Long-Term Superior Performance of a Stem Cell/Hepatocyte Device for the Treatment of Acute Liver Failure

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Cell-based technologies to support/restore organ function represent one of the most promising avenues in the treatment of acute liver failure (ALF). Recently, mesenchymal stem cells (MSCs) have been reported as a new therapeutic for inflammatory conditions. Here, we demonstrate the efficacy of MSCs, when cocultured with hepatocytes, to provide combination hepatic and antiinflammatory therapy in the setting of ALF. MSCs were shown to have multiple beneficial effects *in vitro* that were relevant in a therapeutic context, including (1) hepatocellular functional support, (2) secretion of molecules that inhibit hepatocyte apoptosis, and (3) modulation of an acute phase response by hepatocytes cultured in ALF-induced serum. In addition, we show that the MSC secretome is dynamically changed in response to serum exposure from ALF rats. We then conducted a therapeutic trial of liver assist devices (LADs). LADs containing cocultures of MSCs and hepatocytes provided a greater survival benefit compared to other coculture and monocellular control LADs. Treatment with MSC-hepatocyte devices was associated with specific improvements in hepatic functional and histological parameters as well as decreasing inflammatory serum cytokine levels, validating a combined therapeutic effect. Moreover, MSC coculture reduced the overall cell mass of the device by an order of magnitude. These findings demonstrate the importance of nonparenchymal cells in the cellular composition of LADs, and strongly support the integration of MSCs into hepatocyte-coculture-based LADs as a potential destination therapy for ALF.

Introduction

ACUTE LIVER FAILURE (ALF) affects approximately 2000 individuals annually in the United States, with mortality rates reportedly as high as 80% without successful liver transplantation. According to the U.S. ALF Study Group (1998–2007), of the 44% of patients who were listed for transplantation, only 25% of the overall group received a graft and 10% died on the waiting list.¹ Of larger clinical significance is acute-on-chronic liver failure where sudden insults can exacerbate chronic liver insufficiency.² Because the donor shortage is still limited, alternative approaches are under investigation to provide temporary support for patients as a bridge to transplantation or recovery.

Cell-based technologies to support or artificially restore organ function represent one of the most promising avenues in the treatment of ALF. These different approaches can be broadly classified as cell transplantation, tissue-engineered grafts, and cell-based extracorporeal devices. Hepatocyte transplantation has been used to treat various liver diseases

in over 25 patients and has been associated with partial improvements, but no significant correction of disease.³ Tissue-engineered grafts^{4,5} have yet to be tested clinically, and it is unclear if the complex organ structure and function can be recapitulated in an artificial graft. Further, these approaches involve implantation of grafts and will encounter major immunological barriers, assuming a nonautologous cell source. Extracorporeal devices, by nature, are not implantable and circumvent the problem of immune rejection. To date, five cellular liver assist devices (LADs) have been clinically tested and appear safe,^{6–12} but none have shown a survival benefit in ALF.¹³

A number of basic, yet critical, design criteria can guide the development of LAD technology, such as (1) a reliable cell source (in clinical studies ~100–500 g/device); (2) the structure, function, and viability of the parenchymal cells seeded in the device under fluid flow (in clinical studies ~20–200 mL/min); (3) the pathogenesis of the target tissue; and (4) the storage and transport of the product within the context of clinical feasibility. The expected cell type used in

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LADs is clearly a human hepatocyte to support the systemic dysfunction that occurs after liver failure. However, a supportive cell type cocultured with hepatocytes in the device can aid in meeting these design criteria in a secondary manner. Ideally, a supportive cell can (1) reduce the hepatic cell mass needed in a device by enhancing hepatocyte function *ex vivo*, (2) provide an independent therapeutic benefit to disease, and (3) enhance the preservability of parenchymal cells during storage. We have an extensive history in studying the interactions between nonparenchymal cells and hepatocytes^{14–16} as well as experience with therapeutic bioreactors for ALF.^{17–19} In this study, we sought to determine if supportive cell types can influence the outcome of LAD treatment.

Herein, we demonstrate that the choice of the supportive cell is essential for the long-term efficacy of an LAD. We have previously observed a short-term survival benefit in rats undergoing ALF after treatment with a mesenchymal stem cell (MSC)-based extracorporeal bioreactor.²⁰ In addition, we showed that the administration of concentrated MSC conditioned medium (MSC-CM) decreases systemic inflammation, enhances hepatocellular replication, and prevents death in ALF-induced rats.^{20,21} On this basis, we hypothesized that MSCs may be a strong candidate as supportive cells in LADs. In the present study, we first determined if MSCs could support hepatocyte functions *in vitro* with an emphasis on optimizing cellular conditions for future LAD treatments. We observed that MSCs can prevent an acute phase response of hepatocytes exposed to ALF serum. Finally, we cocultured hepatocytes with MSCs in LADs and demonstrated long-term efficacy of this treatment compared to a gold-standard coculture scheme known to provide a high-level hepatocyte function. MSC-hepatocyte devices improved both hepatic and inflammatory parameters, signifying the first combination therapeutic approach in a single-LAD treatment.

