Mesenchymal Stem Cell–Derived Molecules Directly Modulate Hepatocellular Death and Regeneration

In Vitro and In Vivo

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Orthotopic liver transplantation is the only proven effective treatment for fulminant hepatic failure (FHF), but its use is limited because of organ donor shortage, associated 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 endogenous repair pathways 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 a D-galactosamine–induced rat model of acute liver injury, we show that systemic infusion of MSC-conditioned medium (MSC-CM) provides a significant survival benefit and prevents the release of liver injury biomarkers. Furthermore, MSC-CM therapy resulted in a 90% reduction of apoptotic hepatocellular death and a three-fold increment in the number of proliferating hepatocytes. This was accompanied by a dramatic increase in the expression levels of 10 genes known to be up-regulated during hepatic replication. Direct antiapoptotic and promitotic effects of MSC-CM on hepatocytes were demonstrated using in vitro assays. 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.

Fulminant hepatic failure (FHF) affects approximately 2500 people in the United States 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 organ donor shortage, financial considerations, and the requirement for lifelong immunosuppression.2 Strategies aimed at restoring liver functions using acellular and hepatocyte-based technologies have shown promising results in animal models and human testing of small patient cohorts, but efficacy has yet to be demonstrated 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 by secreting a number of trophic molecules, including soluble extracellular matrix glycoproteins, cytokines, and
growth factors. 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. We recently demonstrated that systemic administration of MSC-derived molecules, either by a bolus of conditioned medium (MSC-CM) or by extracorporeal support using a bioreactor, significantly improved short-term survival in a D-galactosamine–induced rat model of FHF. 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 current study, we investigated whether systemic infusion of MSC-CM leads to a hepatoprotective response in the acutely injured liver, specifically by inhibiting cell death and stimulating reparative programs. In a sublethal regimen of D-galactosamine induction, we demonstrate a significant survival benefit and a prevention of liver enzyme release after MSC-CM treatment. MSC-CM therapy led to a 90% reduction in apoptotic hepatocytes and a threefold increase in the number of proliferating hepatocytes in vivo. In addition, we detected increased gene expression, ranging from 4-fold to 27-fold, of 10 genes known to be up-regulated during hepatocyte replication. Using in vitro assays, we demonstrate that secretions from MSCs have a direct inhibitory effect on hepatocyte death and a stimulatory effect on their proliferation.

Materials and Methods

Unless otherwise stated, materials were purchased from Sigma-Aldrich (St. Louis, MO).

Animals. Male Sprague-Dawley rats weighing 250 to 300 g were used for the FHF experiments. Hepatocytes were isolated from 150-g to 200-g female Lewis rats. 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 provided by the Tulane Center for Gene Therapy. MSCs were cultured and characterized for surface marker expression and adipogenic and osteogenic differentiation capacity as described previously. For the generation of MSC-CM, cells were allowed to grow to 70% to 80% confluence (approximately $1 \times 10^6$ 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 units (Millipore, Bedford, MA) with a 3-kDa cutoff.

FHF Induction and Treatment. FHF was induced by two injections of D-galactosamine, delivered intraperitoneally with a 12-hour interval between injections. 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 so as to ensure that a subgroup of vehicle-treated animals would survive long enough to be analyzed for comparison. After 24 hours, 0.9 mL MSC-CM or 0.9% NaCl solution (vehicle control) was infused into the penile vein. We used four animals per group for tissue collection after sacrifice at 36 hours after treatment and 10 animals 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.

Quantification of Aminotransferase Release. Serum samples were stored at −80°C until analysis. Measurement of alanine and aspartate aminotransferase (ALAT and ASAT, respectively) levels were carried out using Thermo Electron Infinity ALAT and ASAT Reagent (Louisville, CO) according to the manufacturer’s directions.

