Stem cells in liver disease
Daan van Poll

Thesis, University Utrecht, with a summary in Dutch
Proefschrift, Universiteit Utrecht, met een in het Nederlands

Cover illustration by Marcelo Lavallen:

"Healing from stem to stern"
Stem cells stimulate healing and repair of the sick liver.
** Two baseballs representing the *November 2004 and November 2007* Boston Red Sox World Series wins (the first and only World Series wins of Boston since Babe Ruth's "Bambino's Curse" in the year 1920). The author created this thesis in Boston between *September 2004 and December 2007*.

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Stem cells in liver disease

Stamcellen voor leverziektes
(met een samenvatting in het Nederlands)

Proefschrift

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Aan Maarten en Clothilde, mijn ouders

Chapter 1

General introduction & thesis outline

End-stage liver disease is still one of the most frequent causes of death in the Western world (*1*). In the vast majority of cases, cirrhosis is the underlying disease resulting in the development of chronic liver failure over the course of many years. In rare instances, acute liver failure ensues in the devastating clinical picture of fulminant hepatic failure, in which liver functions shut down over the course of days to weeks. Unless the hepatic functions are restored, death results.

Liver cirrhosis is the end-result of a series of pathophysiological events, usually triggered by chronic alcoholism, viral hepatitis or metabolic disorders, ultimately leading to irreversible scarring and chronic organ dysfunction (*2*). In response to unresolved inflammation the normally quiescent stellate cells present in the sub-endothelial space of Disse become activated and start producing excess extracellular matrix, defining fibrosis of the organ (*3*). Where the first stages of fibrosis are reversible (*4*), eventually extensive scarring replaces functional tissue, severely inhibiting hepatocyte-function in the irreversible process of cirrhosis.

Fulminant hepatic failure, affecting 2,500 people each year in the United States, is often caused by acetaminophen-intoxication or viral hepatitis, but in many cases the etiology remains a mystery (*5, 6*). Massive hepatocyte death occurs rapidly, overwhelming the regenerative capacity of the liver (*7*) and leading to a widespread inflammatory response. Mortality rates for fulminant hepatic failure remain high, even when emergency liver transplantation is available *(8, 9).*

Orthotopic liver transplantation is currently the only effective treatment for both liver cirrhosis and fulminant hepatic failure. However, a severe donor shortage, rejection issues and the high costs and complexity of this procedure severely limit its application. As a result, many patients die while waiting for a suitable organ (*10*). Even when transplantation is successfully performed, the imperative immunosuppressive treatment regimen that follows implies serious side-effects, including increased risk of malignancy, diabetes and renal dysfunction (*11, 12*). A large proportion of the grafts deteriorates over time because of recurrent disease or rejection episodes. Although success-rates continue to climb and 90% of patients survive the first year after transplantation, ten years down the line only half of the patients are still alive (*11*). Alternative treatments are not available.

In the search for alternative treatment options, three different strategies are currently being explored. The first is aimed at the reversal of the disease process and stimulation of natural healing processes. In the case of cirrhosis, reversal of the extensive remodeling and restoration of tissue architecture and function are the goals of such therapy. Currently, no treatment options are available which can effectively reverse matrix deposition and loss of tissue architecture *(12)*. In fulminant hepatic failure, injury-reducing therapy to

prevent massive cell death and inflammation and to stimulate regeneration are attractive therapeutic targets. Such a treatment could alleviate the need for organ replacement altogether. Although increased survival and reduced cell death have been observed in rodent models of acute liver injury after treatment with hepatocyte growth factor *(13)* or vascular endothelial growth factor *(14)*, no effective treatment strategy that protects the liver or stimulates repair is clinically available. A second strategy in the quest for new treatments is liver replacement using engineered devices, ideally creating an artificial organ that performs all essential liver functions *(15)*. However, no survival benefit of treatment using such a device could be shown in a large, multicenter, prospective, randomized trial in patients with acute liver failure *(16).* Lastly, much research is being devoted to increase the donor pool for liver transplantation. The availability of porcine organs for clinical transplantation could abruptly end the organ shortage. The development of knockout animals lacking xenogeneic surface antigens is currently bringing this potential donor source closer to clinical reality *(17).* Stem cell technologies are at the core of several promising research avenues in all of these three fields.

One particular stem cell type retaining enormous potential in the development of therapy aimed at reversing liver disease processes is the mesenchymal stem cell. Where traditionally the potential of stem cell types is regarded to lie in the ability to give rise to cell types that need replacement, MSCs have tissue protective and reparative properties that may be much more valuable in the treatment of many currently untreatable disorders (*18*). MSCs are usually isolated from the bone marrow, where they constitute an adult stem cell pool that safeguards the local connective tissue homeostasis and supports hematopoietis via a variety of secreted growth factors, cytokines and other signaling molecules. Research in recent years has shown that transplantation of these cells provides a marked therapeutic effect in models of disease as diverse as liver fibrosis *(19*, *20*), myocardial infarction (*21*), stroke (*22*), kidney failure (*23*) and lung injury. Originally, researchers believed that transplanted MSCs differentiated into lost cell types and hereby contributed to organ function and overall health of the animals. Evidence is accumulating, however, that MSC therapy is effective by virtue of many secreted trophic factors that reduce tissue injury while enhancing repair programs (*14*). A thorough description of the known effects on tissue protection and repair in models of vital organ injury and the investigated mediators is provided in **chapter 2** *(24)*

Much scientific effort in recent years has been invested in the second above described avenue for development of new treatment strategies for severe liver dysfunction: organ-replacing devices. In a landmark paper titled "Tissue Engineering" published 15 years ago, Robert Langer and Joseph Vacanti speculated that permanent liver replacement therapy using implantable devices, using a combination of engineering and biology principles, was among the realm of future therapeutic options for liver disease (*25*). However, no significant survival-benefit of any so far developed bioartificial liver has been shown in clinical trials (*16*). A major challenge lies in the array of complex liver-specific functions that need to be replaced, including plasma

protein synthesis and detoxification. The lack of a reliable source of hepatocytes, necessary to perform these tasks, forms the bottleneck in the further development of effective liver-replacing devices. Embryonic stem cell technology may one day be the answer to this cell source problem.

Embryonic stem cells, derived from the inner cell mass of several day old embryo's, have the unique potential to multiply indefinitely combined with the ability to differentiate into any adult cell type (*26*). These properties render this cell type into a potentially endless source of fully functional hepatocytes, an essential step toward the development of tissue engineered bioartificial livers. However, progress in this field is painstakingly slow and several essential issues remain unsolved, raising questions about the feasibility of using embryonic stem cell technology to overcome the hepatocyte-source problem.

Fetal liver cells form another source of stem/progenitor cells with the potential to form an available source of hepatocytes (*27*). Fetal liver cells are expanded with relative ease in culture while many of the essential differentiation steps towards fully functional, mature hepatocytes have already been taken (*28*). Nonetheless, an important issue that needs be addressed to render these cells suitable for tissue engineering applications is the low level of expression of liver-specific functions by fetal liver cells.

Last year, scientists received the Nobel Prize for Medicine for their discoveries of principles for introducing gene modifications in mice by the use of embryonic stem cell technology (*29*). Using embryonic stem cell technology, the first knockout mouse was produced in 1987 (*30*). This has quickly developed into a popular and widely used technique to create laboratory animals that lack expression of specific genes. Knocking out species-specific epitopes that induce rejection of xenografts in humans could render livers from other species (e.g. pigs) suitable for clinical transplantation, potentially relieving the donor scarcity (*31, 32*).

Transplantation of xenogeneic organs into humans normally results in the rapid destruction of the graft in a process that is triggered by naturally occurring antibodies reactive to xenogeneic epitopes, named hyperacute rejection (*33*). In pigs, a potentially unlimited donor source, eighty percent of the xenoreactive antibodies naturally present in human serum are directed against a single epitope: galactose-(1,3)galactose (Gal) (*32*). Transplantation of kidneys and hearts from Gal-knockout pigs into non-human primates has successfully prevented hyperacute rejection resulting in recipient survival of several months (34-*36*). Whether this translates into acceptance of Gal-knockout porcine liver allografts in primates is a question that remains to be resolved.

Thesis outline

The central theme of this thesis is the development of new treatments for liver failure using stem cell technology. In **chapter 2**, we provide an overview of the known therapeutic effects of mesenchymal stem cells, a particularly interesting adult stem cell type with tissue protective and reparative properties. The broad therapeutic potential is demonstrated by reviewing the relevant literature of mesenchymal stem cell therapy in disease models of the liver. Studies performed in other vital organs are included to provide the overall knowledge on how mesenchymal stem cell therapy protects from injury and stimulates repair.

In this thesis, the following specific questions are addressed:

I. ***How do mesenchymal stem cells inhibit liver fibrosis?*** In **chapter 3**, we investigate whether mesenchymal stem cells can inhibit functions of activated stellate cells, the principal mediator of fibrosis and cirrhosis. Using in vitro studies, we determine the effects of mesenchymal stem cells on matrix secretion, proliferation and programmed cell death of activated stellate cells. The specific mediators involved are identified.

II. ***Is intra- and extracorporeal mesenchymal stem cell therapy effective in fulminant hepatic failure?*** In a rat model of fulminant hepatic failure, we investigate the therapeutic effect of various modes of mesenchymal stem cell therapy (**chapter 4**). Rats are treated with 1) an extracorporeal MSC-based bioreactor through which part of the circulation is diverted, or infused with 2) the cells, 3) homogenates of the cells or 4) concentrates of their secretions. We determine whether modulation of the cellular immune response is involved in the therapeutic effects. Proteomic analysis and separation of the secretions are performed to investigate what mediators are responsible for the observed effects.

III. ***Can mesenchymal stem cell therapy reduce programmed cell death and enhance regeneration in fulminant hepatic failure?*** In **chapter 5**, we investigate the efficacy of mesenchymal stem cell-secreted molecules in reducing hepatocyte apoptosis and enhancing liver regeneration *in vivo*. *In vitro* studies are employed to investigate whether direct effects on cell death and proliferation are involved.

IV. ***Is efficient isolation of mesenchymal stem cells from umbilical cord blood possible using osmotic selection?*** We determine whether it is possible to isolate mesenchymal stem cells from human umbilical cord blood using a straightforward method based on differential osmotic lysis in **chapter 6**. The remarkable ability of mesenchymal stem cells to withstand osmotic stress is exploited to develop an efficient isolation-method.

V. ***Can liver-specific functions of fetal liver stem/progenitor cells be boosted by exposure to a mature liver environment?*** Fetal hepatocytes, in contrast to adult hepatocytes, are highly proliferative in culture but express only low levels of liver-specific functions. In **chapter 7**, we determine whether coculture with adult hepatocytes enhances liver specific functions in fetal liver cells to make them more suitable for tissue engineering applications.

VI. ***Is the galactose-(1,3)galactose knockout model sufficient to eliminate xenoreactivity of pig microvascular liver endothelial cells to primate serum?*** In **chapter 8**, we develop an *in vitro* model of hyperacute rejection of the porcine liver xenograft in primates. Using this model, we determine to what extent the level of xenoreactivity of liver sinusoidal endothelial cells isolated from galactose-(1,3)galactose knockout pigs to human and baboon serum is inhibited when compared to wildtype.

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Chapter 2

Mesenchymal stem cell therapy for protection and repair of injured vital organs

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Abstract

Findings in recent years have caused a profound shift in what is considered to be the therapeutic promise of mesenchymal stem cells (MSCs) in diseases of vital organs including the heart, liver, brain, kidneys and lungs. Originally, research focused on MSCs as a source of regenerative cells via differentiation of transplanted cells into parenchymal or non-parenchymal cell types. It is now clear that trophic modulation of inflammation, cell death, fibrosis and tissue repair is a major component of MSC-therapy. Delivery of only the growth factors, cytokines and other signaling molecules secreted by MSCs is often sufficient to accomplish the therapeutic effects and continuous treatment using MSC-based extracorporeal bioreactors has led to improved results. In this review, we provide an overview of the current knowledge on trophic mechanisms of MSC therapy in disease models of vital organs. Studies are reviewed by mechanism of therapy: modulation of apoptosis, inflammation/immune response, regeneration, fibrosis and angiogenesis. Important issues regarding the optimal delivery methods of MSC therapy will be discussed and critical gaps in our knowledge hampering experimental progress and clinical implementation are identified.

Introduction

There has been a recent paradigm shift in what is considered to be the therapeutic promise of mesenchymal stem cells (MSCs) in diseases of vital organs. The term MSC was originally tailored for the regenerative capacity of this cell type through its ability to differentiate into mesodermal cell types including adipocytes, chondrocytes, osteocytes and stromal cells (1-4). When cultured MSCs were also observed to adopt characteristics of cardiomyocytes (5), hepatocytes (6, 7) and several other cell types (8, 9), many investigators put this presumed widespread regenerative potential to the test in injury models of vital organs. Despite a lack of evidence for replaced organ function through differentiation of transplanted MSCs into functional cells, a clear therapeutic effect was consistently reported in models ranging from myocardial infarction to stroke (10-14). Common findings in these therapeutic trials were: (1) inhibition of inflammatory responses; (2) reduction in apoptosis; (3) prevention of fibrosis; (4) stimulation of endogenous regenerative programs; and (5) neovascularization. Studies in the past years have shown that paracrine cytokines, growth factors and other secreted signaling molecules are responsible for these trophic effects.

MSCs are traditionally isolated from the bone marrow where these cells functionally support hematopoiesis by the secretion of cytokines, growth factors and extracellular matrix as well as by repopulating connective tissue cell types (3, 4, 15-18). However, cells with MSC characteristics are reportedly present in virtually every organ (19), although more rigorous analysis of in vivo self-renewal and potency was not determined in the study. This raises the possibility that MSCs constitute a normally quiescent pool of reparative stem cells, ready to be activated as a source of trophic factors and support in the event of injury. Mobilized and subsequently recruited MSCs may also play a role, as supported by observations that stressful events (e.g. hypoxia) result in the mobilization of MSCs into the systemic circulation (20). This hypothesis is supported by the observation that mesenchymal stem cell-like cells commonly referred to as pericytes are located almost exclusively in the perivascular space of most blood-vessels (21). In addition, molecules locally upregulated in the event of injury facilitate the recruitment of circulating MSCs (table 1) (22-24). However, the physiological role of endogenous MSCs in tissue repair remains elusive due to a lack of unique MSC markers and knockout models (25, 26).

Table 1. Receptors and enzymes expressed by MSCs that facilitate engraftment

MSC Receptor / Enzyme	Tissue Ligand / Substrate	Function	Reference
c-met	HGF	Chemotaxis	(90, 91)
CXCR4	SDF-1	Chemotaxis	(90, 92-95)
CX3CR1	Fractalkine	Chemotaxis	(94)
MMP2	Collagen, gelatin	Engraftment	(90, 93)
MMP9	Collagen, gelatin	Engraftment	(93)
CD44	Hyaluronic acid	Chemotaxis	(22, 96)
PDGF-receptor(ab)	PDGF-BB	Chemotaxis	(91, 97)
PDGF-receptor(ab)	PDGF-AB	Chemotaxis	(91, 95)
EGF-receptor	EGF	Chemotaxis	(91)
FGF-receptor-2	FGF2	Chemotaxis	(91, 98)
IGF-receptor	IGF-1	Chemotaxis	(91, 95, 97, 99)
RANTES-receptor	RANTES	Chemotaxis	(95)
MCP-1-receptor	MCP-1	Chemotaxis	(100)
CCR2/3/4	CXCL8	Chemotaxis	(95, 100)

Table 1. Chemokine receptors and matrix degrading enzymes expressed by MSCs that facilitate migration and engraftment of MSCs to sites of tissue injury and the corresponding chemokines and substrates in these tissues. HGF, hepatocyte growth factor, SDF-1, stromal cell-derived factor-1; MMP, matrix metalloproteinase; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; IGF, insulin-like growth factor; MCP-1, monocyte chemoattractant protein-1;

Despite these gaps in our knowledge, it is clear that administration of MSC products has several potent therapeutic effects involving protection and repair of injured tissues. In this review, we provide an overview of the current knowledge on trophic mechanisms of MSC therapy in disease models of vital organs. An integrated view of MSC therapy compiled from data from disease models of the heart, liver, lung, kidney and brain, is presented with emphasis on the role of specific mediators (Fig. 1). Important engineering criteria regarding the optimal delivery methods of MSC therapy will be discussed and critical gaps in our knowledge hampering experimental progress and clinical implementation are identified.

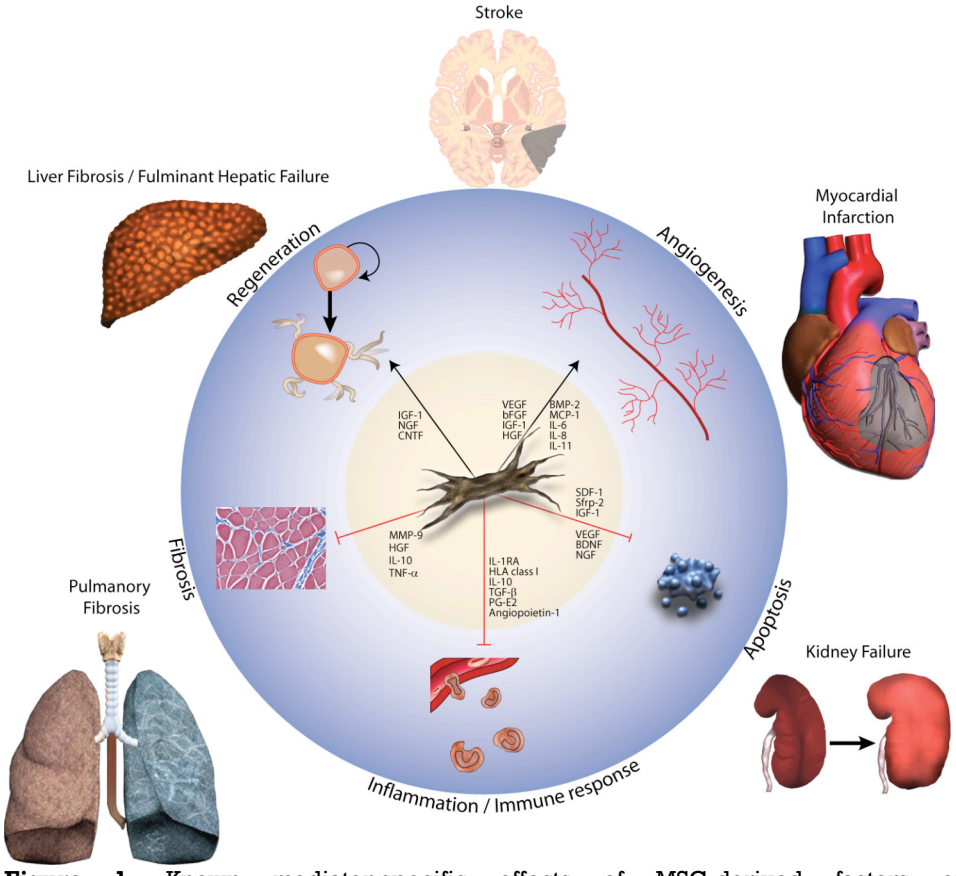

Figure 1. Known mediator-specific effects of MSC-derived factors on pathophysiological processes in tissue injury. For each growth factor, cytokine, chemokine or other mediators a summary of the observed effects and the model in which this was investigated is provided. VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; IGF-1, insulin-like growth factor-1; HGF, hepatocyte growth factor; BMP-2, bone morphogenetic protein-1; MCP-1, monocyte chemoattractant protein-1; IL, interleukin; SDF-1, stromal cell-derived factor-1; Sfrp-2, secreted frizzled protein-2; BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor; IL-1RA, interleukin-1 receptor antagonist; HLA, human leukocyte antigen; TGF-β, transforming growth factor-β; PG-E2, prostaglandin-E2; MMP, matrix metalloproteinase; TNF-α, tumor necrosis factor-α; CNTF, ciliary neurotrophic factor.

Apoptosis

During disease, protection of injured cell mass by inhibition of programmed cell death can potentially preserve organ function. Several studies have shown that MSCs have the ability to inhibit apoptosis of various types of host cells through paracrine mechanisms.

A group of researchers led by Dzau used a set of elegant experiments to demonstrate that the cardioprotective effect of MSC transplantation in a rat myocardial infarction model was largely ascribed to the anti-apoptotic effects of MSC molecules. Local administration of MSCs into the infarcted rat myocardium resulted in a dose-dependant normalization of cardiac function (27). Genetic engineering of MSCs to overexpress a pro-survival gene, Akt-1, prevented massive death of transplanted cells and resulted in dramatically improved results. These studies showed a remarkable improvement of cardiac function in a relatively short amount of time after graft administration, suggesting a role for soluble cues rather than replacement of cardiomyocytes by infused MSCs. In further experiments using conditioned medium from cultured MSCs, paracrine factors secreted by MSCs were shown to be responsible for the therapeutic effects. Conditioned medium directly inhibited apoptosis of cardiomyocytes cultured under hypoxic conditions (28). Moreover, injection of the conditioned medium into infarcted rat hearts reduced the number of apoptotic cardiomyocytes, reducing infarct size and preserving heart function. Silencing studies using RNA-interference identified secreted frizzled related protein 2 (Sfrp-2) as a key anti-apoptotic mediator (29). Several randomized prospective clinical trials investigating the effects of MSC transplantations on myocardial function and perfusion are currently being performed (www.clinicaltrials.gov).

Investigations by other researchers and in other organ systems have demonstrated that several other mediators secreted by MSCs play a role in inhibition of programmed cell death. Koc et al. observed enhanced anti-apoptotic effects of MSCs modified to overexpress stromal-derived factor-1 (SDF-1), identifying the SDF-1 – CX chemokine receptor-4 (CXCR4) axis as an important mechanism of myocardial preservation via downregulation of pro-apoptotic Bax protein (30, 31). Others demonstrated that MSC-derived insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF) have cardiomyocyte apoptosis-inhibiting effects using in vitro studies (32). IGF-1 was also shown to have important pro-survival effects in cultured proximal tubular epithelial kidney cells, crucial in the development of acute kidney failure (33).

Anti-apoptotic effects due to paracrine MSC factors have also been observed in injury models of the liver, kidney and brain. Increased levels of the pro-survival neurotrophins brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) were observed in rats subjected to stroke and treated with MSC infusions, suggesting a role for these molecules in reducing cell death in the ischemic brain (34). In a rat model of toxin-induced fulminant hepatic failure, MSC therapy using cells isolated from human bone marrow samples significantly reduced mortality and was correlated with a reduction in the number of apoptotic hepatocytes (35). In this study, several different

modes of MSC therapy were assessed, including: (1) transplantation of whole cells; (2) infusion of cell lysates; (3) infusion of MSC-derived conditioned medium (MSC-CM); and (4) a MSC-based extracorporeal device. Animal survival was found to be a function MSC-CM dose, but above a certain threshold the conditioned medium became ineffective. These results suggest important pharmacodynamic characteristics of MSC-CM or the presence of molecules that are harmful at high concentrations (e.g. tumor necrosis factor (TNF)-α)) (36). A separate cohort of animals was treated using an MSC-based extracorporeal device that allowed for MSCs to dynamically supplement the animal's plasma continuously during a 10-hour treatment. The strongest effect on hepatocellular damage and survival was observed in the animals treated with this MSC-based bioreactor. A third group of animals was unsuccessfully treated with systemically delivered MSCs, although immunorejection or increased pulmonary lodging of large, xenogeneic cells may have been responsible for the lack of effect.

Immune response

MSCs have potent inhibitory effects on immune cells. Secreted molecules inhibit lymphocyte proliferation, B-cell differentiation and the formation of natural killer cells, dendritic cells and cytotoxic T-lymphocytes (37-40). Human leukocyte antigen (HLA) class I molecules, interleukin (IL)-10, transforming growth factor (TGF)-β and prostaglandin (PG)-E2 have been proposed as responsible mediators, but contradicting reports exist (41-48). The potency of MSC immunosuppression is illustrated by the successful treatment of severe steroid-resistant acute graft-versus host disease (GVHD) after bone marrow transplantation (49). Several patients have now been successfully treated with systemic MSC infusions, resulting in a significant survival-benefit in a randomized controlled trial (50). For an extended literature review on the immunomodulatory properties of MSCs in a bone marrow transplantation frame we refer to an excellent review written by Le Blanc and Ringden (51).

Infiltrating immune cells are essential in the response to tissue injury, but resolution of the inflammation is critical to prevent chronic damage and control harmful immune cell-mediated cytotoxicity (52, 53). Dampening effects on immune cells have been described in several models of vital organ injury. In a rat model of fulminant hepatic failure, we showed that the number of infiltrating leukocytes was dramatically reduced after systemic administration of concentrated conditioned medium from MSC cultures (35). Fractionation of the conditioned medium resulted in preservation of the therapeutic effects only for the heparin-binding fraction, suggesting an important role for known heparin ligands such as growth factors and chemotactic cytokines. Several groups have described a similar inhibition of accumulating immune cells in different models of lung injury (54, 55). Mei and co-workers observed enhanced anti-inflammatory effects of genetically

engineered MSCs to overexpress angiopoietin-1, suggesting a role for this molecule in the immunomodulatory effects of MSCs.
Besides the cellular immune response, the cytokine response is an important component of the inflammatory response to tissue injury. Control of this response is critical for resolution of the inflammatory infiltrate and cytotoxic effects of pro-inflammatory cytokines as described for TNF-α and IL-1 (56). MSC therapy induces local and systemic downregulation of pro-inflammatory cytokines but also upregulation of anti-inflammatory cytokines including IL-10 in models of lung injury, kidney and liver failure (57-60). MSC-secreted interleukin-1 receptor antagonist (IL-1RA) is an important factor in the inhibition of pro-inflammatory cytokine production (61).

Fibrosis

Unresolved inflammation leads to fibrosis and scar formation. The deposition of large amounts of extracellular matrix inhibits organ function and regeneration. MSCs have the potential to inhibit scar formation and extracellular matrix accumulation in disease models of myocardial infarction, liver fibrosis, lung fibrosis and stroke. Several groups have demonstrated that administration of MSC therapeutically inhibits fibrosis formation in rodent models of toxin-induced chronic liver injury (62, 63), triggering the start of a randomized controlled clinical trial evaluating the effect of intraportally infused MSCs on patients with decompensated liver cirrhosis (www.clinicaltrials.gov).
Transplanted MSCs were shown to express high levels of MMP-9, which directly degrades extracellular matrix (64). However, indirect immunomodulatory effects on resident cell types likely play a role in the anti-fibrotic effects of MSCs. Using in vitro studies, we dissected the dynamic interactions between MSCs and activated stellate cells, the principal mediator of matrix deposition in liver fibrosis. We identified IL-10 and TNF-α as key molecules inhibiting matrix deposition by stellate cells, while MSC-derived HGF induced apoptosis in these cells (36). Such alterations in the secretome of MSCs in response to stress-signals (65) is likely to have implications for the techniques required to obtain MSC-conditioned medium preparations with an optimal therapeutic effect.
MSC therapy also inhibits matrix deposition and scar formation in models of heart infarction, lung fibrosis and stroke. Paracrine mechanisms were identified in a study effectively using a monolayer sheet of fat-derived MSCs to prevent detrimental myocardial remodeling after infarction, which is associated with improved compliance and elasticity of the infarct zone (66, 67). In a rat model of brain ischemia, the reduction in scar volume was accompanied by a decreased number of macrophages in the scar wall, again suggesting an indirect mechanism through immunomodulation of scar-promoting cell types (68).

Regeneration

Enhanced regeneration through stimulation of endogenous repair programs, either through activation of a local adult stem cell pool or via enhanced replication of differentiated cell types, has been identified as another mode of MSC therapy. Investigators implanting human MSCs into the hippocampus of severe combined immunodeficient (SCID)-mice demonstrated enhanced proliferation and differentiation of endogenous neural stem cells and suggested a direct effect of cytokines produced by the MSCs including NGF and ciliary neurotrophic factor (CNTF), both neurotrophins known to enhance neurogenesis (22, 34, 69). In coculture studies soluble factors were shown to be responsible for increased oligodendrogenesis from neural stem cells, but a screen for candidate molecules could not identify a specific mediator (70).
Enhanced proliferation of endogenous non-stem cell types has been demonstrated in experimental liver and kidney injury (59, 71, 72). Secreted factors were shown to be sufficient to enhance liver regeneration in a model of acute liver failure in which animals were treated with the MSC-conditioned medium (submitted manuscript). Animal studies of cisplatin-induced acute kidney failure identified IGF-1 as a crucial MSC-derived mitogenic stimulus for proximal tubular epithelial kidney cells resulting in improved organ function (33).

Angiogenesis

Liver regeneration (73), recovery from ischemic heart disease (74), stroke (75) and many other forms of healing (76, 77) are associated with the formation of new blood vessels. Although MSCs have the capacity to differentiate into endothelial cells (78), this phenomenon is not sufficient for the formation of fully functional blood vessels, which requires many well-coordinated steps. However, MSC have the capacity to provide trophic support for the entire process of angiogenesis and collateral formation. Using conditioned medium from cultured MSCs, Kinnaird and colleagues provided evidence that secreted factors are sufficient to improve new vessel formation and improve outcome in a rodent model of limb ischemia, demonstrating independent roles for both VEGF and bFGF (79). Others have confirmed the importance of these two molecules, but in vitro evidence suggests that IGF-1, hepatocyte growth factor (HGF), bone morphogenetic protein-2, monocyte chemoattractant protein (MCP)-1 and interleukins 6, 8 and 11 are also involved (32, 80, 81). MSC therapy results in a significantly increased capillary density in models of myocardial infarction and dilated cardiomyopathy (31, 82, 83).

Potential side-effects of MSC-therapy

After the treatment of 9 patients suffering from GVHD with MSC infusions, 3 developed viral infections (50). Although these patients were at increased risk

for developing opportunistic infections through the nature of their disease, concerns were raised that immunosuppression by the MSCs had caused a reduction of immunosurveillance to pathogens, as supported by in vitro observations that lymphocyte proliferation by herpesviruses is suppressed by MSCs (50).

Another potential serious side-effect is cancer. Theoretically, MSCs could be tumorigenic through direct transformation, suppression of the anti-tumor immune response, direct trophic effects on the tumor or metabolization of chemotherapeutic agents. MSCs have the potential to transform into sarcoma when Wnt signaling is suppressed (84). Animal studies by Djouad and colleagues have revealed enhanced tumor growth after MSC transplantation when tumor cells were implanted (47, 85). It is unclear whether MSCs facilitated the tumor growth via immunomodulatory or trophic effects and effects. Karnoub et al demonstrated that MSCs within tumor stroma promote breast cancer metastasis via cancer cell-induced de novo secretion of the chemokine CCL5 in MSCs (86), In addition, bone marrow stromal cells, highly enriched for MSCs, promote survival of B- and plasma-cell malignancies by inducing hedgehog signaling (87). Although potent anti-tumor effects of MSCs have also been observed, patients with increased risk may not be suitable candidates for MSC therapy and a potential effect on spontaneous tumor formation should be taken into account when assessing trials until more conclusive experimental data becomes available.

Concluding remarks

MSCs secrete an array of trophic mediators with therapeutic potential in diseases of several vital organs through modulation of cell death, inflammation, fibrosis, regeneration and angiogenesis. Experimental success has been obtained using several different modes of MSC therapy: cell transplantation, incorporation of MSCs into biocompatible devices and delivery of only the MSC-secreted molecules. However, important questions regarding the optimal delivery method of MSC therapy remain. Transplanted MSCs have the potential benefit of homing to the site of injury and continued delivery of the trophic signals. However, long-term engraftment levels are low (88) and invasive methods for local delivery of the cells is often necessary to circumvent pulmonary lodging (89). On the other hand, delivery of only MSC-derived products (e.g. conditioned medium) has the potential of becoming an off-the-shelf therapy. Intravenous injections of MSC-CM are easy to titrate, have short-term effects that quickly fade by natural metabolism or degradation of molecules inside the body, and can be sufficient for treatment of acute insults. MSC-based bioreactor treatment offers a treatment option with continued delivery of MSC-products that are secreted dynamically in response to circulating cues from the sick subject, which can significantly alter the secretome of MSCs. Thorough head-to-head comparisons of treatment methods will be necessary to determine which treatment strategy has the greatest potential in specific disease states.

Efforts to identify the specific mediators responsible for MSC-therapy have revealed several growth factors, cytokines, soluble receptors and cytokine antagonists as effective molecules, but many unknowns remain. Systematic analysis of the proteome secreted by MSCs, for example with the use of mass spectrometry combined with immunological methods, is necessary to create a better understanding of the active ingredients. Effectiveness of specific compounds will need to be unraveled with the help of in vitro assays for specific trophic effects, in which high throughput screening of candidate molecules is possible in a controlled environment. Better insight into the responsible mediators secreted by MSCs will not only help to understand how MSC-based therapies work, but may eventually lead to the development of an optimized cocktail of the crucial compounds. Eventually, MSCs may only be necessary to synthesize the raw materials from which this cocktail is extracted.

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Chapter 3

Immunomodulation of activated hepatic stellate cells by mesenchymal stem cells

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Abstract

Bone marrow-derived mesenchymal stem cells (MSCs) have been reported to prevent the development of liver fibrosis in a number of pre-clinical studies. Marked changes in liver histopathology and serological markers of liver function have been observed without a clear understanding of the therapeutic mechanisms by which stem cells act. We sought to determine if MSCs could modulate the activity of resident liver cells, specifically hepatic stellate cells (SCs) by paracrine mechanisms using indirect cocultures. Indirect coculture of MSCs and activated SCs led to a significant decrease in collagen deposition and proliferation, while inducing apoptosis of activated SCs. The molecular mechanisms underlying the modulation of SC activity by MSCs were examined. IL-6 secretion from activated SCs induced IL-10 secretion from MSCs, suggesting a dynamic response of MSCs to the SCs in the microenvironment. Blockade of MSC-derived IL-10 and TNF-α abolished the inhibitory effects of MSCs on SC proliferation and collagen synthesis. In addition, release of HGF by MSCs was responsible for the marked induction of apoptosis in SCs as determined by antibody-neutralization studies. These findings demonstrate that MSCs can modulate the function of activated SCs via paracrine mechanisms and provide a plausible explanation for the protective role of MSCs in liver inflammation and fibrosis, which may also be relevant to other models of tissue fibrosis.

Introduction

Liver fibrosis, the precursor to cirrhosis, is the result of an imbalance in extracellular matrix (ECM) synthesis and degradation, mediated primarily by activated hepatic stellate cells (SCs). Following liver injury, SCs undergo a phenotypic switch from a quiescent, vitamin-A storing cell into a proliferative, α-smooth muscle actin (SMA) positive, myofibroblast-like cell, which shows an upregulation in collagen synthesis *(1)*. *In vivo* activation of SCs is divided into a fibrogenic and hyperplastic response *(2)* that is mediated by many autocrine and paracrine signals. Spontaneous resolution of liver fibrosis has been reported in different models of chronic liver injury *(3, 4)*. This resolution has been correlated with decreased synthesis of type I collagen and tissue inhibitor of matrix metalloproteinases (TIMP) 1 and 2 transcripts, with a concomitant decrease in the number of α-SMA positive SCs *(4).* Yet, it remains unclear whether the decrease in the number of activated SCs is due to selective apoptosis *(5, 6)* or reversion to a quiescent state by microenvironmental cues *(7-9)*.

A new technique in the treatment of inflammatory conditions involves the infusion of bone marrow-derived mesenchymal stem cells (MSCs). Recent studies have demonstrated that MSCs can be of therapeutic benefit in the prevention of fibrotic lesions, such as pulmonary fibrosis after bleomycin challenge *(10)*, and in the protection of cardiac function after a myocardial infarction *(11).* In particular, studies using MSCs for cellular cardiomyoplasty showed that paracrine factors produced by MSCs may contribute to their therapeutic benefit *(11)*. Systemic delivery of MSCs prior to, and during the induction of experimental liver fibrosis significantly inhibits changes in liver histology and clinical serum parameters *(12-14)*, but the preventative mechanisms have yet to be elucidated.

Here, we demonstrate that MSCs indirectly modulate the activity of activated SCs *in vitro* via paracrine stimulation with specific cytokines and growth factors. Suppression of proliferation and collagen synthesis was mediated by MSC-derived interleukin (IL)-10 and tumor necrosis factor (TNF)-α. IL-10 secretion, in particular, was found to be a dynamic response to IL-6 secreted by activated SCs. In addition, secretion of HGF by MSCs led to the apoptotic death of activated SCs.

Materials & methods

Materials were purchased from Sigma-Aldrich, St Louis, MO unless otherwise stated.

MSC isolation, ex vivo expansion and characterization

Human MSCs were isolated and cultured as previously reported *(15)*. The surface antigen profile as analyzed by flow cytometry (FACS Calibur, Becton Dickinson) was consistently CD14-, CD34-, CD45-, CD105+, CD106+ and CD44+. Cells were shown to have adipogenic and osteogenic differentiation potential (Fig. 1) and were used during passages 4-7.

