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Discarded Livers Find a New Life: Engineered Liver Grafts Using Hepatocytes Recovered From Marginal Livers

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Abstract

Treatment for end-stage liver failure is restricted by the critical shortage of donor organs; about 4000 people die in the USA while waiting for a transplant-able organ. This situation has been a major driving force behind the rise of tissue engineering to build artificial tissues/organs. Recent advancements in creating transplant-able liver grafts using decellularized liver scaffolds bring the field closer to clinical translation. However, a source of readily available and highly functional adult hepatocytes in adequate numbers for regenerative liver therapies still remains unclear. Here, we describe a new method to utilize discarded livers to make transplantable new liver grafts. We show that marginal donor livers damaged due to warm ischemia could be treated with machine perfusion to yield 39 million viable hepatocytes per gram of liver, similar to fresh livers, and these cells could be used to repopulate decellularized liver matrix (DLM) scaffolds to make transplantable liver grafts. The hepatocytes from recovered livers sustained their characteristic epithelial morphology while they exhibited slightly lower protein synthesis functions both in plate cultures and in recellularized liver grafts. The dampened protein synthesis was attributed to residual endoplasmic reticulum stress found in recovered cells. The results here represent a unique approach to reengineer transplantable liver grafts solely from discarded organs.

Keywords

Machine perfusion; Hepatocytes; Liver recellularization; Endoplasmic reticulum stress

Treatment for end-stage organ failure is restricted by the critical shortage of donor organs with the organ waiting list currently at 123 000 requests, a number that far exceeds the

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supply of available organs and that continues to grow by 5% each year. This situation has been the major driving force behind the rise of whole-organ engineering that aims to build transplantable organ substitutes to address the void in organ replacement therapies (1). One limiting aspect in whole-liver engineering is the lack of a reliable cell source for primary adult hepatocytes. Pluripotent stem cells have the potential to generate an abundant supply of functional hepatocytes for use in cell-based therapies (2). However, while a number of protocols have derived “hepatocyte-like” cells from a pluripotent state, prohibitive limitations such as low yield and incomplete maturation still persist (3). Moreover, the protocols that describe the generation of the differentiated cells take 2 to 4 weeks, substantially increasing the time, cost, and complexity of engineering whole livers for transplantation (3). A human-sized liver graft will need at least 10% of the native liver mass, which corresponds to tens of billions of hepatocytes; a scale that is not currently achievable using available technologies in stem cell expansion and differentiation (4).

Interestingly, there is an excess number of organ donors, most of which are not considered for transplantation because their organs are damaged. For example, accident victims who arrive at the hospital after cardiac death are not eligible donors because of excessive ischemic damage. By some estimates, the potential number of donors after cardiac death (DCD) is over 200 000 per year in the USA, and about 6000 are considered only marginally damaged (5). Recently, we have developed techniques to recover marginal ischemic rat livers, which result in primary hepatocytes with yield and function comparable to those obtained from fresh livers (6). We also demonstrated that ischemic whole rat livers can be decellularized to create whole-liver scaffolds, and furthermore, that adult hepatocytes can be seeded into these scaffolds, remaining viable and providing essential liver functions in vitro (7,8). In this same study, we evaluated various recellularization methodologies and established that perfusion cell seeding led to a uniform distribution of hepatocytes within the parenchymal space. The recellularized grafts can later be heterotopically transplanted in recipient rats and remain functional in a 24 h ex vivo whole blood perfusion model (7,9). In tandem, these novel technologies provide a unique opportunity to create transplantable liver grafts solely from donor livers that are discarded because they are not found suitable for transplantation. In this study, we report on machine resuscitation of marginal rat livers for high yields of viable primary hepatocytes for the repopulation of decellularized liver matrix (DLM) scaffolds. The liver grafts recellularized with recovered hepatocytes function at levels comparable or lower than those recellularized with hepatocytes from fresh livers. Residual endoplasmic reticulum (ER) stress was evaluated as a possible reason for dampened protein synthesis functions.

Materials and Methods

Animals and liver harvest

All animal care, handling, and surgical procedures were performed in accordance with the guidelines set by Institutional Animal Care and Use Committee (IACUC) at Massachusetts General Hospital. Livers were harvested according to the technique of Delriviere et al. (10) and described previously (6). As the control (Fresh), livers were immediately prepared for hepatocyte isolation. For the experimental ischemia group (Recovered), livers were placed in

a temperature-controlled chamber filled with saline and maintained at 34°C for 1 h for induction of warm ischemia during which time they were cuffed. This model has been previously developed as a DCD model (11) which mimics the temperature profile in vivo and is twice the currently acceptable limit of 30 min in transplant centers. Livers harvested for decellularization were gently flushed with saline and stored in saline at 4°C until utilized.

