogeneic and xenogeneic encapsulated pancreatic islets transplanted into rat peritoneum [8,9]. The protective effect has been attributed variously to Sertoli cell production of Fas-ligand (CD95L), which interacts with Fas-expressing T-cells [12,13], and to release of TGF-beta [14]. Here

we applied this approach to prolonging survival of a xenogeneic hepatocyte-derived cell line in rat peritoneum. Cells of the human hepatocyte-derived cell-line HepG2 were encapsulated with or without rat Sertoli cells in alginate-PLL beads, a technique which immobilizes cells and encourages the formation of close cell-to-cell contact and maintenance of three-dimensional architecture in hepatocytes [15,16]. This form of alginate encapsulation imposes a physical barrier to cell ingress, but does not impede macromolecular egress.

Methods

Animals

Wistar rats were obtained from Charles River U.K. Ltd., Manston Road, Kent, UK. Males, 250–300 g were used for splenic white cell isolation. Males age 15–20 days were used for Sertoli cell isolation. All animals were maintained and experiments conducted in the Biological Services Unit at Hammersmith Campus, Imperial College School of Medicine. Animal experiments and care were as approved by the UK Home Office, and accorded with the *Principles of laboratory animal care* (NIH publication 86-23, revised 1985).

HepG2 cells, obtained from ECACC (Wiltshire, UK), were seeded as monolayers in 175 cm^2 triple-flasks and 80 cm^2 tissue culture flasks, at a density of 5 million cells in 50–75 ml and 300 000 cells in 10 ml, respectively, cultured at 37 °C in a humidified atmosphere of 95% air:5% CO_2 in supplemented α minimum essential medium, α-MEM (Gibco, Paisley, UK) and the medium replenished every 48 h.

Alginate-PLL beads

The method of Selden *et al.* [15] was used to prepare alginate beads which were coated with PLL (MWt cut-off 150 000) according to the procedure described by King *et al.* [17]. Briefly, alginic acid sodium salt from *Macrocystis pyrifera*, [medium viscosity, (Sigma Aldrich Poole, UK)] was gradually dissolved in 0.15 M NaCl (pH 7.4) to give a 2% w/v solution and sterilized. In some experiments the alginate preparation was mixed with cells at 0.5×10^6/ml [HepG2 cells only (HepG2) or HepG2 and Sertoli cells at a ratio of 20:1 (HepG2:Sertoli)]. Alginate was diluted with complete α-MEM to yield 1% alginate. This suspension was loaded into a syringe fitted with a 23G co-axial cannula. The cannula system was connected to an air supply, and the syringe piston controlled by an adjustable valve. An air-flow of 1300–1400 cc/min and a liquid flow of 1.5 ml/min yielded micro-droplets of 400–500 μm diameter. Droplets were collected in the divalent cation solution, calcium chloride (0.102 M in 0.15 M NaCl, pH 7.4). Following sufficient gentle stirring in the cationic solution (15 min) beads were washed with isotonic sodium chloride, incubated with 0.1% 2-(N-Cyclohexylamino)-ethanesulfonic acid (CHES) buffer solution, then incubated with 0.01% PLL solution for 8 min, washed with 0.1% CHES solution, incubated in 0.03% alginate solution for 10 min, washed in sodium chloride solution and finally put into culture medium. Beads were collected into a 175 cm^2 tissue culture flask. Beads containing HepG2 cells with/without Sertoli cells were cultured for 8 days prior to implantation in transplantation experiments, to allow expansion of the HepG2 cell population and allow expression of differentiated hepatocyte function [15], or used as stimulators in leukocyte proliferation assays. The 8-day incubation allows HepG2 cell proliferation, and results in optimum *per cell* performance reflected by protein synthesis *in vitro* at rates reflecting those found *in vivo* [17].