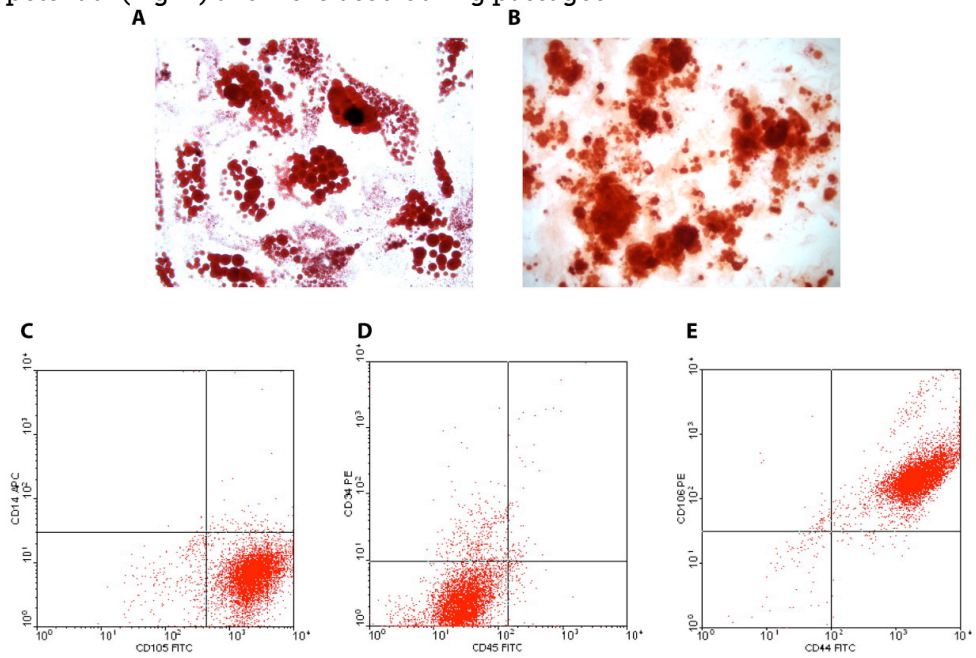

Figure 1. Phenotype and multipotency of bone marrow-derived MSCs. Passage 4-7 cells were exposed to osteogenic and adipogenic differentiation conditions for 14 days and assessed by (a) von Kossa, (b) Oil Red-O and (c) safranin-O staining, respectively. Surface antigen profile of MSCs was (c) CD14- / CD105+, (d) CD34- / CD45-, (e) CD106+, CD44+ as determined by flow cytometry.

SC isolation and culture

Immortalized human SCs were derived as previously reported *(16)*. Primary rat SCs were isolated from 150-200 g female Lewis rats using a two-step step collagenase perfusion *(17)* followed by a Percoll density gradient separation as previously described *(18)*. SCs were activated by culturing them for 10-14 days on tissue culture plastic in DMEM supplemented with 10% FBS before use in experiments. Characterization by immunofluorescence for desmin and α-SMA revealed a purity of >96%.

Coculture Systems
For direct coculture of MSCs and SCs, cells were seeded at a 1:1 ratio in each well of a six-well plate (Corning Costar, Acton, MA). An indirect coculture system between SCs and MSCs was assembled using Transwell membranes (24 mm diameter, 0.4 μm pore size; Corning Costar, Acton, MA). Approximately 1.0 x 10^5 SCs were placed in the lower chamber with either 0-1.0 x 10^5 MSCs placed on the membrane insert. Cocultures were maintained in SC medium for 4 days.

Cytokine treatment, neutralization and protein quantification
Human MSCs were treated with IL-6 (2.5 ng/ml; R&D Systems, Minneapolis, MN), IL-1 (5 ng/ml; R&D Systems, Minneapolis, MN), or tumor necrosis factor-α (TNF-α; 25 ng/ml; R&D Systems) supplemented MSC expansion medium for 24 h. MSCs cultured in expansion medium served as a negative control. After treatment, cells were harvested and analyzed for changes in gene expression.
Quantification of human TNF-α, IL-10 and rat IL-6 and HGF was determined using an ELISA as per vendor's instructions (Endogen, Rockfor, IL). Supernatants were samples after 48 h of coculture and stored at -20°C until analysis.
Neutralization of specific cytokines was performed during indirect cocultures. For all neutralization experiments, the ratio of MSCs to SCs was 1:1. Anti-human IL-10 (BioLegend, San Diego, CA), TNF-α, (BioLegend), or HGF and anti-rat IL-6 (Cell Sciences, Canton, MA) were diluted in SC medium based on the half maximal inhibition concentrations given by the manufacturer. Fresh medium with neutralizing antibodies was added after 48 h of coculture.

Total RNA isolation and RT-PCR
RNA was extracted from 0.1-1.0 x 10^6 MSCs using the Nucleospin RNA purification kit (BD Biosciences, Palo Alto, CA) per the manufacturer's instructions. Approximately 1 μg of total mRNA was reverse transcribed to cDNA using the One-Step RT-PCR Kit (Qiagen, Valencia, CA) per manufacturer's instructions and amplified in a Perkin Etus Thermal Cycler 480. Cycling conditions were: (1) 50 °C for 30 s; (2) 95 °C for 15 min; (3) 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; and (4) a final extension step at 72 °C for 10 min. Primers used for amplification were: IL-10 (364bp) AAGCCTGACCACGCTTTCTA, GTAGAGCGGGGTTTCACCA; and GAPDF (238 bp) GAGTCAACGGATTTGGTCGT, TTGATTTTGGAGGGAGCTCG.

Collagen synthesis
Collagen synthesis was quantified using an ELISA for procollagen type-I C-peptide (Takara-Bio Inc, Shiga, Japan). After the coculture period, the Transwell insert containing MSCs was removed and the medium on the SCs was replaced with fresh medium. Twenty-four hours later, medium was collected and procollagen type-I C-peptide concentration was measured by ELISA.

Proliferation assay
The proliferative capacity of SCs in culture was evaluated by measuring incorporation of 5-bromo-2'-deoxyuridine (BrdU). SCs were incubated with 10 μM BrdU for 24 h. Cells were collected by trypsinization and fixed in 70 % ethanol for 45 min at room temperature. Subsequently, cells were subsequently incubated in 4 M HCl and a solution of 10 % FBS in PBS for 15 min. After incubation with Alexa-Fluor 488 conjugated anti-BrdU antibody cells were analyzed by flow cytometry.

Apoptosis Assay
Quantification of cell apoptosis/necrosis was determined using the Annexin-V Fluor kit (Roche, Indianapolis, IN) as per vendor instructions and analyzed by flow cytometry. Serum deprived SCs served as a positive control for apoptosis.

Statistical Analysis
Results were analyzed using the unpaired Student's t-test. A p-value of $<.05$ was considered statistically significant. Results are provided as mean ± s.e.m.

Results & discussion

MSCs inhibit collagen synthesis in activated SCs

The reversal of experimental liver fibrosis in vivo has been highly correlated with a decrease in tissue collagen content *(1)*. A similar decrease in tissue collagen has been reported after MSC infusion into animals with fibrotic livers *(13, 14)*. We evaluated whether MSCs can reduce secretion of procollagen type I C-peptide (PIP) in SCs. Levels of PIP-secretion by activated SCs (101 ± 11 pg/10^6 cells/day) were significantly reduced at a MSC:SC coculture ratio of 1:10 (41 ± 18 pg/10^6 cells/day; p=0.0491), with a 66% reduction at a 1:1 coculture ratio (34 ± 5 pg/10^6 cells/day; p=0.004; Fig. 3A). A 25% reduction of PIP-secretion was observed in the immortalized human SCs at a 1:1 coculture ration (787 ± 40 to 588 ± 51 pg/10^6 cells/day; p = 0.02). In coculture with a control cell type no reduction in secretion of PIP was observed (Fig. 2). These results suggest that soluble factors released by MSCs inhibit the synthesis of collagen in activated SCs.

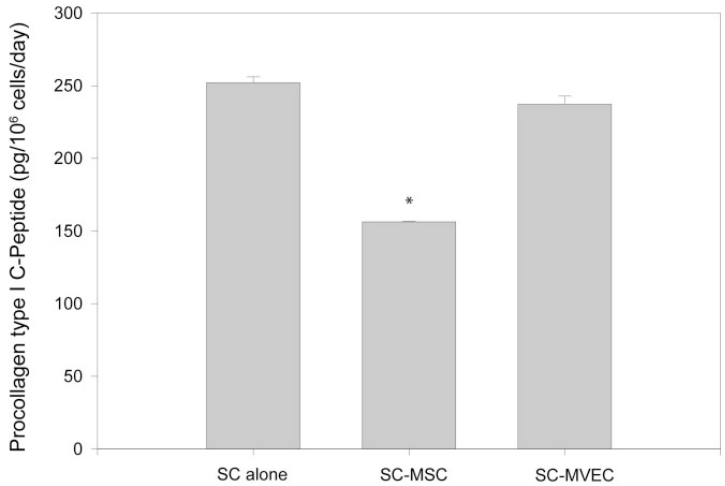

Figure 2. Inhibition of SC PIP secretion is an MSC-specific effect. PIP secretion from SCs decreases as a function of MSCs and not human umbilical vein endothelial cells. Data represent the mean of two experiments performed in triplicate. Error bars are standard deviation. ** p<0.01 compared to SC alone.

MSCs inhibit proliferation and induce apoptosis in activated SCs

The decrease in hepatic fibrosis observed after transplantation of MSCs is accompanied by a reduction in the number of α-SMA+ (activated stellate) cells *(12, 14)*. It is unclear whether this observation is due to a decrease in the proliferative capacity, increased apoptotic cell death or a reversion of activated SCs to a quiescent phenotype *(1)*. We examined MSC paracrine factors can reduce the number of activated SCs by any of these mechanisms.

At a 1:1 coculture ratio, the number of SCs entering the S-phase of the cell cycle, quantified by flow cytometric analysis of BrdU-incorporation, was

significantly decreased from 30 ± 9% to 15 ± 4% (Fig. 3B; p=0.043). No significant difference in proliferation could be detected at lower MSC:SC ratio's. To determine whether MSCs also have the capacity to reduce activated SC numbers by inducing their apoptosis, we quantified the number of Annexin-V expressing cells. In SCs cultured alone, there was a basal level of apoptosis (16%, Fig. 3B) consistent with previous reports *(19)*. Coculture with MSCs at a 1:1 ratio increased apoptosis levels approximately 2.5-fold to 43%, Fig. 3B, p=0.0007). Coculture with fibroblasts resulted in a level of apoptosis that was similar to SCs alone (32%, Fig. 4), demonstrating that the pro-apoptotic effect was MSC-specific. Significant SC death also occurred at MSC:SC coculture ratios of 1:100 and 1:10 (26%, p=0.009 and 33%, p=0.004 respectively, fig. 3B). Flow cytometric analysis did not reveal a decrease in levels of α-SMA expression after coculture with MSCs (Suppl. Fig. 5).

Taken together, these results suggest that MSC have the capacity to inhibit proliferation and induce apoptosis in SCs through secreted factors, while SCs do not revert to a quiescent state. In the context of relatively low levels of MSC engraftment in vivo, a 1:1 ratio of MSCs to SCs may not be a realistic model for the differences seen in α-SMA+ cells in vivo. Induction of apoptosis, which was observed at low MSC:SC ratio's may therefore be a more important mechanism.

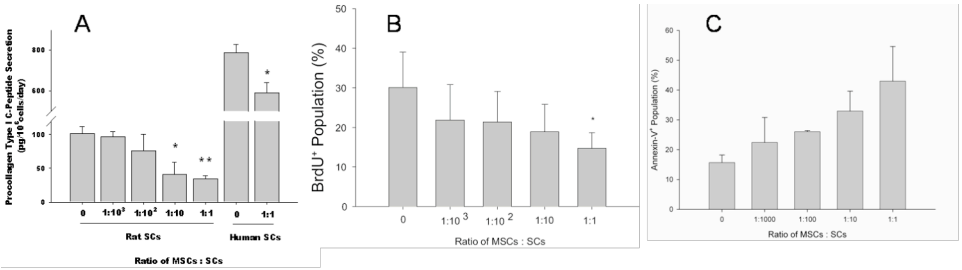

Figure 3. Inhibited collagen synthesis and proliferation and increased apoptosis in SCs cocultured with MSCs. Activated stellate cells were with cultured with and without MSCs at increasing ratios, separated by Transwell membranes. Procollagen type-I C-peptide (PIP) secretion from rat and human SCs in Transwell coculture with MSCs was measured by ELISA (A). BrdU incorporation (B) and Annexin-V reactivity (C) were analyzed in rat SCs by flow cytometry. Data represent the mean of two experiments performed in triplicate. Error bars are standard deviation. $*p < 0.05$, $**p < 0.01$ compared to SCs alone (MSC:SC = 0).

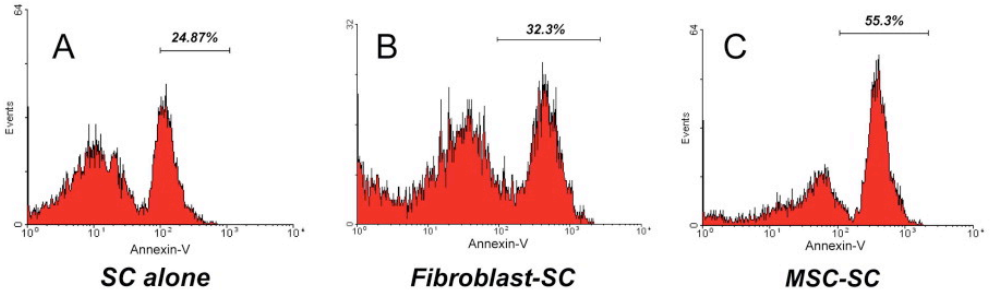

Figure 4. Induction of SC apoptosis is an MSC-specific effect. Annexin-V reactivity of SCs after indirect coculture with MSCs. MSCs induce apoptosis in activated SCs. Annexin-V reactivity of SCs cultured alone (A), cocultured with human foreskin fibroblasts (B) or MSCs (C) after 5 days of culture as measured by flow cytometry. One representative histogram is shown for each condition, with population percentages gated after exclusion of SC autofluorescence.

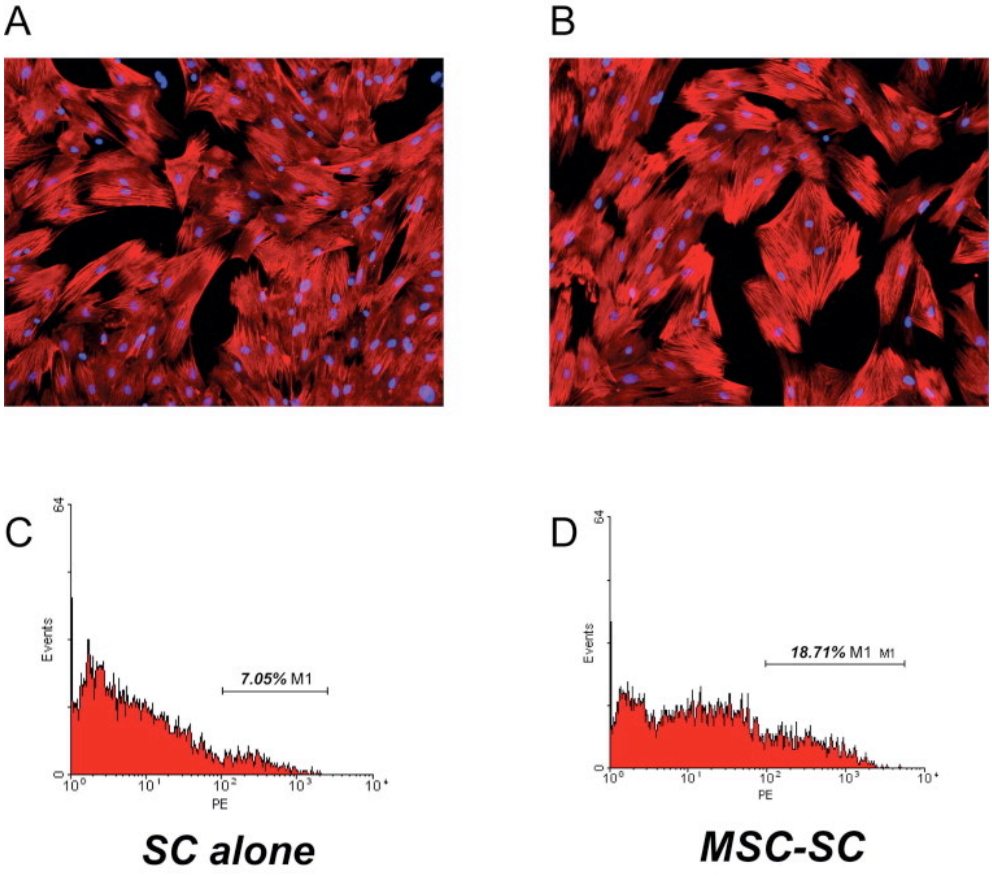

Figure 5. SCs do not revert to a quiescent phenotype after coculture with MSCs. Immunocytochemistry of α-SMA expression of SCs alone (A) and after coculture with MSCs (B). Flow cytometry analysis of α-SMA expression of SCs alone (C) and after coculture with MSCs (D). Percentages are based on autofluorescence gates.

MSCs secrete IL-10 when stimulated by IL-6 and constitutively secrete TNF-α

In an effort to understand the molecular basis of the observed effects of MSCs on SCs, we evaluated whether MSCs secrete immunomodulatory molecules when exposed to cytokines known to be involved in liver fibrosis. Using species-specific reagents, MSCs were found to express and secrete increased levels of the anti-inflammatory cytokine IL-10 in response to IL-6 secreted by the SCs, suggesting a dynamic response of MSCs to a pro-inflammatory environment. In addition, MSCs were found to constitutively secrete TNF-α (Fig. 6A-E).

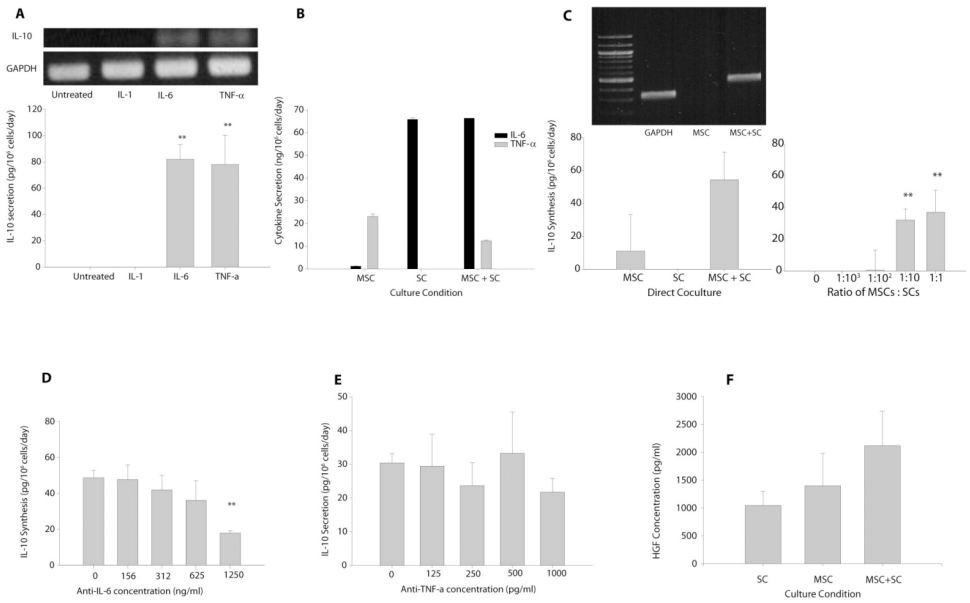

Figure 6. Cytokine expression and secretion by MSCs and SCs. IL-10 mRNA and protein secretion levels of cytokine-treated MSCs. MSCs were treated with IL-1 (5 ng/ml), IL-6 (2.5 ng/ml), or TNF-α (25 ng/ml) for 24 hours. IL-10 mRNA levels were determined by RT-PCR analysis and protein secretion by ELISA on cell culture medium. GAPDH served as an internal control for RT-PCR analysis. One representative RT-PCR result is shown (A). Cytokine secretion in monoculture and direct coculture of SCs and MSCs (1:1 ratio). IL-6 and TNF-α concentrations were measured by ELISA with species-specific antibodies after 2 days of culture. (B). IL-10 mRNA levels and protein secretion in direct and Transwell coculture with activated SCs. ELISA measurement of IL-10 secretion after 2 days of monoculture or 1:1 direct coculture of MSCs and SCs. Detection of IL-10 mRNA transcripts by RT-PCR in MSCs after 4 days of 1:1 Transwell coculture with activated SCs. GAPDH served as an internal control. IL-10 secretion as a function of MSC number (C). Inhibition of IL-10 secretion by MSCs in direct (1:1) coculture with activated SCs after treatment with anti-IL-6 neutralizing antibody. IL-10 secretion from MSCs is reduced after neutralization of SC derived IL-6 (D). Neutralization of TNF-α during indirect coculture with SCs and MSCs does not alter MSC-derived IL-10 secretion (E). HGF secretion after 2 days of monoculture or in direct coculture of SCs and MSCs at a 1:1 ratio (F). Results are the mean of two experiments performed in triplicate. Error bars are standard deviation. **p<.01 compared to control.

Paracrine stimulation with IL-10 and TNF-α from MSCs synergistically inhibits collagen synthesis and proliferation in SCs.

Autocrine IL-10 stimulation in SCs has been shown in vitro to modulate the expression of collagen type I and favor a more quiescent state *(8, 9)*. However, expression of IL-10 is suppressed upon activation in vivo. TNF-α has also been observed to be an anti-proliferative/fibrogenic stimulus for activated SCs *(20)*. Thus, we determined the role of these expressed cytokines on proliferation and collagen synthesis of SCs.

Partial reversal of high levels of PIP-secretion was observed after neutralization of IL-10 or TNF-α at antibody concentrations of 1000 ng/ml (Fig. 7A) and 500 ug/ml (Fig. 7B) or greater, respectively. Neutralization of both IL-10 and TNF-α in coculture led to a synergistic rise in PIP levels from 79 ± 9 pg/10^6 cells/day to 215 ± 20 pg/10^6 cells/day (p=0.002) at maximal antibody concentrations (Fig. 7C).

TFN-α and IL-10 from MSCs were also found to be involved in the inhibition of proliferation of activated SCs. Maximal neutralization of IL-10 led to an increase in the number of BrdU-positive cells from 12% to 15% (p=0.0197, fig. 7D), while anti-TNF-α induced an increase to 33% (p=0.035, fig. 7E). Neutralization of both cytokines synergistically increased proliferation to 44% (p=0.009, fig. 7F). These results indicated that IL-10 and TNF-α secreted by MSCs are both involved in inhibition of collagen synthesis and proliferation in activated SCs.

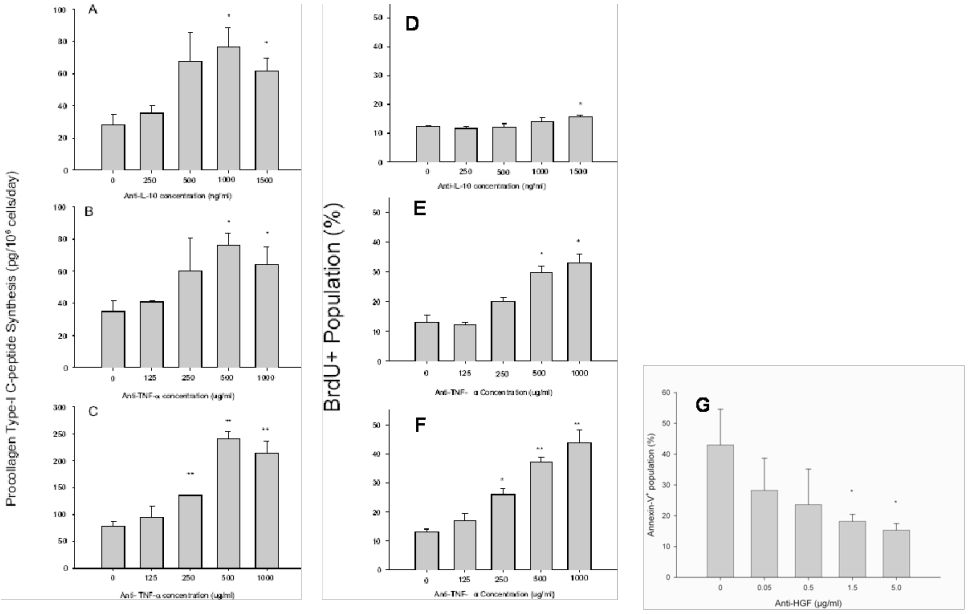

Figure 7. Reversal of inhibited collagen synthesis and proliferation in SCs cocultured with MSCs by neutralizing IL-10 and TNF-α and reversal of induced apoptosis by neutralizing HGF. Activated stellate cells were cultured with MSCs separated by Transwell membranes at a fixed ratio of 1:1. PIP-secretion with increasing concentrations of neutralizing antibody for IL-10 (A), TNF-α (B) or TNF-α with a fixed concentration of anti-IL-10 at 1500 ng/ml (C) was measured by ELISA. BrdU staining was measured by flow cytometry with increasing concentrations of neutralizing antibody for IL-10 (D), TNF-α (E) or TNF-α with a fixed concentration of anti-IL-10 at 1500 ng/ml (F). Annexin-V reactivity was analyzed in rat SCs by flow cytometry at increasing concentrations of neutralizing anti-HGF (G). Data represent the mean of two experiments performed in triplicate. Error bars are standard deviation. **p* < 0.05, ***p* < 0.01 compared to SCs alone (MSC:SC = 0).

MSC-derived HGF induces apoptosis in activated SCs.

Given the observation that a relatively small number of MSCs could cause SC apoptosis, we analyzed the Transwell coculture supernatants for potent pro-apoptotic signals. ELISAs for nerve growth factor and MMP-9, proteins previously reported to induce apoptosis *(5)* in SCs were negative (data not shown). In contrast, we detected a considerable amount of hepatocyte growth factor (HGF) that was produced at approximately equivalent levels by SCs and MSCs (Fig. 6F). HGF has been shown to have a pro-apoptotic effect on liver myofibroblasts *(21)*, which we have reproduced *in vitro* (Fig. 8). Neutralization of HGF resulted in a normalization of apoptosis levels to non-coculture levels from 43 to 15 % (p=0.04, Fig. 7G). No reduction was not observed with an isotype control antibody at the same concentration (data not shown). These data support a role for MSC-derived HGF in accelerating the rate of SC apoptosis.

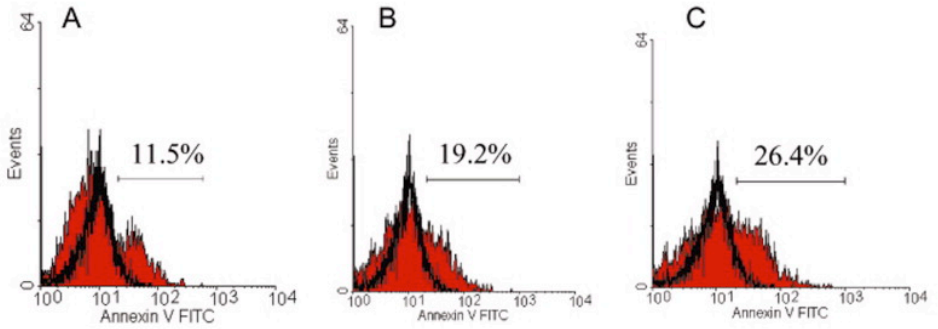

Figure 8. Pro-apoptotic effect of HGF on activated SCs. SC s were stimulated with increasing concentrations of HGF: (A) 0 ng/ml, (B) 10 ng/ml, (C) 1 μg/ml. The apoptotic fraction was determined using flow cytometry for Annexin V.

Discussion

Prior studies have shown that transplantation of a CD45- population of bone marrow cells prevented histopathological changes during chronic exposure to hepato-toxins *(12-14).* These observations were correlated with a co-localization of transplanted cells and SCs, a reduction in the number of α-SMA+ cells, decreased tissue collagen content, and increased gelatinase gene expression. These *in vivo* findings provided a rationale for the therapeutic benefit of MSCs, although evidence supporting this mechanism *in vitro* does not exist. Thus, we investigated the effects of paracrine factors secreted by MSCs on activated SCs, the primary extracellular matrix-producing cell type in the liver.

We observed that MSCs inhibit the proliferative and fibrogenic function of activated SCs in a paracrine manner as a function of MSC number. This inhibition was caused by MSC-derived IL-10 and TNF-α, which acted synergistically. The secretion of IL-10 by MSCs was found to be a dynamic response to IL-6 secretion by activated SCs. Secretion of IL-10 by MSCs in response to TNF-α was observed after exogenous stimulation, but not during mono- or coculture. This result is presumably due to the high levels of stimulation used *in vitro* (25 ng/ml) compared to the low levels measured in coculture (~2.5 ng/ml) indicating a potential threshold concentration of TNF-α necessary for IL-10 expression. Furthermore, MSCs induced apoptosis in activated SCs that is, in part, mediated by HGF. These results support the hypothesis that the therapeutic effect of MSCs may be due to paracrine factors that modulate the proliferation, viability, and function of resident SCs.

Based on these findings, we propose a model describing the cross-talk between MSCs and SCs, and the resulting SC phenotype (Fig. 9). Our studies demonstrate that MSCs act through multiple mechanisms to coordinate a dynamic, integrated response to fibrosis. It is also likely that similar immunomodulatory mechanisms influence the phenotype of resident hepatocytes, Kupffer cells, sinusoidal endothelial cells, and immune cells that infiltrate the liver during inflammation, warranting future studies to examine these multi-cellular effects.

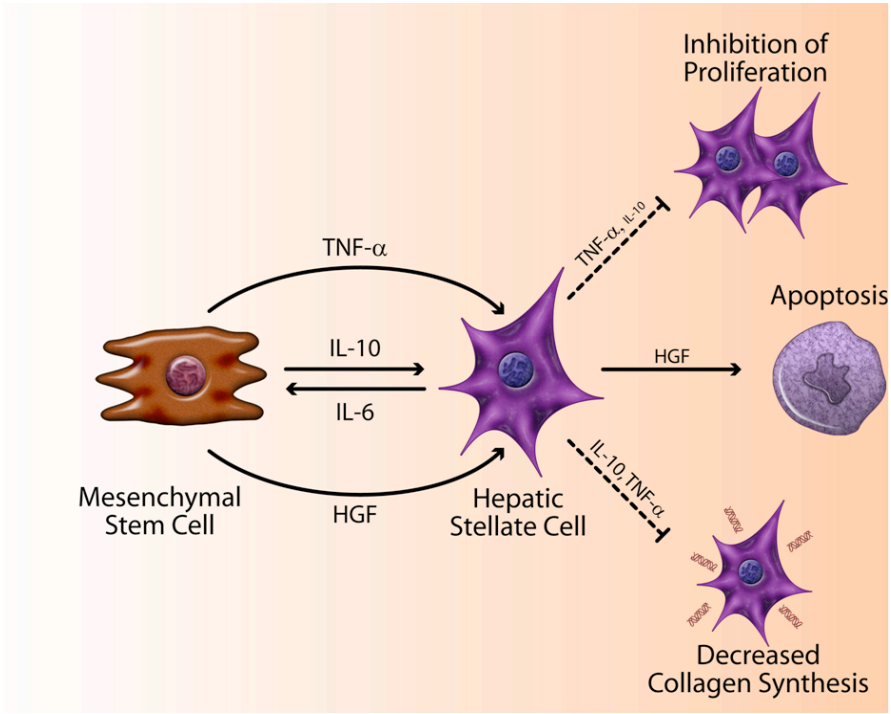

Figure 9. A schematic model of the paracrine effects of MSC-derived factors on activated SCs. Autocrine factors synthesized by SCs are not represented. Release of IL-6 by activated SCs leads to the secretion of IL-10 by MSCs. Induced IL-10, along with constitutively secreted TNF-α, inhibits SC proliferation and collagen synthesis. The marginal effect of IL-10 on SC proliferation is denoted by the smaller font size. SCs undergo apoptosis after coculture with MSCs due to increased levels of HGF.

Several prior studies have documented the presence of host bone marrow-derived cells in the liver and mobilization of stem cell progenitors during liver fibrosis. Endogenous bone marrow-derived, CD45- cells have been observed in human livers during fibrosis *(22),* although their role in disease progression was not determined. A recent study by Russo et al. demonstrated that bone marrow-derived MSCs actively contributed to the SC population of the liver during the progression of fibrosis, although the total SC number during fibrosis did not change *(23).* In another report, the bone marrow was found to renew the myofibroblast population, not SCs, but CD45+ fibrocytes which localized around fibrotic nodules *(24).* We hypothesize that MSCs may be mobilized in response to injury and displace the scar-forming SC mass, but their therapeutic gains may be subdued after chronic insults. Our investigation documents an acute, one-way response of MSCs to activated SCs. However, studies of long-term coculture of MSCs and SCs focused on the reciprocal reaction in the phenotype of MSCs may add to a more global assessment of a potential *in vivo* outcome.

In conclusion, immunomodulation of SCs by MSC soluble factors provides the first mechanistic evidence that MSCs can exert a protective role through paracrine signaling to liver SCs. These findings may be relevant to the beneficial effects of transplanted MSCs in various models of acute and chronic liver injury.

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Chapter 4

Mesenchymal stem cell-derived molecules reverse fulminant hepatic failure

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Abstract

Modulation of the immune system may be a viable alternative in the treatment of fulminant hepatic failure (FHF) and can potentially eliminate the need for donor hepatocytes for cellular therapies. Multipotent bone marrow stromal cells (MSC) have been shown to inhibit the function of various immune cells by undefined paracrine mediators in vitro. Yet, the therapeutic potential of MSC-derived molecules has not been tested in immunological conditions in vivo. Herein, we report that the administration of MSC-derived molecules in two clinically relevant forms, intravenous bolus of conditioned medium (MSC-CM) or extracorporeal perfusion with a bioreactor containing MSCs (MSC-EB), can provide a significant survival benefit in rats undergoing FHF. We observed a cell mass-dependent reduction in mortality that was abolished at high cell numbers indicating a therapeutic window. Analysis of the MSC secretome using a protein array screen revealed a large fraction of chemotactic cytokines, or chemokines. When MSC-CM was fractionated based on heparin binding affinity, a known ligand for all chemokines, only the heparin-binding eluent reversed FHF indicating that the active components of MSC-CM reside in this fraction. Finally, we demonstrate using computed tomography of adoptively transferred leukocytes that MSC-CM functionally diverts immune cells from the injured organ, further supporting the role of chemokines and altered leukocyte migration as a novel therapeutic modality for FHF. These data provide the first experimental evidence of the medicinal use of MSC-derived molecules in the treatment of an inflammatory condition.

Introduction

Clinical trials testing the efficacy of bioartificial liver support in treating fulminant hepatic failure (FHF) have provided some promising results, yet the current generation of devices has not demonstrated sufficient efficacy and reliability for routine use, primarily due to a lack of a functionally stable, human hepatocyte source [1]. We have previously shown that immunomodulation via interleukin-1 receptor antagonism in the form of: (a) the recombinant protein, (b) adenoviral vector gene therapy, or (c) transfected hepatocytes seeded in an extracorporeal device, can provide a survival benefit in an animal model of FHF [2, 3]. However, due to concerns associated with gene transfer in vivo or ex vivo and the expense of recombinant protein production, we sought to identify a natural human "cellular equivalent" of immunomodulation that could be incorporated into bioartificial liver assist devices.

Within the bone marrow space, multipotent stromal cells, also referred to as mesenchymal stem cells (MSCs), are known to be the precursor cell for stromal tissues that support hematopoiesis [4]. The immunomodulatory function of MSCs was first reported after it was observed that they could evade immunosurveillance after cell transplantation [5]. This ability of MSCs to alter an immune response has been exploited for therapeutic purposes as demonstrated by the case of a patient suffering from steroid-refractory graft-versus-host disease who was successfully treated by the infusion of haploidentical MSCs [6]. In vitro studies have subsequently shown that MSCs actively inhibit the function of several immune cells through secreted cytokines, growth factors and enzymatic action, although controversy exists on the identity of the responsible mediators [7-11]. The fortification of the soluble microenvironment by MSCs can also affect non-hematopoietic cells as well. MSCs used for cellular cardiomyoplasty after an ischemic event revealed that MSC-derived soluble molecules inhibited hypoxia-induced apoptosis of cardiomyocytes during the acute phase of injury resolution [12, 13]. Taken together, these studies indicate that MSCs can independently affect immune and tissue cells by paracrine means.

On this basis, we hypothesized that the paracrine function of MSCs may be of therapeutic value when used in the setting of acute organ failure, wherein parenchymal cell loss is integrated with a local and systemic inflammatory response. Here, we report a significant survival benefit in the treatment of FHF after intravenous delivery of MSC biomolecules in the form of bolus injections of conditioned medium (MSC-CM) or a MSC-based extracorporeal bioreactor (MSC-EB). The survival benefit of MSC-CM was a function of the cell mass used for media conditioning and was found to have an optimum. In addition, we performed a proteomic screen of the MSC secretome and detected high levels of potent chemotactic cytokines. Fractionation of MSC-CM based on affinity to heparin sulfate, a known ligand for all chemokines, revealed that the therapeutic activity of MSC-CM was restricted to the heparin bound fraction, further supporting the role of chemokines as responsible mediators. Finally, we show that MSC-CM actively promotes the emigration of adoptively transferred leukocytes from the injured liver, which may be the

functional consequence of introducing chemokines into the circulation without directionality to an injured site.