Recovery of livers for hepatocyte isolation and preparation of DLM scaffolds

The methods for machine perfusion of livers for recovery from warm ischemia have been reported previously (12) and DLM scaffolds were prepared according to (7).

Hepatocyte culture

Hepatocytes isolated from Fresh and Recovered livers were cultured in collagen sandwich static culture (13) or seeded in DLM scaffolds by perfusion to form recellularized liver grafts (7). The culture medium consisted of high glucose DMEM (Invitrogen) supplemented with 10% FBS (Fisher), 2% penicillin-streptomycin (Pen-strep) (Invitrogen), 0.5 U/mL insulin (Novolin), 7.5 µg/mL hydrocortisone (Pfizer), 14 ng/mL glucagon (Bedford Laboratories), 20 ng/mL epidermal growth factor (EGF) (Invitrogen). Medium was changed daily and sampled for functional analysis for 7 days.

For recellularized liver grafts, the median lobe of a DLM scaffold was resected and connected to the perfusion system through portal vein cannulation. The liver was kept immersed in the perfusate in a perfusion chamber while perfused through the portal vein. Portal pressure was maintained between 10 and 15 cm H₂O during a 30-min perfusion with medium. Next, approximately 80 million hepatocytes with over 90% viability were infused into the circuit in four subsequent injections at 10-min intervals as described previously (7,8). The cells were recirculated in the system for 40 min at 37°C. At the end of 40 min, the perfusate was collected, the viability and number of cells that were not retained in the scaffold was determined using a hemacytometer and trypan blue exclusion. Total number of cells retained in DLM was the difference between the initial number of cells seeded and the number of cells present in the perfusate at the end of seeding.

Next, the recellularized liver graft was transferred to a sterile closed chamber and connected to the same machine perfusion setup described above (Fig. 1A). The complete system was placed in an incubator for temperature control, and the oxygenator was connected to a gas cylinder that contained atmospheric mixture. The medium was changed daily for a total of 7 days.

Analysis of hepatocyte functions, histology, and gene expression

Media samples from plate cultures and recellularized liver grafts were analyzed for albumin content using enzyme linked immunosorbent assay as described previously (14) using a polyclonal antibody to rat albumin (Cappel Laboratories, Aurora, OH, USA). Urea content was measured with diacetylmonoxime with a commercially available kit (StanBio Laboratory, Boerne, TX, USA). The data reported were normalized to the initial cell seeding density.

DLM scaffolds and recellularized liver grafts were fixed with 10% formalin, embedded in paraffin, and processed for staining with hematoxylin and eosin (H&E). The slides were imaged using Nikon Eclipse 800 and SPOT camera (Melville, NY, USA).

Gene expression analysis was carried out using Two Step RT-PCR Kit (Invitrogen) following RNA extraction using TRIzol, the qPCR reaction done using the Stratagene mx3005P.

Statistical analysis

Statistical analysis of the data was performed using one- or two-way ANOVA and Student's *t*-test with a confidence interval of 95%. Errors in figures and text represent standard error of the mean.

A more detailed description of methods can be found in the Supporting Information.

Results

Recovery of damaged livers to yield hepatocytes and scaffolds

Cadaveric livers that were exposed to 1 h warm ischemia were resuscitated using subnormothermic perfusion that involves recirculation of basic oxygenated media for 3 h (Fig. 1A). At the end of perfusion, hepatocytes were isolated using the two-step collagenase technique. After Percoll purification, the recovered livers produced 39 million hepatocytes per gram of liver tissue which is statistically not different from the yield of fresh livers ($P = 0.182$) (Fig. 1B). We had shown that warm ischemic livers without perfusion yield less than 2 million viable hepatocytes per gram of liver (12). These results suggest that machine perfusion is crucial in recovering damaged livers due to ischemia to yield high numbers of viable primary hepatocytes, which can be used in liver tissue engineering applications.

Livers that are damaged beyond recovery for isolation of viable hepatocytes were used to obtain DLM scaffolds. The process involves perfusion of the livers with a detergent solution to remove dead cells leaving the extracellular matrix scaffold intact. Based on the protocols we developed previously (7), we prepared scaffolds that appeared white in color as opposed to dark red of normal livers (Fig. 1C) and H&E staining of histology sections confirmed the removal of the cells (Fig. 1D). Corrosion casting of the DLM scaffolds demonstrated that the vascular architecture of the native liver is preserved after cell removal, and venous (blue), portal (red), and biliary (yellow) vascular trees were maintained throughout the scaffold (Fig. 1E). Furthermore, we observed preservation of ECM-bound growth factors including VEGF, bFGF, HGF, and EGF, although at levels lower than native livers (Fig. 1F).