Sertoli cell isolation

The method of Galdieri *et al.* [18], modified by Onoda *et al.* [19] was followed. Briefly, 15–20 day male Wistar rats were killed and the testes removed and placed in a sterile beaker containing Dulbeccos Modified Eagles Medium (DMEM; Life Technologies Ltd., Paisley, UK), decapsulated, minced, and incubated for 15 min in a shaking water bath at 37 °C, first with trypsin (1.5 mg/ml), and DNAase 20 μg/ml, and then with collagenase 2 mg/ml plus hyaluronidase 2 mg/ml. Aggregates of cells, sedimented initially under gravity and then by centrifugation at 80 g for 4 min, were collected discarding the supernatant, which contains predominantly peritubular cells. Further trypsin digestion (0.5 mg/ml, EDTA 0.1 mM) yielded a Sertoli-rich preparation which was washed in 10% foetal calf serum/DMEM and pelleted by centrifugation at 150 g for 5 min. The pellet was re-suspended in serum free defined medium (SFDM: DMEM + penicillin 100 U/ml, fungizone 0.25 ug/ml, streptomycin 100 μg/ml, follicle stimulating hormone 100 ng/ml, insulin 2 U/ml, epidermal growth factor 10 ng/ml, human transferrin 5 μg/ml, vitamin A 50 ng/ml, vitamin E 200 ng/ml, hydrocortisone 10^{-9} M, testosterone 10^{-7} M, estradiol 10^{-8} M, glutamine 2 mM, sodium selenite 5 ng/ml and cytosine arabinoside 3 μg/ml) and plated in six-well plates in SFDM at a density of 2×10^6 cells/well for 72 h (5% CO_2/95% air at 34 °C). Fifty microlitre of 20 mM Tris-HCL buffer (pH 7.5) was added to cells for 2.5 min to lyse the germ cells remaining within the isolate.

In vitro leukocyte responses to HepG2 cells

Spleens were harvested from young adult rats and mechanically disaggregated in DMEM, red cells were lysed in distilled water containing ammonium chloride 0.15 M,

potassium bicarbonate 1.0 mM, disodium EDTA 0.1 mM and seeded into 96 well plates at 100 000 cells per well. In some experiments leukocytes were seeded onto plates containing a monolayer of HepG2 cells. In other experiments, leukocytes were added to alginate beads (either empty, or containing HepG2 cells or HepG2/Sertoli cells) on a cell strainer. Lymphocyte proliferation induced in response to HepG2 cells or beads was assessed after a total culture time of 120 h by addition of 1 μCi [^{3} H] methylthymidine to leukocytes for the last 18 h, after they had been physically separated from the stimulating cells or beads. Cells were harvested on a semi-automatic cell harvester.

Transplantation experiments

Five millilitre of alginate-PLL bead suspension, or similar beads containing HepG2 cells or HepG2/Sertoli cells after 8 days incubation, were injected intraperitoneally into young male Wistar rats under isofluorane anaesthesia (10 animals in each group). Animals were killed either 1 or 4 weeks after transplantation. In a further set of experiments similar beads were transplanted into three similar groups of 10 animals, 48 h after they had been administered the first of two injections of TAA (400 mg/kg) 18 h apart. This regime of TAA induces acute hepatitis with a mortality of approximately 70% by 96 h after administration of TAA.

Removal of transplanted beads

Recipient animals were anaesthetized using isoflurane inhalation. The abdomen was opened and the beads removed using a 18 G blunted needle. The beads were fixed in formal saline or were separated and prepared for electron microscopy.

Electron microscopy

Cells recovered from beads were fixed with 2 ml of cacodylate buffer pH 7.2 containing 0.075% glutaraldehyde.

Results

Rat lymphocyte responses to HepG2 cells *in vitro* and modulation by Sertoli cells and Sertoli conditioned medium

Initial experiments demonstrated the presence of a rat lymphocyte proliferative response to cultured HepG2 cells. As HepG2 cells proliferate during the course of the experiment, the nonadherent leukocyte population was separated from the HepG2 layer after 2 days, and incubated for a further 3 days to demonstrate a proliferative response (data not shown).