Materials & methods

Materials were purchased from Sigma-Aldrich, St. Louis, MO unless otherwise stated.

Cell Culture
Human MSCs were isolated from commercially available bone marrow aspirates (Clonetics-Poietics, Walkersville, MD) and grown as previously reported. Cells were used for experiments during passages 3-7. NIH 3T3-J2 fibroblasts were a kind gift from Dr. Howard Green and cultured according to donor's protocols.

Preparation and delivery of cells, cell lysates and conditioned medium
Cellular lysates were prepared by sonication (VWR Scientific, West Chester, PA). The dose of sonicated cells administered was $2x10^6$ cells per subject, which represented the same cell mass used for extracorporeal bioreactor or cell transplant experiments. Conditioned medium was prepared by collecting serum-free medium (supplemented with 0.05% bovine serum albumin to prevent protein aggregation) after 24 hour culture of different cell masses. The majority of experiments were performed with the optimal cell mass of $2x10^6$ cells. The medium was then concentrated, approximately 25 fold, using ultrafiltration units (Amicon Ultra-PL 3, Millipore, Bedford, MA, USA) with a 3 kDa molecular weight cut-off. For fractionation experiments, concentrated medium was passed over a heparin-agarose column and the flow-through and eluted fractions were collected and reconcentrated using the same ultrafiltration system. A total volume of 500 μl containing cells, cellular lysates, vehicle (PBS) only or 900 μl of conditioned medium was infused in the penile vein 24 hours after induction of FHF using the same anesthesia protocol described for the cannulation procedure.

Liver Failure Induction and Extracorporeal Bioreactor Support
The induction of fulminant hepatic failure and extracorporeal device operation is previously reported [2]. Briefly, male Sprague-Dawley rats weighing between 280 and 370 grams were anaesthetized 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. Twenty-four hours later, 1.2 g/kg Gal-N freshly dissolved in physiological saline and adjusted to pH 7.3 with 1 N NaOH was injected i.p., followed by a second equal injection 12 hours later. Twenty-four hours after the first injection of Gal-N, the arterial and venous lines were connected to an extracorporeal circuit. Plasma was separated using a plasma separator (MicroKros, pore size 0.2 micron). Plasma was perfused through the polycarbonate, flat-plate bioreactor and subsequently reunited with the cellular components of the blood and returned to the animal (Fig. 1). The extracorporeal bioreactor was operated for 10 hours. Animals that died during reactor operation and failed to receive adequate treatment (MSC-EB, N=3 and Fibroblast-EB, N=2) were censored from analysis. Animal survival

was monitored every 12 hours. Plasma or whole blood was analyzed for liver injury biomarkers using a microfluidic metabolic assay (Picollo, Abaxis, Union City, CA).

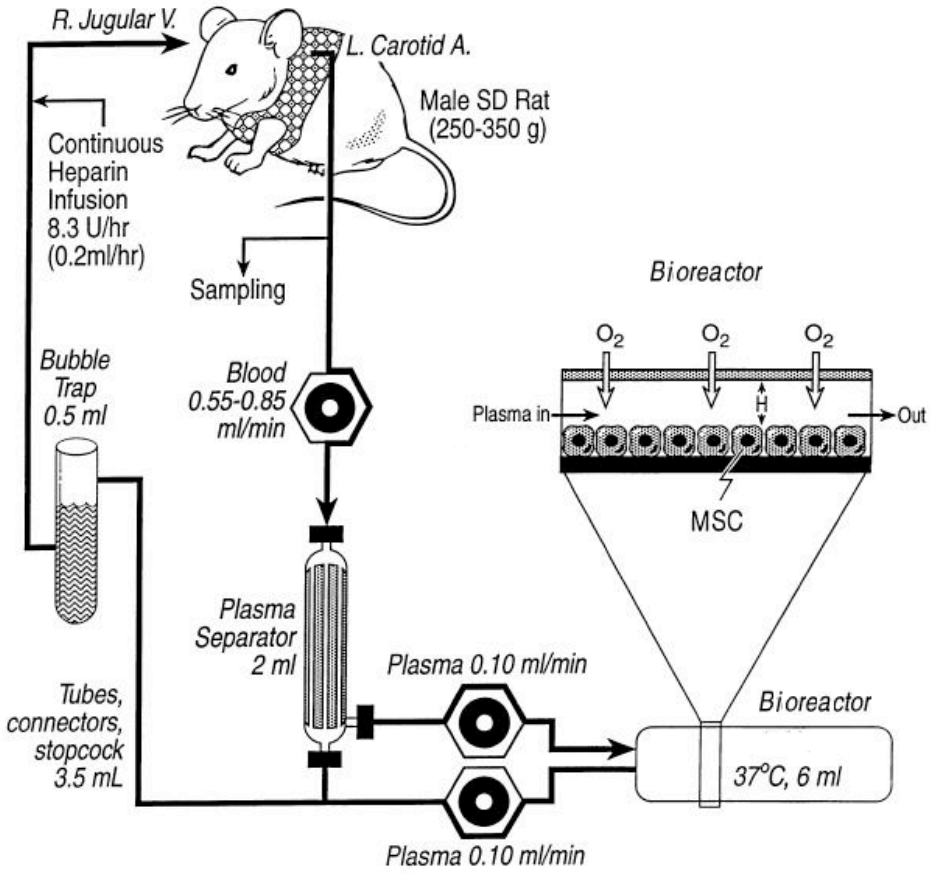

Figure 1. Experimental setup for extracorporeal MSC-based bioreactor support.

Liver histology

Liver tissue was harvested from rats induced with a sub-lethal regimen of Gal-N (0.6 g/kg), 36 hours after treatment with MSC-CM. Tissue was fixed in 10% buffered formalin, embedded in paraffin, sectioned to 4 μm thickness, and stained with hematoxylin and eosin.

Adoptive transfer of radiolabeled leukocytes

Leukocytes were isolated from whole rat blood by NH_4Cl erythrocyte lysis. Cells were pelleted, washed once with PBS and resuspended in 0.9% saline containing the In^{111} oxine isotope (GE Healthcare Biosciences Corp., Piscataway, NJ). Cells were labeled at 92% efficiency with high viability. Approximately $15x10^6$ cells were infused into the penile vein of Gal-N injured

(0.6 g/kg) directly after treatment with vehicle or MSC-CM. SPECT images were captured using a M.CAM gamma camera setup (Siemens Medical Systems, Malvern, PA) at 0, 3 and 24 hours after leukocyte infusion.

Protein array of MSC supernatants

Supernatants were prepared by collecting serum-free medium after 24-hour culture of approximately $2x10^6$ MSCs. These were analyzed for a panel of specified proteins using an antibody array (RayBio Human Cytokine Antibody Array C Series 2000, RayBiotech Inc., Norcross, GA) as specified by the vendor.

Results

MSC-derived components reverse FHF

We first assessed various MSC treatment modalities such as cell transplantation, delivery of cellular lysates or conditioned medium to assess the most efficacious therapy. Sprague-Dawley rats were intraperitoneally administered a total of two injections of 1.2 g/kg of a hepatotoxin, D-galactosamine (Gal-N), each separated by 12 hours [14]. With this regimen of liver failure induction, we have previously shown that death occurs 36-72 hours after injection of the first dose and is associated with significant hepatocellular necrosis concomitant with leukocyte infiltration and an increase of inflammatory cytokines in liver tissue. Animals were treated 24 hours after FHF induction with intravenous injections of whole cells or cell lysates. No significant benefit was seen after the intravenous infusion of $2x10^6$ human MSCs 24 hours post-induction, which is most likely due to poor engraftment, entrapment in the alveolar capillary bed and/or immune rejection of the cells (Fig. 2). In contrast, treatment with cellular lysates, derived from the same cell mass used for transplantation, showed an increased survival trend compared to vehicle ($P < 0.47$) and fibroblast lysate ($P < 0.36$) controls.

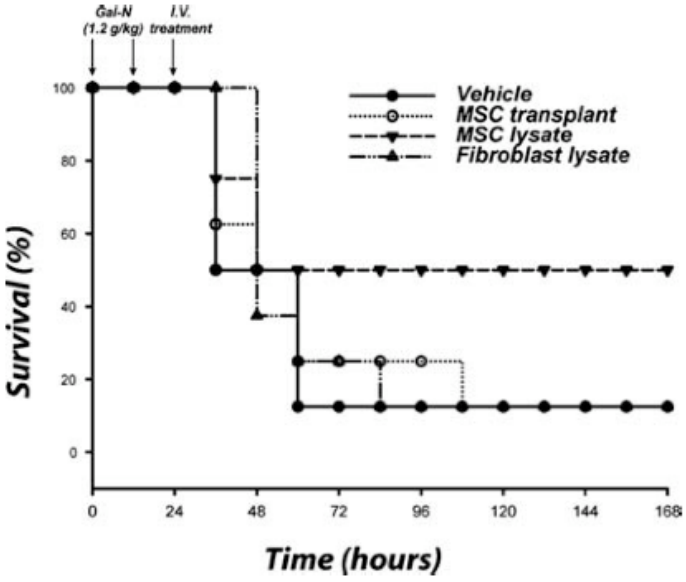

Figure 2. Infusion of MSC lysate provides a survival trend in an animal model of FHF. Sprague-Dawley rats were administered lethal intraperitoneal injections of a hepatotoxin. Animals were treated with i.v. injections of MSCs, or MSC lysates from the same cell mass (2×10^6 cells). Controls received vehicle or NIH 3T3-J2 fibroblast components. Kaplan-Meier survival analysis of Gal-N administered rats treated with cell transplants or lysates. Time points of interventions are stated above survival plots. Results are cumulative data of two independent experiments (N=8 per each group) using different batches of MSCs. P-value determined by Log Rank Test.

We then determined if the efficacy observed with lysates could be reproduced by using the secreted molecules from MSCs. A longitudinal study using MSC-CM from the same cell mass (i.e., $2x10^6$ human MSCs) revealed a distinct survival benefit compared to vehicle ($P < 0.032$) and fibroblast ($P < 0.026$) concentrated medium (Fig. 3A). In addition, we monitored 72-hour survival of FHF-induced rats as a function of MSC mass from which medium was conditioned (Fig. 3B). Interestingly, the effect of MSC concentrate was abrogated at higher cell masses indicating a therapeutic window of effectiveness. Moreover, the observation that xenogeneic MSC lysates and supernatants decreased animal mortality suggests that these factors can cross histocompatability barriers.

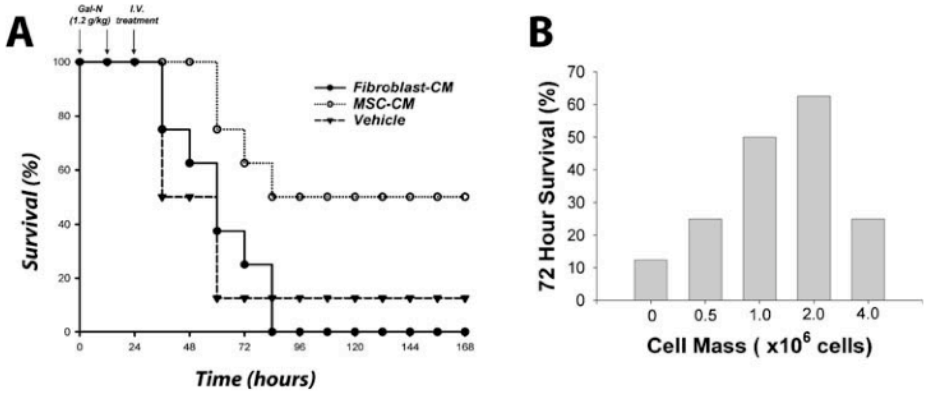

Figure 3. MSC-CM reverses FHF in a cell mass-dependent manner. (A) Kaplan-Meier survival analysis of Gal-N administered rats treated with concentrated MSC-CM. (B) Dose response of animal survival 72 hours after liver failure induction as a function of MSC mass from which MSC-CM was derived. Controls received vehicle or fibroblast conditioned medium (fibroblast-CM). Time points of interventions are stated above survival plots. Results for both panels are cumulative data of two independent experiments using different batches of MSC-CM (N=8 per each group). P-value determined by Log Rank Test.

Combined metabolic and secretory function in MSC-EB support provide hepatoprotection and survival benefit

Based on these results, we then developed a MSC-EB to combine the effectiveness of intracellular and secreted molecules of MSCs in a single device. Animals were treated 24 hours after FHF induction with a human MSC-EB connected to the systemic circulation of the animal. Bioreactors seeded with fibroblasts (fibroblast-EB) and acellular (acellular-EB) bioreactors served as controls. After 10 hours of extracorporeal perfusion, animals were taken off assist support and monitored for survival. Plasma was obtained at the start of, and 24 hours after, bioreactor treatment and analyzed for hepatocyte enzyme release. Liver serologies, including aspartate aminotransferase ($P < 0.02$) and alanine aminotransferase ($P < 0.001$) were improved in animals treated with the MSC-EB (Fig. 4A). These data demonstrate a hepatoprotective effect of

device therapy as shown by the reduction in biochemical markers of hepatocyte death. More importantly, 71% of animals treated with the MSC-EB survived, compared to 14% (Fig. 4B) in both acellular ($P < 0.037$) and fibroblast controls ($P < 0.05$).

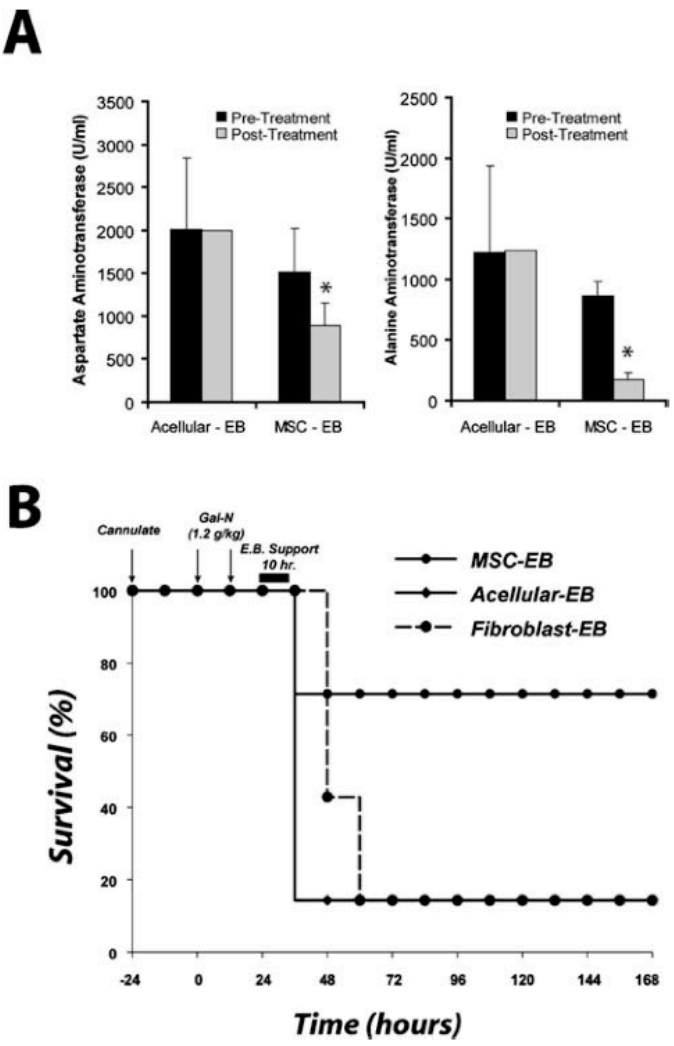

Figure 4. MSC-EB support reduces liver injury biomarkers and increases survival. Animals were treated with an MSC-EB, using a 3T3 fibroblast-based bioreactor (fibroblast-EB) and an acellular bioreactor (acellular-EB) as controls. (A) Serum biomarkers of liver injury, aspartate aminotransferase and alanine aminotransferase preceding and 24 hours after treatment with a MSC-EB (n=5) or an acellular-EB (n=3). Due to mortality, n=1 in the acellular group after treatment. (B) Kaplan-Meier survival analysis of Gal-N administered rats treated with EBs. Time points of interventions are stated above survival plots. Each result for (B) was an independent experiment using different batches of MSCs. P-value determined by student's t-test analysis for panel (A). P-value determined by Log Rank Test for panel (B).

MSC-CM therapy inhibits panlobular leukocyte invasion, bile duct duplication and hepatocellular death

In order to determine histopathological changes after MSC-CM therapy we used a sublethal regimen Gal-N to induce acute liver injury, thereby ensuring survival in our control-treated group for comparison. It should be noted that even at this Gal-N dose, mortality occurred in a vehicle-treated group (N = 1) confirming that the extent of injury in this model can still be fatal. Gal-N injured rats were treated with vehicle (N = 4) 24 hours after injury and their livers were harvested 36 hours thereafter for pathological analysis. Microscopic evaluation of liver tissue from vehicle treated rats revealed profound hepatocellular apoptosis, bile duct duplication and panlobular mononuclear leukocyte infiltration with cytoplasmic vacuolization and severe distortion of tissue architecture (Fig. 5 A,C). MSC-CM treated rats showed no signs of disseminated inflammation (Fig. 5B), although minor periportal edema and fibrin deposition consistent with tissue repair was observed (Fig. 5D).

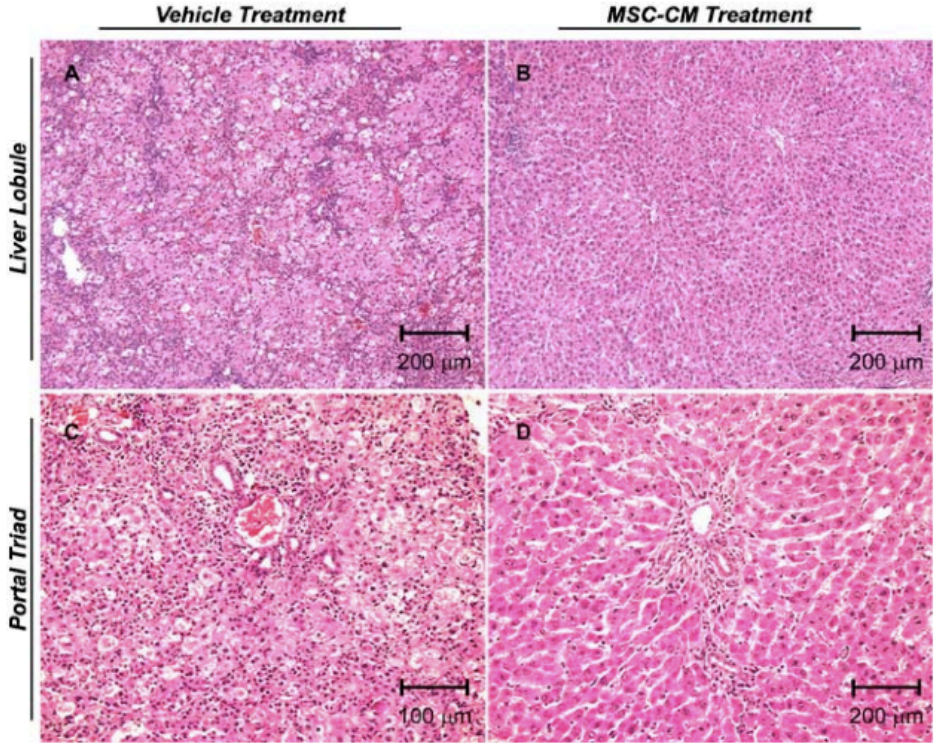

Figure 5. MSC-CM treatment inhibits immune cell infiltration and hepatobiliary cell change in Gal-N injured liver tissue. Representative H&E histology sections of liver tissue from Gal-N injured rats 36 hours after treatment with vehicle (A,C) or MSC-CM (B,D). Scale bars are indicated on the micrographs. Images (A,B) and (C,D) are captured at 10x and 20x magnification, respectively.

MSC-CM treatment alters immune cell migration to the liver

We hypothesized that intravenous delivery of chemokines, without directionality to an injured site per se, may cause a 'chemokine storm' that diverts immune cell migration away from an inflamed, target organ. To test this theory, we adoptively transferred radiolabeled leukocytes, directly after MSC-CM or vehicle treatment, into Gal-N (0.6 g/kg) injured rats and monitored leukocyte trafficking using single photon emission computed tomography (SPECT) over time (Fig. 6A). Qualitatively, more leukocytes were seen in the liver post-infusion and 3 hours thereafter in MSC-CM treated animals (Fig. 6B-C) compared to vehicle treated animals (Fig. 6E-F), which shows that there was no detriment in leukocyte homing. However, by 24 hours post-infusion there was a distinct decrease in signal intensity in the liver of MSC-CM treated animals (Fig. 5D), whereas vehicle controls had increased intensity in the liver (Fig. 6F). These results suggest that there was a selective pressure upon leukocytes to emigrate from the liver due to MSC-CM, unlike control conditions where leukocytes eventually migrated to the injured organ. These data support our hypothesis that altered leukocyte migration may be a potential target of MSC-CM therapy, however other explanations cannot be ruled out without more comprehensive investigations.

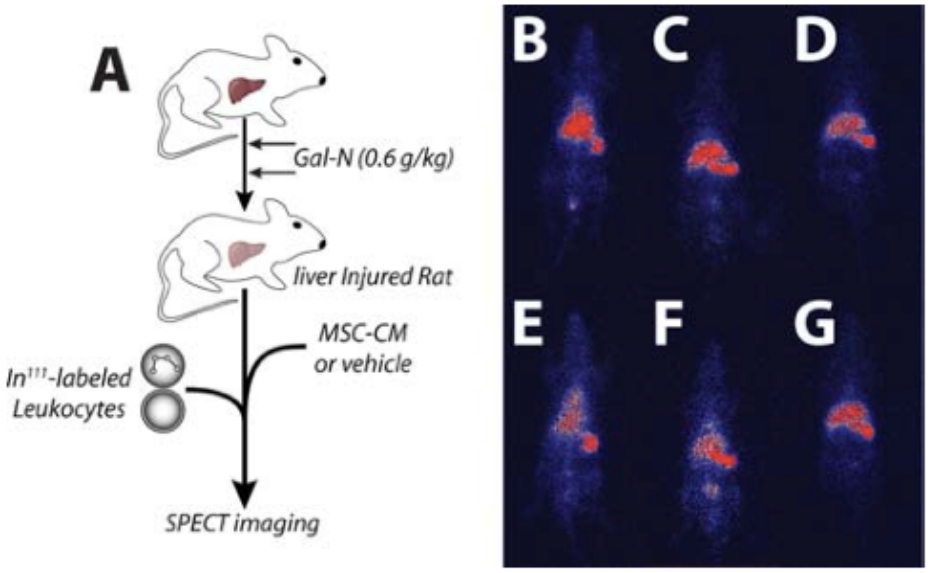

Figure 6. Alteration in leukocyte migration after MSC-CM treatment. (A) Experimental design of adoptive transfer study. Gal-N injured rats were treated with vehicle or MSC-CM followed by infusion of In^{111}-labeled leukocytes. SPECT images were acquired at t = 0, 3, and 24 hr. for MSC-CM (B-D) and vehicle (E-G) treated rats, respectively.

MSC-CM is composed of many chemokines that correlate with therapeutic activity

In an effort to understand the molecular mediators of the observed effects of MSC therapy, we examined MSC-CM using a high-density protein array. MSC-CM contained 69 of the 174 proteins assayed (Fig. 7A), which included a broad spectrum of molecules involved in immunomodulation and liver regeneration. Cluster analysis revealed that a large fraction (30%) of MSC-CM was composed of chemokines (Fig. 7B), many of which were expressed at very high relative levels. We decided to fractionate MSC-CM based on functionality using affinity-based methods rather than other arbitrary molecular criteria such as size or hydrophobicity. MSC-CM was passed over an affinity column impregnated with heparin sulfate, a known ligand for all chemokines and separated into bound and unbound fractions. Each fraction was infused into FHF-induced rats with overall survival as the study endpoint. We observed that the therapeutic activity of MSC-CM was restricted to the heparin bound fraction, providing a strong correlation between chemokines and the survival benefit after MSC-CM infusion in FHF-induced rats (Fig. 7C).

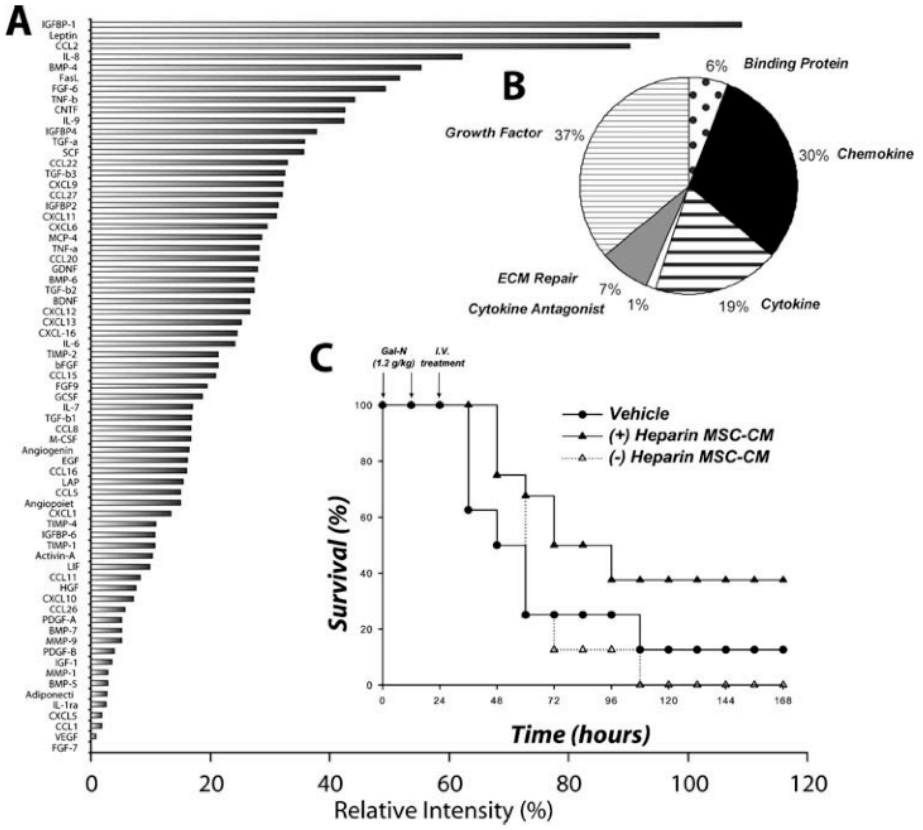

Figure 7. MSC-CM is composed of high levels of chemokines that correlate with survival benefit seen in FHF. Serum-free MSC-CM was analyzed using an antibody array for 174 specified proteins. (A) Densiometry of spotted antibody array results. Data are presented as spot intensity relative to the negative control and normalized to positive control. (B) Pie chart showing cluster analysis of MSC secreted proteins based on reported function. MSC-CM was fractionated over a heparin-agarose column into heparin bound and unbound fractions. (C) Kaplan-Meier survival analysis of Gal-N administered rats treated with the (+) heparin MSC-CM and (-) heparin MSC-CM. Time points of interventions are stated above survival plots. Results for (C) are cumulative data of two independent experiments using different batches of MSC-CM (N=8 per each group). P-value determined by Log Rank Test.

Discussion

Our findings demonstrate that MSC-derived molecules can protect against hepatocyte death and increase survival in Gal-N induced FHF. Specifically, we have shown that an intravenous bolus of MSC-CM during active disease can reverse organ failure. The efficacy of MSC-CM was found to be a function of the cell mass from which medium was conditioned, suggesting important pharmacological aspects of this treatment. Treatment with a MSC-EB, which combined both the secretory and metabolic functions of MSCs in a single device, provided the greatest benefit in survival and potentially illustrates a platform to study stem cell function and the bone marrow microenvironment ex vivo. These results are significant because we have identified a non-hepatic source of human cells with minimal metabolic demands that can be expanded to clinical scale for liver assist devices and shown that MSC treatment can cross histocompatability barriers. Analysis of MSC-CM revealed high levels of potent chemokines that, when fractionated based on heparin binding affinity, correlated with the therapeutic activity of MSC-CM. We are currently testing individual chemokines and combinations thereof to demonstrate a casual relationship. Moreover, we provide evidence showing altered immune cell migration after MSC-CM treatment supporting our theory of 'chemokine storm' as a potentially novel treatment paradigm for FHF. Whether this strategy of MSC-CM or MSC-EB therapy is a relevant means of treatment for other organ failure and inflammatory conditions should be experimentally explored.

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Chapter 5

Mesenchymal stem cell-derived molecules modulate hepatocellular death and regeneration in a rat model of fulminant hepatic failure

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Hepatology. ***In press.***

Abstract

Orthotopic liver transplantation is the only proven effective treatment for fulminant hepatic failure (FHF), but its use is limited because of donor shortage, high costs and the requirement for lifelong immunosuppression. FHF is usually accompanied by massive hepatocellular death with compensatory liver regeneration that fails to meet the cellular losses. Therefore, therapy aimed at inhibiting cell death and stimulating repair could offer major benefits in the treatment of FHF. Recent studies have demonstrated that mesenchymal stem cell (MSC) therapy can prevent parenchymal cell loss and promote tissue repair in models of myocardial infarction, acute kidney failure and stroke through the action of trophic secreted molecules. In this study, we investigated whether MSC therapy can protect the acutely injured liver and stimulate regeneration. In the D-galactosamine induced rat model of FHF, we show that systemic infusion of MSC-conditioned medium (MSC-CM) results in a 90% reduction of apoptotic hepatocellular death and a 3-fold increment in the number of proliferating liver cells. This was accompanied by 4-fold to 27-fold increases in expression levels of 10 genes known to be upregulated during liver regeneration. Direct anti-apoptotic and pro-proliferative effects of MSC-CM on hepatocytes were demonstrated using in vitro studies. Conclusion: These data provide the first clear evidence that MSC-CM therapy provides trophic support to the injured liver by inhibiting hepatocellular death and stimulating regeneration, potentially creating new avenues for the treatment of FHF.

Introduction

Fulminant hepatic failure (FHF) affects approximately 2,500 people in the US annually with mortality rates exceeding 30% even in highly specialized centers (1). Orthotopic liver transplantation is the current gold standard of care, but its use is limited because of donor shortage, financial considerations and the requirement for lifelong immunosuppression (2). Strategies aimed at replacing liver functions using acellular and hepatocyte-based devices have shown promising results in animal models and small series of patients, but efficacy has never been shown in controlled clinical trials (3, 4). FHF is typically associated with massive hepatocellular necrosis with a compensatory regeneration of the organ that fails to meet the cellular losses (5). Therefore, effective treatment strategies aimed at protecting the dying liver cell mass and stimulating regeneration could offer major benefits in the treatment of FHF.

Bone marrow-derived mesenchymal stem cells (MSCs) are known to naturally support hematopoiesis through secretion of an array of trophic molecules, including growth factors and cytokines (6, 7). Recent studies in models of myocardial infarction, acute kidney failure and stroke have shown that MSC therapy has the potential to inhibit cell death and stimulate endogenous regeneration programs (8-11). We recently demonstrated that systemic administration of MSC-derived molecules, either by a bolus of conditioned medium (MSC-CM) or extracorporeal support with a MSC bioreactor, significantly improved short-term survival in a D-galactosamine induced rat model of FHF (12). We observed alteration in immune cell function after MSC-CM therapy, however the effect of MSC-CM on resident liver cells was not fully explored.

In the present study, we investigated whether systemic infusion of MSC-CM can protect the acutely injured liver and stimulate reparative programs in the D-galactosamine model of FHF in the rat. We observed a 90% reduction in apoptotic hepatocellular death and a 3-fold increment in the number of proliferating liver cells in vivo after MSC-CM treatment. In addition, we detected increased gene expression, ranging from 4-fold to 27-fold, of several genes known to be upregulated during hepatocyte replication. Using in vitro assays, we definitively show that secretions from MSCs have a direct inhibitory effect on hepatocyte death and a stimulatory effect on their proliferation.

Materials & methods

Animals
Male Sprague-Dawley rats weighing 250-300g were used for the FHF experiments. Hepatocytes were isolated from 150-200g female Lewis. Animals (Charles River Laboratories, Boston, MA) were cared for in accordance with the guidelines set forth by the Committee on Laboratory Resources, National Institutes of Health.

MSC-CM
Human MSCs were kindly provided by the Tulane Center for Gene Therapy. MSCs were cultured and characterized for surface marker expression and adipocytic and osteogenic differentiation capacity as we described previously (13). For the generation of MSC-CM, cells were allowed to grow to 70-80% confluence (approximately $1x10^6$ MSCs per 175 cm^2 flask), washed thoroughly and cultured in 15 ml serum free Dulbecco's Modified Eagle's Medium supplemented with 0.05% bovine serum albumin. Conditioned medium was collected 24 hours later and concentrated 25-fold using ultrafiltration with a 3kD cutoff.

FHF Induction and Treatment
FHF was induced by two injections of D-galactosamine, delivered i.p. with a 12-hour interval between injections (Sigma Aldrich, St Louis, MO). We chose a dose of 0.6 g/kg D-galactosamine per injection to induce FHF with an intermediate level of mortality based on our previous studies (14). After 24 hours, 0.9 ml of MSC-CM or 0.9% NaCl solution (vehicle control) was injected through the penile vein. We used 4 animals per group for tissue collection after sacrifice at 36 hours after treatment and 10 per group for survival analysis. In a separate group of animals (n=4 per group), blood samples were collected at 12, 36, 60, 108 and 156 hours after treatment by tail snip for analysis of liver enzyme release levels.

Aminotransferase (ALAT and ASAT) Release
Serum was stored at -80° and stored until analysis for alanine and aspartate aminotransferase levels using Thermo Electron Infinity ALAT and ASAT Reagent (Louisville, CO) according to the vendor's instructions.

Cytokine Levels
Quantification of serum levels of rat interleukin (IL)-1β, tumor necrosis factor-α (TNF-α), IL-6, IL-2, IL-1 receptor antagonist (IL-1ra) and IL-10 was determined using enzyme-linked immunosorbant assay (ELISA) per manufacturer's instructions (R&D Systems, Minneapolis, MN).

Liver Histology
Formalin-fixed, paraffin-embedded liver samples were sectioned at 4 μm and stained with hematoxylin & eosin (H&E). Histological assessment was performed by a blinded observer, who scored the liver sections using the

following criteria: normal histology "0"; minor hepatocellular death and inflammation "1"; widely distributed patchy necrosis and inflammation "2"; complete disruption with panlobular necrosis and inflammation "3"; mortality "4".

Immunohistochemistry

Four-micron thick sections of formalin fixed tissue were deparaffinized, rehydrated and blocked in 3% hydrogen peroxide in ethanol for 15 minutes after baking at 60° for 1 hour. For proliferating nuclear cell antigen (PCNA) immunohistochemistry, sections were treated in 10mM Citrate Buffer at pH 6.0 using a digital pressure cooker, blocked with 1.5% horse serum for 15 minutes and incubated with mouse monoclonal anti-PCNA (Clone 24, BD, San Jose, CA) at a 1:500 dilution for 1 hour at room temperature. Primary antibody was detected using Vectastain Elite ABC kit (Vector laboratories, Burlington, CA). For Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) we used the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon International, Temecula, CA) according to the vendor's instructions. The sections were developed using 3,3'-diaminobenzidine and counter-stained with hematoxylin.

Digital Cell Quantification

Quantification of cell numbers in stained liver sections was performed in 10 random 40x images per animal using the public software ImageJ (http://rsb.info.nih.gov/ij/). Positive cells were quantified using appropriate criteria for corresponding sizes of the nuclei. Nuclei of area greater than 700 $pixel^2$ were analyzed to specifically identify hepatocytes from resident non-parenchymal and infiltrating inflammatory cells (15).

Gene Expression

RNA was extracted from liver tissue homogenates using the Nucleospin RNA purification kit (BD, Palo Alto, CA) per manufacturer's instructions. Approximately 100 ng-1 μg of total mRNA was reverse transcribed to cDNA using the TwoStep reverse transcriptase-polymerase chain reaction (RT-PCR) Kit (Qiagen, Valencia, CA) and amplified in a Perkin Etus Thermal Cycler 480. Cycling conditions were: 1) 25 °C for 10 minutes; 2) 42 °C for 60 minutes; 3) 94 °C for 10 minutes. cDNA was either analyzed by endpoint or kinetic RT-PCR using the same cycling conditions for amplification: 1) 94 °C for 10 minutes; 2) 30-40 cycles of 94°C for 30 seconds, 55 °C for 30 seconds and 72 °C for 1 minute; 3) 72 °C for 10 minutes. Primers used for amplification were designed using the public software algorithm Primer3 (Supplementary Table 1). Quantitative RT-PCR data was analyzed using the 2^{-DDCt} method and displayed as relative fold changes normalized to vehicle treatment.

Supplementary table 1. RT-PCR Primer sequences.

