The Nitric Oxide Donor S-Nitrosoglutathione Reduces Apoptotic Primary Liver Cell Loss in a Three-Dimensional Perfusion Bioreactor Culture Model Developed for Liver Support

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Introduction: Artificial extracorporeal support for hepatic failure has met with limited clinical success. In hepatocytes, nitric oxide (NO) functions as an antiapoptotic modulator in response to a variety of stresses. We hypothesized that NO administration would yield improved viability and hepatocellular restructuring in a four-compartment, hollow fiber-based bioreactor with integral oxygenation for dynamic three-dimensional perfusion of hepatic cells in bioartificial liver support systems.

Methods: Isolated adult rat liver cells were placed in culture medium alone (control) or medium supplemented with various concentrations of an NO donor (S-nitrosoglutathione [GSNO]) in the bioreactors. Media samples were obtained from the cell perfusion circuit to monitor cellular response. After 24 and 72 h, histology biopsies were taken to investigate spontaneous restructuring of the cells. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed to quantify apoptotic nuclei.

Results: Control bioreactors exhibited $47.9 \pm 2.9\%$ (mean $\pm$ standard error of the mean) apoptotic nuclei. In contrast, NO-treated bioreactors exhibited a biphasic response. Fewer apoptotic nuclei were seen in the 200 and 500 μM GSNO groups ($14.4 \pm 0.4\%$). No effect was observed in the 10 μM GSNO group (47.3%), and increased TUNEL staining was observed in the 1000 μM GSNO group (82.6%). Media lactate dehydrogenase levels were lower in bioreactor groups treated with 200 or 500 μM GSNO (310 ± 38 IU/L) compared with the control group (919 ± 188 IU/L; $p < 0.05$). Protein synthesis was not affected, as measured by albumin levels in the media (115 ± 19 $\mu\text{g/day/cell}$ inoculum in GSNO-treated bioreactors at 24 h vs. 110 ± 13 in controls; $p = 0.851$). Histologically, all of the bioreactor groups exhibited liver cell aggregates with some attached to the bioreactor capillaries. Increased numbers of cells in the aggregates and superior spontaneous restructuring of the cells were seen at 24 and 72 h in the bioreactor groups treated with either 200 or 500 μM GSNO compared with the control groups.

Conclusion: Addition of an NO donor reduces adult rat liver cell apoptosis during the initial 24 h after cell inoculation within a three-dimensional perfusion bioreactor system for liver support and promotes liver cell aggregation and spontaneous restructuring of the cells at 24 and 72 h. GSNO-treated bioreactors remain metabolically active and show significantly lower levels of cellular injury as compared with controls. Further studies will be required to evaluate the impact of NO treatment of liver support bioreactors for clinical studies.

Introduction

ALTHOUGH ORTHOTOPIC liver transplantation remains the treatment of choice for end-stage liver disease, there are some circumstances, such as acute fulminant hepatic failure, in which temporary extracorporeal liver support may provide a viable option.^{1,2} To date, artificial liver support, for example, using ion exchange resins, has met with limited

clinical success, suggesting the development of bioartificial systems using hepatic cells.³ Various bioreactor culture models for primary cells, including our own,¹ have entered clinical investigation. *In vitro* primary liver cell culture has yielded significant advances of possible applicability to liver bioreactors. Unfortunately, the use of cultured primary hepatocytes is plagued by a number of deficiencies including the length of culture survival, dedifferentiation with loss of

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phenotypic actions, and ischemia-reperfusion stress from the cell isolation and procurement process.⁴

Within the field, a number of bioengineering and cell source issues are still being debated.⁵ We have addressed some of the bioengineering issues by developing an interwoven hollow fiber membrane-based, four-compartment bioreactor.^{6,7} This technology enables high-performance mass exchange, including integral oxygenation, with low solute gradients on a scale of 500 g of liver cells, which restructure and form neo-tissue including neo-sinusoids and neo-space of Dissé. However, an important issue that still limits the long-term utility of liver bioreactors is the apoptotic reduction of primary hepatocyte viability within the initial 24 h after cell stress, due at least in part to the isolation process and *in vitro* procurement.