Materials and Methods

Animals

Female Lewis rats weighing 180–200 g and male Sprague-Dawley rats weighing 280–350 g (Charles River Laboratories, Boston, MA) were used for hepatocyte isolation and ALF induction, respectively. The animals were cared for in accordance with the guidelines set forth by the Committee on Laboratory Resources, National Institutes of Health, and Subcommittee on Research Animal Care and Laboratory Animal Resources of Massachusetts General Hospital. All animals had free access to food and water, both before and after the operation.

Hepatocyte isolation

Hepatocytes were isolated as previously described.²² Typically, $150\text{--}250 \times 10^6$ hepatocytes were isolated from a single liver, with viability greater than 90%. Hepatocyte culture medium consisted of Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 14 ng/mL glucagon, 0.5 U/mL insulin, 20 ng/mL epidermal growth factor, 7.5 $\mu\text{g/mL}$ hydrocortisone (MGH Pharmacy, Boston, MA), 200 $\mu\text{g/mL}$ streptomycin, and 200 U/mL penicillin (Invitrogen).

MSC and fibroblast culture

Human MSCs were kindly provided by the Tulane Center for Gene Therapy (New Orleans, LA). MSCs were cultured and characterized for surface marker expression and adipogenic and osteogenic differentiation capacity as previously reported.²³ Cells were used for experiments during passages 3–7. NIH 3T3-J2 fibroblasts were a kind gift from Dr. Howard Green (Harvard Medical School, Boston, MA) and cultured according to donor's protocol and were used as control.

Serum collection from saline- and galactosamine-treated animals for cell culture study

Rat's carotid arteries were cannulated 24 h before treating; 1.2 g/kg of D-galactosamine (GalN) was injected intraperitoneal to induce ALF, while saline was injected as control. Twenty-four hours after induction, blood was drawn from carotid artery and the serum from both GalN-injected rats (GalN-serum) and saline-injected wild-type rats (WT-serum) were stored at -80°C until used for cell culture studies.

In vitro cocultures

Approximately 7.5×10^5 primary hepatocytes were seeded in each well of a six-well plate (Corning Costar, Action, MA) pretreated with a type I collagen coating as previously described.²² Mesenchymal cells were seeded at various ratios (MSCs or fibroblasts:hepatocytes at 2:3, 1:5, 1:50, and 1:500) 6 h after hepatocyte seeding. Cultures were incubated with hepatocyte culture medium, and samples were collected over time. For an acute phase response study, hepatocyte medium was supplemented with 10% GalN-serum after 7-day coculture. After 24, 48, 72, and 96 h of serum exposure in independent wells, fresh medium was supplied. After an additional 24 h of coculture in hepatocyte medium, medium samples were collected in each time course from each group. Medium samples were stored at -80°C for subsequent analysis.

Cytochrome P450 activity

Approximately 5×10^5 primary hepatocytes were seeded in each well of a six-well plate pretreated with a type I collagen coating; 1×10^5 mesenchymal cells or fibroblasts were seeded 6 h after hepatocyte seeding. Cultures were incubated with hepatocyte culture medium for 7 days. To probe for cytochrome P450 IA1 (CYPIA1) activity, we measured ethoxresorufin-O-deethylase activity as described by Behnia *et al.*²⁴ Briefly, 10 μM of nonfluorescent ethoxresorufin, a specific substrate for rat CYPIA1, was added to the culture medium. The rate of appearance of the fluorescent resorufin product secreted into the surrounding medium, which reflects the activity of CYPIA1 in the culture, was measured using an Fmax fluorescence microplate reader (Molecular Devices, Sunnyvale, CA) at 544 nm excitation and 590 nm emission wavelengths.

Fluorescence live-dead staining

Indirect cocultures of MSCs or fibroblasts and hepatocytes were assembled using Transwell membranes (24-mm diameter, 0.4- μm pore size; Corning Costar, Action, MA). Approximately 5×10^5 hepatocytes were placed in the lower

chamber with 1×10^5 MSCs or fibroblasts placed on the membrane insert. After 24 h of coculture, hepatocytes were exposed to hepatocyte medium containing 10% GalN-serum or WT-serum for 24 h. After 24 h of indirect coculture hepatocytes were stained using a fluorescent Live-Dead Viability Assay (Molecular Probes, Eugene, OR) and captured on a Zeiss 200 Axiovert microscope (Thornwood, NY). Viable and nonviable cells were quantified in 10 random images per well by freely available Image J software.

