Supplementary Materials and Methods

Cell Isolation and Culture

The isolation and characterization of mesenchymal stem cells (MSCs) from bone marrow was as reported previously.[1,2] Briefly, human bone marrow was aspirated during fracture surgeries from normal donors with informed consent, and approval from the institutional review board of the Taipei Veterans General Hospital was obtained before commencement of the study. Mononuclear cells were obtained by negative immunoselection followed by density gradient centrifugation and plated in expansion medium. Expansion medium consists of Iscove's modified Dulbecco's medium (IMDM; Sigma-Aldrich, St Louis, MO) and 10% fetal bovine serum (Hyclone, Logan, UT) supplemented with 10 ng/mL fibroblast growth factor–basic (Sigma-Aldrich), 100 U penicillin, 1000 U streptomycin, and 2 mmol/L L-glutamine (Sigma-Aldrich). MSCs used in this study were clonally derived, and their immunophenotype as well as differentiation potentials into osteoblasts, adipocytes, chondrocytes, and hepatocytes were previously characterized and reported.[3] For generation of MSC-derived hepatocytes, hepatic induction was performed for 3 weeks using the 2-step protocol that we previously reported.[3]

Cell Transplantation

Cell transplantations and surgical procedures were performed under 3% isoflurane inhalation anesthesia. Direct intrasplenic injections were made after laparotomy, and systemic transplantations were made percutaneously via the tail vein.

Partial Hepatectomy

For partial hepatectomy, an upper midline incision was made from the xiphoid inferiorly and the abdominal cavity exposed with an Alm retractor. The anterior lobes to be resected were gently lifted with a moistened cotton swab, and a silk suture was placed under the lobe proximal to the origin. The ends of the suture were tied over the lobe near the inferior vena cava, and the lobe was resected just distal to the suture. The procedure was performed for the anterior lobes, resulting in approximately 60% hepatectomy.

Cell Labeling With QuantumDots

Cells were labeled with QTracker585 (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions.

Preparation of Liver Cell Suspension

Cells were prepared as reported previously.[4] Liver tissues were minced in collagenase buffer containing 3 mg/mL collagenase (Sigma-Aldrich) for 40 minutes at 37°C under gentle agitation. Cells were collected by centrifugation, resuspended in FACS buffer (BD Biosciences, San Jose, CA), and successively passed through 23- and 26-gauge needles and a 40-μm cell strainer (BD Biosciences) to yield a single cell suspension, and red blood cells were removed by ACK lysing buffer. Cell viabilities were >93% by trypan blue dye exclusion, and total cells produced from each liver were $>5 \times 10^7$. For flow cytometry and fluorescence-activated cell sorting, cells suspensions were incubated with biotinylated anti-human β_2-microglobulin antibodies for 30 minutes at 4°C, rinsed with phosphate-buffered saline, and then incubated with streptavidin-QDot585 conjugates for 30 minutes at 4°C. Cells were rinsed with phosphate-buffered saline and resuspended in FACS buffer (BD Biosciences, Flanders, NJ) and analyzed by FACSCalibur (BD Biosciences) for flow cytometry or by FACSAria for cell sorting (BD Biosciences).

Total RNA Isolation and Real-Time Reverse-Transcription Polymerase Chain Reaction

Total RNA was extracted from cells using RNeasy (Qiagen, Chatsworth, CA), and reverse transcription was performed using BD Sprint PowerScript (BD Biosciences). For real-time reverse-transcription polymerase chain reaction (PCR), complementary DNA was analyzed by ABI 7700 Sequence Detector (Applied Biosystems, Foster City, CA). The amount of target template was normalized by the housekeeping gene GAPDH. Primers for GAPDH are forward CCAGGTGGTCTCCTCTGACTTC and reverse GTGGTCGTTGAGGGCAATG; for Bcl-2 are forward GGCTGGGATGCCTTTGTG and reverse CAGCCAGGAGAAATCAAACAGA; for Bcl-xL are forward CAGAGCTTTGAACAGGTAGTGAATG and reverse TCTCAGCTTTCCACGCACAGT; for Akt are forward CTTCTATGGCGCTGAGATTGTG and reverse CCCGGTACACCACGTTCTTC.