In hepatocytes, nitric oxide (NO) functions as an anti-apoptotic modulator in response to a variety of stresses. The antiapoptotic action of NO in hepatocytes includes the upregulation of antiapoptotic proteins, the downregulation of pro-death proteins (BNIP3), the direct modification of caspases by S-nitrosation, and signaling through the cyclic guanosine monophosphate (GMP) second messenger to block proximal apoptotic signaling.⁸ NO can be delivered through various means, the most convenient being in the form of chemical compounds that spontaneously decompose to yield NO (NO donors).⁹ S-nitrosoglutathione (GSNO) is a relatively stable and long-lasting NO donor that is produced endogenously from the activity of the NO synthase and that has been used pharmacologically in both experimental animals and humans.^{10,11}

Our long-term goal is to improve the viability of bioartificial liver support systems for clinical application. Based on the above considerations, we hypothesized that an initial exposure of primary liver cells to GSNO during the cell inoculation phase would result in improved functional parameters of extracorporeal liver bioreactors in which these cells were seeded. More specifically, we hypothesized that the presence of GSNO throughout the initial 24 h of liver cell bioreactor culture after isolation, including spontaneous restructuring of the cells *in vitro*, would be cytoprotective by inhibiting apoptosis. We used a scaled-down version of our three-dimensional (3D) perfusion culture model derived from our larger, clinical scale bioreactors, which were tested in animal studies and used in clinical feasibility studies with porcine or human hepatic cells.^{12,13} Our results demonstrate decreased apoptosis in bioreactors exposed to GSNO and establish a potential role for NO in the development of improved bioartificial liver support systems.

Materials and Methods

Reagents

All reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated. GSNO was purchased from Alexis Biochemicals (San Diego, CA).

Animals

All animals used in the experimental protocols were housed in accordance with the National Institutes of Health animal care guidelines following approval of the University of Pittsburgh Institutional Animal Care and Use Committee. All animals were kept in 12-h light/dark facilities and received food and water *ad libitum*. Male Sprague-Dawley rats

weighing approximately 250–300 g each were purchased from Harlan Sprague Dawley (Indianapolis, IN) and used for liver cell harvest. All animals were acclimatized for at least 7 days before liver cell isolation.

Liver cell isolation

Liver cells were isolated from normal rats by an *in situ* collagenase (type IV; Sigma-Aldrich, St. Louis, MO) perfusion technique, modified as described previously.¹⁴ Isolated liver cells were washed twice by centrifugation (85 g, 5 min, 4°C). The pellet was resuspended in a bicarbonate-buffered culture medium according to the Williams Medium E formulation that was supplemented with amino acids, essential fatty acids, insulin, glucagon, transferrin, selenium, L-glutamine, 3% fetal calf serum, gentamycin, and amphotericin B (Heparmed VITO; Biochrom, Berlin, Germany). Viability was typically greater than 90% as determined by trypan blue exclusion assay. With each cell isolation and bioreactor culture, parallel two-dimensional (2D) cultures were performed in collagen-coated culture dishes (BD Biocoat; Beckton Dickinson, Bedford, MA).

Bioreactor

The bioreactor used was an 8-mL cell compartment, scaled-down version (BR008; StemCell Systems, Berlin, Germany) of a clinical scale bioreactor with a 500-mL cell compartment (BR500; StemCell Systems). This bioreactor incorporates three interwoven hollow fiber membrane systems resulting in a four-compartment technology. This configuration enables high-performance mass exchange, including integral oxygenation, with low solute gradients. Three independent hollow fiber membrane systems (compartments 1–3) were interwoven into repetitive subunits. The extracapillary space formed a compartment in which the cultured cells were seeded (compartment 4). Figure 5 depicts an illustration of the technology that underlies this bioreactor system. Two of the medium membrane systems were made of microporous polyether-sulfone capillaries (Membrana, Wuppertal, Germany) with a molecular weight cutoff of 400,000 Da. Hydrophobic, multilaminate, hollow fiber membrane systems (MHF; Mitsubishi, Tokyo, Japan) enable oxygen supply and carbon dioxide removal. The interwoven capillary network of this multicompartment bioreactor serves three functions: medium inflow, cell oxygenation/carbon dioxide removal, and medium outflow. As a result of interweaving and decentralized medium, plasma exchange gas supply was provided for the cultured cells and scale-up or scale-down was possible. Sterilization was performed with ethylene oxide gas at 60°C and degassing with air.