Albumin enzyme-linked immunosorbent assay and urea assay

Albumin concentrations were determined by a competitive enzyme-linked immunosorbent assay (ELISA) using a polyclonal antibody to rat albumin (Organon Teknika, West Chester, PA) as described previously.²² O-Phenylenediamine and hydrogen peroxide solution were purchased from Sigma (St. Louis, MO). Chromatographically purified rat albumin was obtained from ICN Biomedicals (Aurora, OH).

Urea concentration was measured using the commercially available Urea Nitrogen Assay Kit (Stanbio Laboratory, Boerne, TX). The absorbance was measured in a Versamax microplate reader (Molecular Devices).

Cytokine quantification assay

Direct cocultures of MSCs or fibroblasts and hepatocytes were maintained in hepatocyte medium for 12 h. Approximately 5×10^5 hepatocytes were seeded with 1×10^5 MSCs or fibroblasts, while hepatocytes only (5×10^5 /well) and no cells were maintained equally as controls. After 12 h of culture, cells were exposed to 10% GalN-serum or WT-serum as previously described. After 24 h of serum exposure, supernatants were sampled and stored at -80°C until analysis. Quantification of rat interleukin (IL)-6 was determined using ELISA as per vendor instructions (R&D Systems, Minneapolis, MN).

Proteomic analysis of MSC-CM

Approximately 2×10^6 MSCs were seeded in a 175-cm² flask and were incubated with GalN-serum or WT-serum for 10 h, before subsequent conditioning in serum-free medium for 24 h. Supernatants were analyzed for a panel of specified proteins using antibody array (RayBio Human Cytokine Antibody Array C series 2000; RayBiotech, Norcross, GA) as specified by the vendor. *p*-Value was determined by Student's *t*-test.

Incorporation of cells into the LAD

The construction of the flat-plate LADs has been described previously.¹⁸ Three experimental groups were established for use in cell-based LAD perfusion studies: (1) hepatocytes only (Hep-LAD, $n=6$); (2) fibroblasts and hepatocytes coculture (Fb + Hep-LAD, $n=10$); (3) MSCs and hepatocytes coculture (MSC + Hep-LAD, $n=9$). In all of the groups, the glass surface of the lower plate of the LAD was precoated with 0.125 mg/mL rat-tail collagen solution. Hep-LADs contained 15×10^6 cells. The coculture devices contained hepatocytes (7.5×10^6) with fibroblasts (15×10^6) or MSCs (1.5×10^6) seeded 1 day after hepatocytes. Cells were cultured in the device for 7 days to stabilize the viability and function of cocultured hepatocytes before therapeutic operation.

Liver failure induction and support by LAD

The induction of ALF and LAD operation is previously reported.¹⁸ Briefly, animals were anesthetized using intraperitoneal injections of ketamine and xylazine at 110 and 0.4 mg/kg, respectively. The left carotid artery and right jugular vein were cannulated, and the animal was placed in a metabolic cage. One day later, 1.2 g/kg GalN was injected intra-peritoneum, followed by a second injection 12 h later. One day after the first injection of GalN, the arterial and venous lines were connected to an extracorporeal circuit. Plasma was separated using a plasma separator (MicroKros, pore size 0.2 μm) and perfused through a polycarbonate, flat-plate LAD and subsequently reunited with the cellular components of the blood and returned to the animal. The LAD was operated for 10 h. Animal survival was monitored every 12 h. Whole blood was obtained at 10 h after LAD treatment and analyzed for liver injury biomarkers using a microfluidic metabolic assay (Piccolo–Abaxis, Union City, CA). Serum samples were stored at -80°C until analysis. Ammonia (NH_3) and prothrombin time (PT) in serum were measured with commercially available kits (Sigma). Quantification of rat tumor necrosis factor (TNF)- α , IL-6, and IL-1 β was determined using ELISA as per vendor's instructions (R&D Systems).

Liver histology

We took histology from each experimental group (Hep-LAD, Fb + Hep-LAD, and MSC + Hep-LAD treatment) at 24 h after the LAD treatment and at 1 month (long-term survival case) after the MSC + Hep-LAD treatment. Formalin-fixed, paraffin-embedded liver samples were sectioned at 4- μm thickness and stained with hematoxylin–eosin. For terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) staining, we used the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI) according to the vendor's instructions. Quantification of cell numbers in stained liver sections was performed in 10 random 40 $\times$ images per animal using the public software ImageJ (<http://rsb.info.nih.gov/ij/>). TUNEL-positive cells were quantified using appropriate criteria for a specific threshold of staining intensity as well as corresponding sizes of the nuclei.

Statistical analysis

Data represent the mean of each experiment $\pm$ SD. Statistical significance was determined by a Student's *t*-test analysis or by a repeated measure one-way analysis of variance followed by Fisher's least-significant-difference for multi-comparison for albumin and urea secretion assay or by Kaplan-Meier analysis and Log Rank test for survival analysis, in which each value was compared with the control values performed with Stat-View (Abacus Concepts, Berkeley, CA) for Macintosh.

