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Hepatic Irradiation Augments Engraftment Of Donor Cells Following Hepatocyte Transplantation

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Abstract

Engraftment of donor hepatocytes is a critical step that determines the success of hepatocyte transplantation. Rapid and efficient integration of donor cells would enable prompt liver repopulation of these cells in response to selective proliferative stimuli offered by a preparative regimen. We have earlier demonstrated that hepatic irradiation (HIR) in combination with a variety of hepatotrophic growth signals, ie., partial hepatectomy and hepatocyte growth factor (HGF) can be used as a preparative regimen for liver repopulation of transplanted hepatocytes. In this study, we investigated the effects of HIR on engraftment of transplanted DPPIV positive hepatocytes in congenic DPPIV-deficient rats. HIR-induced apoptosis of hepatic sinusoidal endothelial cells (SEC) within six hours of HIR, resulted in dehiscence of the SEC lining in 24hrs. Although there was no change of the number of Kupffer cells after HIR, colloidal carbon clearance decreased 24 hours post HIR, indicating a suppression of phagocytic function. DPPIV+ donor cells were transplanted 24h after HIR (0–50 Gy). There was a HIR dose-dependent increase in the donor hepatocyte mass engrafted in the liver parenchyma. The number of viable transplanted hepatocytes present in hepatic sinusoids or integrated in the parenchyma was greater in the HIR-treated group at 3 and 7dys after transplantation compared with the sham controls. Finally, we validated these rodent studies in cynomolgous monkeys, demonstrating a single 10Gy dose of HIR was sufficient to enhance engraftment of donor porcine hepatocytes. This data indicates that transient disruption of the SEC barrier and inhibition of the phagocytic function of Kupffer cells by HIR enhances hepatocyte engraftment and the integrated donor cell mass. Thus, preparative HIR could be potentially useful to augment hepatocyte transplantation.

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Keywords

Hepatocyte transplantation; Hepatic irradiation; Hepatocyte engraftment

Hepatocyte transplantation (HT) is of potential has the potential therapeutic value to be of major value in the treatment of inherited and acquired liver diseases (1,2). As the host liver remains intact, HT places less metabolic burden on the engrafted tissue than does orthotopic liver transplantation. Although extensive preclinical studies and clinical trials have demonstrated the safety and feasibility of HT, its widespread clinical application has been hindered by several obstacles. Universal shortage of donor livers greatly limits the availability of high quality hepatocytes for transplantation. Moreover, clinical experience has shown that the number of hepatocytes that can be engrafted safely during a single infusion is insufficient for completely curing liver-based inherited metabolic diseases (3). Therefore, several laboratories, including ours, have explored strategies to increase the efficiency of hepatocyte engraftment and to induce proliferation of the engrafted cells (4–6).

After introduction of hepatocytes into the portal venous system by intrasplenic injection or portal vein infusion, the transplanted cells accumulate in periportal vessels and hepatic sinusoids (7,8). The majority of these cells dies, probably from relative hypoxia, anoikis due to lack of cell adhesion (9), and phagocytosis by activated macrophages (7). A fraction of the hepatocytes crosses the sinusoidal barrier, entering the space of Disse and integrating into liver cords by 7 days. Hepatic nonparenchymal cells are major modulators of initial hepatocyte engraftment. First, the hepatic sinusoidal endothelial cells (SEC), pose a physical barrier to the passage of the transplanted hepatocytes into the space of Disse, as suggested by the enhancement of hepatocyte engraftment by cyclophosphamide and doxorubicin, agents that disrupt hepatic SECs (8,10). Second, Kupffer cells and probably other inflammation-mediating cells may clear the transplanted cells, reducing the number of hepatocytes available for engraftment. Kupffer cell depletion and administration of anti-inflammatory agents have been reported to enhance hepatocyte engraftment modestly (7,11).