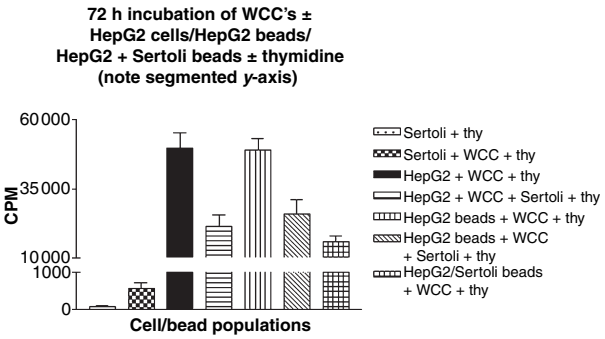


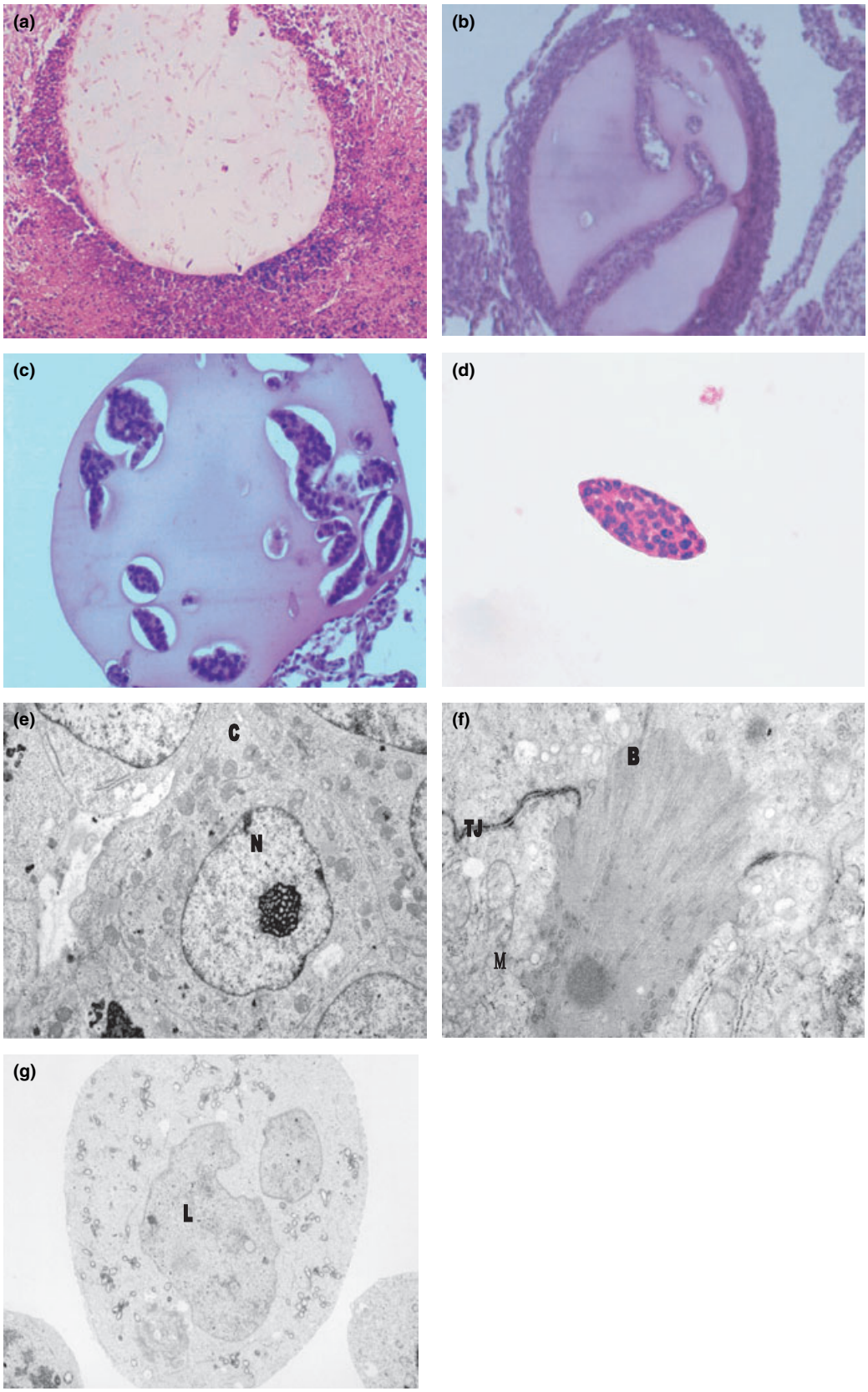
Figure 1 Thymidine incorporation after incubation of isolated rat leukocytes with HepG2 cells with/without Sertoli conditioned medium, HepG2 containing beads with/without Sertoli conditioned medium and beads that contain both HepG2 and Sertoli cells. The graph illustrates significant reduction in c.p.m. if Sertoli medium or cells are present within the incubation medium (*$P < 0.05$). Typical results from one experiment (observations in quadruplicate, repeated on three occasions; mean ± $SD_{(n-1)}$).

