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Successful Transplantation of Reduced Sized Rat Alcoholic Fatty Livers Made Possible by Mobilization of Host Stem Cells

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Abstract

Livers from Lewis rats fed with 7% alcohol for 5 weeks were used for transplantation. Reduced sized (50%) livers or whole livers were transplanted into normal DA recipients, which, in this strain combination, survive indefinitely when the donor has not been fed alcohol. However, none of the rats survived a whole fatty liver transplant while six of seven recipients of reduced sized alcoholic liver grafts survived long term. SDF-1 and HGF were significantly increased in reduced size liver grafts compared to whole liver grafts. Lineage-negative Thy-1+CXCR4+CD133+ stem cells were significantly increased in the peripheral blood and in allografts after reduced size fatty liver transplantation. In contrast, there were meager increases in cells reactive with anti Thy-1, CXCR4 and CD133 in peripheral blood and allografts in whole alcoholic liver recipients. The provision of plerixafor, a stem cell mobilizer, salvaged 5 of 10 whole fatty liver grafts. Conversely, blocking SDF-1 activity with neutralizing antibodies diminished stem cell recruitment and four of five reduced sized fatty liver recipients died. Thus chemokine insufficiency was associated with transplant failure of whole grafts which was overcome by the increased regenerative requirements promoted by the small grafts and mediated by SDF-1 resulting in stem cell influx.

Keywords

fatty liver; reduced sized graft; host stem cells; SDF-1; HGF; plerixafor

Introduction

Improved survival rates have created an increasing imbalance between organs available for transplantation and the number of patients awaiting an organ. Several thousand people die on the liver transplant list every year triggering interest in the use of marginal donors and extended criteria organs. Fatty livers are known to tolerate cold preservation (1, 2) and warm (3) ischemia/reperfusion injury poorly. These grafts are believed to have impaired

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regenerative ability (4, 5). Currently, fatty livers are used with caution because even mild steatosis, defined as <30% of hepatocytes containing fat, is associated with graft dysfunction, particularly when additional risk factors are present (6, 7). Moderate (30%–60%) and severe (>60%) steatosis are major risk factors for graft failure (8–11). While only about 11% of young people have steatosis, the prevalence exceeds 40% in people over 60 years of age (1, 12, 13). Given the prevalence of obesity and hepatic steatosis in the general population, the ability to utilize fatty livers for transplantation would significantly increase the organ pool if new strategies improving their function could be discovered.

We have reported (14) that Lewis rat livers transplanted orthotopically into DA hosts result in lifelong acceptance despite MHC differences, an early rejection crisis, and the absence of immunosuppression. The mechanism of acceptance is due, at least in part to recruitment of host stem cells into liver allografts. Host bone marrow derived c-Kit⁺ CXCR4⁺ CD34⁺ stem cells were observed in the transplanted liver and many of these host cells were demonstrated to contain albumen. Transplantation of reduced size liver grafts accelerated host stem cell recruitment and regeneration. We reasoned that this liver transplant model could be applied to study the outcome of both whole and reduced size fatty livers from alcohol fed Lewis rats to normal DA hosts. We have reported previously that transplantation of whole fatty livers from alcohol fed rats resulted in liver graft failure and recipient death (15). We hypothesized that regenerative stimuli coming from the small liver graft might result in an influx of host stem cells which could replace/repair damaged hepatocytes and result in increased animal survival post-transplant.

Materials and Methods

Rat Strains and Care

Lewis (LEW, RT1¹) and dark agouti (DA, RT1A^a) rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and used at 8–12 weeks of age. Animals were maintained in the specific pathogen-free facility of the Johns Hopkins Medical Institutions. Animals were cared for according to NIH guidelines and under a protocol approved by the Johns Hopkins University Animal Care Committee. Lewis (RT1¹) rats, initially weighing 170 g, were fed a nutritionally adequate liquid diet containing 7% ethanol (Bioserv, Frenchtown, NJ) for 5 weeks, a period sufficient to develop alcoholic fatty liver.