It should be noted that agents that disrupt the endothelial cells or inhibit Kupffer cell function do not, by themselves, induce subsequent proliferation of the transplanted cells. Because adult hepatocytes retain the capacity to undergo multiple rounds of cell division in response to mitotic stimuli and the liver to bodyweight ratio is maintained within strict limits by robust physiological mechanisms (12,13), repopulation of the liver by transplanted hepatocytes can occur only if the transplanted cells proliferate at the expense of the host hepatocytes. Recent studies on the wing development of *Drosophila melanogaster* have shown that when two cell populations with different proliferative potential exist in the same compartment, growth of the rapidly proliferating cells lead to the death of the slower-growing cells, which would have survived in the absence of the more robustly proliferating cells (14,15). Similar mechanisms may exist in mammalian liver, so that if the engrafted hepatocytes possess a greater proliferative capacity than the host hepatocytes, the engrafted cells would grow preferentially in response to mitotic stimuli, progressively competing out the host hepatocytes (16). This has been achieved by preparatively treating rodent hosts with retrorsine or lasiocarpine, which are pyrrolizidine alkaloids that induce a long-lasting cell cycle block, and 66% hepatectomy which provides a strong mitotic stimulus to hepatocytes (17). Because of the carcinogenic potential of the pyrrolizidine alkaloids, we have pursued an alternative approach that consists of preparative hepatic irradiation (HIR), which reduces mitotic capacity of the host hepatocytes (18). The proliferative stimulus was provided by partial hepatectomy (18,19), portal vein ligation (20), induction of apoptosis of host hepatocytes by expressing Fas ligand (21) or by expression of hepatocyte growth factor (HGF) in the host liver (22,23). These preparative regimens led to the proliferation of the

transplanted hepatocytes that engrafted initially as single cells, so that each engrafted cell gave rise to progressively enlarging clusters, which eventually replaced the great majority of the host hepatocytes.

In the current study, we have focused on the effect of various doses of hepatic irradiation on the initial engraftment of hepatocytes. We hypothesized that HIR enhances initial integration of hepatocytes and their subsequent preferential proliferation by different mechanisms, so that the dose requirement for these two effects could be different. However, the rapidity and extent of eventual hepatic repopulation would depend on the total number of hepatocytes that are engrafted initially. Results of our rodent experiments demonstrate for the first time, that at low doses, HIR causes short-term disruption of hepatic sinusoidal endothelial cells and inhibition of the phagocytic function of Kupffer cells, culminating in marked increase in the initial engraftment of transplanted hepatocytes. Furthermore, towards the development of clinical protocol of preparative HIR, we examined whether our findings were valid in non-human primates, and demonstrated that at HIR doses as low as 10 Gy, there was a dramatic increase in the engraftment of donor porcine hepatocytes in cynomolgus monkeys, suggesting that augmentation of hepatocyte engraftment by low-dose HIR may be sufficient for the treatment of many inherited liver diseases, without the need for subsequent preferential proliferation of the engrafted cells.

Materials and methods

Animals

Wild-type (Charles River Laboratories, NY) and dipeptidyl peptidase IV (DPPIV) deficient (Marion Bessin Liver Research Center) male F344 rats weighing 250 – 300 g were housed in the Institute for Animal Studies at the Albert Einstein College of Medicine. Male cynomolgus monkeys (5–9 kg body weight) were housed in the animal care facility at the University of Nebraska Medical Center. Female piglets, 6–8 weeks of age (8 kg to 15 kg), obtained from Larson Farms Inc. (Sargeant, MN), were kept in the animal care facility at the Mayo Clinic. The animal protocols were approved by the respective Institutional Animal Care and Use Committees.

Hepatic irradiation (HIR)

HIR in rodents was performed as previously described (18). Briefly, DPPIV deficient rats underwent laparotomy, followed by intraoperative irradiation of all hepatic lobes, except for the caudate lobe. HIR (0–50 Gy) was delivered using a Philips orthovoltage unit operating at 320 kVP, 5 mA, and 0.5 mm copper filtration at 200 cGy/min after shielding of other abdominal organs with 2 mm thick lead shields. Control DPPIV-deficient rats received a sham procedure, where a laparotomy was performed but no HIR was delivered.

HIR (10 Gy) was delivered in anesthetized monkeys, using 6 MV photon energy beams after 3-dimensional computerized tomographic treatment planning via a combination of coplanar and non-coplanar portals that were shaped by multi-leaf collimation to limit the dose to normal structures like kidney and small bowel.