Evaluation of recovered hepatocyte functions

We evaluated the function of the hepatocytes isolated from recovered livers in static plate culture and in perfused recellularized livers. In static collagen sandwich culture, the recovered cells formed and maintained cuboidal morphology for at least 7 days similar to fresh cells (Fig. 2A). The hepatocytes from recovered livers secreted albumin and urea throughout 7 days in culture although at levels lower than cells obtained from fresh livers ($P < 0.0001$ for both albumin and urea) (Fig. 2B).

Next, we used hepatocytes from recovered livers to recellularize DLM scaffolds. Approximately 80 million live cells were seeded into the DLM scaffolds via perfusion through the portal vein and 94.06 ± 3.4% of the cells were retained in the DLM scaffolds. This number corresponds to about 8% of native liver cell mass. We used median left lobe for recellularization experiments to achieve around 32% of native liver density. Similar to hepatocytes from fresh livers, the recovered hepatocytes repopulated the parenchyma, forming hepatocyte plates around vascular structures in the DLM scaffold (Fig. 2C, Supporting Information Fig. 1). The recellularized grafts using recovered cells produced albumin at levels lower than those using fresh cells corroborating the plate cultures throughout 7 days culture with a statistically significant difference ($P < 0.0001$). Overall, in recellularized liver grafts, the albumin production rate was 11.956 ± 1.29 µg/million cells/day by recovered cells and 18.646 ± 1.55 µg/million cells/day by fresh cells compared to 140 µg/million cells/day by the normal adult rat liver (15) corresponding to approximately 10% of the native liver function. The urea production was similar by both cells types ($P = 0.4928$) (Fig. 2D).

ER stress in recovered cells

Ischemia followed by reperfusion causes ER stress, which is marked by an accumulation of newly synthesized and unprocessed proteins within the ER (16–18). We hypothesize that lower albumin production by recovered cells could be a result of residual ER stress due to warm ischemia. To evaluate this possibility, we tested for activation of ER stress pathways in isolated hepatocyte samples. As a result of ER stress, cells adapt an unfolded protein response (UPR) that helps dampen the stress and UPR initiation are associated with three well-characterized transmembrane protein sensors, IRE1α, ATF6, and PERK. Once activated, IRE1α catalyzes the splicing of X box binding protein-1 (*Xbp-1u*) to *Xbp-1s*, and *Xbp-1s/Xbp-1u* ratio is a reliable indirect measure of the level of IRE1α activation and early ER stress response (19). C/EBP homologous protein (*Chop*) is another UPR target gene that is involved in ER stress-mediated apoptosis. ER stress is also marked by an increase in glucose-regulated protein, *Grp78*, expression which is a central regulator of the UPR stress sensors as well as an ER chaperone to assist protein folding (20).

We found that *Xbp-1* is present in its spliced iso-form, *Xbp-1s*, in recovered cells around 30-fold higher than in fresh cells ($P = 0.0012$) (Fig. 2E). In recovered cells, the expression of both *Grp78* and *Chop* was higher than in fresh cells and the difference was statistically significant ($P = 0.001$ and $P = 0.0006$, respectively) (Fig. 2F). These results indicate the activation of UPR and the presence of ER stress in hepatocytes isolated from machine-resuscitated warm ischemic rat livers. The residual ER stress may be responsible for the low production albumin by recovered hepatocytes.

Discussion

Discarded donor livers may provide either viable liver cells or scaffolds to engineer liver grafts. Cells were procured by resuscitating marginally damaged rat livers with machine perfusion and liver matrix scaffolds were produced by whole-organ decellularization. The

recovered hepatocytes were then used to repopulate DLM scaffolds to produce grafts with comparable functionality to ex vivo plate cultures.