Liver Transplantation

Orthotopic whole liver transplantation or reduced sized (50%) small liver transplants from alcohol fed Lewis to normal DA rats were performed under isoflurane (Abbott Laboratories, North Chicago, IL) inhalation anesthesia according to methods described previously (14). Small liver transplantation consisted of removal of the left lateral lobe, the left portion of the median lobe, and the anterior and posterior caudate lobes. This reduced the liver mass by about 50%. The livers were flushed in situ with 10 mL cold saline via the portal vein, explanted, and immersed in cold saline solution for 1 hour. The host liver was excised by ligation and division of the right adrenal and lumbar veins. The hepatic artery was ligated and divided. The bile duct was cannulated by insertion of a tube 2mm in length (outer diameter 1.2 mm) via choledocotomy and secured with a circumferential 8-0 silk suture. The

inferior vena cava and the portal vein were cross-clamped with microvessel clips. The suprahepatic vena cava was pulled down using a cotton tape passed around the host liver and cross-clamped with a baby Statinsky clamp. The vessels were divided and liver was removed. The donor suprahepatic vena cava was anastomosed end-to-end with the host suprahepatic vena cava with running 8-0 prolene. The portal vein and infra hepatic vena cava were anastomosed using the cuff technique described before. The common bile duct of the donor was cannulated with the small tube residing in the host bile duct and tied in place. The hepatic artery was not reconstructed.

Preparation of non-parenchymal cell suspensions from rat liver with Collagenase D treatment

The liver was excised and cut gently into small pieces with a scalpel blade. The pieces were put in a beaker containing 5 mL of HEPES buffer (10 mM HEPES-NaOH pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂). The collagenase D solution was added to make the final Collagenase D concentration 2 mg/mL. The sample of rat liver was incubated for 45 minutes at 37 degrees under slow continuous rotation. Dissociated tissue was removed and the remnants were pressed through a 100 µm mesh placed over a 50 mL tube. The cells were suspended in 30–40 mL of Hank's balanced salt solution (HBSS) and centrifuged at 400×g for 6 minutes. The supernatant was then aspirated completely. The cell pellet was re-suspended in 5 mL HBSS and pressed through a 70 µm mesh placed over a 50 mL tube. The same procedure was repeated 3–4 times with the remaining liver fragments and the cells were released and washed twice after re-suspension in 4 degree HBSS. After repeated washes in HBSS the final pellet was re-suspended in FBS containing 10% dimethyl sulfoxide (DMSO) and was then frozen and kept in –80 degree until analysis.

Preparation of Cell Suspensions from Blood

Mononuclear cells were isolated from peripheral blood and spleen by Ficoll-Hypaque (1.077 g/liter) density gradient centrifugation. Isolated cells were frozen in FBS containing 10% DMSO and kept at –80 degrees until analysis.

Flow Cytometry

Single-cell suspensions (1×10^6) of blood and liver were analyzed for expression of lineage negative c-kit⁺, CXCR4⁺ and CD133⁺ stem cell markers. All antibodies used were from commercial sources: cKit (1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA), CXCR4 (1:100, ab2074 Abcam, Cambridge, MA), CD133(1:100; ab19898, Abcam) and FITC anti rabbit IgG, PE anti rabbit IgG, Day Light 647 anti Goat IgG (1:200 Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) and strept avidin-Per CP (BD Pharmingen). Nonspecific antibody binding was blocked with donkey, mouse, and rat serum (Sigma) for 30 minutes. Cells were incubated with antibodies for 1 hour at 4°C, and the positive cells were counted by flow cytometry (fluorescence activated cell sorting [FACS]) using CELLQuest software (Becton-Dickinson).

Oil Red O Staining

Liver tissues for histology of reduced and whole grafts were recovered from the right portion of median lobe. Lipid accumulation was determined by way of Oil Red O staining. Oil Red O (CI26125) was dissolved in isopropanol (0.5:100) for stock solution. Formalin fixed cryostat liver sections (8–10 µm) were incubated with freshly prepared Oil Red O working solution (stock solution: distilled water, 3:2) for 15 mins, rinsed with 60% isopropanol and lightly stained nuclei with alum haematoxylin.