Western Blot Analysis

Protein concentration was determined and 20–25 μg of protein was separated on 12% sodium dodecyl sulfate/polyacrylamide gel electrophoresis and blotted onto a polyvinylidene difluoride membrane (GE Healthcare Bio-Sciences, Piscataway, NJ). Nonspecific bindings were blocked by 3% skim milk in TBST buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.1% Tween 20, pH 7.4). The membrane was sequentially hybridized with the primary antibody (copper/zinc superoxide dismutase and manganese superoxide dismutase, 1:5000, Upstate Biotechnology, Charlottesville, VA; catalase, 1:3000, Calbiochem, San Diego, CA; GAPDH, 1:5000, Chemicon, Temecula, CA; albumin, 1:4000, Abcam, Cambridge, England; cytokeratin-18, 1:4000, Abcam; α_1-antitrypsin, 1:2000, Abcam; glutamine synthetase, 1:2000, Abcam; hepatocyte nuclear factor 4, 1:2000, Abcam; cytochrome P450 2C8/9/12/19, 1:4000, Abcam) and with horseradish peroxidase–conjugated secondary antibody (GE Healthcare Bio-Sciences) at room temperature for 1 hour. Washes were performed between incubations. Protein

intensity was determined with enhanced chemiluminescence reagent (PerkinElmer, Wellesley, MA).

Histologic and Immunofluorescence Analysis

Tissues were fixed in 3.7% formaldehyde, dehydrated, embedded in paraffin blocks, and sectioned at 3–4 μm. For histology, sections were stained with H&E (Sigma-Aldrich). For immunofluorescence staining, sections were blocked with 5% goat serum in phosphate-buffered saline (Sigma-Aldrich) and sequentially incubated with mouse anti-human albumin antibody (Sigma-Aldrich) and with Cy3-conjugated goat anti-mouse antibody (Sigma-Aldrich). Washes were performed between incubations. Imaging was performed with the SPOT RT Imaging System (Diagnostic Instruments, Sterling Heights, MI) under an epifluorescence microscope.

Bromodeoxyuridine Incorporation and Immunohistochemical Analysis

Bromodeoxyuridine (BrdU) dissolved in saline was injected into mice intraperitoneally at 12 hours after cell transplantation. BrdU was injected at 50 mg/kg body wt twice daily up to the time the mice were killed. Liver tissues were embedded in OCT compound and cryosectioned at 4–5 μm. For immunohistochemical staining of BrdU, endogenous peroxidase activity was blocked with H_2O_2 for 10 minutes at 37°C and rinsed with phosphate-buffered saline. DNA was denatured in 2N HCl for 30 minutes at 37°C, rinsed with phosphate-buffered saline, and then enzymatically pretreated with 0.1% trypsin for 20 minutes at 37°C. Tissues were blocked for nonspecific binding by 5% goat serum in PBS and then incubated with anti-BrdU antibody (1:1000; Sigma-Aldrich) for 2 hours at 37°C, followed by biotinylated secondary antibody (1:1000; Sigma-Aldrich) for 40 minutes at room temperature and with avidin/horseradish peroxidase conjugate for 30 minutes at room temperature. Positively stained cells were detected using 3,3′-diaminobenzidine tetrahydrochloride substrate (Sigma-Aldrich). Imaging was performed with the SPOT RT Imaging System.

Genomic DNA PCR

Genomic DNA was extracted from cells isolated by fluorescence-activated cell sorting for expression of human β_2-microglobulin using the Illustra tissue and cells genomicPrep kit (GE Healthcare Bio-Sciences) as per the manufacturer's instructions. Total cellular DNA was analyzed by PCR for human- and mouse-specific β_2-microglobulin using a published method.[5] Primers that specifically amplify human β_2-microglobulin (forward: GTGTCTGGGTTTCATCCATC, reverse: GGCAGGCATACTCATCTTTT) were selected based on the published sequence and were shown to amplify human, not mouse, DNA. Primers that specifically amplify mouse β_2-microglobulin (forward: AGACTTTGGGGGAAGCAGAT, reverse: CAGTCTCAGTGGGGGTGAAT) were selected based on the published sequence and were shown to amplify mouse, not human, DNA.