Results

MSCs stabilize long-term hepatocellular function independent of transdifferentiation

To determine if MSCs can provide nonparenchymal support to hepatocytes, cocultured cells were simultaneously seeded at a 1:5 ratio of hepatocytes to mesenchymal cells (MSCs or fibroblasts). After 12 days of coculture hepatocytes showed a typical cuboidal morphology with distinct cell–cell

borders when cocultured with either fibroblasts or MSCs (Fig. 1A). The cultures naturally assembled into homotypic clusters of hepatocytes surrounded by heterotypic interactions with mesenchymal cells.

Hepatocytes in monoculture are known to exhibit poor metabolic function when seeded on collagen-coated surfaces.²² When cocultured with mesenchymal cells hepatocyte function becomes stabilized; however, it was unknown if MSCs could exhibit similar nonparenchymal cell support. We varied the hepatocyte:nonparenchymal cell ratios to determine if hepatocyte function was dependent on the number of nonparenchymal cells. When cocultured with fibroblasts in heterotypic contact, hepatocyte function as measured by albumin secretion became stabilized in long-term culture, which is consistent with prior reports.¹⁶ Albumin secretion at the end of the culture period at a 1:5 ratio of hepatocytes: fibroblasts was $191 \pm 12 \mu\text{g}/10^6$ hepatocytes/day. Similarly,

cocultures with MSCs and hepatocytes at a 1:5 ratio led to an albumin secretion rate of $125 \pm 10 \mu\text{g}/10^6$ hepatocytes/day. In both cases, albumin secretion was found to be a function of cell number; however, at a ratio of 1:50, MSCs failed to support long-term function (Fig. 1B). Urea synthesis was used as another indicator of hepatocyte metabolism and showed similar trends between both groups (Fig. 1B). Without coculture, hepatocytes became dedifferentiated over time and had no significant metabolic function. Overall, these *in vitro* studies suggest that both MSCs and fibroblasts can support long-term hepatocyte function.

Analysis of CYP1A1 activity showed differences between the two coculture groups, with respect to hepatocyte detoxification. MSC coculture led to significantly higher resorufin production after 7-day coculture compared to hepatocytes alone ($p = 0.025$). Fibroblast coculture showed a trend toward increased CYP1A1 activity, but was not statistically

FIG. 1. Mesenchymal stem cells (MSCs) stabilize hepatocellular function when they are cocultured with hepatocytes in a similar fashion to fibroblasts. (A) Phase contrast micrographs of hepatocytes seeded with fibroblasts or MSCs and cultured for 12 days in hepatocyte culture medium (Scale bars, 100 μm). (B) Trends of albumin secretion and urea synthesis from rat hepatocytes alone or cocultured with MSCs or fibroblasts in hepatocyte medium for 12 days. Ratios are shown as hepatocytes:MSCs or fibroblasts. (C) Cytochrome P450 IA1 assay after 7 days coculture with MSCs (MSC + Hep) compared to hepatocytes only (Hep) or cocultured with fibroblasts (Fb + Hep) as controls. * $p = 0.025$; N/S, no significant difference.

FIG. 2. MSCs prevent an acute phase response of hepatocytes cultured in acute liver failure serum. **(A)** Live and dead staining of hepatocytes cocultured with MSCs or fibroblasts after exposed to serum from D-galactosamine-injected rats (GalN-serum) or saline-injected wild-type rats (WT-serum) (scale bars = 100 μm). Coculture in WT-serum is not shown. **(B)** The viability of hepatocytes cocultured with MSCs (MSC + Hep) when they were exposed to GalN-serum or WT-serum determined by image analysis compared to hepatocytes only (Hep) or cocultured with fibroblasts (Fb + Hep) as controls ($*p < 0.01$). **(C)** Trends of albumin secretion and urea synthesis from rat hepatocytes cocultured with MSCs (MSC + Hep) or fibroblasts (Fb + Hep) in hepatocyte medium for 7 days followed by culturing in hepatocyte medium containing 10% GalN-serum for additional 96 h. Monocultures of hepatocytes (Hep) in hepatocyte medium for 1 day followed by culturing in hepatocyte medium containing 10% GalN-serum for additional 96 h served as controls ($*p < 0.01$, $**p = 0.0018$). **(D)** IL-6 levels in medium containing GalN-serum or WT-serum while no cells (No cell) served as a measure of the baseline level of IL-6 in GalN-serum. $*p < 0.05$ for IL-6 secretion compared to hepatocytes-only (Hep) or fibroblast-hepatocyte cocultures (Fb + Hep).

significant when compared to hepatocytes alone ($p = 0.077$) (Fig. 1C).