Rat leukocytes were exposed to alginate capsules containing HepG2 cells, and empty alginate beads. The full beads initiated a significant proliferative response in the leukocytes, tested after they had been separated from the beads (to HepG2 cells 45 920 ± 4898 c.p.m. 3H thymidine incorporated, to empty beads 1445 ± 215 c.p.m.). Subsequently conditioned medium from Sertoli cells was shown to significantly inhibit but not abolish the proliferative response to HepG2 cells encapsulated in alginate (25 812 ± 5330 c.p.m. with Sertoli medium, 49 024 ± 4168 c.p.m. without). Co-encapsulation of Sertoli cells with HepG2 cells in a ratio of 20:1 HepG2:Sertoli cells also resulted in a diminution of the proliferative response to encapsulated cells (Fig. 1).

Histological appearances of beads containing HepG2 cells after intraperitoneal transplantation in rats

Alginate/PLL beads empty of cells were recovered at 1 week and 1 month after transplantation from normal animals (Fig. 2a). Beads remained intact, with a minor surrounding inflammatory cell infiltrate. In beads containing HepG2 cells, these appeared viable and healthy on histological grounds at week 1. By 4 weeks, beads containing HepG2 cells were often fragmented, and only a few cells were present, showing marked cytoplasmic vacuolation, and there was an intense leukocytic reaction both surrounding and infiltrating into the bead (Fig. 2b).

In contrast HepG2/Sertoli beads recovered from a normal animal at week and 1 month demonstrated surviving HepG2 cells with a healthy appearance at both time points and a less florid host white cell reaction (Fig. 2c and d). Electron microscope (EM) studies demonstrated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that HepG2 cells maintained normal cell structure with abundant organelles, golgi apparatus, endoplasmic reticulum and tight junctions (Fig. 2e and f). Electron microscope, EM confirmed survival of Sertoli cells from these beads at 1 month. Figure 2g demonstrates a Sertoli cell recovered from a bead in a normal animal, showing the large lipid vacuole that characterizes this cell type.

In animals implanted with empty beads 48 h after induction of hepatic failure by TAA, mortality was 80% over 5 days and 100% at 1 week. Survival was higher in the animals receiving beads containing cells, and highest in those receiving HepG2/Sertoli cells (Fig. 3). Notably however the experiment was not designed to investigate survival advantage to the animals. With the high mortality in all groups, observations on the fate on implanted beads were limited in number. In the 1-week beads originally containing HepG2 cells alone, there were small numbers of surviving cells showing severe damage and a marked leukocyte infiltrate, at light and EM level (Fig. 4a and c). EM displayed marked cell vacuolation, and disruption of intracellular architecture. In contrast in beads containing HepG2/Sertoli cells, at 1 week HepG2 cells appeared healthy at both light and EM level (Fig. 4b, d and e).