Estimation of Cell Frequencies by Real-Time PCR

Frequencies of human cells in recipient mouse liver were estimated using a published method.[6] The amount of DNA was first determined by absorbance and then by real-time PCR for the mouse albumin gene to normalized genomic DNA. PCR assays were performed in reactions containing SYBR Green PCR Master Mix (Applied Biosystems), 900 nmol/L of forward and reverse primers, and 200 ng of target template. Standard curves were generated by serially diluting human genomic DNA prepared from MSCs into samples containing 200 ng of genomic DNA from mouse liver and bone marrow. Primers for mouse DNA (forward: GAAAACCAGGCGACTATCTCC, reverse: TGCACACTTCCTGGTCCTCA) amplify a specific intron sequence of the single copy serum albumin gene. Primers for human DNA (forward: CATGGTGAAACCCCGTCTCTA, reverse: GCCTCAGCCTCCCGAGTAG) amplify human Alu repetitive sequence.

Exogenous Oxidative Stress and Cell Viability Assay

To assay for cell viability in the presence of exogenous oxidative stress, MSCs and MDHs were seeded at 4×10^3 cells/well in 96-well plates and serum deprived in IMDM for 2 days before the assay. Paraquat was supplemented in IMDM at concentrations between 1 and 4 mmol/L, and paraquat-containing medium was replaced on a daily basis. Cell viability was assay using MTS reagent (Promega, Madison, WI) as per manufacturer's instructions at 0 to 5 days posttreatment with paraquat, and readings were measured at 490 nm on a Bio-Rad Model 680 microplate reader (Hercules, CA). MTS reagent only served as blank control, and results were expressed as relative viability to day 0. For cell viabilities in the presence of hydrogen peroxide, hydrogen peroxide was added into IMDM at concentrations between 100 and 800 μmol/L and replaced daily. For cell viabilities in the presence of superoxide anions, cells were cultured in the presence of xanthine oxidase and xanthine was supplemented into medium at concentrations between 100 μmol/L and 1 mmol/L for 30 minutes. Cell viabilities were determined as described previously. For hepatocyte viability under coculture, 2×10^5 normal NOD-SCID hepatocytes were seeded in the lower level of Transwell chambers in IMDM. On day 0, paraquat was supplemented into IMDM at a final concentration of 4 mmol/L; at 24 hours after addition of paraquat, 1×10^5 MDHs or MSCs preseeded in Transwell chambers were placed into the well containing paraquat-treated hepatocytes for subsequent coculturing. An empty Transwell chamber served as non-cocultured control. Viability and proliferation of

NOD-SCID hepatocytes were determined by MTS assay, and results were expressed as relative viabilities to day 0.

In Vivo Reduced Glutathione/Oxidized Glutathione Assay

Whole blood was collected from the animals at 24-hour intervals, and reduced glutathione and oxidized glutathione levels were determined using the GSH/GSSG Ratio Assay Kit (Calbiochem, San Diego, CA) as per the manufacturer's instructions.

Albumin Enzyme-Linked Immunosorbent Assay

Each well of a microtiter plate was coated with 200 μL of human albumin-specific antibody (Sigma-Aldrich) at 0.2 μg/mL for 1 hour at 37°C and washed 3 times with PBS-T. Samples were applied to the wells and incubated for 1 hour at room temperature and washed 3 times with PBS-T. Subsequently, wells were incubated with detection antibody at 0.2 μg/mL for 30 minutes at room temperature and washed 3 times with PBS-T. Tetramethylbenzidine substrate was added into each well and allowed to develop for 30 minutes and read on a Bio-Rad Model 680 microplate reader. Concentrations were determined against the standard curve.

Image Analysis

Image analysis and the percentages of necrosis were measured using ImageJ (National Institutes of Health). Data are presented as mean ± SD of 10 determinations.

References

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