Immunohistochemistry

Five µm serially cut, frozen sections were fixed with acetone at (–20°C) for 10 minutes and dried for 1 hour at room temperature. The streptavidin-biotin-peroxidase method with the DAKO Kit (Carpinteria, CA) was used to detect antigens. After inactivation of endogenous peroxidase and blocking of nonspecific antibody binding, the specimens were treated with biotinylated antibodies specific for c-Kit (1:200), CXCR4 (1:200), CD133 (1:200), CD34 (1:100; R&D Systems, Minneapolis, MN), Thy-1 (1:100; BD Pharmingen), RT1A^a (1:100, C3; BD Pharmingen), SDF-1 (1:200; Santa Cruz) or HGF(1:200; Santa Cruz) at 4°C overnight. Subsequently, sections were incubated with streptavidin-biotin-peroxidase complex reagent for 30 minutes at room temperature. Diaminobenzidine tetrahydrochloride was used as the chromogen, and hematoxylin was used for counterstaining.

Immunofluorescence Staining

Frozen sections (5-µm) were fixed with acetone (–20°C) for 10 minutes and dried for 1 hour at room temperature. A PBS containing 1% BSA and 5% normal Donkey serum was used for blocking nonspecific background and dilution of antibodies. Sections were incubated for overnight at 4°C with a mixture of a FITC-labeled rat monoclonal antibody to RT1A^a (1:100) and rabbit antibody to rat CD133 (1:100) or goat anti-albumin (1:100, Santa Cruz Biotech). Cy3 donkey anti-rabbit or donkey anti-goat IgG (1:100; Jackson ImmunoResearch Inc.) for 1 hour at room temperature. Cell nuclei were stained blue with DAPI. Tissue sections were analyzed by confocal fluorescence microscopy.

Semi-quantitative reverse transcription (RT)-PCR analysis

Liver specimens were kept frozen at –80°C until homogenized for RNA extraction using the TRIzol Reagent (Invitrogen, Carlsbad, CA). First-strand cDNA synthesis was then performed on 5µg of total RNA using the Superscript First-Strand Synthesis system for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Polymerase chain reactions (PCR) contained 1 µL of deoxynucleoside triphosphate mix (10 mM each dNTP), 1 µl of 10 µM each primer (Table 1), 0.4 µL (5 IU/µL) of Platinum *Taq* polymerase (Invitrogen, Carlsbad, CA), 1.5 µl of 50 mM MgCl₂ and 2 µL total DNA as template in a 50 µL reaction solution. Thermal cycling was started with one cycle at 94°C for 4 minutes. This was followed by 25–35 cycles at 94°C for 30 seconds, 59°C for 30 seconds, 72°C for 30 seconds, and 72°C for final extension for 10 minutes. PCR products were electrophoresed on 1.2% agarose gels and visualized with GelStar® Stain(Lonza Rockland Inc., Rockland, ME). The primer sets for amplification of SDF-1 were 5'-tgagattgccagcacaag-3' and 5'-ctctcggaaggaatctgtc-3'. The primer sets for amplification of HGF were 5'-

acctgaaggtcagatttg-3' and 5'-gggtgctgactgcatttctca-3'. The primer sets for control amplification of beta-actin were 5'-cactgccgcacatctcttct-3' and 5'-agccaccaatccacacagag-3'.

Western blot analysis

Tissues were homogenized in cellLyticTMMT lysis buffer (Sigma-Aldrich Saint Louis MI) at 4°C, vortexed and centrifuged 16,000 rpm at 4°C for 10 minutes. The supernatants were mixed in Nupage[®]LDS sample loading buffer, boiled for 10 minutes at 70°C, and then subjected to SDS-PAGE. After electrophoresis, proteins were transferred onto PVDF membranes using iBlot[®] Dry blotting system according to the manufacturer's protocol. Nonspecific binding sites were blocked by TTBS (0.05% [vol/vol] Tween 20 in Tris-buffered saline [pH 7.4]) with 0.5% Western Blocking Reagent (Roche Applied Science Indianapolis, IN) according to the manufacturer's protocol. Blots were incubated with primary antibody for 12 hours SDF-1 (1:100) or HGF(1:100). The expression of β -actin (1:1000; Cell Signaling Technology, Inc. Danvers, MA), a constitutively expressed housekeeping protein, was used as a loading control. Membranes were washed at least three times with TTBS and incubated with a 1:20,000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) for 45 minutes. Protein bands were visualized by an enhanced chemiluminescence reaction using Immun-StarTM WesternCTM Chemiluminescence Kit (Bio-Rad Laboratories, Hercules, CA) and detected by Bio-Rad Imager ChemiDocXRS (Bio-Rad Laboratories, Hercules, CA).