Gene	Primer-Probe Sequence 5'-3'	Amplicon Size
TNF-a	F: actcccagaaaagcaagcaa R: cgagcaggaatgagaagagg	211
IL-6	F: ccggagaggagacttcacag R: cagaattgccattgcacaac	134
HGF	F: cgagctatcgcggtaaagac R: tgtagctttcaccgttgcag	165
HB-EGF	F: gcctcctgtaattgctctgc R: gccaaaaatcctggagcata	207
EGF	F: acaccgaaggtggctatgtc R: tagagtcagggcaaggcagt	195
SCF	F: caaaactggtggcgaatctt R: gccacgaggtcatccactat	217
OSM	F: caactgggtgctttcagaca R: aacccatgaagcgatggtag	253
Amphiregulin	F: gtctttgtctccgccgtaag R: ctgaacttctggagccttcg:	244
Timp3	F: tgtacaccccagcctctttc R: cttctcgccaagacctcaac	182
TGF-a	F: gcaagttctgcctgttcctc R: gcactgaaccaacccacttt	161
GAPDH	F: atgacatcaagaaggtggtg R: cataccaggaaatgagcttg	177

Supplementary Table 1. RT-PCR Primer Sequences.

Hepatocyte Isolation and Culture Media

Hepatocytes were isolated using a two-step collagenase perfusion procedure as described previously (16). Viability was greater than 90% as determined by trypan blue exclusion. Hepatocyte culture medium consisted of Dulbecco's Modified Eagle's Medium supplemented with 10% FBS 14 ng/ml glucagon, 0.5 U/ml insulin, 20 ng/ml epidermal growth factor (EGF), 7.5 μg/ml hydrocortisone, 200 μg/ml streptomycin and 200 U/ml penicillin. Culture conditions were hepatocyte medium (control), hepatocyte medium mixed at a 50:1 ratio with the 25-fold concentrated MSC-CM (2% MSC-CM) and at a 12.5:1 ratio for 8% MSC-CM.

Hepatocyte Apoptosis In Vitro

Hepatocytes were cultured for 7 days in 12-well plates at a density of $1x10^5$ cells/cm^2 in a collagen gel sandwich configuration in hepatocyte medium as described previously (16). Actinomycin D was added at a concentration of 0.2

μg/ml for 1 hour, followed by TNF-α (30 ng/ml) with or without MSC-CM at 2% or 8% for 8 hours (17). Experiments were performed in triplicate.

Fluorescence Live-Dead Staining

After induction of apoptosis, hepatocytes were stained using a fluorescent Live-Dead Viability Assay (Molecular Probes, Eugene, OR) according to the manufacturer's instructions and captured on a Zeiss 200 Axiovert microscope. Viable and non-viable cells were quantified in 4 random images per well using the digital image analysis methods described above. The viable fraction was defined as the number of viable cells divided by the sum of the viable and non-viable cells.

Hepatocyte Proliferation In Vitro: Culture System

Hepatocytes were seeded at low density (1.25×10^3 cells/cm^2) on a feeder layer of 3T3-J2 fibroblasts (8×10^4 cells/cm^2) that had been growth-arrested by exposure to 12 μg/ml mitomycin-C for 2.5 hours. Hepatocytes were allowed to proliferate with daily medium changes. Two separate experiments were performed in duplicate.

Hepatocyte Proliferation In Vitro: Assays

Cells were cultured with 10 μM of the DNA synthesis marker bromodeoxyuridine (BrdU; Sigma). After 48 hours, cultures were fixed in 70% ethanol for 45 min and treated with 4N HCl and 0.2% TritonX-10. After incubation in blocking buffer for 30 min, cells were incubated for 60 min with anti-BrdU-Alex594 (Invitrogen, Carlsbad, CA) and rabbit anti-rat albumin at 37°C, followed by FITC conjugated anti-rabbit IgG (ICN Pharmaceuticals, Aurora, OH) at room temperature. BrdU positive cells in each hepatocyte colony were counted in fluorescence microscopy images. Albumin content in supernatant samples was determined by ELISA using purified rat albumin and a peroxidase-conjugated antibody. Urea content was determined with a commercially available kit (StanBio Laboratory, Boerne, TX) using the manufacturer's instructions.

Statistics

Data are expressed as the mean ± standard error of the mean. Statistical significance was determined by a two-tailed Student's t-test and by Kaplan-Meier analysis and a Log Rank test for survival analysis. A P-value of <0.05 was used for statistical significance. Statistical image analysis was performed after determining the data could be fit with a normal distribution. A two-tailed Student's t-test was employed after the exclusion of outliers that were less or greater than 2 standard deviations away from the median.

Results

MSC-CM Therapy Inhibits Liver Damage and Improves Survival

When massive hepatocyte death overwhelms the regenerative capacity of the liver during FHF, death ensues. Liver enzyme release levels measured in the peripheral blood provide a good estimation of the amount of ongoing liver damage. Two of four vehicle-treated animals died between 12 and 36 hours following treatment. In the surviving animals, the peak in liver damage was measured at 36 hours after the systemic treatment, both in the control and the MSC-CM treated group. However, the maximum ALAT and ASAT levels were reduced by 67% (P=0.012) and 65% (P=0.009) respectively in the MSC-CM treated animals (Fig. 1A). No significant differences were observed at any of the other timepoints. In the rats treated for survival analysis, a significant survival benefit was observed for the MSC-CM treated animals (Fig. 1B). Only one animal died during the observation period in the MSC-CM group, versus 50% of the control rats (P=0.046). Supplementary Figure 1 describes a significant survival benefit for MSC-CM treated animals when compared to either conditioned medium derived from normal human dermal fibroblast cultures (P=0.031) or vehicle treatment (P=0.040). This demonstrates that the therapeutic effect is not specific to secretions of human proteins from any mesodermal cell type but is MSC-specific. Overall, these results show that MSC-CM protects the liver from excessive damage and reduces mortality associated with this model of D-galactosamine induced FHF.

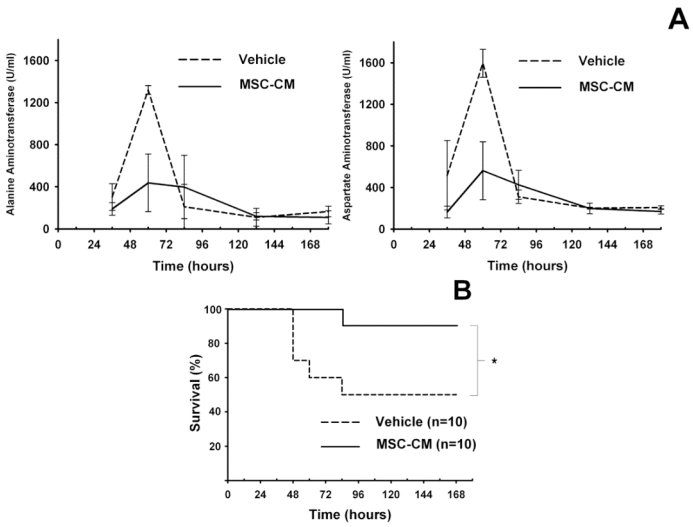
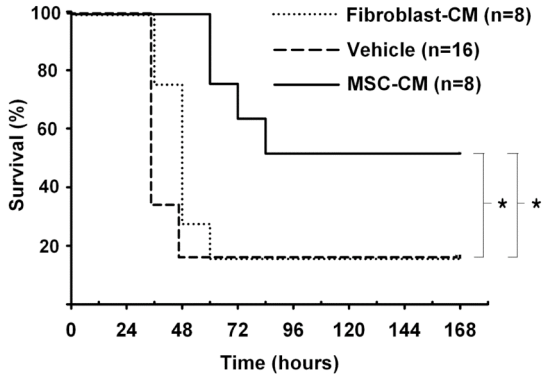


Figure 1. Infusion of MSC-CM inhibits liver damage and provides a survival benefit in a rat model of FHF. FHF was induced in Sprague-Dawley rats using D-galactosamine (0.6 g/kg twice i.p.). Animals were treated with systemic infusions of concentrated MSC-CM or with vehicle 24 hours after the induction of FHF. (A) Liver enzyme release levels in peripheral blood samples collected at 12, 36, 60, 108 and 156 hours after the systemic treatment. (B) Kaplan-Meier survival analysis of D-galactosamine rats. * P=0.046 (Log Rank Test). MSC-CM, mesenchymal stem cell-conditioned medium; FHF, fulminant hepatic failure.



Supplementary Figure 1. The survival benefit provided by MSC-CM treatment in D-galactosamine induced FHF is MSC-specific. Kaplan-Meier survival analysis of D-galactosamine (1.2 g/kg) rats after systemic treatment with MSC-CM, concentrated medium derived from normal human dermal fibroblast cultures or vehicle. * $P<0.05$ (Log Rank Test). MSC-CM, mesenchymal stem cell-conditioned medium; FHF, fulminant hepatic failure.

MSC-CM Treatment Downregulates Systemic Inflammation

Massive liver injury results in a local and systemic inflammatory response that can ultimately lead to multi-organ failure and death. Analysis of serum cytokine levels (Fig. 2) revealed a non-significant decrease for IL-1β ($P=0.054$), but significantly lower levels of TNF-α (64%; $P=0.0002$) and IL-6 (54%; $P=0.0002$) following MSC-CM treatment. These are all pro-inflammatory cytokines known to be upregulated during liver injury. Levels of IL-2 did not change ($P=0.43$). In contrast, the concentration of soluble IL-1ra was 87% lower in MSC-CM treated animals ($P=0.0002$). Levels of the anti-inflammatory cytokine IL-10 were increased 4-fold in MSC-CM treated animals ($P=0.032$). Taken together, these data show that infusion of molecules secreted by MSCs alters the systemic cytokine profile associated with FHF to a more anti-inflammatory state.

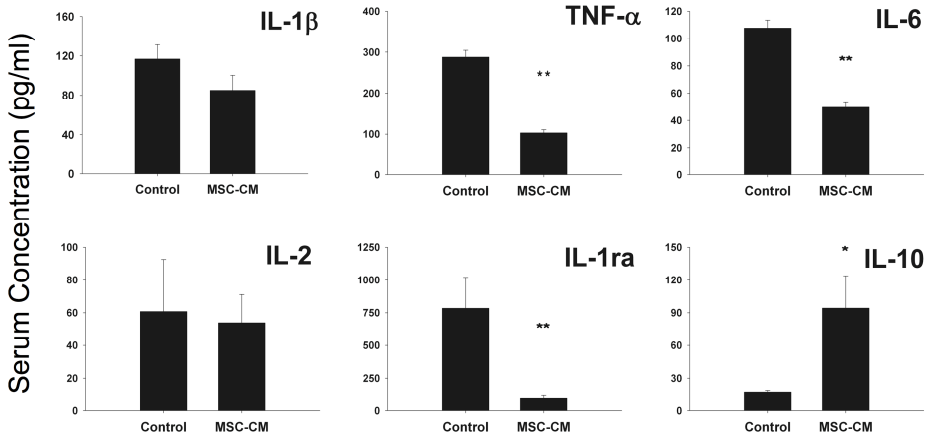


Figure 2. MSC-CM treatment alters systemic inflammatory cytokine profile in D-galactosamine induced FHF. Serum samples were collected from D-galactosamine animals 36 hours after treatment with a systemic injection of MSC-CM (n=4) or vehicle (n=3) and analyzed by ELISA. Data shown are mean ± standard deviation of experiments performed in triplicate. * P<0.05, ** P<0.001. MSC-CM, mesenchymal stem cell-conditioned medium; IL, interleukin; TNF-α, tumor necrosis factor α; IL-1ra, interleukin-1 receptor antagonist.

MSC-CM Therapy Improves Gross and Histopathological Liver Appearance and Reduces Leukocyte Infiltration

D-galactosamine induced FHF is accompanied by characteristic changes in gross appearance of the liver consisting of increased pallor and a soft and shrunken consistency (18). One of four control animals died before animals were sacrificed. Necropsy was not performed on this animal, but based on our prior experience with this model we expect that gross appearance was abnormal. Two of the three remaining control livers were pale, soft and shrunken (Fig 3A). The liver of one vehicle treated rat appeared normal. In contrast, none of the four MSC-CM treated livers demonstrated gross pathological changes (Fig. 3B). Microscopic evaluation of H&E stained liver sections revealed profound hepatocellular death with cytoplasmic vacuolization, panlobular mononuclear leukocyte infiltration and severe distortion of tissue architecture in vehicle-treated animals as previously described (12). In contrast, livers of MSC-CM treated animals demonstrated minor periportal immune cell infiltration with edema and fibrin deposition, characteristic of tissue repair (Fig. 3C-D).

Semi-quantitative histological examination confirmed significant differences between the groups (Fig. 3E). The average score in the MSC-CM group was 1.5 ± 0.6 and 3.0 ± 0.8 for vehicle treated animals (P=0.024). A 58% decrease in the number of infiltrating immune cells was observed after MSC-CM infusion (33 ± 9.3 compared to 84 ± 37 in controls; P=0.004; Fig. 3F). These

results demonstrate that MSC-CM therapy inhibits the development of histopathological changes and immune cell infiltration in the liver in D-galactosamine induced FHF.

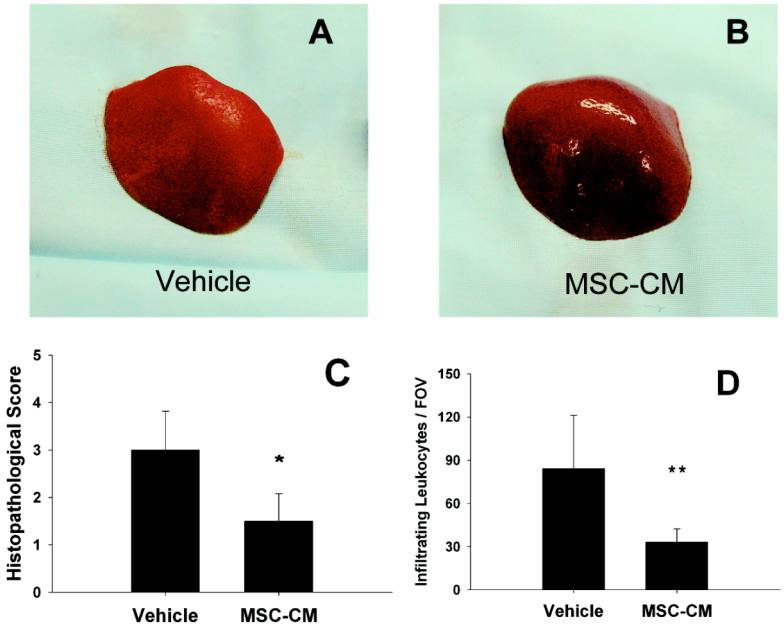


Figure 3. MSC-CM treatment improves gross and histopathological appearance of D-galactosamine livers and reduces the number of infiltrating leukocytes. FHF rats were sacrificed 36 hours after systemic vehicle or MSC-CM treatment. Livers were inspected and samples were subjected to histological analysis after hematoxylin & eosin staining. Gross appearance of representative liver lobes after (A) vehicle and (B) MSC-CM treatment. Representative histopathological images (40x) after vehicle (C) and MSC-CM (D) treatment. (E) Scores determined by semi-quantitative histological exam. (F (or H)) Quantification of infiltrating immune cells. Data shown are mean ± standard error of the mean of 10 random high power fields per animal. * P=0.024, ** P=0.004. MSC-CM, mesenchymal stem cell-conditioned medium; FHF, fulminant hepatic failure; FOV, field of view.

MSC-CM Inhibits Hepatocellular Apoptosis in vivo

To determine whether MSC-CM infusion decreases apoptotic cell death, the number of TUNEL-reactive hepatocyte nuclei in liver sections was determined. In sections from vehicle-treated rats, many large, apoptotic hepatocyte nuclei were observed (Fig. 4A), whereas only few were present after MSC-CM treatment (Fig. 4B). Quantification revealed a 90% reduction in TUNEL-positive hepatocyte-nuclei (8.3 ± 12 / field of view) when compared to control animals (81 ± 52) (P=0.009; Fig. 4C), confirming that MSC-CM therapy effectively reduces hepatocellular death in this model of acute liver injury.

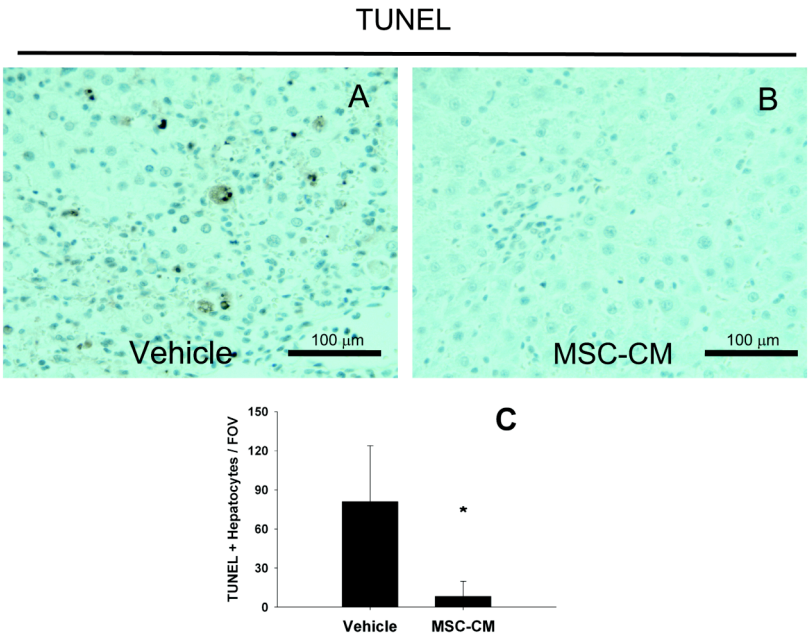


Figure 4. Infusion of MSC-CM decreases levels of apoptosis in livers of D-galactosamine treated FHF rats. Liver sections were stained by TUNEL (dark brown nuclei, large for hepatocytes) and counterstained with hematoxylin (light blue). Representative 40x images from (A) vehicle treated and (B) MSC-CM treated rats. Bar = 100 μm. (C) Quantification of TUNEL-positive hepatocyte-nuclei by digital image analysis. Data are reported as mean ± standard error of the mean for 10 random fields per animal. * P=0.009. MSC-CM, mesenchymal stem cell-conditioned medium; TUNEL, Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling; FOV, field of view.

MSC-CM Inhibits Hepatocyte Apoptosis in vitro

Inhibition of hepatocellular death by MSC-CM therapy in vivo can either be a direct effect of trophic molecules preserving liver cells or an indirect effect, for example via inhibition of the immune response. Therefore, we tested the ability of MSC-CM to directly inhibit apoptosis in cultured primary hepatocytes. When the culture medium was supplemented with 2% MSC-CM a 22% increase in the fraction of viable cells was observed (P=0.005; Fig. 5). With 8% MSC-CM, no significant increase in hepatocytes viability was seen (P=0.15). Overall, these experiments provided evidence that at low concentrations, MSC-CM has a direct anti-apoptotic effect on hepatocytes.

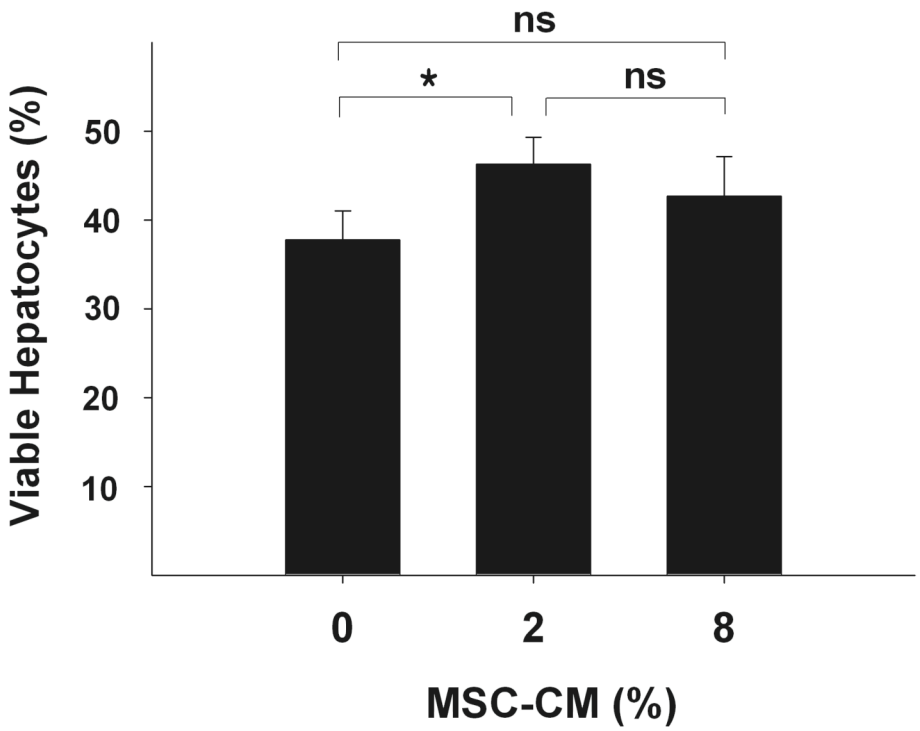


Figure 5. MSC-CM inhibits in vitro hepatocyte-apoptosis at low concentrations. Primary rat hepatocytes were cultured in a collagen double gel. Apoptosis was induced using Actinomycin D and TNF-α. During exposure to TNF-α, hepatocytes were cultured in hepatocyte medium only or hepatocyte medium supplemented with 2% or 8% of 25x concentrated MSC-CM. Hepatocytes were stained using a fluorescent Live Dead Assay. Cell death was quantified using digital image analysis of 4 images per well. Experiments were performed in triplicate. Data are shown as mean ± standard deviation. * P=0.005. MSC-CM, mesenchymal stem cell-conditioned medium.

MSC-CM Enhances Liver Regeneration

Stimulation of endogenous repair programs represents another potential mechanism of an MSC-induced therapeutic effect. To determine the effect of MSC-CM on liver regeneration, PCNA reactive hepatocytes were quantified and compared to vehicle-treated animals. Qualitatively, few PCNA-reactive hepatocytes were seen in control livers (Fig. 6A). Many were observed in MSC-CM treated animals (Fig. 6B). We measured a 3-fold increase in the number of proliferating liver cells (15 ± 5.3 in the MSC-CM treated group compared to 4.5 ± 2.5 / field of view in controls; P=0.04; Fig. 6C). Evaluation of expression levels of 10 genes known to be upregulated during liver regeneration revealed visibly stronger bands in MSC-CM treated samples by endpoint RT-PCR (Fig. 6D). Quantitative analysis demonstrated that expression levels all 10 genes were significantly enhanced after infusion of MSC-CM (Fig. 6E). Increases ranged from 4-fold to 27-fold. These results

demonstrate that administration of MSC-derived soluble factors enhances liver regeneration programs during FHF.

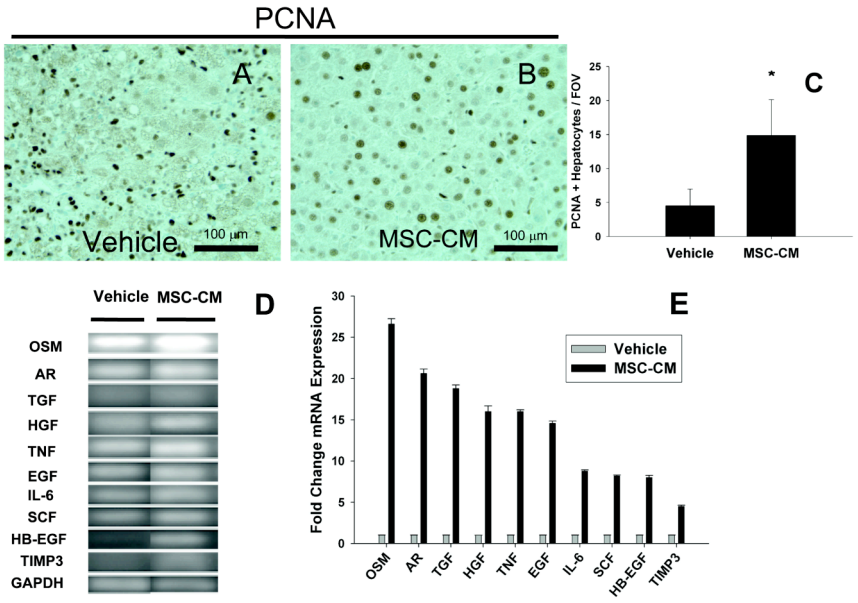


Figure 6. Infusion of MSC-CM enhances liver regeneration in D-galactosamine induced FHF. Liver samples of D-galactosamine rats were analyzed 36 hours after treatment with MSC-CM or vehicle. Sections were stained for PCNA (dark brown nuclei, large for hepatocytes). Representative 40x image from (A) control and (B) MSC-CM treated animals. Bar = 100 μm. (C) PCNA-reactive hepatocyte-nuclei were quantified by digital image analysis. Data are reported as mean ± standard error of the mean for 10 random fields per animal. * P=0.04. (D) Endpoint RT-PCR analysis of 10 genes known to be upregulated during liver regeneration. (E) Quantification of changes in gene expression by kinetic RT-PCR after MSC-CM treatment. PCNA, proliferating cell nuclear antigen; MSC-CM, mesenchymal stem cell-conditioned medium; FOV, field of view; OSM, oncostatin M; AR, α-1β adrenergic receptor; TGF, transforming growth factor-α; HGF, hepatocyte growth factor; TNF, tumor necrosis factor-α; EGF, epidermal growth factor; IL-6, interleukin 6; SCF, stem cell factor; HB-EGF, heparin-binding epidermal growth factor-like growth factor; TIMP-3, tissue metalloproteinase 3.

MSC-CM Stimulates Hepatocyte Proliferation in Vitro

Hepatocyte duplication, a major component of liver regeneration, is regulated by a complex interaction of paracrine and endocrine signals involving non-parenchymal liver cell types as well extra-hepatic organs (19). To determine whether MSC-derived factors can directly enhance hepatocyte replication we

explored the effect of MSC-CM on the in vitro proliferation of primary hepatocytes. Proliferation of rat hepatocyte colonies on a feeder layer of growth-inhibited 3T3-fibroblasts was visualized by double immunofluorescence staining for BrdU and albumin (Fig. 7A). With 2% MSC-CM supplementation, a 79% increase in BrdU-positive hepatocytes was observed (93 ± 12 per field of view with MSC-CM vs. 52 ± 14 in control cultures; P=0.001; Fig. 7B). When medium was supplemented with 8% MSC-CM, no significant increase was measured (59 ± 14 BrdU; P=0.37). In parallel to these findings, the total amount of albumin secreted and urea synthesized per well was increased in 2% MSC-CM supplemented cultures. Albumin levels were 29 ± 2.4 μg/ml/d, compared to 20 ± 1.2 μg/ml/d under control conditions (P=0.006; Fig. 7C). No significant difference compared to control was observed in 8% MSC-CM conditions (23 ± 2.2 μg/ml/d; P=0.14). Urea synthesis (Fig. 7D) shifted from 69 ± 8.1 μg/ml/d in control cultures to 90 ± 10 μg/ml/d in 2% MSC-CM conditions (P=0.019), but was not significantly altered in the presence of 8% MSC-CM (53.1 μg/ml/d; P=0.063). Higher levels were observed for 2% MSC-CM supplementation when compared to 8% MSC-CM for all three assays indicating a therapeutic window of effectiveness.

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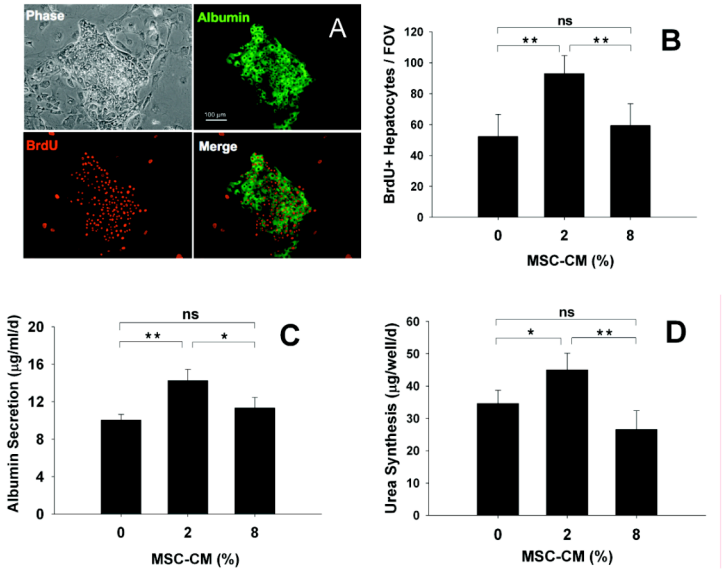

Figure 7. Low concentrations of MSC-CM enhance proliferation and functions of cultured hepatocytes. Hepatocytes were cultured at low density (1.25 x 10^3 cells/cm^2) on a feeder layer of growth-arrested 3T3-J2 fibroblasts. Cells were cultured in hepatocyte medium only or hepatocyte medium supplemented with 2% or 8% of 25x concentrated MSC-CM. (A) Morphology, albumin staining, BrdU uptake and merged image of proliferating hepatocytes after 14 days of culture in 2% MSC-CM. Hepatocyte colonies increased in size during culture periods. (B) Quantification of BrdU-positive hepatocytes by image analysis. (C) Albumin secretion and (D) urea synthesis. Data shown are mean ± standard deviation of two separate experiments in duplicate. * $P<0.05$, ** $P<0.01$; ns = not significant. MSC-CM, mesenchymal stem cell-conditioned medium; FOV, field of view.

Discussion

Inhibition of cell death is a therapeutic effect of MSC therapy that has been observed in models of myocardial infarction, acute kidney failure and stroke (8, 9, 11). In this study, we provide the first clear evidence that delivery of MSC-secretions has the potential to dramatically reduce cell death in the acutely injured liver.

The 90% reduction in the level of hepatocellular apoptosis that we observed in the livers of rats undergoing FHF was much more dramatic than the decrease in cell death measured in cultured hepatocytes. It is likely that the inhibition of the local and systemic immune response after MSC therapy, processes which are known to enhance levels of hepatocellular death in liver injury (20, 21), resulted in a further reduction of hepatocellular death in D-galactosamine treated animals. In a previous study, we showed that altered leukocyte trafficking is a potential therapeutic mechanism of MSC-CM therapy in the D-galactosamine induced model of FHF (12). Nevertheless, the direct anti-apoptotic effect in vitro shows that MSC-CM has a direct preserving effect on hepatocytes.

Local downregulation of pro-inflammatory cytokines and upregulation of anti-inflammatory cytokines, such as IL-10, after MSC transplantation has been described in models of lung and kidney injury (11, 22). Our results demonstrate that these alterations occur on a systemic level. Mortality from FHF is often caused by the uncontrolled systemic inflammatory response that accumulates in multi-organ failure (1). Downregulation of this response may contribute to improved survival of FHF after MSC-CM treatment.

Stimulation of endogenous regeneration programs represents an important avenue of trophic support by MSC therapy, previously described in models of acute kidney failure and stroke (11, 23). Our results demonstrate that MSC therapy can increase the number of proliferating hepatocytes 3-fold in the regenerating, injured liver and that delivery of only the secreted factors from MSC is sufficient to accomplish this effect. However, the direct stimulatory effect of MSC-CM that we observed on the in vitro proliferation of hepatocytes was abrogated when high concentrations of MSC-CM were used. This correlates with observations in previous survival studies in D-galactosamine induced FHF that supra-therapeutic concentrations of MSC-CM leads to a loss of the therapeutic effect (12). MSC secretions contain a complex mixture of cytokines, growth factors and chemokines, including small amounts of TGF-β, a compound with a marked inhibitory effect on hepatocyte-proliferation (12, 24, 25). It is possible that at higher concentrations of MSC-CM the negative effect of for example TGF-β overrules therapeutic effects exerted by trophic components of MSC-CM. Similarly, the effects of TNF-α, present at low concentrations in MSC-secretions (13), become more pronounced at high concentrations of MSC-CM. This may explain why increasing the level of MSC-CM from 2% to 8% has no added anti-apoptotic effect on hepatocytes.

We can only speculate what specific mediators present in MSC-CM are responsible for the reduction in cell death and stimulation of regeneration. In a recent investigation, we performed protein-array analysis of MSC-CM and

detected 69 of 174 assayed proteins, the majority of which were growth factors, cytokines and chemokines (12). Several of the detected molecules have known anti-apoptotic and liver regeneration-stimulating effects. Vascular endothelial growth factor, for example, is known to induce hepatocyte growth factor (HGF) secretion by stellate cells, which in turn induces expression of hepatocyte-mitogenic TGF-α. HGF, present in MSC-CM, is also known to inhibit apoptosis in the injured liver. Insulin-like growth factor binding proteins and IL-6 are other examples of MSC-secreted molecules with apoptosis-reducing effects in liver injury (19). Using in vitro studies, we previously showed that MSCs inhibit activated stellate cell-functions via secreted IL-10, TNF-α and HGF (13). Although it is unlikely that such effects on stellate cells, the principal mediators of hepatic fibrosis, were important in the therapeutic benefit achieved with MSC-CM in the FHF model described in the present study, such investigations support the notion that at least several molecules are involved.

Systematic proteomic analysis combined with fractionation studies of MSC-CM is necessary to identify key therapeutic components, but also potentially harmful components. Such investigations will ideally lead to the development of a balanced cocktail of trophic compounds with an optimized therapeutic effect in FHF.

The vast majority of reports describe cell transplantation as the primary mode of MSC therapy, although Dzau and colleagues have described a therapeutic effect of locally administered MSC-CM in a rat model of myocardial infarction (8). In rodent models of chronic liver disease, investigators have shown that systemic MSC transplantations can result in anti-fibrotic effects (26, 27). Compared to MSC-CM, cell transplantation has the potential advantage of regeneration via stem cell differentiation and sustained release of active molecules by long-term engrafted cells. Although differentiation of MSC into cells with an hepatocyte-like phenotype has been described, differentiation into fully functional hepatocytes is at most a rare phenomenon (28). Moreover, engraftment levels of MSCs are often insignificant (29, 30) and long-term actions may be deleterious in acute organ injury. The liver is not suitable for local injections and portal vein injection to circumvent pulmonary lodging and embolus-formation by transplanted cells is invasive and often lethal in rodents. For these reasons, systemic infusion of MSC-CM represents an effective alternative of delivering the therapeutic effects of MSCs without the aforementioned problems.

In conclusion, the present studies shows that systemic MSC-CM therapy has profound inhibiting effects on hepatocellular death, enhances liver regeneration programs and improves survival in rats undergoing D-galactosamine induced FHF. Furthermore, we demonstrate direct anti-apoptotic and stimulatory effects of MSC-CM on cultured hepatocytes. This work creates potential new avenues for the treatment of this devastating disease.

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Chapter 6

Osmotic selection of human mesenchymal stem/progenitor cells from umbilical cord blood

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Abstract

The isolation of undifferentiated adult stem/progenitor cells remains a challenging task primarily due to the rare quantity of these cells in biological samples and the lack of unique markers. Herein, we report a relatively straightforward method for isolation of human mesenchymal stem cells (MSCs) based on their unusual resistance to osmotic lysis, which we term ''osmotic selection'' (OS). MSCs can remarkably withstand significant exposure to hypotonic conditions (> 30 min) with only a reversible impairment in cell proliferation and with no loss of stem cell potential after exposure. Comparison of MSCs to other circulating nonhematopoietic cells revealed a time regime, by which purification of these cells would be attainable without considerable cell loss. OS showed a 50-fold enrichment of fibroblast colony-forming units from umbilical cord blood samples when compared to commonly employed techniques. After upstream processing, isolated cells using OS were immunophenotyped to be CD14-,CD34-,CD45-, CD44+, CD105+, and CD106+, and displayed multipotent differentiation. Preliminary investigations to determine mechanisms responsible for osmolytic resistance revealed MSCs to have an ineffective volume of 59%, with the ability to double cell volume at infinite dilution. Disruption of filamentous actin polymerization by cytochalasin D sensitized MSCs to osmotic lysis, which suggests a cytoskeletal element involved in osmolytic resistance.

Introduction

Maureen Owen (1), based on the work of Alexander Friedenstein (2), proposed the existence of nonhematopoietic cells in the bone marrow with the ablutie to give rise to mesenchymal cells. These findings were further popularized by Arnold Caplan in the last two decades (3). Initially dubbed the "Caplan cell," these cells were isolated from bone marrow mononuclear cells based on adherence to tissue culture plastic. In addition, they displayed a fibroblastoid morphology and expressed antigens that react with SH2 (CD105) and SH3 (CD73) monoclonal antibodies. In recent years, the Caplan cell has been redefined as a mesenchymal stem cell (MSC). MSC have the ability to reproducibly give rise to adipocytes, osteoblasts, and chondrocytes in vitro (4) and rescue mesenchymal tissue disorders in humans (5). Despite lack of consensus on the relevant cell surface markers of MSCs from other species, expression of CD29, CD44, CD73, CD90, CD105, and CD106 and expression of CD11b, CD14, CD18, CD34, CD36, and CD45 have been determined to be important positive and negative markers of human MSCs, respectively (6).

The ability to readily expand in culture, while maintaining a self-renewing phenotype, has made MSCs a candidate for many cell-based therapies. Though the bone marrow has been established as the primary source of MSCs, due to the invasive nature of bone marrow aspirator, identification of other abundant and reliable sources of MSCs has become a priority. The isolation of MSCs from peripheral sources, such as umbilical cord blood (UCB) (7, 8) and adipose tissue (9), has been reported, though other contradictory studies report the absence of MSCs in the these biological samples (10, 11). We conjectured that the successful isolation of these rare cells may strongly depend on the sensitivity of the technique used, hereby leading to contradictory reports. To date, the most definitive isolation criterion of MSCs is their ability to differentially adhere to tissue culture substrates.