Hepatocyte isolation and transplantation

For rodent studies, donor hepatocytes were isolated from wild-type F344 rats with a modified collagenase perfusion method, as described before (24). Twenty-four hours after HIR and sham operation, 1×10^7 hepatocytes (>80% viability) were injected intrasplenically in anesthetized animals. For primate studies, porcine hepatocytes were isolated, as previously described (25) and either transplanted immediately upon isolation or within 24 hours of isolation after standard cold preservation.

Rodent studies

DPPIV-deficient F344 rats received either HIR (5, 15, 30 and 50 Gy) or sham operation. To determine the effect of HIR on SECs, animals were sacrificed at various time points (2h, 6h, 1, 2, 3, and 7 days; n = 2 – 4 for each time group) and blood was collected for serum chemistry, followed by perfusion of the liver with PBS, and preparing liver tissues from each lobe for cryostat sections and paraffin embedding. Animals that received HT (n=2 for each time point) were sacrificed 1, 3, and 7 days after HT for histological examination of the transplanted hepatocytes in the liver.

Detection of apoptosis

Terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate (dUTP) nick end labeling (TUNEL) staining was performed using ApopTag in situ apoptosis detection kit (Chemicon International, Inc., Temecula, CA), according to manufacturer's instructions. TUNEL-positive apoptotic cells were counted in 20 high power fields (HPF, $\times 400$) from each slide.

Transmission electron microscopy (TEM)

Twenty-four hours after HIR, the rat liver was fixed by perfusion through the portal vein with 0.134 mol/L cacodylate buffer (pH 7.4), followed by 50 ml of a buffer containing 1.5 % glutaraldehyde, 2% paraformaldehyde, 0.067 mol/L cacodylate, and 1% sucrose (pH 7.4) (8). The left lobe was excised and cryopreserved in sucrose gradient (7.5% to 2.3%). Cryosections were postfixated in osmium tetroxide, stained en bloc with 1% uranyl acetate, dehydrated and embedded in epoxy resin. Thirty consecutive sinusoidal areas in 50 nm liver sections were examined using a JEOL transmission electron microscope (Olympus, Tokyo, Japan).

Serum chemistry

Serum levels of alanine aminotransferase (ALT) were measured in a clinical biochemical laboratory as markers of hepatocellular injury. Serum levels of hyaluronic acid (HA) were measured by the sandwich enzyme-binding assay (Corgenix Inc., Westminster, CO) as markers of SEC function.

Kupffer cell assays

Kupffer cells were visualized in 5 μ m cryostat sections using mouse monoclonal ED2 anti-macrophage antibody by the indirect immunoperoxidase technique (26). In each slide, the number of Kupffer cells was counted in 20 consecutive periportal or pericentral areas in a high power field (HPF, $\times 400$). In order to determine the phagocytic activity of Kupffer cells, colloidal carbon clearance test was performed 24 hours after HIR or sham operation, according to protocols published before (27,28). Kupffer cells containing cytoplasmic carbon particles were counted in 20 consecutive periportal or pericentral areas under a HPF ($\times 400$) in each fresh-frozen liver section. Some animals received intravenous gadolinium chloride (GdCl_3 , 10mg/kg body weight) to eliminate Kupffer cells.

Detection of transplanted hepatocytes in rodents

To analyze survival and engraftment of DPPIV positive transplanted cells *in situ*, 5 μ m cryostat sections were stained for DPPIV histochemistry and DPPIV enzyme activity was measured from liver homogenates, using previously published protocols (18). The sections were further stained for ATPase activity to colocalize bile canalicular ATPase and DPPIV activity in integrated donor hepatocytes, using published protocols (8). In each liver section, the number of DPPIV positive transplanted cells was counted in 10 low power fields (LPF,

100×) and in 40 – 80 consecutive periportal areas in HPF (×400). The number of transplanted cell foci per 100 cm² of liver was determined as described before (8).

Porcine albumin ELISA

The in vivo function of engrafted hepatocytes in primates was determined by assaying porcine albumin in recipient plasma every 2–3 days by a sandwich enzyme-linked immunosorbent assay (ELISA) using rabbit anti-pig albumin antibody as the capture antibodies (1:500; Accurate Chemical and Scientific Corp., Westbury, NY) and a horseradish peroxidase-conjugated goat anti-porcine albumin antibody (1:10,000; Bethyl Laboratories, Inc., Montgomery, TX) for colorimetric detection of porcine albumin.