Administration of neutralizing anti-SDF-1 antibodies or stem cell mobilizing agent Plerixafor

Neutralizing anti-SDF-1 antibodies were purchased from R&D System (Minneapolis, MN). To inhibit SDF-1, anti-SDF-1 antibodies (500 μ g/kg body weight) diluted in 0.25ml PBS were administered intraperitoneally to recipient rats at 6 and 24 hours after small liver transplantation. Control animals received same amount of mouse IgG at the same times. In selected experiments, hepatic non-paranchymal cells were isolated from anti-SDF-1 treated rats on day 2 after transplantation.

To determine if whole fatty liver grafts could be salvaged by stem cells, DA recipients of Lewis whole fatty livers were given Plerixafor (Mozobil, Genzyme Inc.) which is an antagonist of the alpha chemokine receptor CXCR4 and acts as bone marrow stem cell mobilizing agent (17). Recipients with fatty whole liver grafts were treated with plerixafor (1mg/kg, s.c.) immediately after reperfusion and on day 2 and 4 post transplantation.

Statistics

Continuous variables were presented as the mean $\pm$ SD. Dichotomous variables were presented as both number and percentage values. Data of flow cytometry were analyzed using the Student's t test (two-tailed), with dichotomous variables analyzed by the Fisher's exact test (two-tailed). The proportion of surviving rats between the treatments groups was compared using Kaplan-Meier method and analyzed by the Chi-squared test. $P < 0.05$ was considered significant.

Results

Improved survival with transplantation of reduced size alcoholic fatty liver grafts compared to fatty whole grafts

Transplanted rats were divided into two groups: 1) reduced sized (small) alcoholic fatty liver grafts (n=7), and 2) whole alcoholic fatty liver grafts (n=10). Most (6 out of 7, 85%) recipients survived when reduced size (50%) alcoholic livers were transplanted, while none of 10 recipients survived when alcoholic whole livers were used as donors for transplantation (Fig. 1A). Liver enzyme elevations were significantly mitigated in reduced size alcoholic fatty liver recipients at 3 days after transplantation (Fig. 1B). The morphology of transplanted livers is shown in Figure 2A, which is representative of our experience. After five weeks of feeding with 7% alcohol rat livers were yellow, soft and 50–60% of hepatocytes contain a single, bulky fat vacuole in the cytoplasm displacing the nucleus to the edge of the cell fulfilling the definition of moderate macrosteatosis. The whole liver transplant remained yellow and patches of necrosis were visible on the surface. By contrast, the small liver transplant was reddish brown and nearly full sized at 3 and 5 days after transplantation. H&E staining of whole liver transplants showed hepatocyte necrosis in central vein areas at 3 days after transplantation, and the necrotic areas were enlarged by 5 days (Fig. 2B). There were no necrotic areas in liver tissue sections from small liver grafts.

Recruitment of lineage negative c-Kit+CXCR4+CD133+ stem cells in reduced size fatty liver transplants

To determine if host stem cells were involved in small liver transplant salvage, we performed flow cytometry measuring the stem cell constituents in peripheral blood and liver allografts after transplantation. Stem/progenitor cells have been phenotypically defined according to the expression of specific surface antigens such as c-Kit, and CXCR4 and CD133. They do not express lineage markers found in maturing blood cells. We have utilized flow cytometry to analyze lineage-negative (Lin-neg) cells according to the intensity of labeling with c-Kit, CXCR4 and CD133 (Fig. 3A). The percentage of triple positive (cKit +CXCR4+CD133+) cells in Lin-neg enriched stem cell populations was significantly greater in peripheral blood in the both recipient groups at 12 hours after transplantation (Fig. 3B). However, the percentage of Lin-neg c-KitCXCR4+CD133+ cells remained at high levels in the animals receiving reduced size fatty livers at 1, 2 and 3 days after transplantation while it fell significantly in the animals receiving whole fatty livers at 1, 2, and 3 days after transplantation. Similar results were found studying cell suspensions made from the transplants namely, c-Kit+CXCR4+CD133+ cells in lineage negative stem cell enriched populations were significantly increased in the liver allografts in the animals receiving small alcoholic fatty livers at 2 or 3 days after transplantation (Fig. 3C), while these cells decreased in the whole liver suspensions.