Current methods of MSC isolation involve density fractionation of mononuclear cells followed by differential adhesion. The purity of the isolated cells can be enhanced using cell elutriation, rosetting, or sorting systems in conjunction with density fractionation. Nonetheless, this can result in nonspecific isolation of mononuclear cells, and activation and/or potential loss of target cells. Osmotic lysis has also been used as a technique as it has the potential to differentially deplete contaminating cell populations and achieve isolation of the desired cell type (12). We hypothesized that resistance to osmotic lysis could also be a potential positive selection mechanism if appropriate processing parameters can be determined, whereby differential lysis of all contaminating cells is achieved. We identified MSCs to be extremely resistant to extended exposure to hypotonic shock. Based on this finding we characterized specific processing variables that were vital to the isolation of MSCs using spiked blood experiments. We applied this variable set to the isolation of these cells from UCB and observed a significantly greater number of cells with a similar immunophenotype to bone marrow–derived MSCs when compared to density separation and cell rosetting methods. The isolated cells from UCB produced more colony-forming units (CFUs) per unit of blood and were shown to be multipotent.

In an effort to uncover molecular mechanisms responsible for osmolytic resistance, we performed osmotic analysis, and attempted to modulate resistance based on filamentous actin polymerization disruption using cytochalasin D. Osmotic analysis showed isolated MSCs to possess an ineffective volume of *60% with the ability to double cell volume at infinite dilution, which is in stark contrast to most other mammalian cells that posses an ineffective volume of less than 30%. Finally, disruption of filamentous actin polymerization by cytochalasin D dramatically sensitized MSCs to osmotic lysis, suggesting that the cytoskeletal network is involved in the resistance to osmotic lysis.

Materials & methods

Human cells

Human MSCs were isolated from commercially available bone marrow aspirates (Clonetics-Poietics, Walkersville, MD) and maintained as previously described.[4] MSCs were used for experiments during passages 4–7. Neonatal fibroblasts (American Type Culture Collection, Manassas, VA), nonsmall cell lung carcinoma cells (American Type Culture Collection), and microvascular endothelial cells (Cambrex, East Rutherford, NJ) were grown according to manufacturer's instructions.

Spiked blood experiments

Approval for the collection of blood from healthy volunteers was obtained from the Institutional Review Board of Massachusetts General Hospital. Blood was drawn using Vacutainer tubes with heparin anticoagulant (Becton Dickinson, San Jose, CA). MSCs were incubated with CellTracker Green (Molecular Probes, Carlsbad, CA) for 15 min and spiked into whole blood at different concentrations. Prepared samples were exposed to deionized (DI) water for different times, and viability and cell number were determined using a coulter counter (Becton Dickinson), either gated between 12 and 25 mM or analyzed by trypan blue exclusion. Images of isolated cells were taken using phase contrast and fluorescence microscopy. After 10–14 days, cultures were fixed in 70% ethanol, stained with Wright-Giemsa, and scored for colony-forming unit-fibroblast (CFU-F).

MTT proliferation assay

MSCs were exposed to either isotonic or hypotonic solutions for different durations and seeded at similar densities in 12-well tissue culture plates. At days 0, 2, 4, and 6 after isolation, we performed a 3-(4,5-Dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT) assay as described by the vendor (Invitrogen, Carlsbad, CA). A reduction in MTT signal compared to isotonic solutions was referred to as reduction in cell proliferation.

Isolation of MSCs from UCB

UCB was received by St. Louis Medical Center in accordance with the guidelines of Institutional Review Board of Massachusetts General Hospital. UCB was diluted 1:1 with 1x phosphate-buffered saline (PBS) and subjected to different upstream processing. Forty milliliter of blood from each sample was used for isolation of MSCs using Ficoll-Paque (Sigma Aldrich, St. Louis, MO) and RosetteSepMSC enrichment kit (StemCell Technologies, Vancouver, BC).

Isolation of MSCs using the hypotonic selection protocol utilized 8 mL of blood. Either 0.5 or 1 mL (1:100 and 1:50 dilutions) of cord blood was aliquoted into 50 mL tubes containing 45 mL of DI water. The tubes were capped and set on a horizontal shaker for either 20 or 30 min. Following shaking, 5 mL of 10x PBS was added to the tubes and set on the shaker for an additional 5 min. The samples were then centrifuged at 350 g for 5 min, and

the pellet was resuspended in 1mL of 1x PBS. This was then used for either culture or phenotypic characterization using flow cytometry.

Multipotent differentiation

The multipotency of MSCs was assessed in vitro by culturing the cells in induction medium for 2–3 weeks as previously reported (4), with medium changes every 3 days. Osteogenic medium consisted of Iscove's modified Dulbecco's medium (IMDM) supplemented with 0.1 mM dexamethasone, 10 mM b-glycerol phosphate, 0.2 mM ascorbic acid (AsA), 100 U/mL penicillin, and 100 mg/mL streptomycin. Adipogenic medium consisted of IMDM supplemented with 0.5 mM 3-isobutyl-1-methylxanthine, 1 mM hydrocortisone, 0.1 mM indomethacin, 10% rabbit serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. For chondrogenesis studies, cells were transferred into a 15 mL polypropylene tube and centrifuged at 1000 rpm for 5 min to form a pelleted micromass, which was then treated with chondrogenic medium. Chondrogenic medium consisted of high-glucose Dulbecco's modified Eagle's medium (DMEM) (Chemicon International, Ternecula, CA) supplemented with 0.1 mM dexamethasone, 50 mg/mL AsA,100 mg/mL sodiumpyruvate, 40 mg/mL praline, 10 ng/mL transforming growth factor b1 (TGF-b1), 50 mg/mL ITS þ premix (Becton Dickinson; 5.35 mg/mL linoleic acid), 100 U/mL penicillin, and 100 mg/ mL streptomycin. Cytological stains were performed as previously reported. Histological sections of chondrocyte micropellets were paraffin embedded and stained with toluidine blue, followed by decolorization with two graded alcohol washes. Sections were then cleared with xylene and imaged using a Nikon Eclipse E800 Upright Microscope.

Osmometry

Cells were exposed to solutions of different concentrations of mannose (0–1000 mOsm). Suspended cells in 1x PBS represented the resting cell membrane diameter. Cells were suspended in a hemocytometer and allowed for significant time to reach a new steady-state volume. Image analysis to determine cell diameter was performed using Metamorph software (Meta Imaging Software, Downingtown, PA).

Disruption of fibrillar actin polymerization

Cells were incubated with 10 mg/mL of cytochalasin D (Sigma Aldrich) while suspended for 75 min. Untreated cells were suspended in medium. Each group was exposed to DI water for certain periods of time, and viability was assessed by trypan blue exclusion.

MSC phenotyping

For each sample, different subpopulations were assessed by flow cytometry (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ). Cells were stained using BD antibodies (BD Biosciences, San Jose, CA). MSC cell-surface markers were visualized using peridin chlorophyll-a–, fluorescein isothiocyanate–, phycoerythrin-, and allophycocyaninconjugated antibodies. Positive markers include antibodies against CD44, CD105, and CD106 C surface antigens. Negative markers were made up of antibodies that react with CD34, CD45, and

CD14 surface antigens. Stained cells were washed in 1% Hanks Azide Buffer (HAB; Hank's balanced salts without calcium or magnesium, plus 1% bovine serum albumin, 0.1% sodium azide, and 1 mM ethylenediamine tetraacetic acid), pelleted, resuspended in HAB, and analyzed on a FACSCalibur flow cytometer (BD Biosciences), using CellQuest software (BD Pharmingen, San Jose, CA). All data correspond to the means of cell concentration or fluorescence intensity.

Results

Identification of osmotic selection processing parameters in spiked blood experiments

As a proof of principle, we examined the membrane integrity of a pure population of bone marrow–derived MSCs exposed to DI water (Fig. 1A). Remarkably, viability was not compromised for the first 10 min of exposure, followed by a gradual cell loss with an approximate 47.5% loss of cell mass after 20 min. Following the initial observation that MSCs can survive extended exposure to hypotonic solution, a comparison study between MSCs and other circulating, nonhematopoietic cells suggested that the resistance of MSCs to osmotic lysis could be exploited as a selection method (Fig. 1B). Neutrophils, the most prevalent subpopulation of leukocytes, as well as monocytes, the traditional contaminating cell in MSC isolations of bone marrow, failed to survive past 10 min in hypotonic conditions. Survival of microvascular endothelial cells, a surrogate for a circulating endothelial progenitor, followed a similar trajectory. Neonatal fibroblasts, a surrogate for circulating fibrocytes, displayed partial resistance to osmotic stress with complete lysis after 30 min of exposure. Likewise, survival of nonsmall cell lung carcinoma followed a similar course and represented an interesting correlation. Cancer cells have been observed to be highly resistant to osmotic lysis, a phenomenon that has deterred its experimental success as a tumoricidal agent following surgical procedures (13). MSCs showed resistance to osmolysis relative to the other cell types with only significant loss of cells after 30 min. More importantly, these data demonstrated that exposure of a cell suspension that included MSCs to hypotonic conditions for greater than 20 min should result in a marked enrichment of MSCs by selectively lysing all other cells. After isolation we observed a partial reduction in proliferation that was soon normalized after long-term culture, suggesting a reversible process of cell stress post-isolation (Fig. 1C).

Determination of an appropriate time regime to recover circulating MSCs with relative specificity led to an assessment of the sensitivity of this method. Bone marrow–derived MSCs, labeled with a cell tracking dye, were spiked into the whole blood of healthy individuals and subjected to different exposure times and ratios of whole blood to hypotonic solution. After upstream processing, a population of pure MSCs remained with trace contaminants and cell casts (Fig. 1E–G). A CFU-F assay revealed a suitable processing time and dilution for subsequent isolation of MSCs from patient blood samples (Fig. 1D). At lesser dilution and exposure to hypotonic solution, the greatest number of CFU-F was observed, which is why greater dilutions were not pursued. Essentially, at longer exposure times and increased dilution, MSCs were lost due to osmolysis and less buffering by intracellular components, respectively. Taken together with the comparison study data, later experiments used an exposure time of 30 min and dilution factor of whole blood to hypotonic solution of 1:50 to enhance the purity of processed samples.

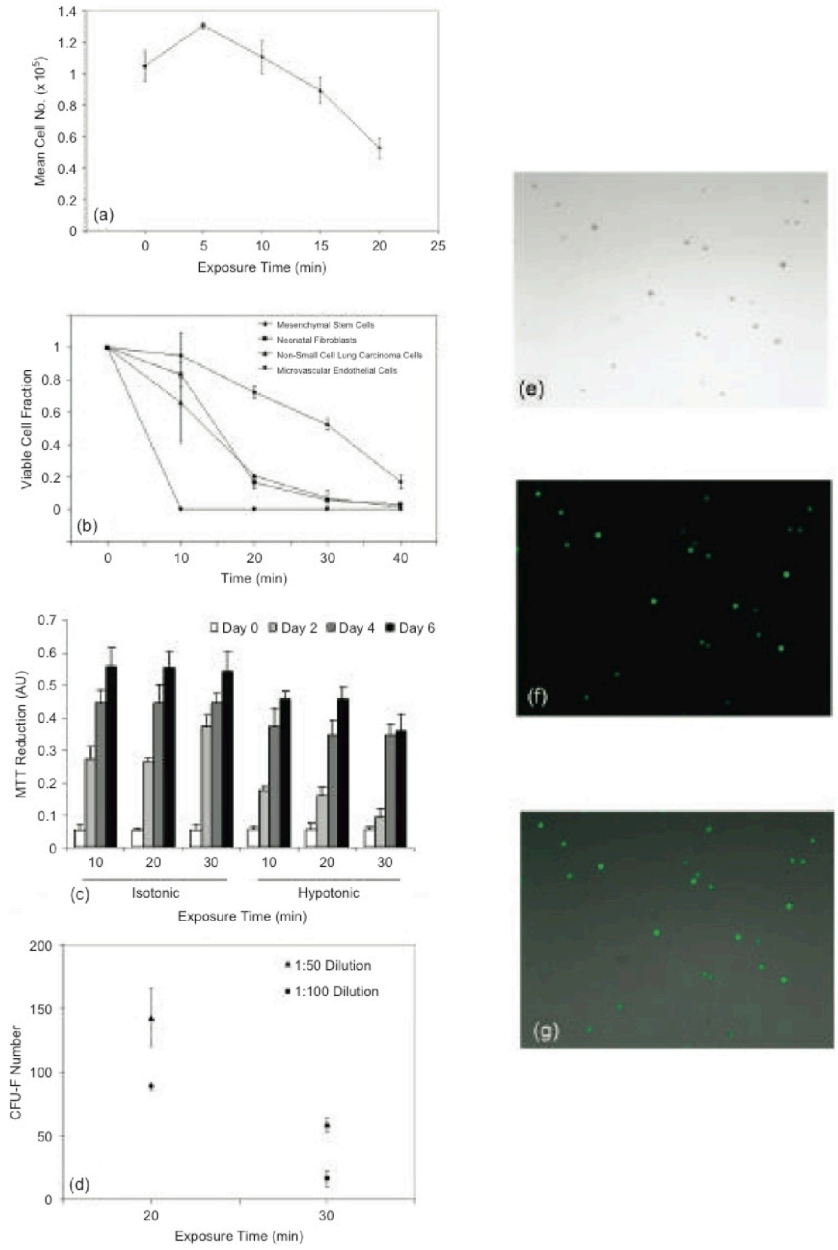

Figure 1. Determination of osmolytic resistance parameters for use in spiked blood experiments. (A) Osmolytic resistance of bone marrow–derived MSCs over time. (B) Comparison of osmolytic resistance between bone marrow–derived MSCs and other circulating, nonhematopoietic cells. Plot shows normalized cell concentration as a function of the exposure time in hypotonic solution. (C) MTT assay of cell proliferation after isolation of MSCs from isotonic or hypotonic conditions after different exposure times. (D) CFU-F number after spiking MSCs into whole blood and recovering cells

using OS. CFU-F compared to different exposure times and whole blood:hypotonic solution ratios. Phase contrast (E), uorescence (F), and merged (G) images of isolated MSCs after spiking into whole blood. Spiked cell concentration was approximately 15% of mononuclear cell fraction. Images taken after exposing the blood preparation to 20 min in hypotonic conditions at a 1:50 ratio of blood to hypotonic solution. Data represent mean ± SEM. Color images available online at www.liebertpub.com/ten.

Isolation of MSCs from UCB using osmotic selection does not cause cell injury Repeatable and reliable isolation of MSCs from cord blood provided a suitable challenge for validation of the osmotic selection (OS) protocol. Based on previously described characterization, UCB samples were exposed to DI water for 30 min at a 1:50 dilution. After return to isotonic conditions, surviving cells were plated on standard tissue culture flasks and expanded. Simultaneously, standard MSC isolation based on published reports was performed using the Ficoll and RosetteStep protocols. Phenotypic characterization of cells isolated using each of the three isolation methods was compared to the antigen expression of bone marrow–derived MSCs. CD44, CD105, and CD106, and CD34, CD45, and CD14 were selected as positive and negative determinants of MSC phenotype, respectively. Flow cytometry analysis of processed samples showed discrepancies in the phenotype of isolated cells. The surface antigen profile of bone marrow–derived MSCs was consistently CD14-, CD44+, CD105+, and CD106+ (Fig. 2A, E). This subpopulation was not observed after cell rosetting (Fig. 2C, G), with only few cells that expressed this phenotype after density gradient centrifugation (Fig. 2B, F). Erythrocyte lysis using ammonium chloride retained cells with a similar antigen profile as density gradient centrifugation (Supplemental Fig. 1, available online at www.liebertpub .com). The significant loss of cells due to rosetting and density gradient centrifugation suggests that these methods would be ineffective in recovering MSCs that are found in biological samples at very low numbers. OS provided a striking enrichment of MSCs, while processing five times less blood (Fig. 2D, H). The predominant phenotype after OS was CD14-, CD34-, CD45-, CD44+, CD105+, and CD106+ (Supplemental Fig. 2, available online at www.libertpub.com).

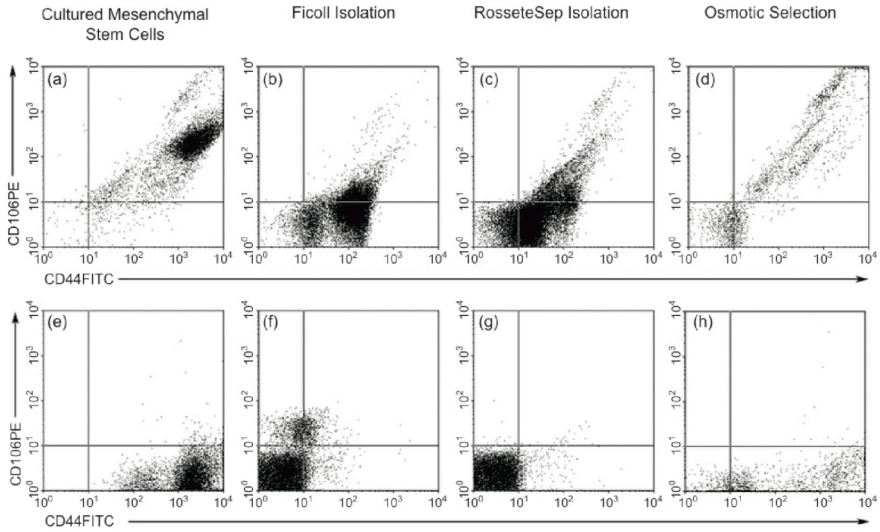

Figure 2. Flow cytometry analysis of isolated cells from UCB using different upstream selection methods. (A–D) Scatter plot of CD14/ CD105 and (E–H) CD106/CD44 expression of recovered cells using (A, E) RosetteSep, (B, F) Ficoll-Paque, and (C, G) OS, respectively. (D, H) Control expression of bone marrow–derived MSCs. RosetteSep and Ficoll-Paque isolations processed 20 mL of UCB, whereas cytotoxic selection processed only 4 mL. Cytotoxicity study was performed at 30 min of exposure time at a 1:50 dilution of blood to hypotonic solution.

To assess whether OS would lead to irreversible cell injury and to ascertain whether these were mobilized progenitor cells, isolated cells were subcultured and tested for proliferation and multipotency. The morphology of isolated cells appeared fibroblast-like with focal adhesions that were reminiscent of MSCs (Fig. 3A). After 14–21 days of culture, a significant number of CFU-F were visible after OS when compared to conventional methods (Fig. 3B). With limited dilution culture (2.25 x 10^2 cells/cm^2), OS provided an approximate 50-fold increase in the number of CFU-F/ mL of blood (Fig. 3C). Assuming approximately 10^2 mononuclear cells/mL, our data suggest that there are approximately 6 cells/mL of UCB (0.006–0.01% of the mononuclear cell fraction) with adherent clonogenic potential. The yield of clones isolated using OS may be considered a more representative number of MSCs in UCB that differs from other reports. Adipogenesis, osteogenesis, and chondrogenesis were all verified after induction in respective differentiation medium (Fig. 3D–F). These results suggest that if there is cellular injury, it may be a reversible condition as judged by the ability of MSCs to expand and differentiate.

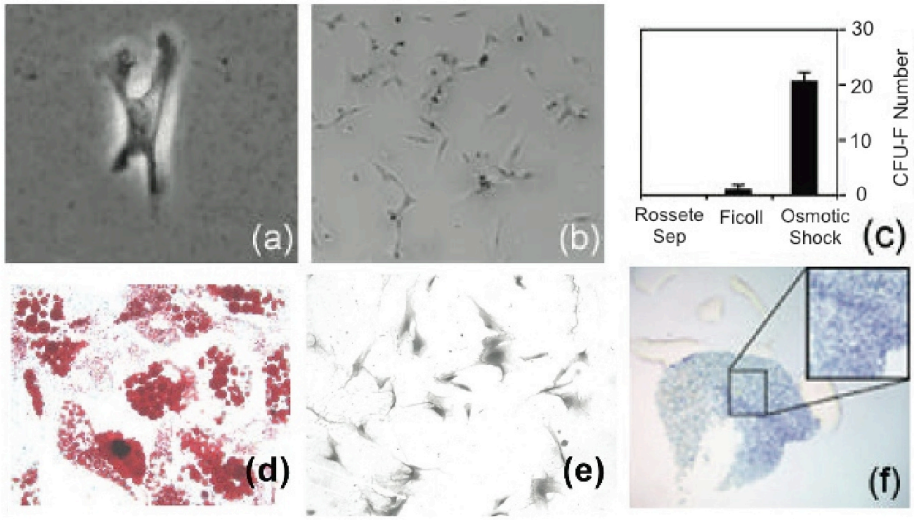

Figure 3. Retention of proliferative and multipotent abilities after OS. (A) Phase contrast micrograph of isolated cell after attachment to culture surface. Original magnification 20x.(B) Wright-Giemsa staining of CFU-F assembly 10 days after OS. Original magnification 4x.(C) Comparison of CFU-Fs using OS, Ficoll-Paque, and RosetteSep. RosetteSep and Ficoll-Paque isolations processed 20 mL of umbilical cord blood, whereas cytotoxic selection processed only 4 mL. Adipogenesis (D), osteogenesis (E), and chondrogenesis (F) assessed by Oil-Red O, von Kossa, and toluidine blue histology. Original magnification 10x. Color images available online at www.liebertpub.com/ten.

MSCs have a high ineffective cell volume and are sensitized to OS after cytoskeletal disruption

Cells exhibit various mechanisms to regulate water traficking. We examined broad aspects of cellular water handling to determine a molecular foundation for the osmotic resistance of bone marrow–derived MSCs. Osmometry analysis of the change in normalized cell volume compared to osmolarity showed distinct differences between MSCs and fibroblasts in the ability to withstand volume changes (Fig. 4A). At infinite dilution, the attainable cell volume of an MSC was about twice its normal volume in isotonic conditions, whereas fibroblasts tripled their normal volume. The ineffective cell volume, defined as the cell's insoluble volume fraction, of MSCs was approximately 59%, a fraction that starkly contrasts fibroblast's ineffective cell volume of 41% and most other cells whose ineffective cell volume is roughly 30–40% (14).

The actin cytoskeleton mediates a variety of essential biological functions in cells, including division, morphological changes, and motility. Studies have suggested that the actin cytoskeleton is involved in water handling and regulation of cell volume. In addition, the cytoskeletal network of a cell is also considered to be a large constituent of its ineffective cell volume. To

determine the role of the actin cytoskeleton in osmolytic resistance of MSCs, we disrupted actin filament polymerization by cytochalasin D. Treatment with cytochalasin D alone was not cytotoxic, but when treated cells were challenged with an hypoosmotic solution, there was a dramatic reversal of the osmolytic resistance in treated MSCs by roughly a log order (Fig. 4B).

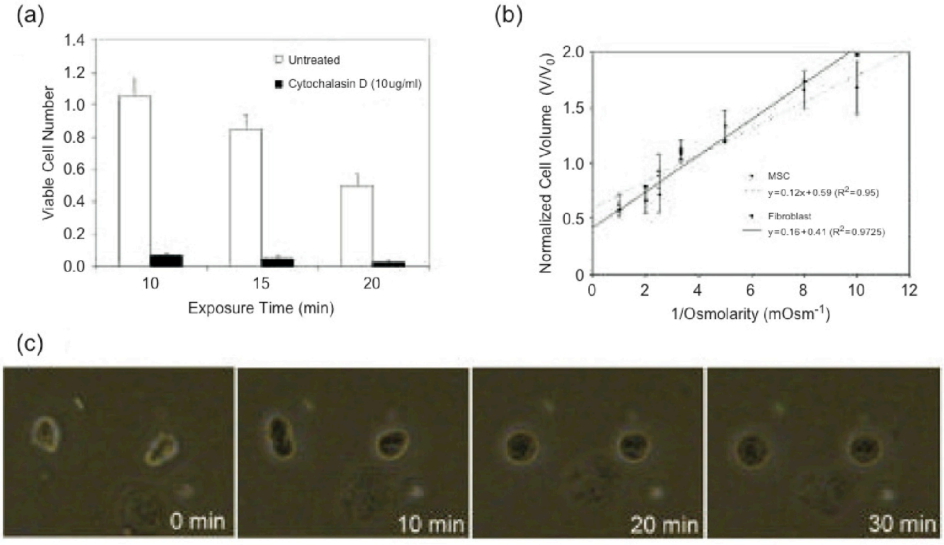

Figure 4. Mechanisms of osmotic resistance of bone marrow–derived MSCs. (A) Osmolytic resistance of bone marrow–derived MSCs with and without disruption of actin filament polymerization by cytochalasin D. MSCs were incubated with cytochalasin D (10 mg/mL) for 75 min and exposed to hypotonic conditions for the stated period of time. Normalized cell concentration data represent mean ± SEM. (B) Boyle-van't Hoff plot of bone marrow–derived MSCs and neonatal human fibroblasts. Normalized cell volume observed during change in solution osmolarity after steady-state cell volume achieved. The regression equation shows that the dependent variable, V/V0, depends on the independent variable, 1/Osm, and the regression residual represents the ineffective cell volume, which in this case is 59% for MSCs and 41% for fibroblasts. Error is small where not shown. (C) MSCs at infinite dilution at various time points show doubling of cell volume following 20 min of exposure. Color images available online at www.liebertpub.com/ten.

Discussion

Given the invasive nature of bone marrow aspiration, the search for MSCs in ectopic sources has intensified. MSCs are present at small percentages in the bone marrow stroma, and it is likely that other sources will be more dilute in MSCs. Therefore, more stringent methods to isolate these rare cells are critical to their recovery from other biological samples. Currently used multistep processing techniques are highly variable, rely on multiple determinants, and often fail to isolate homogeneous populations. Here we describe a single-step method to isolate MSCs based on their relative resistance to osmotic lysis, and apply this technique to the isolation of MSCs from UCB at far greater and purer yields than commonly employed methods. We also provide an initial framework to understand the molecular basis of osmolytic resistance that correlates to substantial ineffective cell volume and cytoskeletal integrity.

Cell sorting relies on inherent physical and biochemical differences between different cells. A cell's behavior in hypotonic conditions is highly variable and correlates to their functional and structural abilities to handle fluid flux. This feature can be exploited if a suitable time regimen can be identified to obtain depletion of contaminating cell types. We have previously shown depletion of erythrocytes following 10-second exposure to DI water (12). Here we subject samples to prolonged exposure to DI water; the key feature of this technique is its specificity and sensitivity for MSCs. When compared to other nonhematopoietic cells that could be causes of contamination, MSCs displayed a greater relative threshold for exposure to osmolytic conditions. An important variable in this differential resistance was the effect of convection on osmolytic resistance; that is, other cell types could withstand similar exposure times as MSCs in purely diffusive settings of water transport. Although the exact nondimensional parameters were not determined, the present work may be an initial effort toward an automated MSC isolation process using fluidic systems.

A major concern in using OS is the integrity of isolated cells. Our studies demonstrate that any dynamic changes in cell morphology and function are fully reversible as assessed by MSC proliferation and differentiation studies using MSCs exposed to OS. This can be attributed, in part, to the intricate osmoregulatory mechanisms of MSCs. Due to constant time-varying internal and external osmotic environments, cells make use of several volume regulatory mechanisms to temporarily counter osmotic gradients that attempt to alter cell volume. These mechanisms include ion and organic osmolyte transport across the cell membrane, de novo formation of organic osmolytes, and altered metabolism of macromolecules (15, 16). MSCs were found to be equipped with atypical ineffective cell volume (* 60%), which is in stark contrast to the ineffective cell volume of most mammalian cells (< 30%). Typically, in most mammalian cells, > 150% increase in cell volume following swelling is necessary for osmotic pressure to be sufficient to result in osmolysis (17). However, in MSCs only a 100% increase in cell volume was observed at infinite dilution. A major component of the ineffective cell volume is the cytoskeleton, a network of self-assembled proteins that are integral to cell shape and function and act as the solid-state foundation for mechanotransduction and intracellular transport. Actin, a key component, polymerizes readily to form filamentous actin, which exists in dynamic equilibrium with monomeric actin. Dynamic transitions entailing changes from one conformation to another are central to mechanically related events, including osmotically induced cell volume regulation. Disruption of filamentous actin polymerization of MSCs using cytochalasin D dramatically increased the susceptibility to hypotonic shock and loss of osmolytic resistance. An anticytoskeletal agent can lead to many other alterations in cell physiology, such as loss of biomechanical integrity, disruption of the glycocalyx, and interference of signaling pathways or vesicle trafficking, that warrant a more rigorous study of the role of the cytoskeleton in osmotic resistance.

Studies concerning the mobilization of MSCs in blood are complementary to new methods to isolate these cells. Our attempts to isolate MSCs from peripheral blood of healthy individuals recovered cells with a phenotype of CD44-, CD105-, and CD106-, which is not consistent with MSCs (data not shown). To date, it is unclear whether MSCs mobilize naturally or in response to tissue injury. Furthermore, determination of factors that can mediate this mobilization will be invaluable to procurement of cells from haploidentical individuals for transplantation.

In conclusion, stem cells may possess distinct survival mechanisms in order to be renewed throughout the life of an organism. Indeed, some of the strongest selection pressures in evolution result from environmental hydro-osmotic stress due to fluctuating salinity and extreme temperatures. The ability to handle dynamic changes in tonicity may be a cytoprotective trait that has evolved in MSCs and is differentially lost in their progeny. We exploited the ability of MSCs to withstand a hypotonic environment as a positive selection marker that resulted in a pure, high yield of MSCs from UCB, and can be amenable to the isolation of these cells from other sources.

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Chapter 7

Elevated hepatocyte-specific functions in fetal rat hepatocytes cocultured with adult rat hepatocytes

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Abstract

Fetal hepatocytes (FHEPs) are a potential source of highly proliferative transplantable cells, but express low levels of liver-specific functions. We hypothesized that the microenvironment of adult hepatocytes (AHEPs) may upregulate these functions. Primary FHEPs were seeded on top of collagen sandwiched AHEPs either directly or separated by a porous Transwell membrane insert. In direct cocultures, albumin (ALB) secretion, urea synthesis and cytochrome P450 (CytP450) activity were all approximately two fold higher than the sum of the corresponding monocultures. Using a Transwell porous insert led to similar results, suggesting a major role for soluble factors. When AHEPs and FHEPs were separated after coculture, initially both AHEPs and FHEPs showed significantly increased ALB secretion when compared to control monocultures, while urea synthesis was significantly increased for the FHEPs only. Functions of previously cocultured FHEPs normalized over the course of a week, but AHEP function remained elevated even after separation. In conclusion, coculturing AHEPs with FHEPs increases expression of liver-specific functions in both cell types. The effect on FHEPs, but not AHEPs, was reversible. Unraveling the underlying mechanisms and optimizing this phenomenon will be useful to make fetal liver cells a potential cell source for hepatic tissue engineering applications.

Introduction

Cell-based therapies are at the core of emerging new strategies for the treatment of hepatocellular disease, including bioartificial liver systems (BAL) and hepatocyte transplantation. Several types of BAL tested in phase I clinical trials have been shown to be safe and biocompatible. Recently, a large, prospective, controlled, randomized trial showed improved survival for BAL-treated patients with hepatic failure awaiting liver transplantation.[1, 2] Hepatocyte transplantation has been shown to be feasible and safe for the treatment of patients with metabolic liver diseases and as a bridge for patients with liver failure awaiting liver transplantation.[3] However, implementation of these experimental treatment modalities in the clinical setting is slow, primarily because of a shortage of transplantable hepatocytes.

Inadequate numbers of primary human hepatocytes are available from donors. Although the extensive replication potential of mature hepatocytes in-vivo has been observed, they cannot be propagated in vitro to divide to a reasonable extent in culture without losing their liver-specific functions.[4, 5] Reversibly immortalized hepatocytes have been shown to provide life-saving metabolic support after transplantation in animal models of acute liver failure, but the risks involved with genetic modification have prevented this technique from entering clinical application.[6] Alternatives include xenogeneic hepatocytes, tumor-derived cell lines and stem cell derived hepatocytes, each carrying its own disadvantages. Porcine liver cells, which are readily available and physiologically similar to human hepatocytes, contain retroviruses in their genome which are capable of infecting human cells.[7] Tumor-derived cell lines provide a theoretically unlimited source of human hepatocytes, but low expression of liver-specific functions renders them unsatisfactory as a source for BAL and their tumorigenicity makes them unsuitable for transplantation.[8] Stem cell differentiation protocols have provided hepatocyte-like cells with expression of lineage-specific markers, but obtaining large numbers of functional hepatocytes from this source is not yet feasible.[9]

Fetal hepatocytes (FHEPs) may provide a source of highly proliferative, transplantable cells, resistant to cryopreservation and ischemic injury.[10-12] Although the liver is the central metabolic organ in the adult, during mid- and late fetal stages its major function is hematopoiesis, and liver-specific functions are only expressed marginally.[13] For this reason, in vitro induction of their maturation and upregulation of their metabolic functions is necessary to develop the potential of FHEPs in cell-based therapies.

In vitro studies have shown that growth factors and hormones including oncostatin M, dexamethasone and nicotinamide and extracellular matrices such as Matrigel induce expression of adult hepatocyte (AHEP) markers and attenuate hematopoiesis.[14-16] Some of these soluble factors are known to increase liver-specific functions when combined with specific polymer scaffolds.[17, 18] Studies investigating the effects of AHEP extracellular matrix on expression of mature hepatocyte markers found that neonatal and fetal rat hepatocytes acquire an adult phenotype in this environment.[19, 20] Cell transplantation experiments have shown that FHEPs mature after engraftment

in the adult liver environment.[21, 22] Despite these efforts, induction of liver-specific functions in fetal hepatocytes remains a major hurdle in this otherwise potentially attractive cell source for functional hepatocytes.

Our hypothesis is that AHEPs provide an environment in which FHEPs will express higher levels of hepatocyte-specific functions. For this purpose, albumin secretion, urea synthesis and cytochrome P450 activity were assessed in cocultures of FHEPs and AHEPs. We found that coculture upregulates liver-specific functions in both FHEPs and AHEPs, and that this effect is preserved even when the two cell types are separated by Transwell membrane inserts, suggesting that soluble factors were largely responsible for the enhanced levels of hepatocyte-specific functions. Furthermore, the functional enhancements were partly reversible, since removing FHEPs from Transwell cocultures with AHEPs resulted in a return of FHEP functions to the levels seen in non-cocultured FHEPs within a week, while separated AHEPs kept secreting increased amounts of albumin.

Materials & methods

Materials

Cell culture medium consisted of Iscove's Modified Dulbecco's Medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen), 500μg/L porcine insulin (Eli Lilly, Indianapolis, IN), 1mg/L glucagon (Bedford Laboratories, Bedford, OH), 1mg/L hydrocortisone (Pharmacia Corporation, Peapack, NJ), 10μg/L epidermal growth factor (Becton Dickinson Biosciences, San Jose, CA), 2000u/L penicillin (Invitrogen) and 2mg/L streptomycin (Invitrogen). Transwell permeable supports with 0.4μm pore size and 6-well plates were purchased from Corning Incorporated, Corning, NY. Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Invitrogen.

Animals

Female Lewis rats were obtained from Charles River Laboratories (Wilmington, MA). Both non-pregnant animals and timed-pregnant animals were maintained on standard chow with a 12-hr light-dark cycle. The first day of gestation was defined as day 0. All animal procedures were performed in accordance with National Research Council guidelines and approved by the Subcommittee on Research Animal Care at the Massachusetts General Hospital.

Hepatocyte isolation

AHEPs were isolated from adult, non-pregnant female Lewis rats weighing between 200 and 250g by a two-step collagenase procedure as described by Seglen and modified by Dunn et al.[23, 24] Fetal liver cells were freshly isolated from pooled livers of fetal Lewis rats on day 18 of gestation as previously described.[25] Liver cells were counted with a hemacytometer, excluding the hematopoietic cells, which were easily distinguished by their smaller size and morphology, from the counts. During a typical FHEP isolation, 1-2 million liver cells were isolated per fetal liver. Cell viability ranged from 90 – 95% based on trypan blue exclusion for the AHEPs, and between 85% and 95% for the FHEPs.

Hepatocyte Culture

Both FHEPs and AHEPs were seeded at a density of 1×10^5 cells/well in 6-well plates. Wells were pre-coated with 0.4mL of a mixture of 9 parts of a collagen type I stock solution (1.25mg/mL) and 1 part of 10x DMEM. Collagen stock solution was prepared from rat-tail tendon by a modified procedure of Elsdale.[26] The collagen mixture was allowed to gel for 60 minutes before cell seeding. Two milliliters of cell suspension were seeded into the wells and incubated in a humidified 5% CO_2 / 95% air incubator at 37°C.

The AHEPs were cultured in a collagen sandwich configuration as described by Dunn et al.[27, 28] Briefly, the medium was aspirated after 24 hours and a second layer of collagen-10X DMEM mixture (0.4mL) was added. After 60 minutes of incubation at 37°C to allow for gelation, two milliliters of fresh culture medium were added.