Bioreactor culture

Liver cell suspensions were inoculated into the bioreactor and cultured throughout a period of 24 or 72 h. The extracapillary space of compartment 4 was injected with 10⁸ liver cells. The cell compartments were continuously perfused with culture media via the polyether-sulfone capillaries hollow fiber bundles with a fresh feed rate of 6 mL/h combined with a recirculation loop at the rate of 30 mL/min. Waste medium was removed from the circuit at 6 mL/h. The total medium content in the circuits with bioreactors, tubes, and bubble traps was 45 mL. The flows of compressed air and carbon dioxide in

the gas compartment were maintained at 20 mL/min. Partial pressures of oxygen and carbon dioxide and acid/base status were regularly measured. The carbon dioxide content was adjusted to maintain the medium pH between 7.35 and 7.45.

Analysis of metabolic activity

The membrane integrity of the cells inside the bioreactors was characterized by measuring lactate dehydrogenase (LDH) release. Synthetic metabolic activity was assessed by measuring the concentration of albumin. All analytes were measured in the recirculating medium using standard clinical analysis methods.

Bioreactor tissue preparation

At the end of 24 or 72 h culture period, the bioreactors were opened, and tissue specimens were immediately fixed in 2% paraformaldehyde. The order of the sampled tissue representing the depth in the bioreactor was noted to address the cell distribution within the bioreactor. After overnight fixation, the samples were embedded in paraffin and 4 μ m sections obtained. Representative materials from various depths of each bioreactor were used for further analysis.

Histological and immunohistochemical analysis

Hematoxylin and eosin staining was performed on sections from bioreactor cultures to evaluate tissue architecture and organization. The ApopTag[®] Peroxidase kit (Intergen Company, Billerica, MA) was used to detect apoptosis by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method. TUNEL-positive apoptotic nuclei were detected by the presence of brown staining. Slides were viewed on a Zeiss Axioskop 40[®] upright research microscope (Chester, VA), and digital images were obtained on a Nikon Coolpix 4500 camera (Melville, NY).

Statistical analysis

The experiments were performed using liver cells freshly harvested from $n = 3$ rats pooled for each pair of bioreactors run. We used $n \geq 24$ rat livers in total and $n = 25$ bioreactor runs, of which $n = 3$ were used to establish the experimental setup. We performed 24-h studies with bioreactors containing no GSNO (24 h control group, $n \geq 4$) and groups with GSNO concentrations of 10, 200, 500, or 1000 μ M to study the effect of GSNO on 24-h apoptosis after cell inoculation following cell isolation ($n = 3$ per group). The control and 200 μ M GSNO group experiments were repeated as 72-h experiments to evaluate 72-h histology ($n = 3$ per group). Histology was performed in three representative high-power fields per slide and averaged and compared for statistical significance using the Student's *t*-test. The null hypothesis was rejected for

FIG. 1. GSNO reduces liver cell apoptosis as determined in % by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay at 24 h in culture. Bioreactors seeded with medium-treated (control) liver cells exhibited $47.9 \pm 2.9\%$ apoptotic nuclei. By comparison, 200 and 500 μ M GSNO-treated bioreactors demonstrated fewer apoptotic nuclei ($14.4 \pm 0.4\%$; $p < 0.05$). GSNO, S-nitrosoglutathione.

p -values < 0.05 . Data were analyzed using SigmaStat[®] Version 3.1 (SPSS, Chicago, IL).

Results

NO inhibits liver cell apoptosis after 24 h in a 3D perfusion culture model

We first assessed the effect of GSNO on liver cell apoptosis 24 h after transitioning the cells into a bioreactor setting. The control bioreactor group exhibited $47.9 \pm 2.9\%$ TUNEL-positive cells (Fig. 1). The bioreactor groups seeded with GSNO-containing medium exhibited a biphasic response. Fewer apoptotic nuclei were seen in the 200 and 500 μ M GSNO groups ($14.4 \pm 0.4\%$) versus the control group (Fig. 2). No effect was observed in the 10 μ M GSNO group (47.3%), and increased TUNEL staining was observed in the 1000 μ M GSNO group (82.6%). The effect of the combined 200 and 500 μ M GSNO groups was statistically significant versus control ($p < 0.05$).