Since some reports state that MSCs can transdifferentiate into hepatocyte-like cells^{25,26} we attempted to verify if this might be an additional reason why markers of hepatocyte function were increased in the cocultures of MSCs and hepatocytes. To determine if MSCs were differentiating to hepatocyte-like cells, we cocultured rat hepatocytes and human MSCs and stained for human albumin. After 7 days of coculture, no human albumin-positive MSCs were identified, demonstrating that MSCs were not significantly differentiating to hepatocytes within this system (data not shown).

MSCs preserve hepatocyte viability and function during an acute phase response to ALF-serum stimulation

GalN is previously demonstrated to induce hepatic failure through a systemic inflammatory cascade.^{20,27} To evaluate

whether MSCs can protect cultured hepatocytes against the effects of inflammatory cytokines present in serum from GalN-induced ALF we incubated hepatocytes with medium containing 10% GalN-serum or WT-serum. First, we examined the viability of hepatocytes 1 day after GalN-serum stimulation at a 1:5 ratio of hepatocytes to mesenchymal cells. The viability of hepatocytes was significantly higher when cocultured with MSCs ($75.5 \pm 12.39\%$) compared to fibroblasts ($57.5 \pm 9.89\%$) ($p = 0.0021$) or hepatocytes alone ($55.4 \pm 6.24\%$) ($p = 0.0002$) (Fig. 2A, B). These data suggest that MSC-secreted factors can prevent hepatocyte death when exposed to inflamed serum.

We next studied if MSCs can support hepatocyte function during GalN stimulation. Hepatocytes secreted similar levels of albumin when cocultured with MSCs or fibroblasts (96 h 112.12 ± 21.32 vs. $129.42 \pm 15.25 \mu\text{g}/10^6$ cells/day, respectively) (Fig. 2C). In contrast, hepatocytes synthesized significantly higher levels of urea when cocultured with MSCs (96 h $237.28 \pm 39.62 \mu\text{g}/10^6$ cells/day) compared to fibroblasts (96 h $175.02 \pm 19.98 \mu\text{g}/10^6$ cells/day) ($p = 0.0018$).

IL-6 is known as an acute phase cytokine secreted by hepatocytes during liver injury or systemic inflammatory conditions.²⁸ We quantified the secretion of IL-6 from rat hepatocytes cultured in WT- or GalN-serum. Using species-specific reagents, IL-6 levels in medium containing 10% GalN-serum were significantly decreased when exposed to cocultures of MSCs and hepatocytes (19.9 ± 10 pg/mL; $p = 0.0412$) compared to hepatocytes alone (83.42 ± 46.5 pg/mL) or cocultures of fibroblasts and hepatocytes (55.15 ± 24.12 pg/mL) (Fig. 2D).

Alteration in MSC secretome after stimulation with liver failure serum

Since MSCs were also exposed to GalN-serum during the coculture period, we next studied the reciprocal response of MSCs to inflammatory signals found in the sera. To this end, we performed proteomic analysis of MSC-CM after stimulation with WT-serum or GalN-serum. GalN-serum stimulation led to a significant decrease in the total secreted protein of MSCs compared to WT-serum stimulation (451 ± 47 μ g/ 10^6

cells/day vs. 362 ± 64 μ g/ 10^6 cells/day; $p = 0.014$) (Fig. 3A). The total number of species detected using a high-density antibody array was not different between serum stimulation groups (WT-serum = 51 species; GalN-serum = 50 species), but 19 species were uniquely detected after WT stimulation compared to 8 species with GalN-serum stimulation (Fig. 3B). In addition, 10 species had significant fold changes after WT-serum stimulation versus 20 species with significant fold changes after GalN-serum stimulation. The specific chemical composition of MSC-CM was found to be dependent on preconditioning. After WT-serum stimulation, MSCs secreted a mixture of growth factors (32%), chemokines (31%), carrier proteins (11%), extracellular matrix-modifying molecules (11%), cytokines (5%), cytokine antagonists (5%), and other constituents (5%; Fig. 3C). In contrast, MSCs stimulated with GalN-serum before conditioning secreted a mixture of cytokines (37%), chemokines (37%), growth factors (13%), and carrier proteins (13%), with no significant secretion of extracellular matrix-modifying molecules, cytokine antagonists, or other constituents (Fig. 3D).

FIG. 3. The MSC secretome in response to inflammatory serum stimulation. (A) Total protein and comparative analysis of MSC-conditioned medium derived from cells stimulated with GalN-serum or WT-serum for 10h before conditioning ($*p < 0.05$). (B) Venn diagram representing the number of unique and shared species dependent on the nature of serum stimulation. Densitometry analysis of antibody array of MSC-conditioned medium collected from cells preconditioned with WT-serum stimulated (C) and GalN-serum stimulated (D) shown as fold changes in relative intensities compared to internal positive and negative control.