FHEPs were co-cultured with the AHEPs in two different ways: (1) in direct contact, or (2) separated by a porous Transwell membrane. FHEPs were isolated and seeded 24 hours after the AHEPs. In the direct coculture experiment, freshly isolated fetal cells were seeded at a density of 1 x 10^5 cells/well on top of the second collagen layer of the AHEP cultures (Fig. 1a). In the Transwell coculture experiments, the fetal cells were seeded onto Transwell permeable supports without collagen, which were then placed on top of the AHEP cultures. After six days of coculture, some of the Transwell supports were removed, washed with phosphate buffered saline (PBS), and placed in empty wells, thus allowing for the subsequent monoculture of the cocultured FHEPs and AHEPs (Fig. 1b). In these experiments, FHEPs seeded on the Transwell membranes and grown as monocultures were used as controls. Cells were cultured for a total of 14 days, with daily medium changes. Medium samples were collected every 48 hours for protein secretion analysis, plus 24 hours after separating the Transwell cocultures. Each experiment was performed in triplicate, and was performed three times using cells from different isolations.

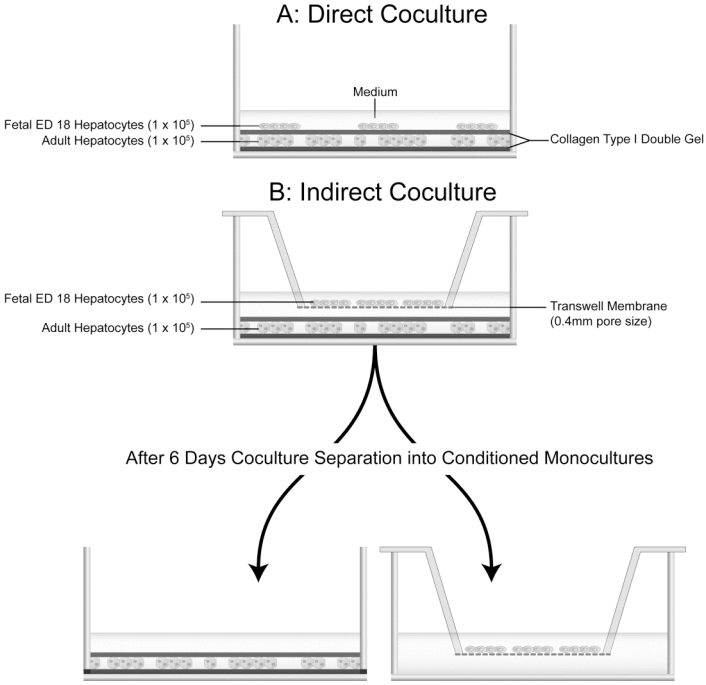

Figure 1. Description of coculture methods used. In the direct coculture system (A) AHEPs were seeded on a collagen type I gel at a density of 1 x 10^5 cells per well in a 6-well plate. Twenty-four hours later, a second gel was applied, after which 1 x 10^5 FHEPs isolated on gestation day 18 were seeded. In the Transwell coculture system (B), AHEPs were cultured as above. After application of the second gel, a Transwell permeable membrane with 0.4μm pores was introduced and 1 x 10^5 FHEPs were seeded on the membrane. On day 6, some of the Transwell inserts were removed to determine the expression liver-specific functions in previously cocultured FHEPs and AHEPs.

Immunofluorescence and microscopy
FHEPs were cultured on a single collagen gel for 48 hours. Cells were fixed for 15 minutes in 4% paraformaldehyde solution prepared in PBS. All steps were performed at room temperature. After washing, cells were incubated with a blocking buffer containing 10% normal horse serum and 0.025% Triton X 100 in PBS for 45 minutes. Subsequently, the cells were incubated with one of the following primary antibodies for 60 minutes: goat polyclonal anti-rat albumin (ALB) at 5μg/mL (ICN Biomedicals) and goat polyclonal anti alpha-fetoprotein (AFP) at 5μg/mL (Santa Cruz Biotechnology, Santa Cruz, CA). After washing three times with PBS, cells were incubated with a 1:200 dilution of FITC-conjugated donkey anti-goat secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 60 minutes. Then, cells were washed 3 times with PBS, incubating with 1μg/mL of propidium iodide (Sigma Aldrich Corporation, St Louis, MO) for 5 minutes during the first wash. Fluorescent and phase-contrast microscopy was performed with a Zeiss Axiovert 200 M inverted microscope.

Albumin enzyme-linked immunoabsorbent assay (ELISA)
ALB concentrations were determined by a competitive ELISA using a polyclonal antibody to rat ALB (Organon Teknika Corporation, West Chester, PA) as described previously.[24] 0-Phenylenediamine and hydrogen peroxide solution were purchased from Sigma. Chromatographically purified rat ALB was obtained from ICN Biomedicals, Aurora, OH. The absorbance was measured in a Versamax microplate reader (Molecular Devices, Sunnyvale, CA).

Urea assay
Urea concentration was measured by the specific reaction of diacetyle monoxime with urea using the commercially available Urea Nitrogen Assay Kit (Stanbio Laboratory, Boerne, TX) in accordance with the manufacturer's instructions. The absorbance was measured in a Versamax microplate reader (Molecular Devices).

Cytochrome P450 assay
Cytochrome P450IA1 isoenzyme (CytP450) activity was determined by fluorometric ethoxyresorufin deethylase activity (EROD) assay (Invitrogen), which measures the rate of formation of resorufin from ethoxyresorufin 48 hours after induction with 2μM 3-methylcholanthrene, per manufacturer's instructions.[29] Cells were cultured for a total of six days before the assay was performed.

Statistical analysis
Statistical significance was calculated using a 2-tailed Student t test for paired data and analysis of variance as applicable. The threshold for statistical significance was considered $p<0.05$.

Results

FHEPs express mature hepatocyte markers

To characterize rat FHEPs, we analyzed the expression of ALB and AFP by rat FHEPs harvested at day 18 of gestation by immunofluorescence. Most FHEPs stained positively for ALB, but were only very weakly positive or negative for AFP (Fig. 2). These results confirmed that the FHEPs isolated at this stage show a high degree of maturation when analyzed for these markers.

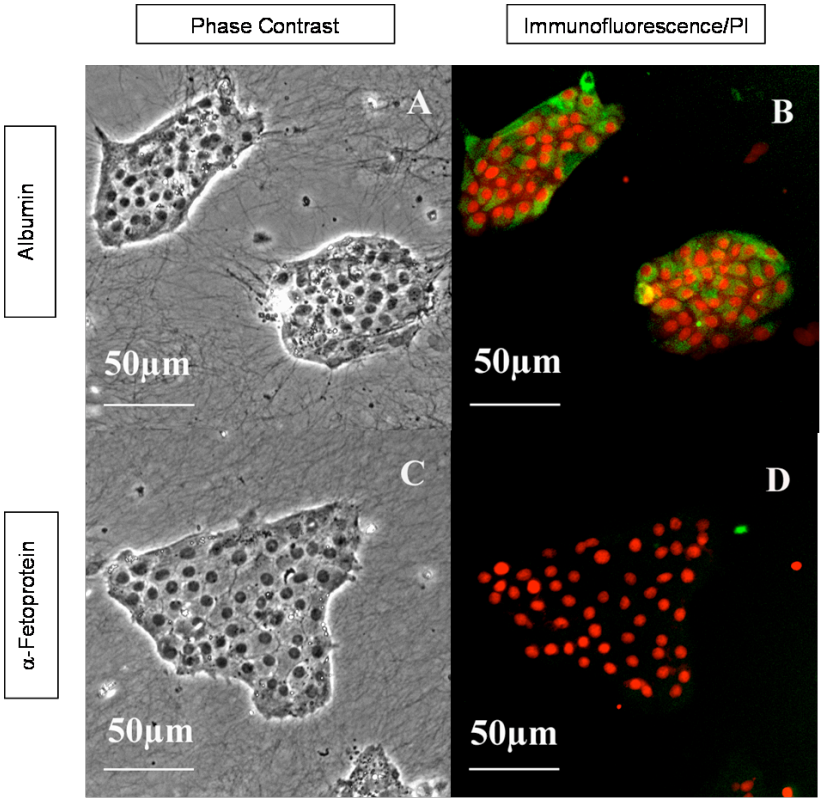

Figure 2. Albumin (ALB) and a-fetoprotein (AFP) expression of FHEPs harvested on gestation day 18. Cells were stained for ALB (A, B) and AFP (C, D) by immunofluorescence with FITC labels. Nuclei were stained with propidium iodide (PI). Original magnification: x 100.

FHEP monocultures exhibit low liver-specific functions

The rate of ALB secretion, urea synthesis and CytP450 activity of FHEPs was determined and compared to AHEPs during a two-week culture period. FHEP ALB production was stable over the two-week culture period and averaged 0.8µg ALB/day/1 x 10^5 plated cells, which was 11% of AHEP production (7.8µg ALB/day/1 x 10^5 plated cells: Fig. 3a). Similarly, urea synthesis by FHEPs was 10% of AHEP rates: 2.5 versus 22.6µg urea/day/1 x 10^5 plated cells (Fig. 3b). CytP450 activity in the FHEPs was 4.4nM resorufin/min/1 x 10^5 plated cells,

which was 26% of the level seen in AHEPs (17.3nM/min/1 x 10^5 plated cells) on day 6 after isolation (Fig. 3c). In short, quantitative analysis confirmed that the expression of liver-specific functions by FHEPs is only a fraction of the level seen in AHEPs. No difference in albumin secretion and urea synthesis was observed between control monocultures of FHEPs cultured on a collagen gel or on the Transwell insert membrane without a collagen gel (data not shown).

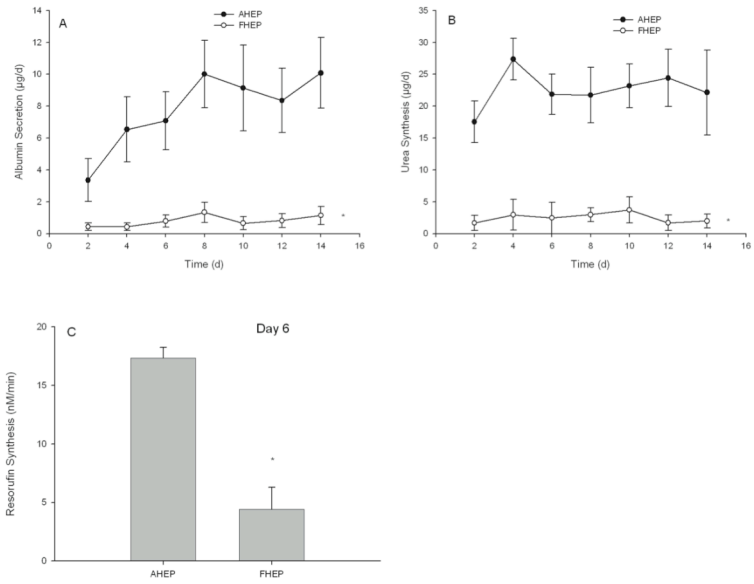

Figure 3. Levels of ALB secretion (A), urea synthesis (B) and cytP450 activity (C) by FHEPs and AHEPs in monocultures. Data shown are averages of three separate experiments performed in triplicates (n=9) ± SE. * statistically significantly different from AHEPs ($p<0.01$).

Coculture of FHEPs and AHEPs leads to synergism in the expression of liver-specific functions

The effects of direct coculture between FHEPs and AHEPs were determined by comparing the liver-specific functions in coculture to the sum of those performed by monocultures in otherwise identical conditions. In the direct coculture system, ALB secretion was found to be significantly higher over the two-week culture period ($P<0.01$), showing a more than two-fold increase (21.1 versus 9.8 µg ALB/day/1 x 10^5 plated cells) on day 10 (Fig. 4a). Urea synthesis was on average 64% higher over the two week culture period in the coculture system ($P<0.01$), peaking at more than two-fold at day 14 (51.7 versus 24.1 µg urea/day/1 x 10^5 plated cells, Fig. 4b). CytP450 activity

measured on day 6 was 74% higher compared to the sum of the monocultures (38.3 versus 22.0 nM resorufin/min/1 x 10^5 plated cells, P<0.01: Fig. 4c). These results suggest a synergistic effect between the FHEPs and AHEPs on the expression of liver-specific functions, although it is unclear how much of the increase was contributed by FHEPs and AHEPs.

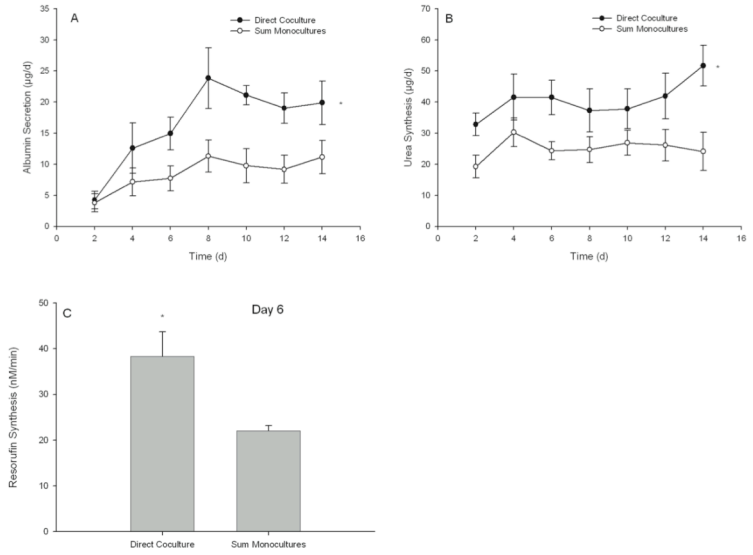

Figure 4. Levels of ALB secretion (A), urea synthesis (B) and cytP450 activity (C) for cocultured FHEPs and AHEPs compared to the sum of these levels for FHEP and AHEP monocultures. Data shown are averages of three separate experiments performed in triplicates (n=9) ± SE. * statistically significantly different from Sum Monocultures (p<0.01).

Soluble factors induce upregulation of functions in cocultures

Phase-contrast microscopy observations showed that in the direct coculture system cells that were initially separated by a collagen gel had migrated to the same focal plane within a couple of days, allowing for potential direct cell-cell contacts (data not shown). In a subsequent set of experiments, the role of soluble factors on the observed functional upregulation was investigated by separating FHEPs and AHEPs using a porous Transwell insert membrane. ALB secretion and urea synthesis were not significantly different from that measured in the direct coculture system (P-values 0.63 and 0.14 respectively: Fig. 5). These results suggest that soluble factors secreted by the cells are likely to be responsible for the enhanced liver-specific functions.

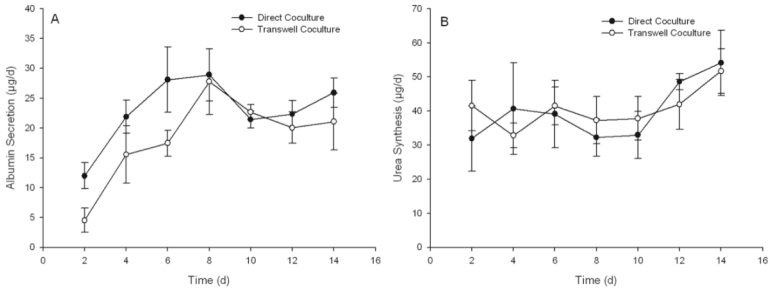

Figure 5. Levels of ALB secretion (A) and urea synthesis (B) for cocultured FHEPs and AHEPs using the direct vs. the Transwell coculture technique. Data shown are averages of three separate experiments performed in triplicates (n=9) ± SE. There were no statistically significant differences between the groups.

Both FHEPs and AHEPs contribute to increased function in cocultures

Transwell inserts were removed from the coculture environment after 6 days to allow for the subsequent monoculture of previously cocultured AHEPs and FHEPs for two purposes: to determine the contribution of each cell type to the increased functions in coculture and to determine whether the increase in functions was reversible or permanent. Both the previously cocultured AHEPs and FHEPs secreted significantly more ALB than their control monocultures, which was maintained for the entire culture period for the cocultured AHEPs ($P<0.01$, $P<0.05$ respectively). The average ALB secretion from the day of separation until day 14 by the cocultured AHEPs was 20.0 µg ALB/day/1 x 10^5 plated cells, which was 94% higher than the 10.3 µg ALB/day/1 x 10^5 plated cells observed in the control AHEPs during this period. The previously cocultured FHEPs, on the other hand, lost function over time. From six days onwards after the separation, no difference in albumin secretion with the control FHEPs was observed (Fig. 6a). One day after separation, the cocultured FHEPs secreted 4.0µg ALB/day/1 x 10^5 plated cells, which represented 34% of the level measured in control AHEPs (11.8µg/day/1 x 10^5 plated cells) and was several-fold higher than in control FHEPs (Fig. 6b).

Urea synthesis was also increased in previously cocultured FHEPs and AHEPs when compared to their control monocultures ($p<0.05$, Fig. 6c). The cocultured FHEPs synthesized 9.5µg urea/day/1 x 10^5 plated cells one day after separation from the coculture, which constituted 34% of the level seen in control AHEPs (27.9µg/day/1 x 10^5 plated cells) and was 2.4-fold higher than in control FHEPs ($p<0.01$: Fig. 6d). As was the case for albumin secretion, urea synthesis by the separated FHEPs decreased over time, normalizing to control levels 8 days after separation. Levels of urea synthesis by separated AHEPs stayed significantly higher over the culture period than levels observed in control AHEPs ($p<0.05$), although the difference was small and not significant one day after separation ($p=0.26$). On average, levels of urea synthesis were 33.9µg urea/day/1 x 10^5 plated cells from the day of separation of the cocultured AHEPs until the end of the culture period, which was 29% higher than the 26.4µg urea/day/1 x 10^5 plated cells observed in the control monocultures ($p<0.05$).

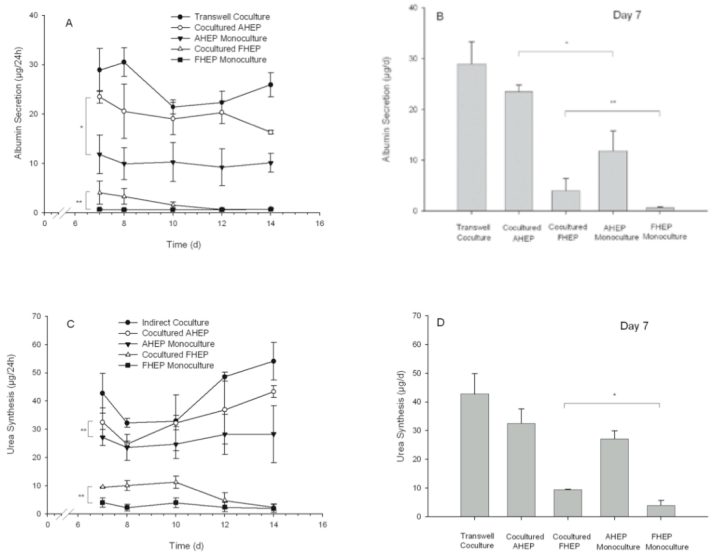

Figure 6. Levels of ALB secretion (A, B) and urea synthesis (C, D) for previously cocultured FHEPs and AHEPs using the Transwell technique after separation into monocultures. Separation occurred on Day 6 of Transwell coculture. Control FHEP and AHEP monocultures as well as control cocultures from the same cell isolations are included for comparison. Panels A and C show the time course after separation into monocultures. Panel B and D provide comparisons among the groups on day 7 (one day after separation). Data shown are averages of three separate experiments performed in triplicate (n=9) ± SE. *statistically significant ($p<0.01$). ** statistically significant ($p<0.05$).

Discussion

The present work shows that FHEPs exhibit increased liver-specific functions during and after coculture with AHEPs. Liver-specific functions of the AHEPs were also increased in this coculture system. Data from Transwell cocultures allowing only free exchange of diffusible media contents also show increased function in cocultures, suggesting that soluble factors are at least in part responsible for this effect. After separation of the FHEPs from the AHEPs in the Transwell cocultures, FHEP function decreased, suggesting that the increase in FHEP function is reversible. On the other hand the enhanced function in AHEPs was maintained even after separation.

Several reports describe that AHEP extracellular matrix is capable if inducing markers of terminal differentiation in fetal [19] and neonatal [20] hepatocytes. However, only expression of markers and no metabolic function was investigated, and no investigations concerning the involvement of soluble mediators were reported. The present study shows that major hepatocyte-specific functions of FHEPs are upregulated in the microenvironment created by AHEPs, and the results are most consistent with the notion that paracrine mediators are involved. Although the specific factors responsible for the observed effects remain to be elucidated, identification of these factors may allow for improved methods to optimize FHEP function, necessary for their potential use in cell-based therapies for liver disease.

After separation from the Transwell cocultures, levels of albumin secretion and urea synthesis by the previously cocultured FHEPs decreased to the levels observed in non-cocultured FHEPs within a week. We conclude that the upregulation is reversible and discontinuation of the stimulus provided by the AHEPs causes the liver-specific functions of FHEPs to normalize over time. Figuring out what specific soluble factors are responsible for the observed effects may allow for prolonged upregulation by continuous stimulation without the use of the scarcely available AHEPs.

Several studies have shown that hematopoietic stem cells have the capacity to contribute to hepatocyte replacement after engraftment in liver tissue, although it remains unclear whether this happens through transdifferentiation or cell fusion.[30-32] At day 18 of gestation, the rat fetal liver still functions as a hematopoietic organ. Theoretically, cells derived from hematopoietic precursors present in the fetal liver cell isolations may have contributed to increased liver-specific functions in coculture. Any significant contribution is unlikely, however, because the appearance of functional bone-marrow derived hepatocytes is an extremely rare phenomenon.[33]

AHEPs also exhibited increases in liver-specific functions after coculture with the FHEPs. Although we cannot exclude that FHEPs secrete factors that stimulate AHEP function, this effect may be also explained by the fact that the FHEPs contain fractions of non-parenchymal liver cells, like stellate cells, which are known to support the function of adult liver cells.[34, 35]

In conclusion, coculturing FHEPs with AHEPs leads to an upregulation of liver-specific functions in both cell types. No direct contact between FHEPs and AHEPs is necessary, suggesting that paracrine factors mediate these effects. Optimizing and unraveling this phenomenon could provide methods to

upregulate liver-specific functions of FHEPs, necessary for the development of fetal liver as a cell source for hepatic tissue engineering applications.

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Chapter 8

Reactivity of liver sinusoidal endothelial cells from galα(1,3)gal-deficient pigs to human and baboon serum

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Xenotransplantation. Submitted.

Abstract

Elimination of galactose-α(1,3)galactose (Gal) expression in pig organs prevents hyperacute xenograft rejection of kidneys and hearts transplanted into non-human primates. However, naturally present antibodies to non-Gal epitopes remain an immunological hurdle due to the induction of endothelial cell damage leading to acute humoral xenograft rejection. The liver is an immunoprivileged organ as shown by the stable acceptance of allogeneic liver grafts in some species and low levels of xenoreactive antibody reactivity of its sinusoidal endothelial cells. Therefore, there is a need to study the extent of human or baboon serum-induced damage in liver sinusoidal endothelial cells (LSECs), the main antigenic component of the liver, originating from Gal-deficient pigs. LSECs were isolated from wildtype and Gal-deficient pigs. IgM and IgG binding to freshly isolated LSECs was analyzed by flow cytometry. Complement activation in cultured cells was quantified in supernatants from serum-exposed LSEC cultures using C3a and CH50 ELISA's. Levels of complement-activated cytotoxicity (CAC) were determined by a fluorescent Live Dead Assay and by the quantification of LDH release. IgM binding to Gal-deficient LSECs was 80% and 87% lower than its binding to wildtype LSECs for human and baboon serum respectively. Levels of IgG binding were low in all conditions and only modestly inhibited for the Gal-deficient cells. Compliment activation measure by C3a and CH50 levels released following exposure to human serum was reduced by 42% ($P < 0.05$) and 52% ($P < 0.005$) respectively. Complement-activated cytotoxicity in Gal-deficient LSECs was reduced by 60% ($P < 0.001$) in human serum and by 72% ($P < 0.001$) in baboon serum when compared to wildtype LSECs. Lactate dehydrogenase release levels were reduced by 37% and 57% respectively ($P < 0.001$). The developed in vitro model can be used to determine reactivity of pig liver microvascular endothelium to primate serum. LSECs from Gal-deficient pigs retain a considerable amount of susceptibility to complement-induced cell damage when exposed to primate serum. Our results suggest that the privileged status of the liver in the allogeneic setting does not translate to the pig-to-primate xenotransplantation arena and that additional strategies may be needed to facilitate porcine liver graft acceptance in humans.

Introduction

End-stage liver disease causes 25,000 deaths each year and is the tenth most frequent cause of death in the United States. Orthotopic liver transplantation is the only available curative option, but with only 7000 available organs per year in the United States many patients die waiting for a donor (1). Living donor liver transplantation only adds about 4% to the donor pool (288 living donors in 2006 [UNOS.org]). Xenotransplantation, using pig organs, could alleviate this shortage. However, transplantation of porcine organs into primates normally initiates a cascade of events that starts with binding of naturally occurring antibodies to xenogeneic epitopes on the endothelium and results in hyperacute xenograft rejection within minutes to hours of transplantation (2, 3). These xenoreactive antibodies predominantly recognize galactose-α(1,3)galactose (Gal), a terminal disaccharide synthesized by α(1,3)galactosyltransferase on the endothelial surface of pigs (4, 5). The development of Gal-deficient pigs by nuclear transfer cloning (6, 7)) revived the concept of xenotransplantation of porcine organs as a potential source of organs for clinical transplantation. Hyperacute xenograft rejection was prevented in pig-to-primate models of kidney and heart transplantation using Gal-deficient organs, resulting in up to several months survival (8, 9). However, Gal-deficient organs are still rejected in weeks to months by acute humoral xenograft rejection, which represents the next major hurdle in pig-to-primate xenotransplantation. Acute humoral xenograft rejection, also named acute vascular rejection, is characterized by the activation of microvascular endothelial cells leading to thrombotic microangiopathy. How exactly the endothelial cells become activated is unknown, but activity of antibodies directed against non-Gal antigens are thought to play an important role (10). Recently, studies of isolated aortic endothelial cells demonstrated that 13% to 36% of the reactivity of human natural antibodies remained in spite of the elimination of the Gal epitope (11). At the same time, over 21% of complement-activated cytotoxicity (CAC) remained, indicating that a considerable amount of humoral immune reactions directed against non-Gal antigens remain even in Gal-deficient conditions. These recent studies raise important questions regarding the compatibility of Gal-deficient organs for liver xenotransplantation.

The liver is an immunoprivileged organ as shown by the stable acceptance of allogeneic liver grafts without immunosuppression in several species (12, 13) and the suppression of rejection of solid tissue grafts which are co-transplanted with a liver allograft from the same donor (14, 15). Hepatocytes, which present their antigens to the systemic circulation by sending processes through the fenestrations of the liver sinusoidal endothelial cells (LSECs) (16), exhibit increased resistance to CAC (17). Porcine LSECs, the main antigenic component of liver allografts, bind significantly lower levels of human natural antibodies compared to aortic endothelial cells (18). These observations raise the possibility that the liver has a unique position in the xenotransplantation arena. Therefore, it is important to know whether LSECs from Gal-deficient

pigs exhibit an increased resistance to mechanisms of humoral xenograft rejection.

In this study, we quantified components of humoral xenograft rejection in human and baboon serum against the microvasculature of the liver in vitro. Significant levels of natural antibody binding and complement activity were observed for both wildtype and Gal-deficient LSECs. Levels of preformed IgM binding were 80% (human) and 87% (baboon) lower in Gal-deficient LSECs and a generally low level of IgG activity against pig LSECs was measured in both human and baboon serum with only modest decreases in Gal-deficient conditions. Complement activity levels were reduced to approximately half of the levels measured in wildtype LSECs. CAC in Gal-deficient LSECs was reduced by 60% in human serum and by 72% in baboon serum when compared to wildtype LSECs.

Materials & methods

LSEC Isolation

LSECs were isolated from the livers of SLA^{dd}, Gal+/+ miniature swine (wildtype), and partially inbred, SLA^{dd}, Gal-/- (Gal-deficient) miniature swine, kindly provided by Dr. David H. Sachs from kidney donor animals. Animals were kept under standard conditions and cared for in accordance with the guidelines set forth by the Committee on Laboratory Resources, National Institutes of Health.

Directly after nephrectomy was performed, the liver was excised and put on ice. After cannulation of the portal vein branch to the left lateral lobe, digestion of the tissue was achieved using a two step-perfusion method. First, the lobe was flushed for 10 minutes with ice-cold 0.9% NaCl solution supplemented with 5% dextrose, 20 U/L heparin and 4 meq/L KCl. Subsequently, the lobe was perfused for 18 minutes at 37 °C with a solution of 0.5% collagenase type IV (Sigma Aldrich, St Louis, MO) in Krebs Ringer Buffer supplemented with 3 mM $CaCl_2$. The lobe, weighing between 175 and 225 grams, was then cut into 12 pieces and the liver cells were gently dispersed into Krebs Ringer Buffer on ice.

After collection of a total of 400 ml of cell suspension, clumps were removed by filtration through a 250 μm and then a 150 μm mesh. Hepatocytes were pelleted by a differential centrifugation step at 50g for 10 minutes at 4 °C. The non-parenchymal cells, present in the supernatant, were collected by centrifugation at 300g for 15 minutes at room temperature. Cells were resuspended in 30 ml of elutriation buffer that consisted of Gay's Balanced Salt Solution supplemented with 1% bovine serum albumin. DNA-se was added (10 μg/ml) and the cell suspension incubated at 37 °C for 15 minutes. All subsequent steps were performed at room temperature. Differential elutriation was performed using an elutriating centrifuge with a JE-5.0 rotor (Beckman Coulter, Fullerton, CA) using a standard chamber at 2,500 rpm. The suspension was introduced at a flow rate of 18 ml/min. This flow-rate was maintained for 15 minutes. LSECs were collected at a flow rate of 38 ml/min. The cells were pelleted at 300g for 15 min and resuspended in microvascular endothelial growth medium EGM-2-MV (Cambrex, East Rutherford, NJ). A differential adhesion step was performed for 5 minutes before non-adhering cells were counted and viability was assessed by trypan blue exclusion. The yield was routinely 20-40 x 10^6 cells with viability exceeding 90%.

LSEC Culture & Characterization

Cells were cultured at a density of 5 x 10^5 cells/cm^2 at 37 °C in a humidified 5% CO2 incubator in 12-well plates that had previously been coated with human fibronectin (R&D Systems, Minneapolis, MN) at 50 μg/ml for 1 hour. LSEC purity was greater than 85% as assessed by FITC labeled acetylated-LDL (Invitrogen, Carlsbad, CA) uptake and the lack of stellate cell auto-fluorescence. Cells were cultured for 2 days before complement activation studies.

Human and Baboon Sera
Human and baboon serum were collected from normal healthy donors and stored at -80 °C until use. Complement was inactivated in selected samples by incubation at 56 °C for 60 minutes.

Antibody Binding
Freshly isolated wildtype or Gal-deficient LSECs (2×10^5) were incubated in human or baboon serum diluted at a 1:2, 1:20 or 1:200 ratio with PBS for 30 min at 37 °C. Cells were washed and collected by centrifugation at 300g for 15 minutes. Binding of IgM and IgG was detected by incubation with a FITC-labeled swine anti-human IgM antibody and a TRITC-labeled swine anti-human IgG antibody (Accurate Chemical Scientific Corporation, the Netherlands) at a 1:100 dilution for 30 minutes on ice. Binding was quantified by flow cytometry using a FACSCalibur (BD, San Jose, CA), analyzing 20,000 events per experiment.

Complement Activation
LSECs were incubated with human or baboon serum diluted 1:2 with PBS for 60 minutes at 37 °C. Supernatants were collected and stored at -80 °C for ELISA. Analysis of C3a anaphylotoxin levels, a non-specific measure of complement activation, was performed using a commercially available kit (BD, San Diego, CA) per manufacturer's instructions. The CH50 Eq Enzyme Immunoassay (Quidel, San Diego, CA) was performed according to the vendor's instructions to quantify the total classical complement activity.

Complement-activated Cytotoxicity
Complement-activated cytotoxicity after incubation with human or baboon serum was assessed using a fluorescent Live-Dead Viability Assay (Molecular Probes, Eugene, OR) according to the vendor's instructions. In this assay, the cytoplasm of live cells accumulates green fluorescent calcein due to esterase activity, while the nucleus of dead cells is labeled fluorescently red by ethidium homodimer due to loss of membrane integrity. Incubation with heat-inactivated serum was used as a negative control. Live and dead cells were captured on a Zeiss 200 Axiovert microscope and quantified in 4 random images per well using the public software ImageJ (http://rsb.info.nih.gov/ij/). Lactate dehydrogenase (LDH) release in supernatants of the serum-exposed cultures was quantified using the LDH Cytotoxicity Assay Kit II (Biovision, Mountain View, CA) according to the manufacturer's instructions.

Statistical Analysis
Data is expressed as the mean ± standard deviation. Statistical significance was determined by a two-tailed Student's t-test. A P-value of 0.05 was considered statistically significant.

Results

Antibody Binding

Binding of preformed xenoreactive antibodies to porcine cell surface antigens is an essential step in both hyperacute xenograft rejection and acute humoral xenograft rejection. To assess the decrease in preformed antibody binding to pig LSECs due to the elimination of the Gal epitope, we analyzed IgM and IgG binding to wildtype and Gal-deficient pig LSECs using FACS. Significant levels of human and baboon serum preformed IgM binding to LSECs isolated from wildtype pigs were observed. For human serum, IgM reactivity decreased in Gal-deficient LSECs by 74%, 81% and 84% at serum dilutions of 1:200, 1:20 and 1:2 respectively. Decreases were 82% 85% and 94% for baboon serum IgM reactivity at serum dilutions of 1:200, 1:20 and 1:2 respectively (Fig. 1a/b). Levels of IgG binding to pig LSECs were low in both human and baboon serum (Fig. 1c/d). Only with 50% serum, a 27% reduction in the level of human IgG binding was observed. No difference was detected at lower serum concentrations and no changes in IgG binding were measured for baboon serum. These results demonstrate that the majority of IgM binding to pig LSECs is prevented in Gal-deficient animals and that levels of preformed IgG binding are less prominent and only modestly affected by knocking out Gal.

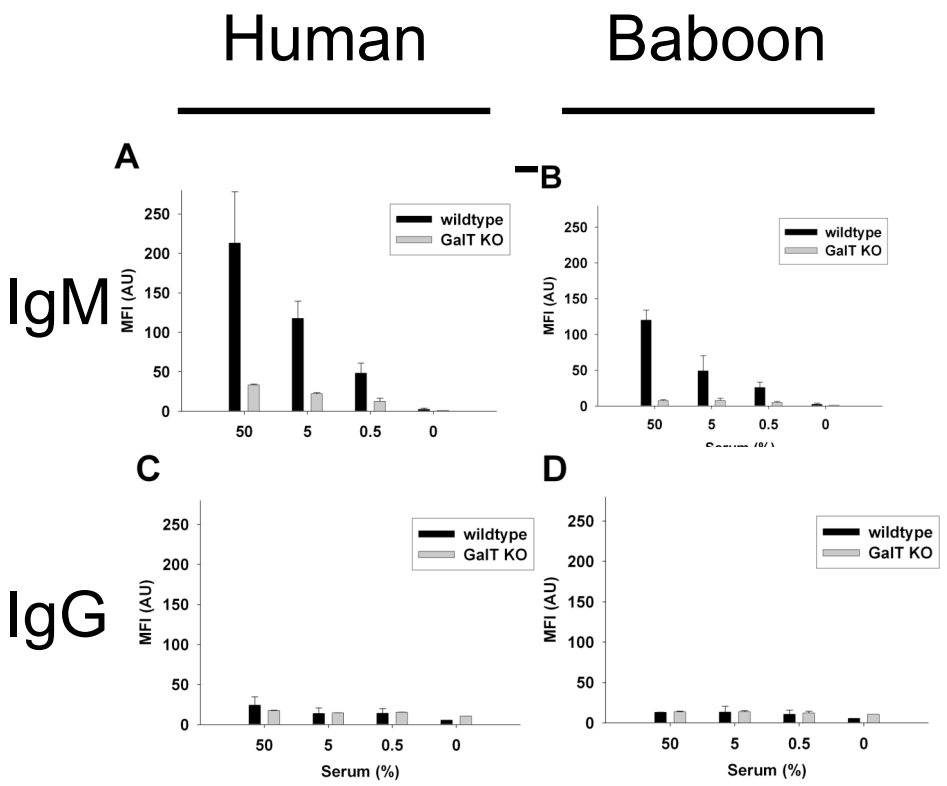

Figure 1. IgM and IgG reactivity of human and baboon serum to pig LSECs from wildtype and Gal-deficient animals. LSECs were isolated and exposed to varying concentrations of human and baboon serum. Binding of IgM and IgG antibodies was detected by the binding of a fluorescently labeled secondary antibody. Analysis was performed using flow cytometry. LSEC, liver sinusoidal endothelial cell; Gal KO, α(1,3)galactosyltransferase knockout.

Complement Activation

Levels of complement activation in serum samples exposed to wildtype and Gal-deficient LSECs was assessed using two different assays. We measured C3a complement levels as an indication of total complement activation (Fig. 2a). C3a levels were reduced by 42% ($P < 0.05$) in pig Gal-deficient LSECs compared to wildtype LSECs. The CH50 assay (Fig. 2b), measuring the classical complement pathway, demonstrated a 52% ($P < 0.005$) reduction in activity in pig Gal-deficient LSECs lacking expression of Gal antigens.