Immunohistochemistry confirmed these findings and showed significant number of c-Kit+, CXCR4+, CD133+, Thy-1+, and CD34+ cells in liver tissue sections recovered from small alcoholic fatty liver allografts at 3 days after transplantation. In contrast, fewer cells stained with these stem cell markers were present in whole alcoholic fatty liver allografts (Fig. 4A).

Immunofluorescence staining demonstrated that the CD133+ cells were host derived as shown in figure 4B.

Increased SDF-1 expression in reduced size fatty liver allografts after transplantation

Semi-quantitative PCR analysis of the liver transplant showed that mRNA expression of the attractor molecule SDF-1 was significantly increased in small alcoholic fatty liver grafts compared to whole alcoholic fatty liver grafts at days 1 and 3 after transplantation (Fig. 5A and Supplemental Supporting Figure S1-A). SDF-1 molecular concentrations determined by western blot (Fig. 5B and Supplemental Supporting Figure S1-B) and ELISA (Fig. 5C) were also significantly higher in liver homogenates in animals receiving reduced size fatty livers compared to the whole fatty liver transplant group, especially at days 1, 2, and 3 after transplantation. Immunohistochemistry also showed that SDF-1 reactivity was dramatically increased in the reduced size alcoholic fatty livers (Fig. 5D), and this reactivity was localized in hepatic sinusoid areas, suggesting that these cells, and not the hepatocytes, produced SDF-1. Alternatively, this chemokine could be adherent to endothelium as a mechanism to attract infiltrating cells.

Increased HGF expression in reduced size fatty liver allografts after transplantation

Semi-quantitative PCR testing for the presence of HGF mRNA in the liver transplant showed that HGF was significantly increased in small fatty liver grafts compared to whole fatty liver grafts at days 3 and 5 after transplantation (Fig. 6A and Supplemental Supporting Figure S1-C). HGF chemokine concentrations in liver homogenates (Fig. 6B and Supplemental Supporting Figure S1-D) were also significantly higher in animals receiving small alcoholic fatty livers compared to the whole alcoholic fatty liver transplant group, especially at days 3 and 5 after transplantation. Immunohistochemistry also demonstrated that HGF increased in the reduced sized fatty liver transplants. These results parallel the findings for SDF.

Reduced size fatty livers failed to survive in anti-SDF-1 neutralizing antibody treated recipients while pharmacological mobilization of host stem cells improved survival of fatty whole liver grafts

To demonstrate the critical role of SDF-1 mediated stem/progenitor cell mobilization in the improved survival of partial fatty liver graft, inactivation of SDF-1 was achieved by intraperitoneal injection of anti-SDF-1 antibodies in five rats at 6 and 24 hours post-transplantation. Five transplanted control rats were injected with the same volume of mouse IgG at the same time points. c-Kit+CXCR4+CD133+ cells in lineage negative stem cell enriched populations were significantly decreased in the reduced size fatty liver allografts in the animals receiving anti-SDF-1 antibodies at 2 days after transplantation (Fig. 7A), while these cells increased in the animals treated with control mouse IgG. The diminished stem cell responses lead to the death of 4 of 5 recipients at seven days while only 1 of 5 died in the control group (Fig. 7B). Thus inactivation of SDF-1 eliminated the survival advantage of reduced size fatty liver allografts.

To determine if whole fatty liver grafts could be salvaged by mobilization of stem cells, recipients with fatty whole liver grafts were treated with plerixafor (1mg/kg, s.c.)

immediately after reperfusion and on day 2 and 4 post transplantation. Five of 10 recipients receiving plerixafor treatment survived whole fatty liver transplantation (Fig. 8) contrasting with the absence of any survival in the ten rats not treated.

DISCUSSION

After five weeks of feeding with 7% alcohol, rat livers were yellow, soft and at least half of the hepatocytes in the central vein area contained fat fulfilling the definition of moderate macrosteatosis. Given this injury recent evidence has shown unusual pathways leading to cell death following an ischemic period (3). Strategies for improving regeneration in lean livers have proved to be ineffective in the presence of fat deposits in hepatocytes (19). Unlike mature hepatocytes in healthy adult liver, most mature hepatocytes in alcoholic fatty livers cannot replicate (20). Thus it is clear that effective regeneration requires replacement of damaged hepatocytes with healthy cells. The studies we report show that this may be accomplished when a reduced size liver is transplanted into a weakly rejecting host. They extend our observations (14) and those of others (18) that regenerative stimuli after partial hepatectomy drive bone marrow stem cells into a liver suffering from toxic or immunological injury. The contribution of bone marrow stem cells to liver regeneration via cell fusion (21) or spontaneous transdifferentiation (22) has been demonstrated in animals.