MSC-hepatocyte composite devices improve liver serologies and provide long-term survival benefit

Our *in vitro* studies suggested that coculture devices seeded with MSCs and hepatocytes may have superior efficacy when compared to fibroblast-hepatocyte devices. Thus, we developed flat-plate LADs that were seeded with different cellular compositions to observe the effect of nonparenchymal support in the efficacy of the overall LAD treatment.

Liver serologies, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST), were improved in the animal groups treated with MSC + Hep-LAD (ALT, 1019 ± 193 U/L to 292 ± 176 U/L; AST, 1824 ± 567 U/L to 759 ± 393 U/L) (Fig. 4A). In addition, serum ammonia (NH_3) levels

and PT were measured as the hepatic functional parameters and were reduced in the same MSC composite group (NH_3 , 2.87 ± 0.32 to 1.55 ± 0.40 $\mu\text{g/mL}$; PT > 120 to 21 ± 6 s) (Fig. 4B). The Fb + Hep-LAD showed similar reductions in liver injury biomarkers and ammonia removal, but failed to show significant differences in PT (PT, 109 ± 10 s to 86 ± 37 s). Devices with only hepatocytes did not have significant improvements in any liver function tests. These data suggest that either composite device could reduce the amount of hepatocyte death and provide partial hepatic support, though MSC composite devices improved all critical hepatic parameters, including coagulation factor synthesis.

MSC + Hep-LADs and Fb + Hep-LADs had distinct survival benefits at a short-term endpoint of 7 days compared to

FIG. 4. MSC-hepatocyte liver assist devices (LADs) reduce liver injury biomarkers, improve hepatic functional parameters, and increase survival. Animals were treated with hepatocyte-only device Hep-LAD ($n = 6$) or composite devices, MSC + Hep-LAD ($n = 9$) or Fb + Hep-LAD ($n = 10$). Serum was collected directly before and after (10 h) device treatment analyzed for (A) biomarkers of liver injury (alanine aminotransferase [ALT] and aspartate aminotransferase [AST]) and (B) functional parameters, NH_3 and prothrombin time (PT). In the two groups treated with composite devices (Fb + Hep-LAD and MSC + Hep-LAD), posttreatment serum biomarker levels were significantly lower than those of pretreatment (Fb + Hep-LAD: ALT $p = 0.0229$ and AST $p = 0.0487$; MSC-Hep: ALT $p = 0.002$ and AST $p = 0.0144$). Also, NH_3 levels were significantly lower than those of pretreatment (Fb + Hep-LAD $p = 0.0114$; MSC + Hep-LAD $p = 0.009$). $*p < 0.05$ compared to Hep-LAD. In MSC + Hep-LAD group, prothrombin time was significantly lower than that of Fb + Hep-LAD ($p = 0.0422$). (C) Kaplan-Meier survival analysis of treated animals for 20 days after a single 10-h LAD support period. The animal group treated with the MSC + Hep-LAD showed significantly higher over all survival rate compared to Fb + Hep-LAD ($p = 0.0283$) and Hep-LAD ($p = 0.0007$), respectively. $*p < 0.05$.

devices seeded only with hepatocytes (MSC + Hep-LAD, 78%; Fb + Hep-LAD, 50%; Hep, 0%; $p < 0.05$) (Fig. 4B). However, significant differences in long-term survival were observed between the composite devices. At a 20-day endpoint, 78% animals treated with MSC + Hep-LADs were alive while only 20% of animals treated with Fb + Hep-LADs survived in the long-term ($p = 0.0283$). These data demonstrate that MSC-hepatocyte cocultures are an optimal cellular composition for LADs to reverse ALF with a long-term survival benefit in this model.

Coculture bioreactor therapy reduces hepatocellular apoptosis in vivo

Histological analysis of liver sections at 24-h post-LAD treatment showed signs of hepatocellular death and sinusoidal hemorrhage after monocellular device therapy. Livers of MSC + Hep-LAD treated rats had no apparent compromise of the vasculature, while livers from Fb + Hep-LAD treated animals showed intermediate damage between these two groups (Fig. 5A). These pathology results are consistent with the increased serological levels of AST and ALT in rats treated with hepatocyte devices without a cocultured mesenchymal cell. At a 30-day timepoint, we observed no fibrotic changes in MSC + Hep-LAD-treated animals, confirming that the organ architecture was not impaired in the long-term after treatment. To determine whether LAD treatment decreases apoptotic cell death, the number of TUNEL-reactive hepatocyte nuclei in liver sections was determined. In sections from Hep-LAD-treated rats, many large, apoptotic hepatocyte nuclei were observed that were qualitatively reduced by either coculture device treatment (Fig. 5B).