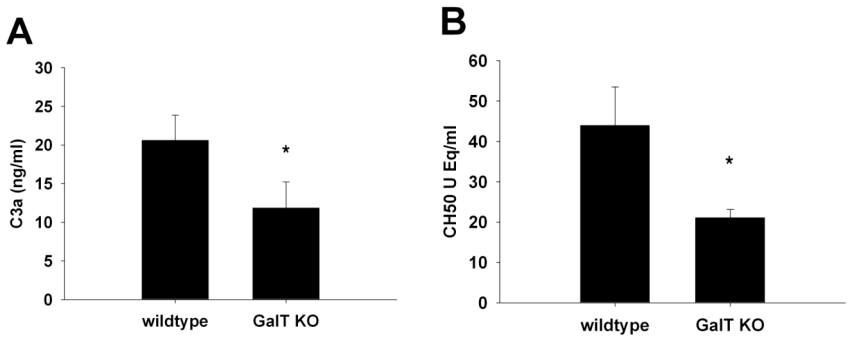

Figure 2. C3a and CH50 complement activity levels in human serum exposed to pig LSEC from wildtype and Gal-deficient animals. LSECs were cultured for 2 days before exposure to human serum. Samples were collected and analyzed for C3a levels and CH50 activity using ELISA. Data shown are mean ± standard deviation of six measurements. * $P < 0.05$, ** $P < 0.005$ (Student's t-test). LSEC, liver sinusoidal endothelial cell; Gal KO, α(1,3)galactosyltransferase knockout.

CAC

To determine whether pig LSECs are susceptible to CAC and to what degree this cytotoxicity is retained for LSECs when Gal expression is blocked, viability and LDH release were measured in pig LSECs following a one hour exposure to human or baboon serum. Many dead cells were observed when pig LSECs were exposed to normal human plasma (Fig. 3a). In contrast, almost no cells died after heat-inactivation of complement in the serum samples (Fig. 3b), demonstrating that pig LSECs are susceptible to CAC in human serum. Significant decrease in the cytotoxicity levels was measured for LSECs isolated from Gal-deficient pigs when compared to wildtype cells (Fig. 3c). The Gal-deficient LSECs demonstrated a 60% lower level of cytotoxicity following exposure to human serum ($P < 0.001$) and a 72% drop following exposure to baboon serum ($P < 0.001$). Levels of LDH release from Gal-deficient LSECs were reduced by 37% and 57% respectively (Fig. 3d; $P < 0.001$). These results show that Gal-deficient LSECs are considerably less susceptible to cytotoxicity in primate serum than LSECs from wildtype animals.

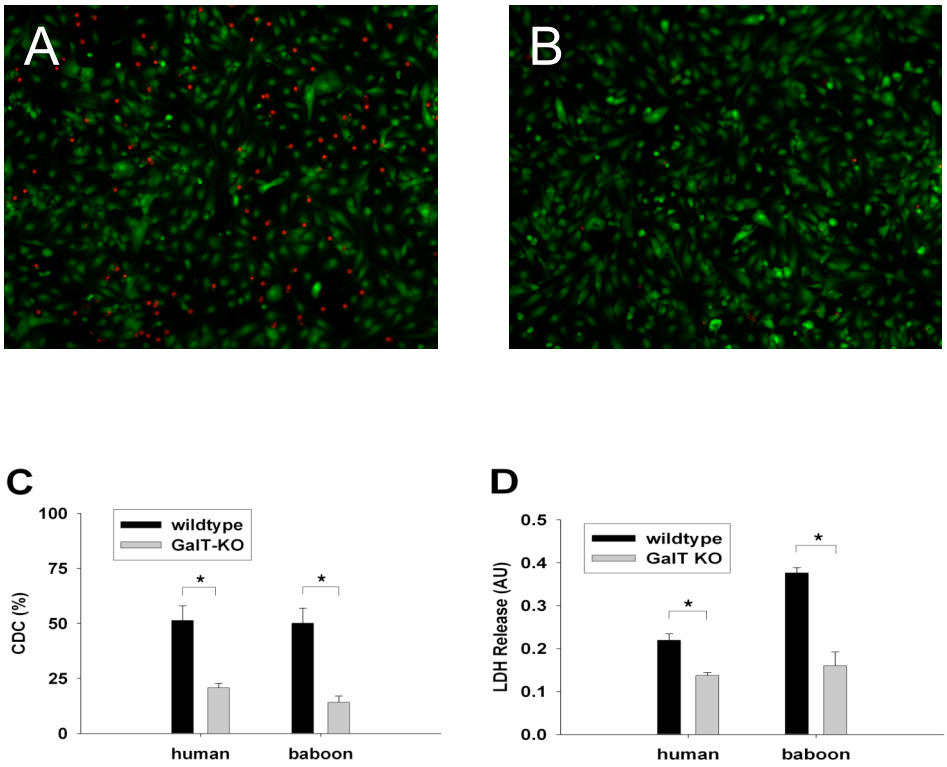

Figure 3. CAC in wildtype and Gal-deficient pig LSECs by exposure to human and baboon serum. Cultured LSECs were exposed to (A) normal and (B) heat-inactivated human serum. Live (green) and dead (red) cells were visualized using a fluorescent Live Dead Assay. (C) Quantification of CAC by image analysis. (D) LDH release. Data shown are mean ± standard deviation of 6 experiments, using 4 images per experiment for image analysis. CAC, complement-activated cytotoxicity; Gal KO, α(1,3)galactosyltransferase knockout; LSEC, liver sinusoidal endothelial cell.

Discussion

A major obstacle in the transplantation of porcine organs to humans is the presence of naturally occurring antibodies to xeno-antigens expressed on the endothelial surface of the blood vessels in the transplanted organ. With the availability of Gal-deficient pigs, much of the attention has been shifted to non-Gal epitopes. In pig-to-primate kidney transplantations using organs from Gal-deficient animals, a strong association between anti-non Gal antibodies and acute humoral xenograft rejection was observed (19). The blood of every adult naturally contains anti-non Gal antibodies, such as IgM and IgG antibodies against N-glucolylneuraminic acid (20), an antigen expressed on porcine endothelial cells but not in humans. The liver, however, is an immunoprivileged organ in the allogeneic transplantation setting. Therefore, we investigated whether the LSEC isolated from Gal deficient pigs maintain an immunoprivileged status when exposed to human or baboon serum.

We demonstrate that LSECs derived from Gal-deficient pigs are less susceptible to mechanisms of humoral xenograft rejection than wildtype LSECs, although significant levels of antibody binding remain even in the absence of the Gal epitope. Baumann and colleagues reported similar reductions in levels of IgM binding in Gal-deficient cells for aortic endothelial cells (11). However, they measured a much more robust degree of inhibition in preformed IgG binding and CAC than what we observed in Gal-deficient LSECs, suggesting that the immunoprivileged status of the liver in the allogeneic setting doesn't translate to pig-to-human liver transplantation.

There are different strategies to circumvent hyperacute xenograft rejection and acute humoral xenograft rejection, including the use of organs from pigs transgenic for complement-regulating factors such as CD55 (human decay-accelerating factor, hDAF), CD46 membrane cofactor protein or CD59. Kidneys and hearts from hDAF transgenic pigs transplanted into non-human primates have shown similar results in rejection prevention when compared to Gal-deficient organs (21-23). Another strategy is to knockout the gene responsible for important non-Gal epitope expression such as N-glucolylneuraminic acid (20), but this genotype has yet to be created in pigs. Our results suggest that elimination of Gal antigens may have to be combined with at least one of these strategies to render pig liver cells resilient to humoral cytotoxic effects of primate serum. Although pig-to-primate liver transplantation remains the ultimate test for organ rejection, our experiments provide valuable information that can be obtained using tissue available from animals that are sacrificed for other purposes. In addition, this in vitro model may be used to investigate the mechanisms behind the immunoprivileged status of the liver. Any other resident or non-liver cell type can be isolated and added to the cultures to examine interactions between liver cell types and their effects on the immunological status of the organ.

In conclusion, our data shows that LSECs from Gal-deficient pigs retain a considerable amount of susceptibility to CAC of primate serum. This suggests that the privileged status of the liver in the allogeneic setting does not translate to the pig-to-primate xenotransplantation arena and that additional

strategies may be needed to facilitate porcine liver graft acceptance in humans.

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Chapter 9

General discussion, conclusions & future perspectives

Mesenchymal stem cells are an exciting and promising cell type in the stem cell research arena, which has quickly developed into one of the fastest developing fields within medical science (*1*). The current body of literature describing MSC therapy as an experimental treatment modality in models of liver disease, myocardial infarction, stroke, kidney failure and lung fibrosis was reviewed in **chapter 2**. By combining the knowledge acquired in models of vital organ disease, we summarized how MSC therapy modulates cell death, fibrosis, inflammation, regeneration and angiogenesis through the action of a large array of cytokines, growth factors and other signaling molecules (*2-6*). In the following, experimental chapters of this thesis, we apply the current understanding of these mechanisms of MSC therapy in an effort to develop new applications of stem cell therapy in the future treatment of severe liver diseases.

As put forth in the introduction, this thesis used a set of *in vitro* techniques combined with intra- and extracorporeal treatment modalities in a rat model of fulminant hepatic failure in order to determine:

I. How mesenchymal stem cells (MSCs) inhibit experimental liver fibrosis.
II. The therapeutic potential of intra- and extracorporeal MSC-therapy in a model of fulminant hepatic failure.
III. The capability of MSC-therapy to therapeutically modulate liver regeneration and apoptotic cell death in fulminant hepatic failure.
IV. The efficiency of osmotic selection as a novel isolation method for MSCs from umbilical cord blood.
V. Whether fetal liver stem/progenitor cell-functions can be boosted by exposure to a mature liver environment.
VI. Whether the galactose-α(1,3)galactose knockout model is sufficient to eliminate xenoreactivity of pig liver microvasculature to primate serum.

The findings are summarized and discussed here.

Liver fibrosis

Stellate cells are the main mediators of the excess deposition of extracellular matrix that defines liver fibrosis, the precursor to liver cirrhosis (*7*). In response to hepatic injury, stellate cells undergo a phenotypic switch from a quiescent, retinoic acid storing cell type into a proliferative, myofibroblast-like cell: the activated stellate cell. It is characterized by excess deposition of

collagen in the subendothelial space of Disse, resulting in impaired metabolite transport, distortion of the hepatic architecture, and ultimately dwindling of liver cell functions and liver failure (*8*).
Recent studies demonstrated that MSCs can be of therapeutic benefit in the prevention of experimental fibrotic lesions in the lung, heart and liver, as we reviewed in **chapter 2** (*9-11*). Systemic delivery of MSCs in models experimental liver fibrosis significantly inhibits pathological changes in liver histology and clinical serum parameters, but the preventative mechanisms remain to be elucidated.
In **chapter 3**, we demonstrated that MSCs modulate the activity of activated stellate cells *in vitro* through paracrine action of several cytokines and growth factors secreted in a paracrine fashion by the MSCs. Suppression of proliferation and collagen synthesis was mediated by MSC-derived interleukin (IL)-10 and tumor necrosis factor (TNF)-α. IL-10 secretion, in particular, was found to be a dynamic response to IL-6 secreted by activated stellate cells. In addition, secretion of hepatocyte growth factor by MSCs led to the apoptotic death of activated SCs.
These results support the hypothesis that the therapeutic effect of MSCs in liver fibrosis are due to paracrine factors that modulate the proliferation, viability and function of resident stellate cells. This immunomodulation of stellate cells by MSC-secreted factors provides the first mechanistic evidence that MSCs can exert a protective role in the fibrotic liver through paracrine signaling. Paracrine anti-fibrotic effects of MSC therapy have been described in the literature, but specific mediators were not identified (*2*). Although progression of liver fibrosis may be inhibited using MSCs or their secreted products (*11*), whether MSC therapy can also reverse the architectural and structural changes that accompany cirrhosis is a question that requires further investigation.

Fulminant hepatic failure

Current bioartificial liver systems for the treatment of fulminant hepatic failure to allow for natural regeneration of the organ or to bridge to orthotopic liver transplantation are based on hepatocyte-based liver function support of the failing liver. However, a large randomized clinical trial testing the efficacy of bioartificial liver support in the treatment of liver failure has not been able to show a significant survival benefit for such treatment, primarily due to a lack of a functionally stable, human hepatocyte source (*12*).

The immune response is known to play an important role in the pathophysiology of experimental fulminant hepatic failure (*13*). Based on our results described in **chapter 3** and several studies reporting inhibition of key immune cell functions by MSC-secreted factors (*14-17*), we hypothesized that the paracrine MSC functions are therapeutic when used in the setting of fulminant hepatic failure.

Systemic delivery of MSCs did not provide a significant survival benefit for

rats undergoing D-galactosamine induced fulminant hepatic failure. Pulmonary lodging or immune rejection of the xenogeneic cells may have caused the observed lack of effect. In contrast, a significant survival benefit in the treatment of fulminant hepatic failure was observed after intravenous delivery of 1) MSC lysate, 2) MSC-conditioned medium, and 3) MSC-based extracorporeal bioreactor support, as described in **chapter 4**. The survival of MSC-conditioned medium treated animals was a function of the cell mass used for media conditioning and was found to have an optimum. Histological analysis showed a remarkable reduction in infiltrating leukocytes. In addition, we showed that MSC-conditioned medium actively promotes the emigration of adoptively transferred leukocytes from the injured liver. A proteomic screen of the MSC secretome revealed the presence of 21 chemotactic cytokines. Fractionation of MSC-CM based on affinity to heparin sulfate, a known ligand for all chemokines, revealed that the therapeutic activity of MSC-CM was restricted to the heparin bound fraction, further supporting the role of chemokines as responsible mediators. One of the chemokines present in the MSC-conditioned medium, stromal-cell derived factor-1 (SDF-1), represents a chemokine with a leukocyte-repelling effect known as fugetaxis (*18*). Although neutralization of this specific chemokine using neutralizing antibodies did not have an effect on survival (data not shown), such mechanisms may explain the observed effects on leukocyte migration.
Our proteomic analysis was based on the non-quantitative analysis of 174 non-specifically chosen human proteins. In addition, heparin sulfate not only binds chemokines, but also other positively charged molecules like hepatocyte growth factor (HGF), which has a known protective effect on endothelial cells in tissue injury (*19*). Therefore, it cannot be ruled out that compounds in the MSC-secretome besides chemokines were indeed responsible for observed therapeutic effects, such as growth factors and cytokines (*20*). It is known that at least several dozen different signaling molecules are produced and secreted by MSCs, but a thorough systematic analysis has yet to be performed (*21*). Preferably, fractionation studies combined with proteomic analysis, for example using mass spectrometry, would be employed to determine which exact molecules are responsible for the observed therapeutic effects.

Apoptosis and liver regeneration

Fulminant hepatic failure is typically associated with massive hepatocellular cell death with a compensatory regeneration of the organ that fails to meet the cellular losses (*22*). Therefore, effective treatment strategies aimed at protecting the dying liver cell mass and stimulating regeneration could offer major benefits in the treatment of FHF.

MSC therapy has been shown to effectively reduce parenchymal cell death through the action of secreted mediators in models of cardiac infarction, kidney failure and stroke (*3, 4*). Enhanced endogenous regeneration has been reported in models of acute kidney failure and ischemic stroke, where insulin-like growth factor-1 (IGF-1) has been identified as a key proliferative signal in

the MSC-secretion mix (*23, 24*). No reports exist on the effects of MSC therapy on liver regeneration and hepatocyte death in models of liver injury. We detected IGF-1 in the secretome of MSCs, however, suggesting that this factor was important in the observed effects on liver regeneration.
In Chapter 5, MSC-based therapy was shown to effectively reduce liver damage by inhibiting hepatocellular apoptosis and to enhance liver regeneration in a rat model of fulminant hepatic failure (**chapter 5**). Administration of the secreted molecules from MSC cultures was sufficient to obtain the therapeutic effects, showing that soluble mediators as opposed to cell-cell contact dependent mechanisms are responsible. In **chapter 4**, proteomic analysis revealed the presence of IGF-1 in MSC-secretions. Imberti reported a crucial role for this molecule as a mediator for renal tubular regeneration in a model of acute kidney failure (*3*), while Togel and colleagues demonstrated that this compound effectively inhibited apoptosis in a separate model of acute organ injury (*25*). Others have shown cytoprotective effects of vascular endothelial growth factor (VEGF) and stromal-cell derived factor-1 (SDF-1) in models of organ failure (*26, 27*). All these molecules were detected in our MSC-conditioned medium using our protein array (**chapter 4)**. Therefore, it is likely that these growth factors contributed to the observed therapeutic effects the MSC-conditioned medium preparations that we used. Mirotsou used a set of elegant experiments to prove that secreted frizzled protein-2 (SFRP-2), a signaling molecule with Wnt-inhibiting activity, plays a key role in myocardiocyte-protective effects of MSC-secretions in a model of myocardial infarction (*6*). This observation suggested that not only growth factors and cytokines, but also more exotic signaling molecules like SFRP-2 are important, adding another level of complexity to the effects of MSC therapy.
Systemic administration of MSC-secretions also resulted in a downregulation of the systemic inflammation that accompanies D-galactosamine liver injury. It is possible that this was an indirect effect of the lower levels of cell death in MSC-treated animals. Conversely, interleukin-1 receptor antagonist (IL-1RA), detected in our MSC-secretions, may have caused direct anti-inflammatory effects, an observation that was previously reported in an experimental lung fibrosis study using MSC-transplantations (*28*).

In **chapter 4**, we showed that MSC therapy reduces immune cell infiltration. In order to determine whether the observed changes in the rate of liver regeneration and hepatocyte apoptosis were direct effects of the administered therapy, or indirect effects via downregulated immune cell function, we dissected the effects using *in vitro* assays. In these experiments, we administered increasing concentrations of MSC-secretions to hepatocyte cultures in which apoptosis was induced by exposure to TNF-α. Similarly, we exploited the efficient hepatocyte regeneration assays developed in our lab to study the direct effects of MSC-derived growth factors and cytokines on liver regeneration. Using these models we proved that secretions from MSCs have a direct inhibitory effect on hepatocyte death and a stimulatory effect on their proliferation.

In our *in vitro* studies, we demonstrated that the therapeutic effects of MSC therapy were abolished when high dosages of MSC-secretions were used. These results confirmed the observations from **chapter 4** that the therapeutic effects of MSC-conditioned medium treatment is a function of the cell mass used for media conditioning and has an optimum, an observation that has not been reported by others but could be explained by the presence of low concentrations of "negative" molecules in MSC-conditioned medium. MSC secretions contain a complex mixture of cytokines, growth factors and chemokines, including small amounts of TGF-β, a compound with a marked inhibitory effect on hepatocyte-proliferation (*29*). It is possible that at higher concentrations of MSC-CM the negative effect of, for example, TGF-β overrules the beneficial effects exerted by trophic components of MSC-CM. Similarly, the cytotoxic effects of TNF-α, present at low concentrations in MSC-secretions and necessary at low levels to initiate hepatocyte duplication during the regenerative response of the liver, may become more pronounced at high concentrations of MSC-CM (*30*). Systematic proteomic analysis combined with fractionation studies of MSC-CM may not only result in the identification of the key therapeutic components, but also of the potentially harmful ones. These harmful components may then be neutralized or eliminated to further enhance therapeutic effects.

MSC-isolation from umbilical cord blood

MSCs are usually isolated from the bone marrow. More recently, investigators have begun to extract MSCs from adipose tissue (*31*). Given the invasive nature of these methods, the search for MSCs in ectopic sources has intensified. MSCs are present at small percentages in the bone marrow stroma, and it is likely that other sources will be more dilute in MSCs (*32*). Therefore, more stringent methods to isolate these rare cells are critical to their recovery from other biological samples. Currently used multistep processing techniques are, however, highly variable, rely on multiple determinants, and often fail to isolate homogeneous populations (*33*).
In **chapter 6,** we have described a single-step method to isolate MSCs based on their relative resistance to osmotic lysis, and applied this technique to the isolation of MSCs from UCB at far greater and purer yields than commonly employed methods. We also provided an initial framework to understand the molecular basis of osmolytic resistance that correlates to substantial ineffective cell volume and cytoskeletal integrity.
MSCs were shown to remarkably withstand significant osmotic lysis (> 30 min) with only a reversible impairment in cell proliferation and with no loss of stem cell potential after exposure. After isolation we observed a partial reduction in proliferation that was soon normalized after long-term culture, suggesting a reversible process of cell stress post-isolation. The significant loss of cells due to rosetting and density gradient centrifugation suggests that these methods would be ineffective in recovering MSCs that may be found in biological samples at very low numbers. Osmotic lysis showed a 50-fold enrichment of

fibroblast colony-forming units from umbilical cord blood samples when compared to these commonly employed techniques.
Investigations to determine the mechanisms responsible for osmolytic resistance revealed MSCs to have an ineffective volume of 59%, with the ability to double cell volume at infinite dilution. Disruption of filamentous actin polymerization by cytochalasin D sensitized MSCs to osmotic lysis, which suggests a cytoskeletal element involved in the osmolytic resistance.
Using the techniques described in this chapter, MSCs may be isolated from any patient at birth using non-invasive and cheap methods (*34*). After expansion in culture, these cells could be cryopreserved so that autologous MSCs are readily available for the treatment of fulminant hepatic failure or other conditions that may be treated using MSCs.

Boosting fetal liver stem/progenitor cell functions

Tissue engineering applications including bioartificial liver systems are at the core of emerging new strategies for the treatment of hepatocellular disease (*35*). However, inadequate numbers of primary human hepatocytes are available from donors and other cell sources. Fetal hepatic stem/progenitor cells may provide a source of highly proliferative liver cells, resistant to cryopreservation and ischemic injury. However, the expression of liver-specific functions of such cells is only a fraction of that expressed by mature hepatocytes (*36, 37*). Therefore, significant upregulation of these functions is needed in order to render fetal hepatic stem/progenitor cells a useful cell source for for example bioartificial liver-assist devices.

In **chapter** 7, we tested the hypothesis that hepatocytes isolated from adult animals provide an environment in which fetal hepatic stem/progenitor cells express higher levels of hepatocyte-specific functions over time. For this purpose, albumin secretion, urea synthesis and cytochrome P450 activity were assessed in cocultures of fetal and adult liver cells separated by Transwell membranes and were traced after separation from these cocultures.
Several investigators have reported that extracellular matrix from adult hepatocytes is capable of inducing markers of terminal differentiation in fetal hepatic stem/progenitor cells (*38-40*). However, metabolic functions were not investigated, and no data concerning the involvement of soluble mediators were reported. Our study showed that major hepatospecific functions of fetal stem/progenitor cells are upregulated by paracrine factors secreted in the microenvironment created by adult hepatocytes. Although the specific factors responsible for the observed effects remained to be elucidated, identification of these factors may allow for improved methods to optimize fetal liver stem/progenitor cell functions. Prolonged upregulation may be feasible by continuous exposure to such factors, which is necessary for their potential use in cell-based therapies for liver disease. However, ethical issues associated with the use of fetal tissues will remain, further complicating the implementation of this cell type in tissue engineering.

Gene-knockout technology for xenotransplantation

Xenotransplantation, using pig organs, could alleviate the shortage of available donor livers. However, transplantation of porcine organs into humans normally initiates a cascade of events that starts with binding of naturally occurring antibodies to xenogeneic epitopes on the endothelium, resulting in hyperacute xenograft rejection within minutes to hours of transplantation (*41, 42*). Because eighty percent of the constitutively present xenoreactive antibodies are directed against the galactose-α(1,3)galactose (Gal) epitope, elimination of the gene responsible for expression of this antigen could result in elimination of hyperacute rejection and bring the possibility of transplantation of porcine livers into humans one step closer (*43, 44*).

In **chapter 8**, we quantified humoral xenograft rejection in primate serum targeted against the microvasculature of the liver *in vitro*. Levels of preformed IgM binding to Gal-deficient liver microvascular cells were $1/5^{th}$ (human) and $1/7^{th}$ (baboon) of the levels measured in wildtype cells. Complement activity levels were reduced to approximately half of the levels measured in wildtype liver microvasculature. Complement-dependent cytotoxicity in Gal-deficient cells was reduced by 60% in human serum and by 72% in baboon serum when compared to wildtype LSECs. In short, we demonstrated that liver microvascular cells derived from Gal-deficient pigs are less susceptible to mechanisms of humoral xenograft rejection than wildtype LSECs, but that significant levels of xenoreactivity remain.

The use of organs from pigs transgenic for complement-regulating factors such as CD55, CD46, CD59 are among the alternative strategies to circumvent hyperacute rejection of porcine organs in primates (*45, 46*). Another strategy is to knockout the gene responsible for important non-Gal epitope expression such as N-glucolylneuraminic acid, but this genotype is not yet available (*47, 48*). Pig-to-primate liver transplantation remains the ultimate challenge for organ rejection. However, our experiments provide valuable information using tissues acquired from animals that had to be sacrificed for other experiments. Our results suggest that elimination of Gal antigens may have to be combined with at least one of the other known strategies for prevention of hyperacute rejection to render porcine liver microvasculature resilient to natural antibody-induced cell lysis. Even if xenotransplantation in a clinical setting becomes possible, moral issues will remain a major hurdle for the application of xenogeneic organs in clinical transplantation. It is likely that a significant number of people will never accept the idea of a porcine organ donor. Nevertheless, it is likely that objections that accompany animal-to-human transplantation will diminish once clinical successes are being reported. In other words, as the Economist recently phrased it: "The 'yuck'-factor that cross-species transplants would probably provoke would surely fade if lives were saved." (49)

Conclusions

The findings presented in this thesis warrant the following conclusions.

I. Mesenchymal stem cells (MSCs) inhibit fibrogenic functions of activated stellate cells *in vitro* through the action of secreted IL-10, TNF-α and HGF.
II. Extracorporeal MSC-based bioreactor treatment improves survival in a rat model of fulminant hepatic failure.
III. A single intravenous injection with the secretions from MSCs, rich in trophic growth factors and chemokines, is sufficient to improve survival, enhance liver regeneration, reduce cell death and inhibit inflammation in a rat model of fulminant hepatic failure.
IV. Osmotic selection is an efficient method for MSC isolation from umbilical cord blood, but results in a temporary inhibition of cell proliferation.
V. Liver-specific functions in fetal stem-progenitor liver cells are reversibly boosted by exposure to a mature hepatocyte environment.
VI. The galactose-α(1,3)galactose knockout model in the pig partially eliminates *in vitro* parameters of hyperacute liver microvasculature rejection in a pig-to-primate xenogeneic setting.

Of the investigated stem cell technologies, MSCs have the most direct promise in the treatment of liver diseases, and more specifically, of fulminant hepatic failure. Both treatment with a complex extracorporeal setup in which MSCs are incorporated into a bioreactor, and the more elegant, off-the-shelf treatment with an intravenous injection of homogenized MSCs, or concentrates of MSC-secretions, improves the survival of D-galactosamine induced fulminant hepatic failure. These therapeutic effects are the result of the action of an array of trophic factors secreted by MSCs that effectively modulate liver regeneration, inflammation and cell death. Although validation in different animals models and thorough molecular studies are imperative, these results may form the basis of a future treatment regimen for patients with fulminant hepatic failure that can currently only be cured by orthotopic liver transplantation.

Future Perspectives

The media, fed by speculative reports from scientists throughout the world, support the public notion that stem cell technology may one day provide an endless source of replacement-organs that have been grown in a laboratory environment. This discipline is commonly referred to as "regenerative medicine". Although embryonic stem cells may, theoretically, be used to create endless amounts of any cell type that is needed in the body, the creation of complex, fully functional tissues such as liver seems light-years away. Nonetheless, the implementation of several other stem cell technologies for the treatment of liver diseases may be closer to clinical reality. One of the most promising ones is the potent mesenchymal stem cell (MSC) therapy, which, as we show in this thesis, therapeutically modulates inflammation, regeneration and cell death in fulminant hepatic failure.

Based on the recent development of hepatocyte-based "bioartificial livers", MSC-based extracorporeal devices hooked up to the circulation of rats were used to treat fulminant hepatic failure. This form of MSC-treatment resulted in a dramatic increase in survival when compared to control animals (this thesis). Treatment with such devices, however, is costly and complicated, and is therefore unlikely to become a clinical reality. Infusion of molecules secreted by MSCs using a simple intravenous injection – a treatment modality which was also shown to be effective in this manuscript – on the other hand, would not require invasive procedures, has the potential to become off-the-shelf therapy and does not expose patients to allogeneic or cellular materials.

Injection of MSC-secretions therefore represents a treatment modality with a large potential in diseases such as fulminant hepatic failure, but possibly also in other forms of organ injury including myocardial infarction, stroke and acute kidney failure. MSC-secretions may be collected from large numbers of cultured cells, concentrated to therapeutic concentrations, and stored until therapy is required. Effects may be titrated by using different dosages of this "drug", much like the administration of conventional intravenous drugs.

In contrast to currently used drug therapies, however, this type of therapy would imply the simultaneous injection of an array of largely unknown compounds (e.g. growth factors, cytokines). Identification of the specific therapeutic compounds therefore seems imperative and may lead to the development of "cocktails" with optimized therapeutic efficacy in different diseases. MSCs may then only be used for the production of the raw materials or secretions from which this therapeutic cocktail is extracted. Patients with currently untreatable conditions such as fulminant hepatic failure, and maybe even liver cirrhosis, may be treated routinely with simple i.v. injections of this mixture of MSC-derived compounds. Such simple injections may ultimately replace the need for orthotopic liver transplantations and safe patients that would otherwise die waiting for a donor.

The scenario described above represents the optimistic view. The pessimistic picture is that MSC therapy, although effective in the D-galactosamine induced rat model of fulminant hepatic failure, is not effective in humans. Regarding

cirrhosis, we have only shown *in vitro* inhibitory effects on its precursor phenomenon, fibrosis. Hence, effective MSC-therapy for this clinically much more relevant disease is even more speculative.
Besides the described problems in boosting liver-specific functions of fetal/stem progenitor cells and the generally slow developments in the tissue engineering field, ethical issues will likely prevent implementation of fetal cell technologies for clinical use. Similar issues apply for embryonic stem cell research and genetic modification techniques for the development of knockout animals for potential xenogeneically transplantable organs, but alternative methods such as intracytoplasmic oocyte injection exist to circumvent these issues. Although several hurdles remain to be taken, clinical transplantation of for example porcine organs may become a possibility. Nevertheless, moral and religious issues are likely to interfere with the widespread implementation of transplantation of for example porcine organs into humans. Mesenchymal stem cell therapy is largely devoid of these issues and has many attractive therapeutic and practical characteristics that could contribute to the implementation of MSC therapy as a clinical treatment modality for fulminant hepatic failure, and potentially a whole array of serious diseases. Let's hope a next BBC-article will describe the successful treatment of liver failure patients with MSC-therapy.

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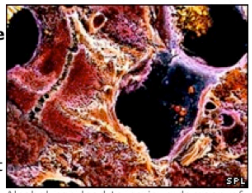
Appendix

Last Updated: Wednesday, 26 September 2007, 07:42 GMT 08:42 UK

Therapy to slow down liver damage

Scientists have developed a new way to treat liver failure by dampening the immune response using stem cells taken from the bone marrow.


Alcohol can lead to serious damage of liver tissue

So far the technique has only been tested in animals, but if it works in humans it could help save lives.

Potentially a patient could be kept alive longer until a donor organ is found - and the liver would be given the maximum chance to repair itself.

The Massachusetts General Hospital work features in the journal PLOS One.

The liver is one of the few major organs that is able to regenerate itself.

> **"This development could potentially reduce the number of donor organs used in urgent transplant procedures"**
>
> Professor Mark Thursz
> British Liver Trust

However, the organ cannot cope with the extensive damage inflicted by diseases like chronic hepatitis, or excessive long-term alcohol consumption.

At present, the only treatment for severe "end-stage" damage is a transplant - but donor organs are limited, and recipients must rely on powerful drugs to suppress their immune response.

Key cells

External liver assist devices have successfully supported some patients, but such machines require a supply of preferably human liver cells, which have been difficult to acquire.

The US researchers used mesenchymal stem cells (MSCs) - cells from the bone marrow that develop into tissues supporting blood cell development in the marrow cavity.

Previous research has shown that MSCs are able to inhibit several immune system activities, apparently by putting a brake on the movement of immune cells to areas of damage.

A supply of MSCs can be extracted from a patient's own marrow and expanded to levels that could be therapeutically useful.

The researchers tested several ways of using the cells to

treat rats with liver failure.

Simply transplanting MSCs into the animals' livers was not effective.

However, two methods of delivering molecules secreted by the cells lessened inflammation within the liver and halted cell death.

Cycling the blood of rats with liver failure through an external bioreactor containing MSCs also greatly reduced signs of liver failure in the animals, and boosted survival rates from 14% to 71%.

Researchers Biju Parekkadan and Daan van Poll said in theory a patient could be injected with a drug containing MSC-derived molecules to try to halt cell damage, and allow regeneration.

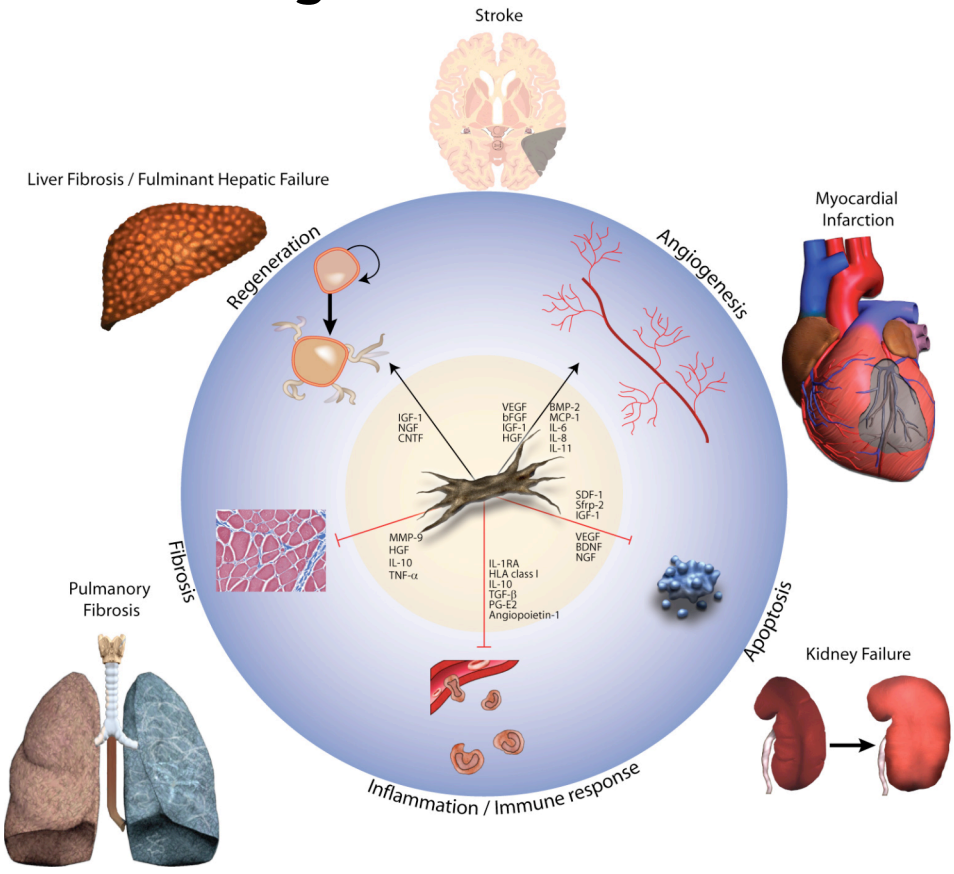
If that was not successful, or the damage was too extensive, then a device similar to the bioreactor could be considered to buy extra time before a transplant.

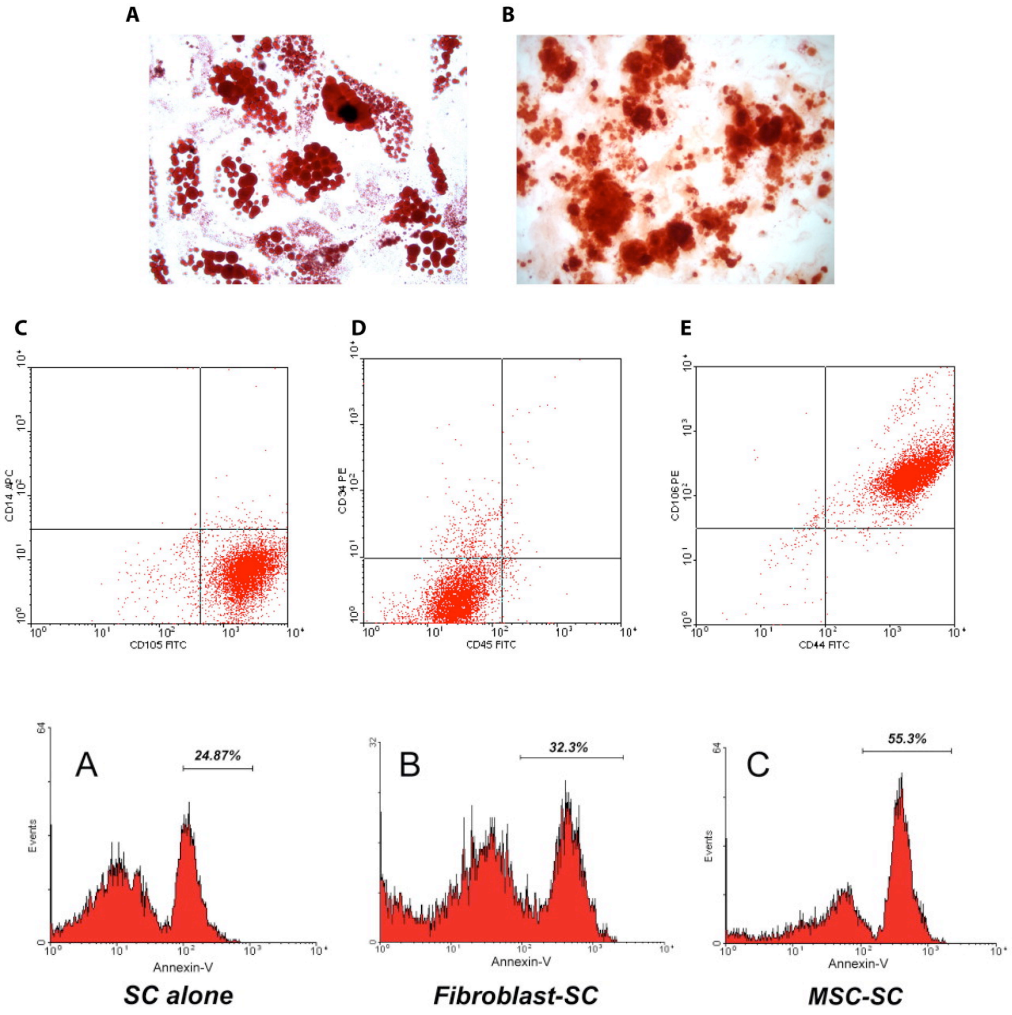
The British Liver Trust warned the research was still at an early stage.