Our present studies were designed to mimic clinical conditions in two important ways: first, the hepatic injury was produced by simple chronic alcohol intake and low grade rejection; and second the regenerating cells came from a normal host bone marrow and not from cells exposed to alcohol, radiation, or the chemotherapeutic injury required for bone marrow transplant. We speculated that the moderate alcohol-injured whole fatty liver was incapable of sustaining to synthesis of important chemokines, including SDF-1 and HGF, needed for stem cell recruitment resulting in uniform failure of whole fatty liver transplants. We proposed that regenerative requirements present in the small grafts might lead to increased production critical chemokines and salvage by host bone marrow stem cells. Indeed, while we found the stem cell response at 12 hours following transplant to be the same in both normal and reduced sized fatty liver transplants, the production of two known stem cell chemokine attractor molecules SDF and HGF was short lived in the whole fatty livers. This was reflected by meager numbers of bone marrow stem cells present in the transplant. However, the reduced size alcoholic fatty liver provided conditions for progressive extrahepatic progenitor cell recruitment by increasing and sustaining SDF-1 and HGF expression. This produced a concentration gradient between liver and bone marrow (BM) facilitating recruitment of hematopoietic stem and progenitor cells (HSPCs) (23, 24). Thus our studies confirmed the reports of others showing that partial hepatectomy increased SDF-1 and HGF production and promoted stem/progenitor cell trafficking (24).

This proposal is strengthened further by growing insight regarding the function of SDF-1, (CXCL12) which is an α -chemokine that strongly attracts hematopoietic stem progenitor cells (HSPCs) possessing the CXCR4 receptor (25, 26). SDF-1-CXCR4 signaling plays an important role in the homing of HSPCs to the bone marrow (27, 28) by tethering the HSPCs to the endothelium (29, 30). It leads to the expression of matrix metalloproteinases (MMPs) (31) and is important for wound healing presumably via attraction of CD34 cells. We found

it localized in the sinusoids of the liver where it can attract stem cells with the CXCR-4 marker. Several studies report increased circulating plasma levels of SDF-1 in autoimmune and viral liver disease in man. There is also increased expression of SDF-1 in the parenchyma of rejecting liver transplants and viral/autoimmune liver diseases (24, 32). Similar observations have been reported in murine liver injury models (33, 34).

Hepatocyte growth factor (HGF) was originally isolated as a mitogen for adult hepatocytes and has been shown to be a potent regulator of HSPC proliferation and differentiation (35), as well as a powerful stimulator of angiogenesis (36). The activity of HGF is mediated primarily through binding to its receptor, a transmembrane tyrosine kinase encoded by the MET proto-oncogene (*c-met*). It has been shown recently that HGF exerts a strong chemotactic effect on mesenchymal stem cells (MSCs) in a wound-healing model (37). In summary, there is mounting evidence that moderate liver injury stimulates the production of SDF-1 and HGF leading to bone marrow stem cell attraction, retention, and differentiation.

The source of cells aiding regeneration is likely to be bone marrow mesangial cells bearing the c-kit, CXCR4, CD133 and Thy-1 surface markers. Triple positive lineage-negative c-Kit +CXCR4+CD133+ cells were significantly increased in the peripheral blood and liver allografts from recipients of reduced size alcoholic fatty livers. These triple positive cells were scarce in whole fatty liver transplants, but were abundant in the reduced size fatty liver allografts. CD133-positive cells carried the host allotype demonstrating their origin from the bone marrow. A variety of bioactive cytokines secreted by the recruited stem cells might be involved in restoring liver function and promoting regeneration (38, 39) in the reduced size fatty liver allografts. However, the contribution of BMSCs to liver regeneration via spontaneous transdifferentiation or cell fusion has been demonstrated in animals and humans (40–43). A recent publication (44) suggested an alternative possibility that the protective effect of bone marrow-derived stromal cells (BMSCs) might involve mitochondrial transfer to injured cells. In a mouse acute lung injury model, the instilled BMSCs attached to alveoli by forming Cx43-based gap junctional channels (GJCs), and released mitochondria-containing microvesicles that the epithelia engulfed. Interestingly, the mitochondrial transfer resulted in increased alveolar ATP concentrations (44). Chronic alcohol consumption causes oxidative mitochondrial DNA damage and reduces liver ATP concentration (45). Whether the protective effect of recipient-derived BMSCs involves mitochondrial transfer to hepatocytes in small fatty livers warrants further investigation.