FIG. 5. MSC + Hep-LAD treatment decreases levels of apoptosis in livers of GalN-treated acute liver failure rats. Representative (A) 20 $\times$ images of hematoxylin-eosin and (B) 40 $\times$ images of terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) stain from 24h after Hep-LAD treatment, 24h after Fb + Hep-LAD treatment, 24h after MSC + Hep-LAD treatment, and 1 month after MSC + Hep-LAD treatment (scale bars = 100 μ m). (C) Quantification of terminal deoxynucleotidyl transferase-mediated nick-end labeling-positive hepatocyte nuclei by digital image analysis. Data are reported as mean $\pm$ SD of the mean for 10 random fields per animal. * $p = 0.0019$, ** $p = 0.0035$. Color images available online at www.liebertonline.com/ten.

Quantification of TUNEL-positive hepatocyte nuclei in MSC + Hep-LADs ($27.6 \pm 25.67/\text{field of view}$; $p = 0.0019$) and Fb + Hep-LAD-treated animals ($38.8 \pm 23.82/\text{field of view}$; $p = 0.0035$) showed significant differences when compared with Hep-LAD-treated animals ($118.4 \pm 36.5/\text{field of view}$). These results confirm that MSC + Hep-LAD and Fb + Hep-LAD therapy effectively reduce hepatocellular apoptosis (Fig. 5C).

MSC-hepatocyte composite devices modulate the acute inflammatory response

Since we observed a reduction in IL-6 secretion by hepatocytes when cocultured with MSCs after stimulation with GalN-serum *in vitro* compared to when cultured alone, we analyzed whether MSC + Hep-LAD treatment provided antiinflammatory support *in vivo*. MSC + Hep-LAD treatment showed significantly lower levels of inflammatory cytokines (TNF- α , 14.6 ± 11.4 pg/mL; IL-6, 72.2 ± 53.5 pg/mL; IL-1 β , 45.2 ± 32.3 pg/mL) compared to treatment with the Hep-LAD (TNF- α , 37.7 ± 9.9 pg/mL; IL-6, 762.7 ± 263 pg/mL; IL-1 β , 170.6 ± 66.3 pg/mL). However, the Fb + Hep-LAD treatment (TNF- α , 26.2 ± 13.9 pg/mL; IL-6, 649.4 ± 462.9 pg/mL; IL-1 β , 205.7 ± 45.2 pg/mL) failed to show significant differences of the cytokine levels compared to the Hep-LAD treatment (Fig. 6). Based on these data, we conclude that the combination of hepatic and antiinflammatory support imparted by MSC + Hep-LAD treatment led to the significant difference in long-term survival compared to Fb + Hep-LADs, which only provided hepatic support.

Discussion

Biomedical devices that incorporate primary cells for therapeutic use often require nonparenchymal cells to support tissue-specific functions *ex vivo*. In this study, we have determined that the choice of the nonparenchymal cell type can be critical to the long-term efficacy of the device. These results may solely rely on the intrinsic differences between MSCs and other mesenchymal cells with respect to their

trophic secretions.^{20,29–32} We previously reported that an extracorporeal bioreactor seeded with MSCs could provide a short-term survival benefit to rats undergoing ALF when compared to an acellular or fibroblast-laden device.²⁰ In addition, we have shown that molecules secreted by MSCs are bioactive and can modulate the immune and hepatocellular response to liver failure.^{20,21} However, these results were predominantly due to immunological/trophic support provided by MSCs and were assessed with a short-term study endpoint. In this study, we determined whether the combination of hepatic and immunological/trophic support would yield greater long-term efficacy of existing LAD prototypes.

Coculture of hepatocytes with nonparenchymal cells has been shown to preserve stereotypical hepatocyte morphology and a variety of synthetic, metabolic, and detoxification functions of the liver.^{16,33–34} Also, there are few reports describing the fate of hepatocytes cocultured with bone marrow-derived stem cells.³⁵ We observed that hepatocellular metabolism in basal conditions became stabilized in long-term culture with MSCs similar to fibroblasts. These *in vitro* results suggested that MSCs and 3T3-J2 fibroblasts may utilize very similar mechanisms to stabilize hepatocellular function, although this warrants further investigation. Since some reports state differentiation of MSCs to hepatocyte-like cells,^{25,26} we demonstrated that MSCs had not significantly differentiated to hepatocytes within this system. However, we cannot rule out that MSCs differentiated into other cell types or underwent possible functional changes in their phenotype during coculture.