GSNO reduces bioreactor liver cell injury without inhibiting biosynthetic function

Media LDH levels, a measure of cellular injury, were lower in GSNO-treated bioreactors (310 ± 38 IU/L) compared with the controls (919 ± 188 IU/L; $p < 0.05$; Fig. 3). Albumin synthesis remained unaltered as measured by albumin levels in the media (115 ± 19 μ g/day/cell inoculum in GSNO-treated bioreactors vs. 110 ± 13 control [$p = 0.851$]).

FIG. 2. Representative sections of TUNEL-stained bioreactor slides demonstrate decreased apoptotic rate with 200 and 500 μ M GSNO treatment versus control (20 $\times$ magnification). Note that the size of the aggregates is smaller in the control group. Color images available online at www.liebertonline.com/ten.

FIG. 3. Bioreactor circulating levels of lactate dehydrogenase (LDH; IU/L) were determined as a marker of hepatic cellular injury. LDH levels are significantly diminished in bioreactors medium exposed to 200 and 500 μ M GSNO versus control ($p < 0.05$).

Histological evaluation of the 24- and 72-h groups exhibited superior tissue restructuring in the presence of GSNO

Upon opening the bioreactor cell compartment at the end of the culture period, tissue formation was observed macroscopically (data not shown). Microscopic evaluation of 4- μ m bioreactor samples stained with hematoxylin and eosin showed formation of cell aggregates in the range of 6 to 300 cells. All of the bioreactors demonstrated formation of liver cell aggregates, some of them attached to the bioreactor capillaries. Increased numbers of cells in the aggregates and superior tissue reformation by the cells were observed in the bioreactors treated with either 200 or 500 μ M GSNO in the 24-h groups and 200 μ M in the 72 h groups, as compared with the control groups. In the control bioreactors, cells attached mainly to the hollow fibers with aggregate formation being less pronounced (Fig. 4).

Our microscopic investigation of each cell preparation before cell inoculation into the bioreactors demonstrated that we did not introduce larger cell clusters into the systems, rather mainly single cells and a few clusters of three to six cells. The larger cell clusters observed after bioreactor cultures were thus formed after cell inoculation into 3D perfusion culture. This conclusion is also supported by our 2D control culture findings. The isolated cells cultured in parallel in 2D culture dishes did not exhibit the formation of such larger clusters in the time frame studied.

Discussion

There is currently interest in establishing the bioartificial systems with primary hepatic cells for extracorporeal liver support.¹⁵ The initial quality of the harvested primary cells—experiencing an isolation and procurement stress—reduces the onset and the magnitude of liver bioreactor performance by reducing cell viability, in part by apoptosis.¹⁶ In this study, we focused on mitigating this early apoptotic cell loss. Our aim in this study was to determine if the NO donor GSNO could, through its antiapoptotic actions on hepatocytes,¹⁴ improve initial liver cell survival in a specific bioreactor. We used the cell model of primary rat hepatocytes and a scaled-down version of a bioreactor that was already used for clinical liver support bioreactor.

There have been many reports on the development of bioreactors for hepatocyte culture for bioartificial livers,¹⁷ and clinical trials for extracorporeal organ support have been initiated.¹⁸ The cell cultures within these devices have often shown improved function and tissue reformation as compared with the 2D cultures. In our study, we used a hollow fiber, membrane-based, four-compartment bioreactor with decentralized mass exchange, including integral oxygenation. We have previously shown that four-compartment bioreactors enable cultures of several weeks' duration, and support a spontaneous restructuring of tissue-like structures by both nonparenchymal and parenchymal cells.⁶ The bioreactor design is based on interwoven sets of semipermeable hollow fibers in a multicompartiment configuration that enable production of a 500 g clinical-scale device, but the design also permits scaling down to a smaller cell mass for *in vitro* studies.¹⁷