But Professor Mark Thursz, of St Mary's Hospital, London and spokesperson for the trust, said: "A long standing goal in hepatology is the suppression of liver cell death until regeneration could occur.

"This development could potentially reduce the number of donor organs used in urgent transplant procedures thereby increasing the number available for the growing number of patients on routine waiting lists."

Color images

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C
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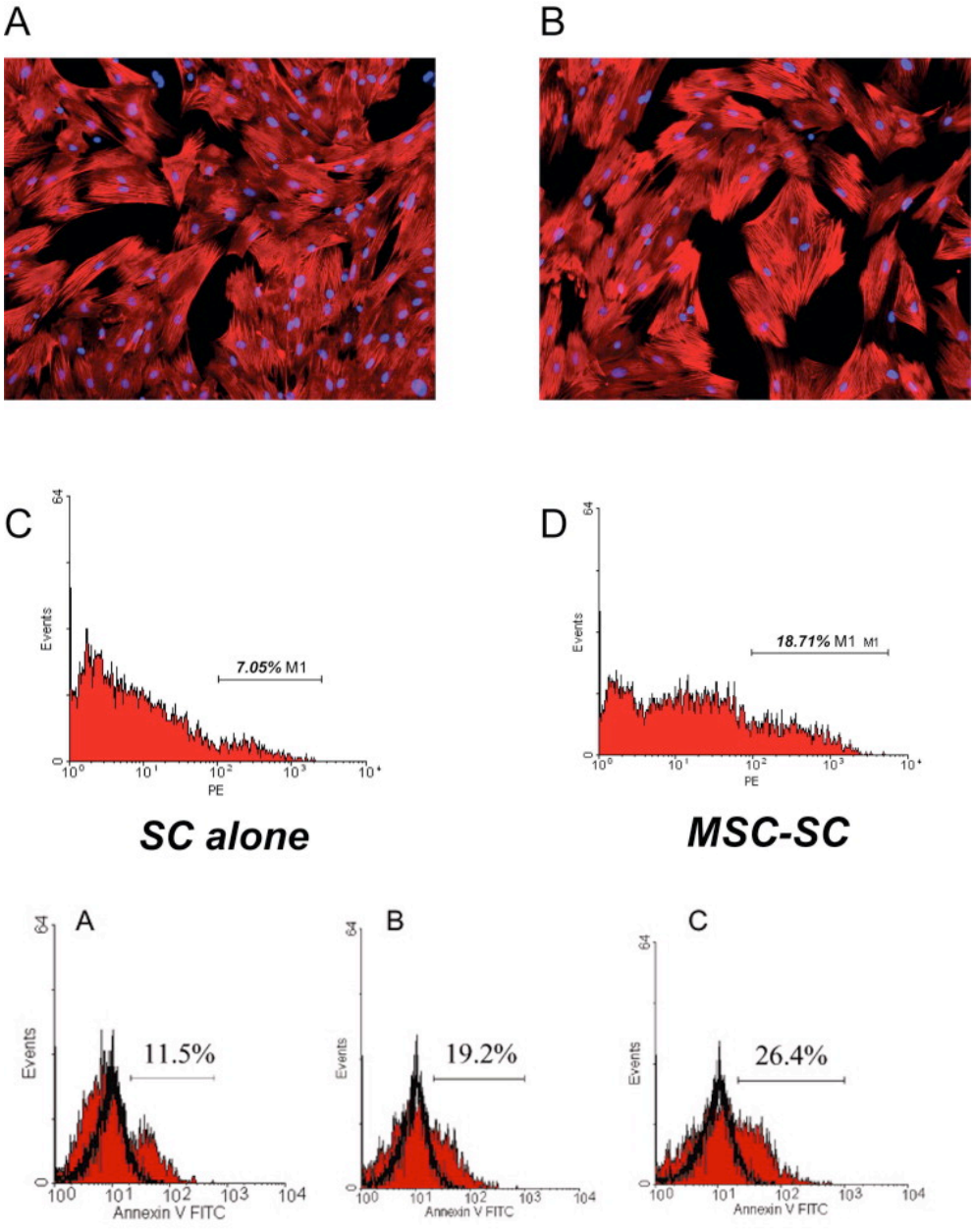
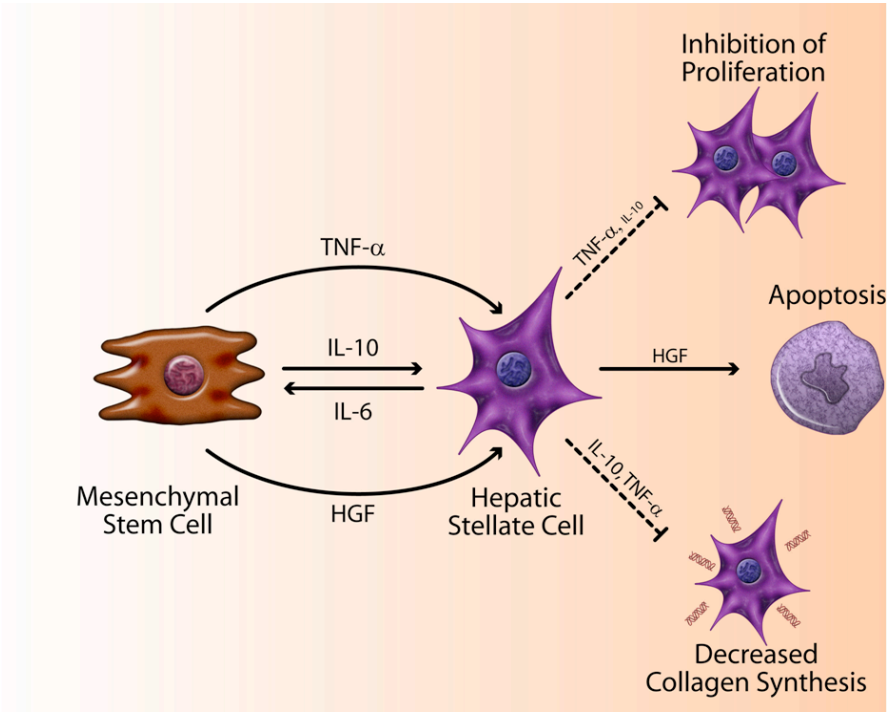
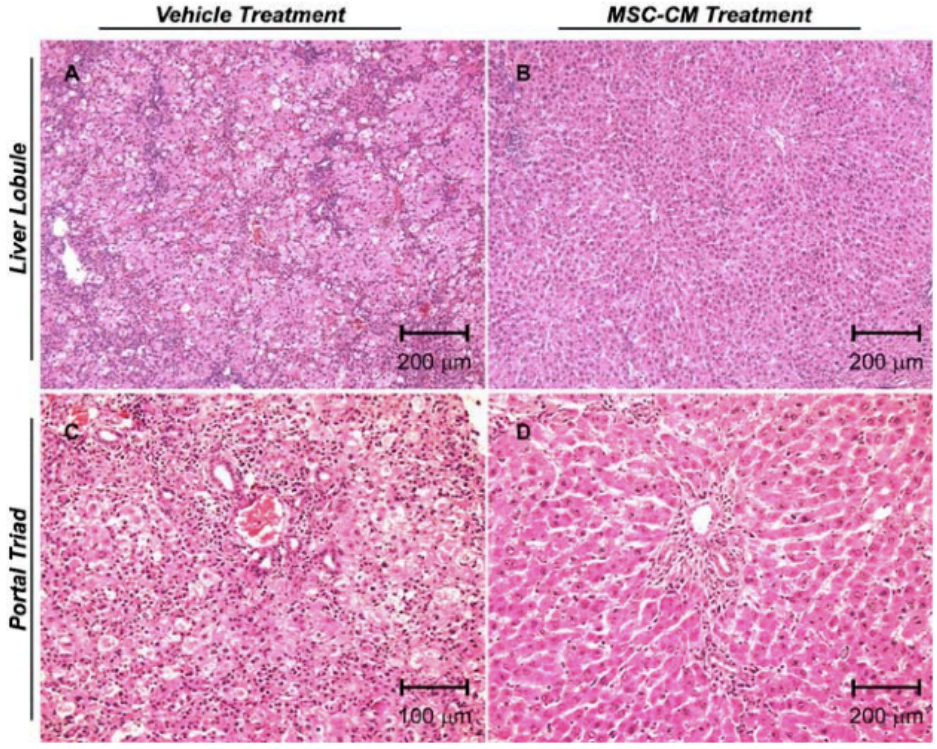
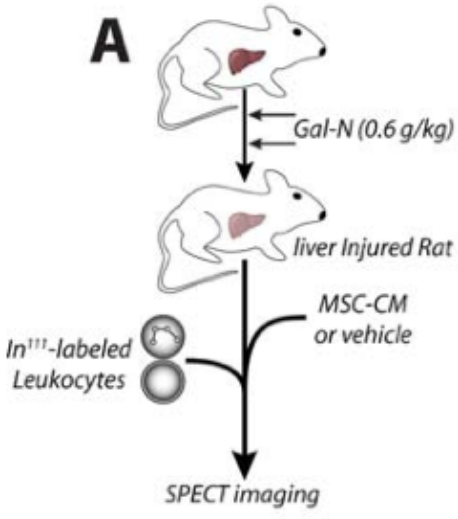
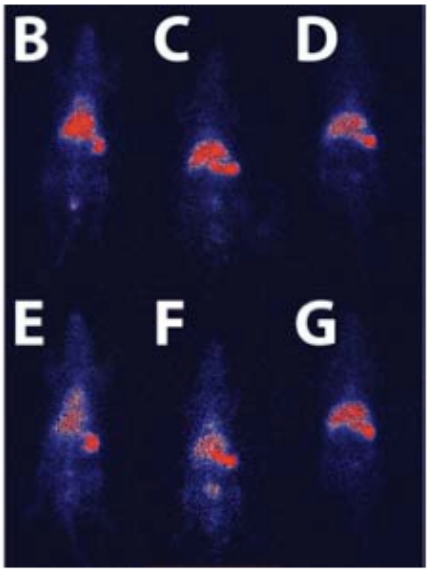
Events
Annexin-V
SC alone
Fibroblast-SC
MSC-SC

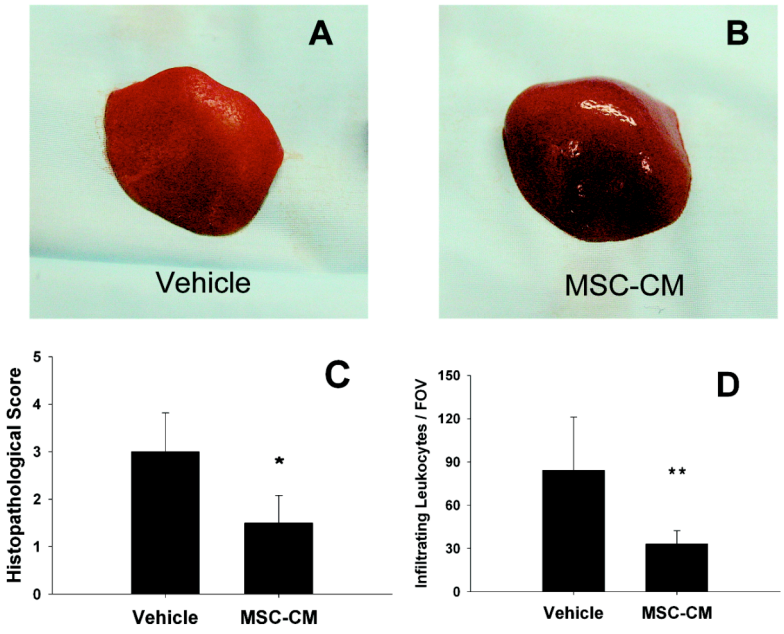

A
B
C
Events
7.05% M1
PE
SC alone
D
Events
18.71% M1
PE
MSC-SC
A
Events
11.5%
Annexin V FITC
B
Events
19.2%
Annexin V FITC
C
Events
26.4%
Annexin V FITC


Inhibition of
Proliferation
TNF-α
TNF-α, IL-10
Apoptosis
IL-10
IL-6
HGF
Mesenchymal
Stem Cell
HGF
Hepatic
Stellate Cell
IL-10, TNF-α
Decreased
Collagen Synthesis

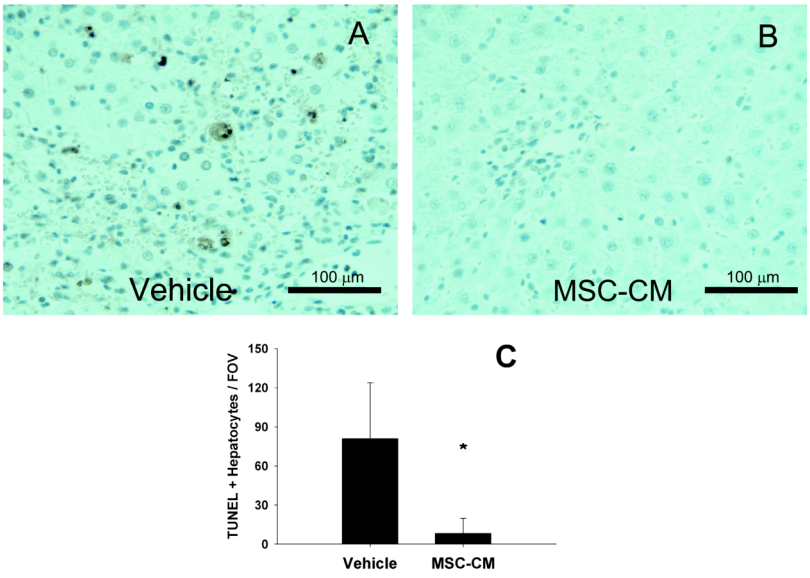

Vehicle Treatment
MSC-CM Treatment
Liver Lobule
Portal Triad
A
B
C
D
200 μm
200 μm
100 μm
200 μm

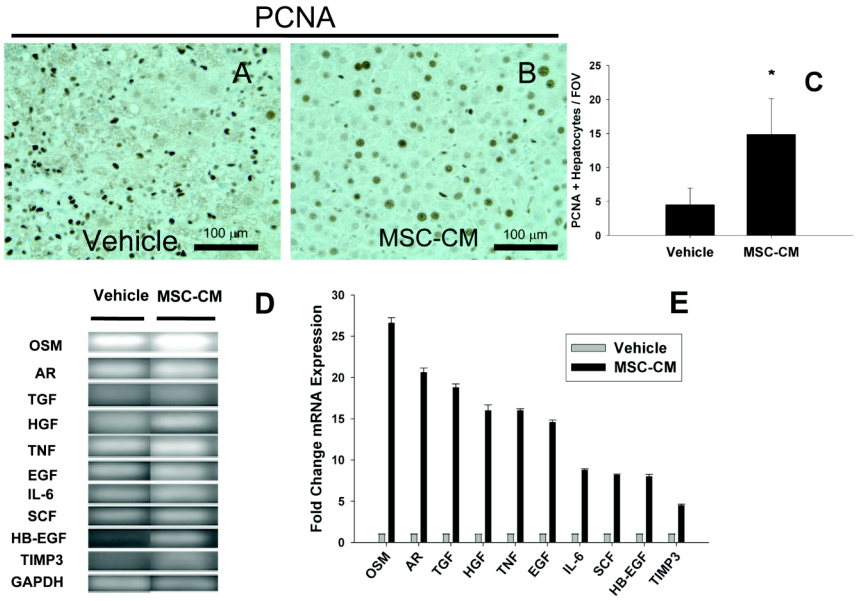

A
Gal-N (0.6 g/kg)
liver Injured Rat
MSC-CM
or vehicle
In111-labeled
Leukocytes
SPECT imaging

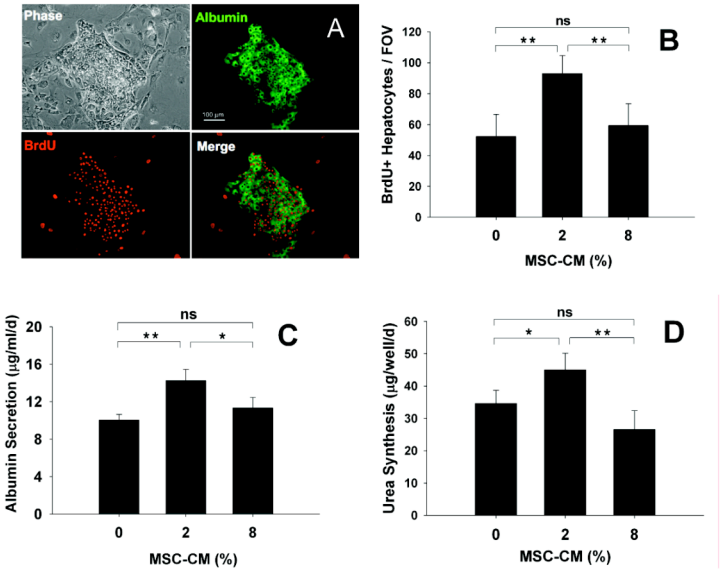

B
C
D
E
F
G

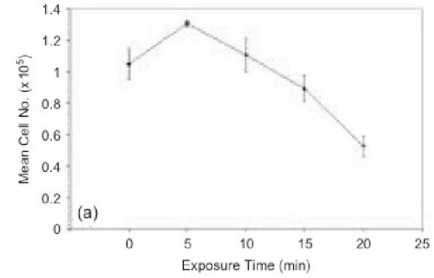

A
Vehicle
B
MSC-CM
C
Histopathological Score
5
4
3
2
1
0
*
Vehicle
MSC-CM
D
Infiltrating Leukocytes / FOV
150
120
90
60
30
0
**
Vehicle
MSC-CM

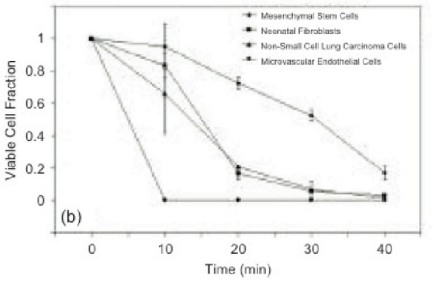
TUNEL

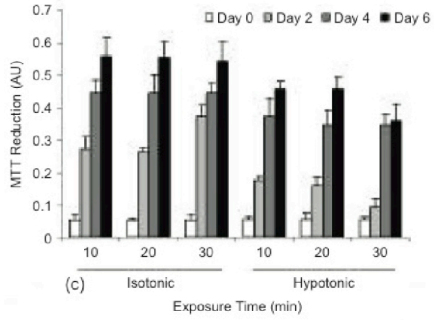

A
Vehicle
100 μm
B
MSC-CM
100 μm
C
TUNEL + Hepatocytes / FOV
150
120
90
60
30
0
*
Vehicle
MSC-CM

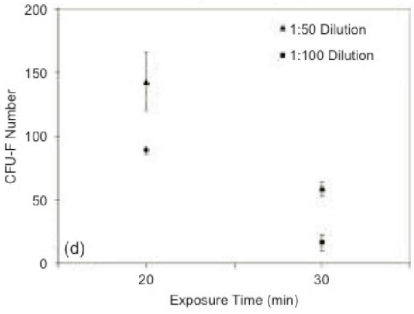

PCNA
A
Vehicle
100 μm
B
MSC-CM
100 μm
C
PCNA + Hepatocytes / FOV
25
20
15
10
5
0
*
Vehicle
MSC-CM
Vehicle
MSC-CM
D
OSM
AR
TGF
HGF
TNF
EGF
IL-6
SCF
HB-EGF
TIMP3
GAPDH
E
Fold Change mRNA Expression
30
25
20
15
10
5
0
Vehicle
MSC-CM
OSM
AR
TGF
HGF
TNF
EGF
IL-6
SCF
HB-EGF
TIMP3


Phase
Albumin
A
100 μm
BrdU
Merge
B
ns
**
**
BrdU+ Hepatocytes / FOV
120
100
80
60
40
20
0
0
2
8
MSC-CM (%)
C
ns
**
*
Albumin Secretion (μg/ml/d)
20
16
12
8
4
0
0
2
8
MSC-CM (%)
D
ns
*
**
Urea Synthesis (μg/well/d)
60
50
40
30
20
10
0
0
2
8
MSC-CM (%)

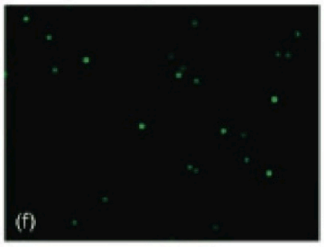

Mean Cell No. (x 10^5)
(a)
Exposure Time (min)

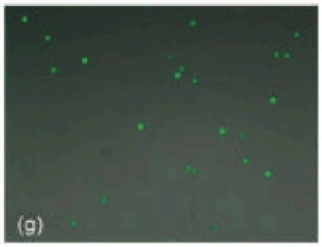

Mesenchymal Stem Cells
Neonatal Fibroblasts
Non-Small Cell Lung Carcinoma Cells
Microvascular Endothelial Cells
Viable Cell Fraction
(b)
Time (min)

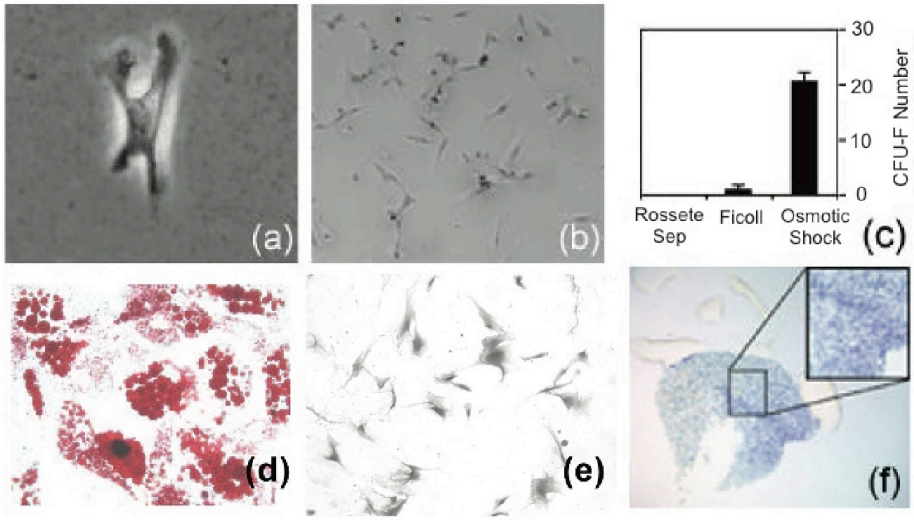

Day 0
Day 2
Day 4
Day 6
MTT Reduction (AU)
(c)
Isotonic
Hypotonic
Exposure Time (min)

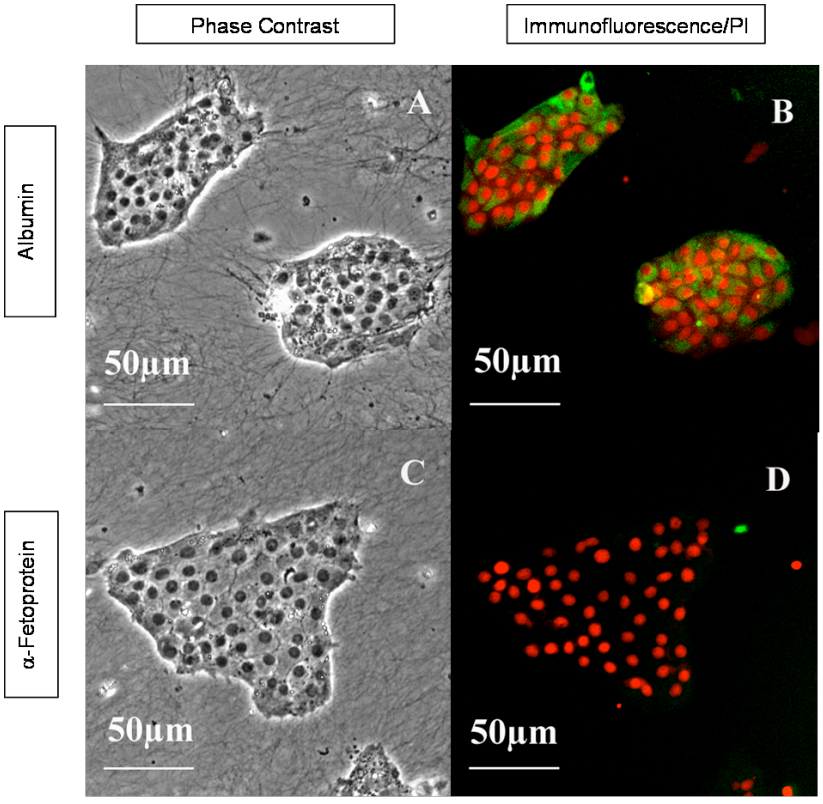

1:50 Dilution
1:100 Dilution
CFU-F Number
(d)
Exposure Time (min)

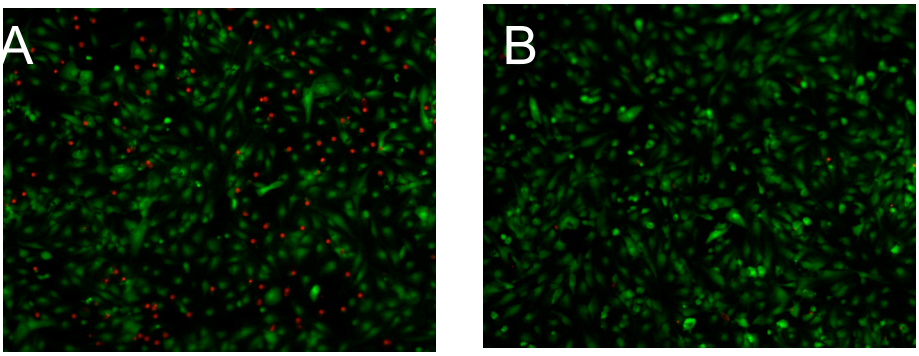

(e)


(f)


(g)


(a)
(b)
30
20
10
0
CFU-F Number
Rossete Sep
Ficoll
Osmotic Shock
(c)
(d)
(e)
(f)


Phase Contrast
Immunofluorescence/PI
Albumin
α-Fetoprotein
A
B
C
D
50µm
50µm
50µm
50µm


A
B

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http://news.bbc.co.uk/2/hi/health/7012371.stm

Curriculum Vitae

written by Roland & Alex, "para-nymphs"

Daan van Poll was born on June 14th 1978 in Haarlem, the Netherlands. Having a ponytail was not exactly an advantage in getting him through the election board of the Stedelijk Gymnasium in Haarlem. Nevertheless, he finished high school in 1995. At this stage he went to study Liberal Arts in the United States at Willamette University, Salem, Oregon for one year. During this year, his parents started to fear that all the good lessons they tried to teach him during childhood were lost. Daans' consecutive joining of an underdog-fraternity even filled his parents with despair. Later, seeing the reprehensibility of his behaviour, he joined ODOLEH, the eldest social fraternity in Amsterdam. To the dismay of his professors and fellow students, Daan spent much of his time as a member of several committees of his distinguished fraternity as a highly talented tennis player during the first years of medical school at the University of Amsterdam,.

Later in his studies, Daan participated in several surgical research projects at the Antoni van Leeuwenhoek Ziekenhuis (Amsterdam, the Netherlands), Royal Prince Alfred Hospital (Sydney, Australia, both under the supervision of prof. dr. B.B.R. Kroon) and at the Academic Medical Centre (Amsterdam, the Netherlands, led by prof. dr. D.J. Gouma). As a Fulbright fellow, Daan started a PhD project under the supervision of prof. dr. I.H.M. Borel Rinkes (University Utrecht), prof. dr. M.L. Yarmush (Harvard Medical School) and prof. dr. R.G. Tompkins (Harvard Medical School) at the department of surgical services / Center for Engineering in Medicine at the Massachusetts General Hospital and Shriners Hospital for Children in Boston in 2004. The PhD project focused on the application of stem cells for the experimental treatment of hepatic diseases and lasted until 2007. Currently, Daan is working as a resident at the surgical department of the Diakonessenhuis in Utrecht, the Netherlands, under supervision of Dr. G.J. Clevers.

Acknowledgements (Dankwoord)

Beste Inne,
Ons eerste gesprek: "Dus je bent jong, vrijgezel, en je wilt graag onderzoek doen in de VS? Stuur me je CV nog maar eens op dan." Sprankelend van de energie liep ik weer naar buiten, en dat is elke keer weer het geval wanneer ik bij jou langs geweest ben. Je bent een inspirator, een voorbeeld, een jaloers-makend fijne promotor, maar bovenal een ongelooflijk prettig mens. Het is een eer en een genoegen om jou als promotor te hebben, en ik hoop nog heel erg veel van je te mogen leren. Dank je wel voor je enthousiaste begeleiding, je nimmer aflatende steun en je vertrouwen.

Dear Ron,
I greatly appreciate your hospitality to receive young Dutch doctors to come and work in your lab, as well as your seemingly infinite enthusiasm and readiness to discuss research projects and share ideas in your office. I feel honoured that you are making it all the way to the tiny country of the Netherlands to attend my defense!

Don Maish,
During our first meeting I was utterly impressed with your preference for Adidas over Armani, but really, it sums it all up. Bling bling doesn't impress you (anymore) as you once spoke out. Your down-to-earth ("sexy-science my a..!"), informal, and father/capo-style approach to leading science and life in general have taught me many things. Thank you for having me in your lab and for offering an inexperienced, at least initially utterly useless doctor a shot at basic science in the Shrine.

Dear Koby,
We both started working in the CEM on September 2nd 2004, but your knowledge about basic science as a bioengineering PhD was light years ahead of mine. Your competence, clarity of mind, and straightforward approach make you the fantastic instructor that you are, and I hope you will employ these skills in a successful lab that you will undoubtedly one day have. It is a pleasure to be able to invite you to take part in my PhD-committee. Enjoy your time in Holland with Michal!

Professoren van Gulik, Metselaar, Verbout, Rothuizen, van Diest en dr. van Hillegersberg,
Het is voor mij een eer dat u de tijd heeft genomen om mijn eerste serieuze poging tot wetenschap met een kritisch oog te bekijken. Hartelijk dank voor het lezen en beoordelen van mijn proefschrift en voor het plaatsnemen in de promotiecommissie.

Gino,
Fijne vent, natuurlijk noem ik je. Thanks for introducing me to IBR!

Biju,
Our friendship and teamwork in the lab have been a rollercoaster ride as life itself can be, but I am happy that we were a team in the lab. You are an unbelievably talented and intelligent investigator with a great spirit. I wish you luck on all your journeys.

Arno, François, Monica & Carlo, Jack, Maria-Louisa, Lynn, Ilana, Chris, Cenk, Hiro, Cheul, Mehmet, Martin, Reza, Mitra, Olivier, Zak, Bob, Linda, Ed, Sam, Owen and Sandra,
All of you at the Shrine and MGH that made my life in the lab possible and enjoyable: it was an unforgettable time, thank you for the support and company.

Herman,
Het was waanzinnig om jou als fellow-Dutchman en "fellow Borel-kid" in het laboratorium te hebben: Wholefoods, Laphroaig, op je dakterras te vertoeven, slechte grappen, véél slechte grappen, je soms bizarre maar altijd vermakelijke teksten, en iemand om het promotietraject in haar volle glorie mee te kunnen delen. De gedrevenheid en het doorzettingsvermogen waarmee je een bijzonder uitdagend en uiteindelijk succesvol promotie-traject van de grond getild hebt en waarmee je straks ongetwijfeld ook je opleiding zal gaan doen in het Zwitserse is bewonderenswaardig. Veel succes, en stay in touch!

Sebas,
Super-souertje, heerlijke gozer die je bent, het was waanzinnig om je in Boston te hebben. Squashen, een biertje of een hap om mooie plannen, vrouwen, of juist sores door te spreken, het was altijd relaxed. Keep rollin', en alle geluk met la Mary!

Appletonstreet-bewoners van het eerste uur: Job, Lucas, Pantsman, Sti-jn, en even later Ferdinand, Joppe, en Inge,
Ongelooflijk mooi was het op Appleton 88. Met het housewarming/toga-feest (wat een bizar succes) dat één dag na mijn aankomst gegeven werd was de toon gezet en is het feest eigenlijk nooit meer opgehouden. Een jaar lang met stuk-voor-stuk te gekke ski-trips, uitstapjes naar New York, diners, Red Sox games, avonden uit en BBQ's volgde, het kón niet op! Ik zou bijna vergeten dat ik dat jaar ook nog hard gewerkt heb, jullie zijn top.

Appleton-Street "the international version": Lukie, miss Myers, la Delphine, mme. Marie, Irina, Grace, il-Mario, Robbert alias de Strijbe..er aka Deef, iron-Mike, Bobby, Lauren, BJ, Kim and Dr. Evil,
In one word: awesome. Two more then: like, totally. Guys, Appleton with you guys was unbelievable. Needless to say more, you were all there to

experience it. Rip Amsterdam apart as soon as you get a chance, take care of the cat (it's embarrassing, but I actually miss that utterly retarded animal), don't kill Rhonda, and keep that house orange and alive!

Most-appreciated guests at Appleton: Marcelo, Jeroen, Nicolas, Marijn, Bram, Yeast, Matthijs, Christiane, Eliza, Rutger&Jarom, Ellen, Henrike, Jaap, Jmi, Juliette, Aleid, Jurriaan, Marjolein, Freddie, Peer, Reinier, Renée, Jill, Selda, Leif-Ann (LA, aka plezier voor 2), Gediz (x6?), Durk, Casper, Dam, Stephan, Roos, Anandi, Svend, Madeleine, Marjolein, Willemijn, Angela and Yannis,
Thanks for coming over and taking your share in making Appleton 88 an awesome place to live!

Tim,
Ger, mattie, wat ben jij toch lekker bezig de laatste tijd, en terecht! Hou dit vast. Het is alleen wel onacceptabel (en verdacht) dat je je vriendin zo verbergt... Gaaf dat je 2 keer langs kon komen.

Joost,
Gio, dat weekje dat jij er was was, pffff, één groot gekkenhuis, plankgas all the way, zoals ik je ken! Blijf gas geven en voor spektakel blijft zorgen, dat zijn we van je gewend. Pik, ik hou van je.

Tom,
Jij was één van die gasten die steevast één keer in de zoveel tijd even belde om kontverhalen te delen en 's te kijken hoe de sfeer in de States was, dat waardeer ik enorm. Het is mooi je weer in de buurt te hebben, laten we er een mooie zomer van maken op Kortenhoef. Met geheven vuist!

Bossie, Jesse, Ron, Woude, Tijl en Vlietje,
Ik heb jullie gemist, het is mooi om weer terug te zijn. Bedankt voor de support.

Lex, paranimf,
Het zijn geen makkelijke tijden, maar ik vind het geweldig dat je straks als paranimf op m'n promotie staat. Hang in there, mooie tijden zijn weer op komst.

Roland, paranimf,
Het was mooi om met je te cruisen en te rellen op die bruiloft en in NY. Mooi dat je m'n promotie op kan luisteren met je aanwezigheid, wat een heerlijke gozer ben je toch.

Dear Hannah,
Times with you were wonderful. Be good to yourself girl.

van Poll-en, Dumoleijns, van Liers en Joustraatjes,
Bonma, Sanne, Quérine, Logaine, Valerie, Marguerite, Willem, Maarten, tante Odette, oom Max, tante Margriet, oom Erik, tante Gabriel en iedereen meer,
Familie is voor altijd, dit boekje mag gewoon in de prullenbak.

Bart,
Broer, en nog veel meer, bedankt voor je support. Gaaf dat jij en Sanne jullie trip onderbreken om bij m'n verdediging te kunnen zijn, alle succes met jullie heftige plannen!

Pa, ma,
Jullie onvoorwaardelijke steun was van onschatbare waarde. Ik hou van jullie.