At 1 month, no HepG2 cells were identifiable in any beads in animals implanted with HepG2-containing beads and all beads identified were infiltrated with leukocytes and appeared heavily fibrosed and attached to adjacent structures (e.g. the liver capsule or the mesentery) (Fig. 4f). In contrast in HepG2/Sertoli containing beads from animals at 1 month after induction of acute hepatic failure (AHF) followed by bead implantation, HepG2 colonies could readily be identified. EM confirmed normal appearances in some surviving HepG2 cells with preserved biliary canaliculi, although the morphology of the surviving Sertoli cells was less well preserved (Fig. 4g and h).

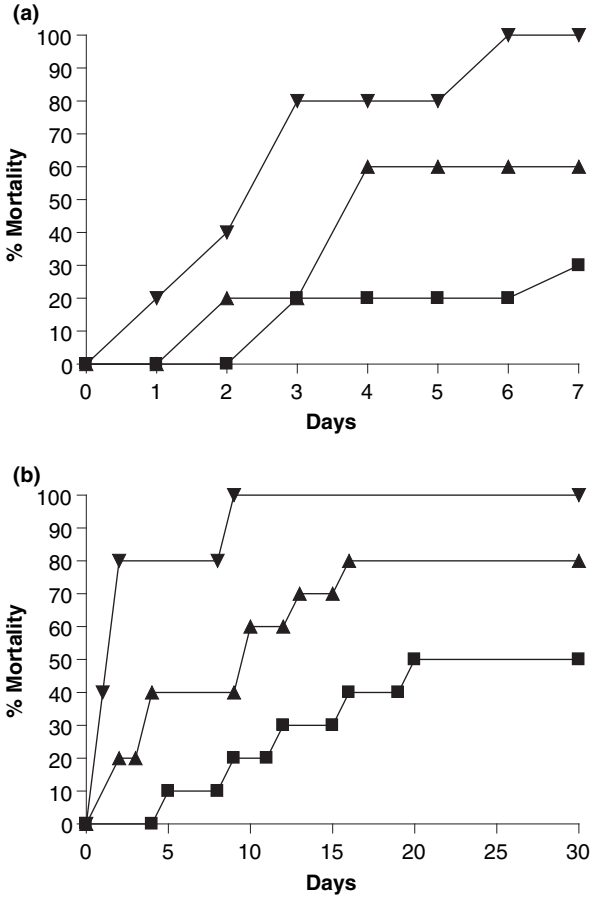


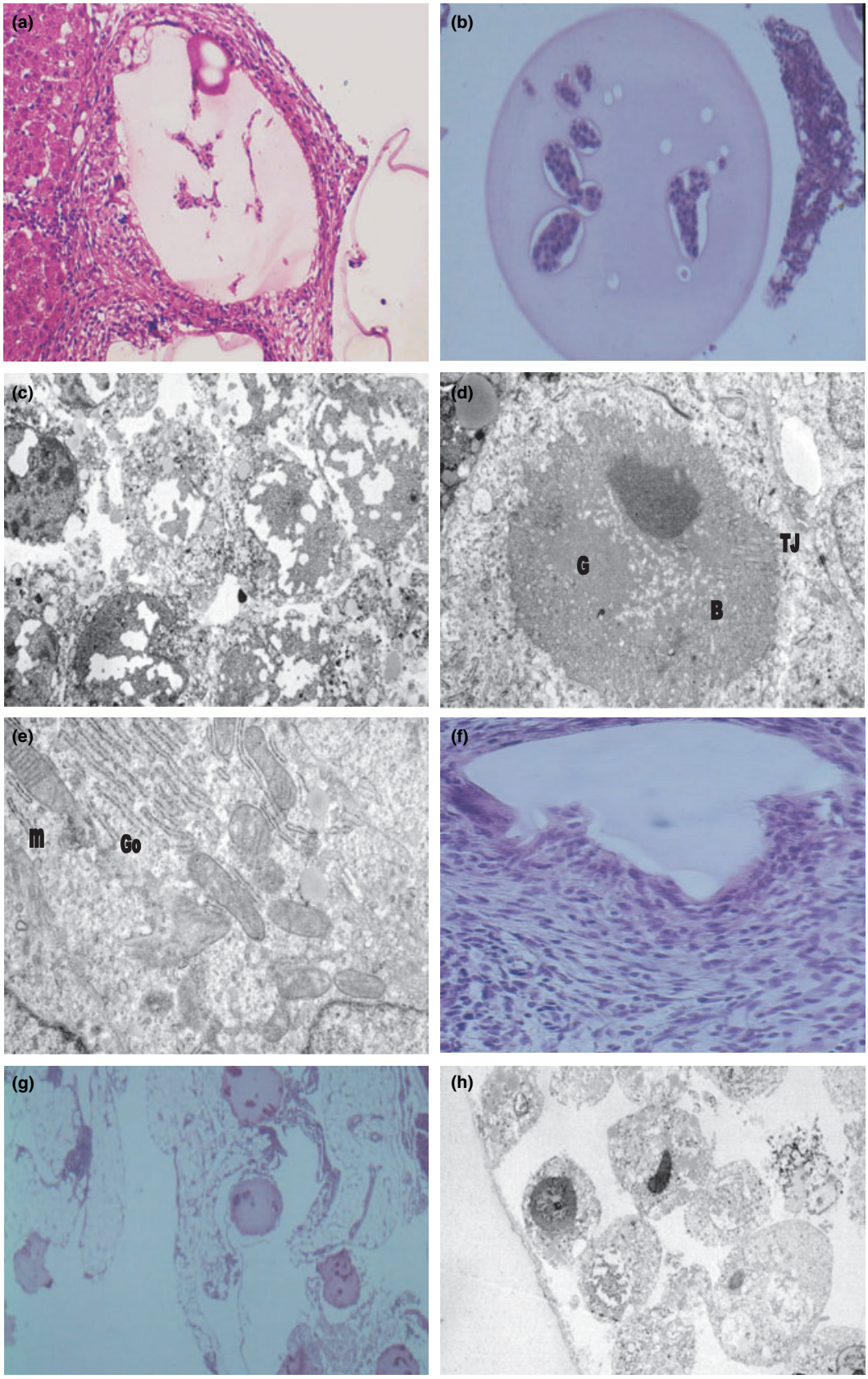
Figure 3 Mortality in animals at 7 days (a) and 30 days (b) post-transplantation after AHF was induced with thioacetamide (TAA). Forty-eight hours following the second dose of TAA, animals received intra-peritoneal transplants of 5 ml of empty alginate coated beads, HepG2 containing beads or HepG2 and Sertoli cell containing beads. There is a lower mortality associated with the HepG2/Sertoli containing beads compared with empty beads (*$P < 0.05$ log rank test). Empty beads = ▲; HepG2 beads = ▼; HepG2/Sertoli beads = ■.

Discussion

This study successfully combined two techniques, encapsulation of cells in alginate-PLL coated beads and manipulation of the attributed immunoprotective effect of Sertoli cells by co-encapsulation to prolong the survival of xenogeneic HepG2 cells transplanted into the peritoneum of the Wistar rat.