In normal physiological processes, apoptosis assists in regulating cell proliferation, as opposed to necrosis that is thought to be uncontrolled.¹⁹ A series of morphological and biochemical changes occurs when cells undergo apoptosis. This process may be induced by various events such as binding of extracellular death signals to host cell receptors, the lack of survival signals, and genetic damage. These events are usually followed by the activation of caspases, which initiate and execute apoptosis.¹⁹ NO exerts both pro- and antiapoptotic effects on diverse cell types and under different physiological and pathological conditions, a phenomenon that has been recognized for over a decade.^{20–24} NO can be delivered in several distinct ways to exert its antiapoptotic effects, including via gene transfer therapy with NO synthases,^{25–33} NO gas, or chemical NO donors.^{27,34,35} Elucidating NO signaling during hepatocyte procurement for liver support appears to be a worthwhile goal,

FIG. 4. Hematoxylin and eosin stain of representative sections from the 200 μ M GSNO (right) and the control group (left) of the primary rat liver cell bioreactors at the completion of a 72-h three-dimensional cell perfusion experiment. In the control group, the cells mainly immobilize on the hollow fiber membranes and exhibit only a few aggregates. The cells form larger aggregates between the hollow fibers in the 200 μ M GSNO group with a more substantial cell-generated architectural matrix (magnification marked in the figure). Color images available online at www.liebertonline.com/ten.

FIG. 5. Schematic drawing of the dynamic three-dimensional perfusion tissue-density bioreactor technology. (A) Housing with tube connectors, partially opened to view the capillary systems. All membrane compartments are interwoven, forming a tight network. Interweaving the smallest capillary units (B) allows scale-up without changing the smallest units, as shown in (C). This design allows for scaling the liver cell culture either up or down by multiplication of these units. The corresponding capillaries of each compartment are bundled to common inflow and outflow heads to be connected to tube systems. Cells are inoculated via 16 open-ended tubes (not shown), allowing cell distribution within the cell compartment. (B) The smallest interwoven capillary membrane unit exhibiting the four compartments. The cells are shown between the capillaries. The cell aggregate size is limited by the spaces between the capillaries. (C) Each capillary membrane compartment can be perfused independently addressing the reduction of substance gradient distances between the capillary units and enhancing mass exchange. Color images available online at www.liebertonline.com/ten.

because pharmacologic manipulation of the responses to these signals may be a way to improve readiness for—and clinical applications of—the bioartificial liver support systems.

An improvement of cell survival and function during the initial cell recovery phase following a procurement injury may also be of interest for other hepatic cell applications in regenerative medicine. An interesting alternative to the use of primary adult hepatocytes for extracorporeal liver support in acute- or acute-on-chronic liver failure is the use of adult liver progenitor cells from adult³⁶ or fetal tissue,⁷ or the generation of hepatic cells from embryonic stem cells. Transitioning such cells into bioreactors for expansion and clinical use also requires cell procurement and thus an *in vitro* injury. Because bioreactors are required for the expansion of progenitor cells, reducing procurement injury of cells for studies

on liver progenitor cell transplantation in chronic liver failure is also of interest.

Despite the amelioration of early hepatocellular apoptosis and improvement in cell aggregation, GSNO supplementation did not appear to enhance metabolic activity as measured by the levels of synthesized albumin in the short term. Future studies are required to determine the potential long-term functional benefits of early improvements in reducing procurement damage. Ongoing device development and improvements in bioreactor manufacturing with potential reductions in production costs will facilitate future research efforts. New prototypes of bioreactors are in development which will assist in accessing the cell compartment to allow for tissue sampling without compromising the entire culture compartment. Given the limitations of cost and experimental design, our current experiment required combinations of the 200 and 500 μM groups for statistical analysis. Further, the mechanism of action of GSNO with regards to reducing procurement injury and apoptosis in a 3D bioreactor culture will require future investigation.

In summary, our results indicate that NO signaling plays a role in the stress-induced cell mass loss during the early phase of transitioning primary liver cells into bioreactors. Addition of an NO donor, GSNO, reduces 24-h rat liver cell apoptosis within a 3D perfusion bioreactor system and promotes liver cell aggregation and tissue reformation *in vitro*. GSNO-treated bioreactors remain metabolically active in terms of albumin release and show significantly lower levels of cellular LDH enzyme liberation. Further studies will be required to evaluate the impact of GSNO treatment of liver bioreactors for clinical applications.

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Disclosure Statement

No competing financial interests exist.

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