Our approach addresses two major bottlenecks in liver tissue engineering by providing a potentially unlimited source of high quality hepatocytes and biological scaffolds. Currently, primary human hepatocytes are derived from suboptimal sources, such as disqualified donor livers, since healthy human livers are prioritized for transplantation. Disqualified donor livers produce limited quantities of cells that are variable in quality (21) and liver resections usually provide hepatocytes in acceptable viabilities but are limited in yield and application (22). Low function and potential risk of malignant transformation must be considered when using immortalized cell lines instead of primary hepatocytes, while ethical considerations persist with regard to the use of fetal liver cells (23), followed by an incomplete understanding of embryonic- and induced pluripotent stem cell-directed differentiation and the related costs of producing sufficient numbers of cells (3). Livers from DCD are an untapped source of functional hepatocytes, and we have shown that warm ischemic rat livers can be resuscitated through subnormothermic machine perfusion for successful transplantation (24) and high yield of viable hepatocytes (12) due to a restoration of cellular ATP reserves during perfusion (12). In this report, we successfully used these cells to repopulate engineered rat liver grafts and showed that the distribution and morphology of cells were similar to hepatocytes obtained from fresh livers. The decellularized liver scaffolds used in this approach have the native liver's vascular architecture intact, allowing continuous perfusion with nutrients to maintain the viability and function of cells. The scaffolds were shown to retain some of matrix-bound growth factors that play a role in hepatocyte function and viability including VEGF, bFGF, HGF, and EGF.

The hepatic functions in terms of urea and albumin production by engineered liver grafts were measured. While urea production by recovered cells was the same as fresh cells in engineered liver grafts and static plate cultures, their albumin production was found to be significantly lower than fresh cells in both recellularized liver grafts and static plate cultures during the first 5 days of culture. Low protein production by recovered cells could be a result of ER stress induced by ischemia-reperfusion injury. It was found that recovered cells had a higher expression of ER stress markers immediately after isolation indicating the protein production machinery in the recovered cells was burdened and hence produced less albumin. Interestingly, by 6 days, the albumin production by recovered cells in recellularized liver grafts but not in static cultures was found to be not significantly different from that by fresh cells indicating possible resolution of ER stress. The native like microenvironment created by the combination of ECM-bound factors, perfusion culture and three-dimensional configuration in recellularized liver grafts may have been responsible for this effect, but this requires further investigation.

The recellularized liver grafts developed here only contain parenchymal cells but lack crucial nonparenchymal cells such as endothelial cells. Endothelial cells are necessary to cover the vascular walls within the DLM scaffold and recreate nonthrombogenic surfaces to allow for uninterrupted blood flow within the graft. This is a critical step in clinical translation as the in vivo success depends on complete coverage of vascular walls by endothelium to prevent blood coagulation.

ER stress in the isolated cells could be resolved by modification of the perfusion or cell culture media through addition of chemical chaperones such as 4-phenyl butyric acid or taurine-conjugated ursodeoxycholic acid, which have been shown to alleviate ER stress in diabetic animals (25). Other strategies include reduction of ischemia/reperfusion injury using actively oxygenated, low viscosity media during perfusion, which has been shown to increase the number of viable hepatocytes after warm ischemia (26).

Conclusion

We successfully demonstrated techniques to prepare transplantable rat liver grafts using damaged livers that are considered unusable and discarded. Future work will include isolation of nonparenchymal cells along with hepatocytes from recovered livers to repopulate and recreate all the liver components in the recellularized liver graft. While promising, these results need to be confirmed in the human scale for clinical translation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Recovery of damaged livers for preparation of liver grafts. (A) Schematic of the perfusion setup. (B) Hepatocyte yield after perfusion recovery (*N.S., $P = 0.182$, $n = 8$). (C) Gross appearance of DLM scaffold before and after decellularization. (D) H&E staining of DLM scaffold before and after decellularization. (E) Corrosion cast of DLM scaffold to confirm the presence of vascular architecture, blue: venous, red: portal, yellow: bile ducts. (F) Growth factors that are retained in the DLM scaffold. Scale bars (C, E) 1 cm (D) 100 μ m. [Color figure can be viewed at wileyonlinelibrary.com]

Fig. 2.

Hepatocyte characteristics in culture. (A) Phase contrast images of cells in static sandwich culture for 7 days. (B) Albumin and urea secretion by recovered and fresh hepatocytes in static sandwich culture ($n=3$). (C) H&E staining of recellularized liver grafts 7 days post perfusion culture. (D) Albumin and urea secretion by recovered and fresh hepatocytes in recellularized liver grafts ($n=6$). Scale bars (A, C) 50 μ m. ER stress markers in isolated hepatocytes. (E) Ratio of *Xbp-1s/Xbp-1u* concentrations. (F) *Chop* and *Grp78* expression normalized to fresh cells ($n=5$, * $P=0.0012$, ** $P=0.001$, $\dagger P=0.0006$). [Color figure can be viewed at wileyonlinelibrary.com]