It is possible that the whole steatotic liver fails not because of chemokine failure but because it produces a high necrotic burden sufficient to cause death. The recipients of smaller grafts may survive because they manage well the lower burden of toxic metabolites associated with steatosis. To test this possibility we treated large fatty liver hosts with plerixafor, a drug known to mobilize stem cells bearing the CXCR-4 marker. Rather than uniform failure, 5 of 10 rats survived, indicating that chemokine failure was at least partly responsible for the failure of whole fatty liver grafts. Conversely, the inactivation of SDF-1 by passive antibody administration attenuated the recruitment of stem cells leading to graft failure in 4 of 5 hosts of small liver grafts. These studies support the concept that the influx of host stem cells is the mechanism permitting survival of small fatty liver grafts, and this trafficking is lacking in the whole fatty as the consequence of the SDF-1, and perhaps other chemokine failure.

The different outcomes observed between the small and whole grafts was dramatic, repeatable, and explained by the findings we report. While our studies raise the important possibility that a reduced sized moderate fatty liver can be used for human liver transplantation, enthusiasm for this approach must be tempered by clinical realities. Our outcomes were dependent upon normal bone marrow stem cell response which may not exist in the patients with chronic liver failure. Increased levels of SDF-1 in patients with chronic liver disease may foretell exhausted bone marrow reserves. For this reason additional new studies designed to evaluate the function of bone marrow stem cells in the chronically ill liver recipients must be undertaken before this approach can be contemplated for clinical application.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Improved survival with transplantation of reduced size fatty liver grafts compared to fatty whole grafts

Reduced size (50%) or whole livers from alcohol fed Lewis rats were transplanted into normal DA recipients. (A) Animal survival. Most (6/7) recipients survived when reduced size (50%) alcoholic livers were transplanted, while none of recipients survived when alcoholic whole livers were used as donors for transplantation. (B) Blood levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) at 3 days after transplantation. Data represent mean $\pm$ SE of n=3 or 4 animals per group. * $P < 0.05$.

Figure 2. Histological changes of alcoholic fatty liver transplants

(A) The gross morphology of small and large (left panels) and oil red O stain (left middle panels) of pre-transplant donor livers. Gross morphology liver allografts at day 3 (right middle panels) or day 5 (right panels) after transplantation. After five weeks of feeding with 7% alcohol rat livers were yellow and at least half of the hepatocytes in the central vein area contained fat. The small liver transplant had gotten larger similar to normal liver size and was reddish brown at 3 and 5 days after transplantation. By contrast, the whole liver transplant was yellow with white patches of necrosis. RM: the right portion of median lobe, LM: the left portion of median lobe. (B) H&E staining of whole liver transplants showed hepatocyte necrosis in the central vein areas at 3 days after transplantation and the necrotic areas were enlarged by day 5. In contrast, there were no necrotic areas in liver tissue sections from small liver grafts. Tissue sections from the RM. Images were photographed with a 40× objective. Representative photographs of n = 3 individual samples per group.

Figure 3. Quantitative analysis of stem cells in peripheral blood and allografts after alcoholic fatty liver transplantation

Flow cytometry analysis of Lineage negative (Lin-) c-Kit+ CXCR4+CD133+ cells in blood and liver allografts after transplantation. (A) lineage-negative (Lin-) bone marrow-derived stem cells were analyzed according to the intensity of labeling with CD133, c-Kit and CXCR4. (B) The percentage of Lin-Triple positive (c-Kit+CXCR4+CD133+) cells was significantly greater in the peripheral blood in the animals receiving reduced size fatty livers at day 1, 2, and 3 after transplantation. (C) The percentage of Lin-Triple positive cells was also significantly increased in non-parenchymal cell suspensions made from the liver

allografts in the animals receiving reduced size alcoholic fatty livers at day 2 and 3 after transplantation. The data presented at 0 day post transplantation was the baseline levels. Quantitative data are represented as group means (bars) (n=3). * $P<0.01$.