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

Liver Histology. Formalin-fixed, paraffin-embedded liver samples were sectioned at 4-μm thickness and stained with hematoxylin-eosin (HE). 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-micrometer-thick sections of formalin-fixed tissue were deparaffinized, rehydrated, and blocked in 3% hydrogen peroxide in ethanol for 15 minutes after baking at 60°C for 1 hour. For CD45 and proliferating cell nuclear antigen (PCNA) immunohistochemistry, sections were treated in 10 mM citrate buffer at pH 6.0 using a digital pressure cooker, blocked with 1.5% horse serum for 15 minutes, and incubated with mouse anti-CD45 (BD, San Jose) or mouse monoclonal anti-PCNA (Clone 24, BD, San Jose) at a 1:200 or 1:500 dilution for 1 hour at room temperature, respectively. Sections for CD45 analysis were washed and incubated with a goat anti-mouse immunoglobulin G secondary antibody for 25 minutes at room temperature. Finally, the sections were washed and mounted with me-
dium containing 4′,6-diaminobenzidine (Vector Laboratories, Burlington, CA). With respect to PCNA analysis, primary antibody was detected using Vectastain Elite ABC kit (Vector Laboratories, Burlington, CA). For terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) staining, 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 counterstained with hematoxylin.

**Digital Cell Quantification.** Quantification of cell numbers in stained liver sections was performed in 10 random 40× images per animal using the public software ImageJ (http://rsb.info.nih.gov/ij/). Positive cells were quantified using appropriate criteria for a specific threshold of staining intensity as well as corresponding sizes of the nuclei. Nuclei of area greater than 700 pixel² were analyzed to specifically identify hepatocytes from resident nonparenchymal and infiltrating inflammatory cells. Infiltrating inflammatory cells were counted by similar thresholding of HE sections and analysis of nuclei with area less than 700 pixel² and were qualitatively verified as CD45+ cells by immunofluorescence.

**Gene Expression.** RNA was extracted from liver tissue homogenates using the Nucleospin RNA purification kit (BD, Palo Alto, CA) per the manufacturer’s instructions. Approximately 100 ng to 1 µg total messenger RNA was reverse transcribed to complementary DNA using the TwoStep reverse transcription polymerase chain reaction (RT-PCR) Kit (Qiagen, Valencia, CA) and amplified in a Perkin Etus Thermal Cycler 480. Cycling conditions were as follows: (1) 25°C for 10 minutes; (2) 42°C for 60 minutes. Complementary DNA was analyzed by either endpoint or kinetic RT-PCR using the same cycling conditions for amplification: (1) 94°C for 10 minutes; (2) 30 to 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 were analyzed using the 2-ΔΔCt method and displayed as relative fold changes normalized to vehicle treatment.

**Hepatocyte Isolation and Culture Media.** Hepatocytes were isolated using a two-step collagenase perfusion procedure as previously described. 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% fetal bovine serum 14 ng/mL glucagon, 0.5 U/mL insulin, 20 ng/mL epidermal growth factor, 7.5 µg/mL insulin, 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 1 × 10⁵ cells/cm² in a collagen gel sandwich configuration in hepatocyte medium as previously described. Actinomycin D was added at a concentration of 0.2 µg/mL for 1 hour, followed by tumor necrosis factor alpha (TNF-α) (30 ng/mL) with or without MSC-CM at 2% or 8% for 8 hours. 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 nonviable cells were quantified in four random images per well using the digital image analysis methods described previously. The viable fraction was defined as the number of viable cells divided by the sum of the viable and nonviable cells.

**Hepatocyte Proliferation In Vitro: Culture System.** Hepatocytes were seeded at low density (1.25 × 10⁴ cells/cm²) on a feeder layer of 3T3-J2 fibroblasts (8 × 10⁴ cells/cm²) 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). After 48 hours, cultures were fixed in 70% ethanol for 45 minutes and treated with 4N HCl and 0.2% TritonX-100. After incubation in blocking buffer for 30 minutes, cells were incubated for 60 minutes with anti-BrdU-Alex594 (Invitrogen, Carlsbad, CA) and rabbit anti-rat albumin at 37°C, followed by fluorescein isothiocyanate–conjugated anti-rabbit immunoglobulin G (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 enzyme-linked immunosorbent assay 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 t test and, specifically, a log-rank test for survival analysis. A P value of <0.05 was used for statistical significance. Statistical image analysis was per-
formed after determining that the data could be fit with a normal distribution. A two-tailed Student $t$ 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 Enzyme Release 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 estimate of ongoing liver damage. Two of four vehicle-treated animals died between 12 and 36 hours after 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, B). No significant differences were observed at any of the other timepoints. In the rats treated for survival analysis using a 0.6 g/kg D-galactosamine induction regimen, a significant survival benefit was observed for the MSC-CM–treated animals (Fig. 1C). Only one animal died during the observation period in the MSC-CM group, versus 50% of the control rats ($P = 0.046$). Supplementary Fig. 1 describes a significant survival benefit for MSC-CM–treated animals when compared with either conditioned medium derived from normal human dermal fibroblast cultures ($P = 0.031$) or vehicle treatment ($P = 0.040$). These results suggest that the therapeutic effect is not specific to secretions of human proteins from any mesenchymal 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.