We demonstrated the expected proliferative response of rat splenocytes to xenogeneic liver-derived cells using the

Figure 2 (a) H&E stained empty agarose mounted alginate/PLL coated bead retrieved from a normal animal after 1 week. Note the intense white cell reaction around the outside of the bead. × 40. (b) H&E stained bead mounted in agarose. Section is from a HepG2 containing bead from a normal animal at 1 month. This clearly shows the thick encasement of white cells that has led to a fibrosed, attached bead. Also note that host white cells have infiltrated the bead. × 40. (c) H&E stained agarose mounted HepG2/Sertoli containing bead. Colonies of normal-appearing HepG2 cells retrieved from a normal animal after 1 week. Note the clumps of white cells attached to the outside of the bead. × 40. (d) H&E stained clump of normal-appearing HepG2 cells recovered from a HepG2/Sertoli cell containing bead from a normal animal after 1 month. Magnification ×100. (e, f) Electron micrographs of hepatocytes from a recovered HepG2/Sertoli bead at 1 month from a normal animal. HepG2 cells with normal cell structure and abundant organelles, golgi apparatus, endoplasmic reticulum and tight junctions (TJ) (C, cytoplasm; N, nucleus; B, biliary canaliculi; M, mitochondria). Magnification ×3600 (e) and 12 000 (f). (g) Electron micrograph of a Sertoli cell from a recovered HepG2/Sertoli bead (magnification ×6000) from a normal animal at 1 month. The Sertoli cell is round and has a central large lipid vacuole that characterizes this cell type and the smaller lipid droplets present within the cell structure.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HepG2 cells. Encapsulation of the HepG2 cells in alginate behind a layer of PLL did not prevent this proliferative response, compatible with other data that such encapsulation does not constrain potentially immunogenic molecules within a diffusion barrier. Sertoli cells, via a soluble mediator, could inhibit this response, although it was not entirely abolished.

Subsequently we transplanted empty capsules, capsules containing HepG2 cells alone, and HepG2 cells plus Sertoli cells, into the peritoneum of rats. Empty capsules elicit an inflammatory response, which has been characterized as cytokine-mediated, in particular via TNF-alpha production locally, and has been attributed to the PLL component of the bead. Manipulation of PLL concentration has been shown to reduce this effect, but in our studies it appeared that the fibrotic reaction was least when capsules containing Sertoli cells were used, suggesting active immunosuppression is a potential means of preventing such fibrosis. The most significant finding, however, was that the survival of encapsulated HepG2 cells was prolonged, and hepatocytic morphology better preserved, when Sertoli cells were also present. In further experiments, we damaged the host liver 2 days prior to implantation, with the hepatotoxic agent TAA. We reasoned that the toxic agent initiating damage to hepatocytes would have been metabolized at this time, but a variety of stimuli to survival and proliferation of liver cells (e.g. Hepatocyte Growth Factor, HGF; albumin–bilirubin complexes [20]) would be present in the circulation, although their effect might also be modified by factors unfavourable to hepatocyte survival that we and others have demonstrated in liver failure plasma [21,22]. Intriguingly, Sertoli cells also carry HGF receptors, so complex pathways of interaction between the milieu of liver failure, Sertoli cell function, and HepG2 cell survival and function may occur [23]. Only in the presence of Sertoli cells, in the group of animals with AHF, was it possible to recover some viable HepG2 cells 4 weeks after transplantation. Without the Sertoli cells, no HepG2 cells were found at 1 month, and indeed at 1 week there was a profound inflammatory response to the HepG2-containing capsules.

The experiments were designed to examine cell survival and histology following transplantation. They were not designed to investigate a survival advantage and were not suitably powered for this end-point. Others have demonstrated beneficial effects of encapsulated and nonencapsulated hepatocytes in animals models of liver failure [24,25]. It is intriguing that in animals implanted with capsules 2 days after the initiation of TAA induced liver failure, during the phase in which hepatocytes are under intense stimulus to proliferate, mortality was reduced in animals that had received the combined HepG2 and Sertoli cell capsules. Further experiments would however be necessary to investigate this further, both to confirm benefit from using this cell line and to demonstrate that Sertoli cells were critically important in altering survival.