We next challenged hepatocytes with GalN-serum stimulation experiments to simulate hepatocytes that were either native to the ALF subject or seeded in a therapeutic LAD that would also be exposed to such serum conditions. Here, we observed significant differences between MSCs and fibroblasts in attenuating an acute phase response. Hepatocytes cultured in GalN-serum-containing medium had decreased viability and upregulated IL-6 secretion that was prevented by indirect coculture with MSCs. Recent studies by our

FIG. 6. MSC-hepatocyte LAD treatment reduces inflammatory cytokines. Serum inflammatory cytokine levels of TNF- α , IL-6, and IL-1 β were measured by enzyme-linked immunosorbent assay directly after operation of cell-based LADs. * $p < 0.05$; N/S, no significant difference.

group have demonstrated that MSC-derived molecules include many growth factors, cytokines, and chemokines that collectively can enhance hepatocyte replication and protect against hepatocyte death. Our *in vitro* analysis further confirms the hepatoprotective effect of MSCs and demonstrates that MSCs can also downregulate an acute phase response indirectly by influencing hepatocytes themselves. Proteomic analysis of MSC-CM after preconditioning of cells with inflammatory sera verified that MSCs altered their secretory profile, suggesting an active change to their diseased microenvironment. These data underscore the point that MSCs, unlike conventional therapeutics, are reactive to disease in real-time. Correlating the dynamics of the secretory changes in MSCs with respect to the pathogenesis of GalN-induced injury may be crucial to understanding this form of therapy. For example, MSC basal secretions are known to contain a large array of active components such as soluble Fas ligand, IL-1ra, and transforming growth factor- α that potentially have direct effects on the immune response, while other growth factors, including fibroblast growth factor-2, insulin-like growth factor binding protein-1, and hematopoietic growth factor, may directly promote hepatocyte function and replication in the early phase of treatment. We speculate that after a certain time within the bioreactor, MSCs may become stimulated by the ALF-serum and secrete higher vascular endothelial growth factor and fibroblast growth factor-7 to promote endothelial cell function and stromal repair. A comprehensive proteomic analysis of MSC-CM is underway to identify the active ingredient(s) within MSC-CM.

We next determined if MSCs could be a substitute for fibroblasts in the development of a coculture LAD. Post-LAD treatment, both composite devices showed reduction of liver injury biomarkers. However, the MSC + Hep-LAD provided a significant long-term survival benefit compared to the Fb + Hep-LAD. The total cell number seeded in the MSC + Hep-LAD was reduced compared to Fb + Hep-LADs, which suggests that MSCs can minimize the overall cell number of the devices, which can allow for smaller devices and decreased dead volume that is outside of the patient and in the perfusion circuit. More importantly, given the added benefits of MSCs, we speculate that MSC + Hep-LADs can potentially reduce the number of primary hepatocytes or allow for less functional hepatocytes (e.g., porcine or human hepatoma cells) to be used for future LADs. In this study, we used different strains of hepatocytes to mimic the clinical situation where allogeneic hepatocytes would likely be used in bioreactors. Further, we used mouse fibroblasts because former studies indicated that the murine embryonic fibroblast cell line 3T3-J2 is superior to other nonparenchymal cells with respect to the induction of liver-specific functions by isolated rat hepatocytes.³⁴ In two prior studies, we compared human MSC-CM to human and mouse fibroblast-CM and verified that the benefit observed by human MSC-CM therapy is not due to species differences.^{20,21} Overall, we can conclude that the secretions from human MSCs can cross histocompatibility barriers allowing for the use of allogeneic MSCs for LADs, which facilitates their use in clinical settings.

To comprehend the effect of MSCs in this model, it is paramount to understand the pathophysiology of GalN intoxication. GalN has been described to cause focal hepatocellular necrosis with panlobular infiltration of polymor-

phonuclear cells in the liver. Other reports also state that GalN can sensitize cells to endotoxins relinquished from the gut, leading to septic-like sequelae, including an uncontrolled systemic inflammatory response syndrome (SIRS) that materializes as multi-organ failure ultimately causing death.³⁶ We have now shown downregulation of IL-1 β , IL-6, and TNF- α by MSC + Hep-LAD treatments, essentially reversing the cytokine storm that is associated with SIRS. Based on these data, we conclude that the MSC + Hep-LAD integrated two therapeutic modalities within a single device to provide a long-term survival benefit: (1) hepatocellular metabolism, synthesis, and secretion, and (2) bioactive molecules secreted by MSCs that have been shown to inhibit hepatocyte apoptosis, enhance hepatocyte replication, and modulate the immune system. Further, these data suggest that MSC-based devices may find utility for other clinical indications, including septicemia, SIRS, and other organ failure syndromes.

In conclusion, these findings demonstrate the importance of nonparenchymal cells in the cellular composition of LADs. In particular, MSCs can not only stabilize primary cell functions in biomedical devices, but also provide an immunomodulatory component of therapy that directly affects systemic inflammation and tissue injury. Further, the incorporation of MSCs may reduce the total number of primary cells required for clinical application, thereby enabling smaller devices and reduced extracorporeal volumes. This experimental evidence supports the notion of LADs containing cocultures of MSCs and hepatocytes as a potential destination therapy for ALF.

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Disclosure Statement

No competing financial interests exist.

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