**MSC-CM Treatment Down-Regulates 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 nonsignificant decrease for IL-1$\beta$ ($P = 0.054$) but significantly lower levels of TNF-$\alpha$ (64%; $P = 0.0002$) and IL-6 (54%; $P = 0.0002$) after MSC-CM treatment. These pro-inflammatory cytokines are known to be up-regulated 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 fourfold in MSC-CM–treated animals ($P = 0.032$). Taken together, these data demonstrate that the infusion of molecules secreted by MSCs alters the systemic cytokine profile associated with FHF to a more anti-inflammatory state.

**MSC-CM Therapy Improves Gross and Microscopic Liver Histopathology and Reduces Leukocyte Infiltration.** D-galactosamine–induced FHF is accompanied by characteristic changes in the 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 pathology of the liver 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 HE-stained liver sections revealed profound hepatocellular death with cytoplasmic vacuolization, panlobular mononuclear CD45-positive leukocyte infiltration (Fig. 3E), and severe distortion of tissue architecture in vehicle-treated animals as previously described.12 In contrast, liv-
ers of MSC-CM–treated animals demonstrated minor periportal immune cell infiltration (Fig. 3F) with edema and fibrin deposition, characteristic of tissue repair (Fig. 3C, D).

Semi-quantitative histological examination of liver tissue confirmed significant differences between the groups (Fig. 3G). The average score in the MSC-CM group was 1.5/10 ± 0.6 and 3.0/10 ± 0.8 for vehicle-treated animals (P = 0.024). A 58% decrease in the number of infiltrating CD45-positive immune cells was observed after MSC-CM infusion (33/10 ± 9.3 compared with 84/10 ± 37 in controls; P = 0.004; Fig. 3H). 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.

**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 with control animals (81 ± 52/field of view; P = 0.009; Fig. 4C), confirming that MSC-CM therapy effectively reduces hepatocellular death in this model of acute liver injury.

**MSC-CM Inhibits Hepatocellular 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 alteration of the immune response to the damaged organ. 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 suggest that at low concentrations, MSC-CM has a direct antiapoptotic effect on hepatocytes.

**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, proliferating chain nuclear antigen (PCNA)-positive hepatocytes were quantified and compared with vehicle-treated animals. Qualitatively, few PCNA-positive hepatocytes were seen in control livers (Fig. 6A). Many were observed in MSC-CM–treated animals (Fig. 6B). We measured a threefold increase in the number of proliferating liver cells (15 ± 5.3 in the MSC-CM treated group compared with 4.5 ± 2.5/field of view in controls; P = 0.04; Fig. 6C). Evaluation of expression levels of 10 genes known to be up-regulated during liver regeneration

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![Graphs showing cytokine levels](image-url)

**Fig. 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 enzyme-linked immunosorbent assay. 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-alpha; IL-1ra, IL-1 receptor antagonist.
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.

**MSC-CM Stimulates Hepatocyte Proliferation In Vitro.** Hepatocyte replication, a major component of liver regeneration, is regulated by a complex interaction of paracrine and endocrine signals involving nonparenchymal cell signaling. MSC-CM treatment improves gross and microscopic 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 HE staining. Gross appearance of representative liver lobes after (A) control or (B) MSC-CM treatment. Microscopic high-power field of liver tissue after (C) vehicle or (D) MSC-CM infusion. Bar = 100 μm. Verification of CD45+ infiltrates by immunofluorescence staining of (E) vehicle or (F) MSC-CM treatment. Bar = 200 μm. (G) Scores determined by semi-quantitative histological examination. (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; HE, hemotoxylin-eosin.

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**Fig. 3.** MSC-CM treatment improves gross and microscopic 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 HE staining. Gross appearance of representative liver lobes after (A) control or (B) MSC-CM treatment. Microscopic high-power field of liver tissue after (C) vehicle or (D) MSC-CM infusion. Bar = 100 μm. Verification of CD45+ infiltrates by immunofluorescence staining of (E) vehicle or (F) MSC-CM treatment. Bar = 200 μm. (G) Scores determined by semi-quantitative histological examination. (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; HE, hemotoxylin-eosin.