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Figure 4. Recruitment of host lineage negative c-Kit⁺CXCR4⁺CD133⁺ stem cells in reduced size fatty liver transplants

(A) Immunohistochemistry staining for c-Kit, CXCR4, CD133, Thy-1 and CD34 in tissue sections from reduced size (upper panels) or whole (lower panels) alcoholic fatty liver allografts at 3 days after transplantation. Positive cells are brown. Representative photographs of $n = 3$ individual transplant samples per group. Images were photographed with a 40 $\times$ objective. (B) Double fluorescence staining for CD133 and RT1A^a at 3 days after transplantation. Sections stained with both anti-CD133 and anti-RT1A^a antibodies show CD133 positive cells (red) stained for recipient RT1A^a (green) in rats receiving reduced size liver allografts. Representative photographs of $n = 3$ individual transplant samples per group. Images were photographed with a 40 $\times$ objective.

Figure 5. Increased SDF-1 expressions in reduced size alcoholic fatty liver transplants
 (A) Semi-quantitative RT-PCR analysis of reduced size or whole alcoholic fatty liver allografts after transplantation. The mRNA expression of the attractor molecules SDF-1 were significantly higher in reduced size liver allografts at day 1, 3, and 5 after transplantation. Representative graphs of three individual samples. (B) Liver lysates were western blotted for SDF-1 with β -actin as internal control. SDF-1 protein concentrations in liver homogenates were also significantly higher in reduced size fatty liver allografts compared to whole fatty liver allografts, especially at days 1, 2, and 3 after transplantation.

Representative graphs of three individual samples. (C) ELISA analysis further confirmed the levels of SDF-1 in liver homogenates were significantly higher in reduced size alcoholic fatty liver allografts compared to whole alcoholic fatty liver allografts at days 1, 3, and 5. The data presented at 0 day post transplantation was the baseline levels of SDF-1 in rats prior to transplantation of alcoholic fatty livers. Three individual transplant samples per group, $*P < 0.01$. (D) Immunohistochemistry for SDF-1. The number of SDF-1 positive cells was dramatically increased by transplantation of reduced size alcoholic fatty livers (day 3). Representative photographs of $n = 3$ individual transplant samples per group. Images were photographed with a 40 $\times$ objective.

Figure 6. Increased HGF expressions in reduced size alcoholic fatty liver transplants

(A) Semi-quantitative RT-PCR analysis of reduced size or whole alcoholic fatty liver allografts after transplantation. The mRNA expression of the attractor molecules HGF were significantly higher in reduced size liver allografts at days 3 and 5 after transplantation. The data presented at 0 day post transplantation was the baseline levels. Representative graphs of three individual samples. (B) Liver lysates were western blotted for HGF, and β -actin used as internal control. HGF protein concentrations in liver homogenates were also significantly higher in reduced size alcoholic liver allografts compared to whole liver allografts,

especially at days 3 and 5 after transplantation. Representative graphs of three individual samples. (C) Immunohistochemistry for HGF. The number of SDF-1 positive cells was dramatically increased by transplantation of reduced size alcoholic fatty livers (day 3). Representative photographs of $n = 3$ individual transplant samples per group. Images were photographed with a 40 $\times$ objective.

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Figure 7. Effect of SDF-1 inactivation on survival of reduced size fatty liver allografts

(A) Flow cytometry analysis of Lineage negative (Lin-) c-Kit+ CXCR4+CD133+ cells in reduced size fatty liver allografts after transplantation. The percentage of Lin-Triple positive cells was significantly decreased in non-parenchymal cell suspensions made from the liver allografts in the animals treated with anti-SDF-1 antibodies at day 2 after transplantation. Quantitative data are represented as group means (bars) (n=3). * $P < 0.01$. (B) Inactivation of SDF-1 eliminates the survival advantage of reduced size fatty liver allografts. * $P < 0.001$.

Figure 8. Pharmacological mobilization of host stem cells improved survival of fatty whole liver allografts

Four of eight recipients receiving plerixafor treatment survived whole fatty liver transplantation (black line) contrasting with the absence of any survival in the ten rats not treated (gray line). * $P<0.001$.