The mechanism of the immunosuppressive action of Sertoli cells and Sertoli conditioned medium was not investigated in this study, and there is evidence that the action may be complex and multifactorial. Studies on immunoprivilege in the testis suggested release of Fas ligand from Sertoli cells to be the mechanism, acting by binding to Fas-expressing activated T-cells [12,26]. D'Alessio *et al.* [27] have now indicated that Fas ligand associated with Sertoli cells in the testis may be synthesized by other cellular elements such as meiotic spermatocytes and haploid spermatids, and Sertoli cells themselves do not express FasL mRNA. Recently Suarez-Pinzon *et al.* [14] indicated that implants of pancreatic islets with Sertoli cells were protected from rejection not by FasL but by transforming growth factor-beta (TGF-beta). Plasma levels of TGF-beta in animals that received the islet and Sertoli cell transplants were significantly higher than in those receiving islets cells, and anti-TGF-beta antibody abrogated the protective effect, whereas the administration of anti-FasL antibody did not. The existence of a mechanism other than

Figure 4 (a) H&E stained section mounted in agarose ×40. Section taken from a HepG2 containing bead retrieved at 1 week from an AHF induced animal. This shows a marked reduction in HepG2 cell number and a very marked infiltrate of pleomorphic white cells. On the left of the section normal looking hepatic parenchyma is seen. (b) H&E stained bead mounted in agarose ×40. Section from a HepG2/Sertoli bead from an AHF animal at 1 week. HepG2 cells appear normal in colonies and the bead has maintained its structure. There is a large mass of white cells in a clump to the right of the bead. This bead had been attached to the mesentery of the small bowel. (c) Electron micrograph of cell from beads containing HepG2 cells from animals in AHF at 1 week (×3000). The HepG2 cells are in very poor condition with marked vacuolation, loss of lipid droplets and glycogen granules. Organelles have ruptured and the cell membrane is also disrupted. (d, e) Electron micrographs of hepatocytes from a HepG2/Sertoli bead from an AHF animal at 1 week (D ×7000, E ×10 000). HepG2 cells appear normal. This bead had been attached to the mesentery of the small bowel. (B, bile canaliculi; G, glycocalyx; Go, golgi apparatus; M, mitochondria; TJ, tight junction and desmosomes). (f) H&E stained bead mounted in agarose ×40. Section is from a HepG2-containing bead from an AHF animal at 1 month. Few beads were present when the abdomen was inspected at retrieval. Amongst the beads recovered none had any remaining HepG2 cells. All beads were heavily fibrosed and attached to the liver capsule or to the mesentery. (g) H&E stained section mounted on agarose (x20) illustrating HepG2/Sertoli beads recovered from an AHF animal at 1 month. This section shows several beads in peritoneal tissue (fat and muscle). The HepG2 colonies appear to be fewer than in the 1 week beads. (h) Electron micrograph of cells from HepG2/Sertoli beads at 1 month from a surviving AHF animal ×3000. Hep H2 cells appear abnormal in some sections, but normal in others. Large numbers are still present.

FasL-Fas interaction underlying the protection noted in this system is supported by our failure to demonstrate FasL in Sertoli-HepG2 capsules by immunoassay after *in vitro* incubation (data not shown). Furthermore, as hepatocytes express Fas, expression of FasL by Sertoli cells in this system would be more likely to lead to deleterious rather than protective effects on hepatocyte survival.

Other potential Sertoli cell products could also play a role – either as immunosuppressants, as stimulators of hepatocyte survival and proliferation acting directly on implanted cells, or as anti-inflammatory substances. Candidates from Sertoli cells under these headings include interleukin (IL)-6 and fibroblast growth factor (FGF) [28,29]. Interestingly, a combined approach of modifying hepatocyte encapsulation procedures, and co-encapsulation with bone marrow stem cells, has recently been reported to lead to prolongation of hepatocyte survival in the peritoneum in an allogeneic rat model, providing another example in which an alternative cell-type may protect implanted liver cells [30]. Having established in this study that Sertoli cell implantation can protect xenogeneic liver-derived cells, mechanistic studies may provide strategies that could be applied clinically to protect hepatocyte implants – xenogeneic or allogeneic – in man.

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