**Fig. 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 40× 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; FHF, fulminant hepatic failure; TUNEL, terminal deoxynucleotidyl transferase-mediated nick-end labeling; FOV, field of view.
min levels were 29 increased in 2% MSC-CM–supplemented cultures. Albumin secreted and urea synthesized per well was insignificant increase was measured (59/1406, 0.019), but was not significantly altered in the presence of 8% MSC-CM (53.1 µg/mL/day; P = 0.063). Higher levels were observed for 2% MSC-CM supplementation when compared with 8% MSC-CM for all three assays, indicating a therapeutic window of effectiveness.

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. 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 that are known to enhance levels of hepatocellular death in liver injury, resulted in a further reduction of hepatocellular death in vivo. In a previous study, we showed that MSC-CM altered leucocyte trafficking in the D-galactosamine–induced model of FHF. Here, we demonstrate a direct anti-apoptotic effect on hepatocytes, suggesting the effect is not limited to the modulation of the inflammatory cascade.

Local down-regulation of pro-inflammatory cytokines and up-regulation of anti-inflammatory cytokines, such as IL-10, after MSC transplantation has been described in models of lung and kidney injury. Our results demonstrate that these alterations occur on a systemic level. Mortality from FHF is partly attributed to the uncontrolled systemic inflammatory response that manifests into multi-organ failure. Down-regulation of this response may contribute to improved survival of FHF after MSC-CM treatment.

Stimulation of endogenous regeneration mechanisms represents an important avenue of trophic support by MSC therapy, previously described in models of acute kidney failure and stroke. Our results demonstrate that MSC therapy can increase the number of proliferating hepatocytes threefold in the regenerating, injured liver and that MSC-secreted factors are sufficient to accomplish this effect. However, the direct stimulatory effect of MSC-CM that we observed on the 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 supratherapeutic concentrations of MSC-CM lead to a loss of the therapeutic effect. MSC secretions contain a complex mixture of cytokines, growth factors, and chemokines, including small amounts of transforming growth factor beta (TGF-β), a compound with a marked inhibitory effect on hepatocyte proliferation. It is possible that at higher concentrations of
Fig. 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 40× 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 up-regulated 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-1, adrenergic receptor; TGF-β, transforming growth factor-beta; HGF, hepatocyte growth factor; TNF-α, tumor necrosis factor alpha; 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; FHF, fulminant hepatic failure.

Fig. 7. Low concentrations of MSC-CM enhance proliferation and functions of cultured hepatocytes. Hepatocytes were cultured at low density (1.25 × 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 25× 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; n.s., not significant; MSC-CM, mesenchymal stem cell conditioned medium; FOV, field of view.
MSC-CM the negative effect of TGF-β, for example, overrules therapeutic effects exerted by trophic compo-
nents of MSC-CM. Similarly, the effects of TNF-α, present at low concentrations in MSC secretions, 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 antiapoptotic 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, most of which were growth factors, cytokines, and chemokines. Several of the detected molecules have known antiapoptotic and liver regeneration–stimulating effects. Vascular endothelial growth factor, for example, is known to induce hepatocyte growth factor secretion by stellate cells, which in turn induces expression of hepatocyte-mitogenic TGF-β. Hepatocyte growth factor, 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. Using in vitro studies, we previously showed that MSCs inhibit activated stellate cell functions by a dynamic, multi-molecular cytokine response during coculture. 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 current 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.

Most 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. In rodent models of chronic liver disease, investigators have shown that systemic MSC transplantations can result in anti-fibrotic effects. Compared with 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 a hepatocyte-like phenotype has been described, differentiation into fully functional hepatocytes is at most a rare phenomenon. Moreover, engraftment levels of MSCs are often insignificant, 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 in acute and potentially chronic settings without the aforementioned problems.

In conclusion, the current study shows that systemic MSC-CM therapy has profound inhibitory effects on hepatocellular death and enhances of liver regeneration programs, and that it ultimately improves survival in rats undergoing D-galactosamine–induced FHF. Furthermore, we demonstrate direct antiapoptotic and promi-
totic effects of MSC-CM on cultured hepatocytes. This work validates that MSC-CM induces an integrated re-
sponse to liver disease and creates potential new avenues for the treatment of this devastating disorder.

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