Statistical Analysis

The results were expressed as mean±S.D. A statistical analysis of the data was made by the Mann-Whitney U test.

Results

HIR induces apoptosis and dehiscence of hepatic SE

Since irradiation induces apoptosis in some cell types, such as lymphocytes, salivary gland acinar cells, and intestinal endothelial cells, we wanted to examine the effect of HIR on hepatic SEC death. SEC apoptosis was determined by TUNEL staining after 50 Gy HIR, a dose that induced extensive liver repopulation by engrafted donor cells. TUNEL staining showed few apoptotic cells in normal liver (Fig. 1A). The number of apoptotic SECs increased significantly 6 hours after HIR (Fig. 1B, C and F). In contrast, apoptotic hepatocytes were seen only occasionally (Fig 1D). Twenty-four hours after HIR, the number of apoptotic cells decreased significantly (Fig 1E), suggesting that the apoptotic cells had been cleared, probably by phagocytic cells, such as Kupffer cells.

TEM of sham-irradiated liver demonstrated an intact lining of the normal hepatic sinusoidal endothelium with its characteristic fenestrae (Fig 2A). In contrast, HIR induced nuclear crenellation and chromatin condensation (6–24 hours after HIR), followed by disruption of endothelial cell lining with exfoliation of endothelial cells from the space of Disse in irradiated livers (24–48 hours) (Fig 2B).

HIR increased serum ALT and HA levels transiently

We determined serum ALT and HA levels, which are markers of hepatocellular and SEC injury, respectively (Fig 2C). Serum ALT is released from dying hepatocytes. HA is cleared from the circulation by SECs by receptor-mediated endocytosis, whereby serum HA levels increase with SEC death. Serum ALT levels increased modestly (~2-fold) after HIR, with peak levels at 6 hours (pre-HIR, 57.0±13.7 U/L versus post-HIR, 138.7±8.4 U/L; $p<0.05$), eventually returning to baseline within 2 days. This indicated that the acute effect of HIR on hepatocellular death was minimal. In contrast, serum HA levels started increasing within 6 hrs and peaked at 1 day after HIR (pre-HIR, 9.2±13.2 ng/ml versus post-HIR, 175.5±63.4 ng/ml, $p<0.01$). HA levels remained significantly elevated for 2 days (180.5±51.4 ng/ml versus pre-HIR levels, $p<0.05$), and returned to pre-HIR values in 7 days. The transient elevation in HA levels suggested that the HIR-induced hepatic SEC damage was reversed in a week.

HIR reduced the phagocytic function of Kupffer cells without affecting their numbers

We next examined the effect of HIR on the number and phagocytic activity of intrahepatic resident macrophages, Kupffer cells. Liver sections were stained with ED2, an anti-

macrophage antibody, to estimate the number of Kupffer cells (Fig. 3 A–D). Although a statistical significant difference was not achieved, Kupffer cells tended to be distributed more in periportal areas than in pericentral areas (periportal, 27.4 ± 5.9 versus pericentral, 19.5 ± 3.5 ; $p=0.127$) in control sham irradiated livers (Fig 3A). HIR did not affect the number of Kupffer cells in either pericentral (Fig 3B) or periportal areas (Fig 3C). As expected, administration of GdCl₃ eliminated Kupffer cells almost completely from the liver (Fig. 3D).

In order to evaluate the phagocytic function of Kupffer cells, India ink was injected intravenously and the clearance of colloidal carbon from the blood was measured. As demonstrated in Fig 3J, the clearance of colloidal carbon was decreased after HIR. Since Kupffer cells primarily phagocytose carbon particles from the blood, the clearance of colloidal carbon from the blood can be expressed as the phagocytic index of these cells. The phagocytic index (Fig 3K) of HIR-treated animals was significantly reduced than the sham irradiated controls (HIR, 0.012 ± 0.007 versus sham irradiated, 0.024 ± 0.007 , $p<0.05$). Moreover, light microscopic examination of the liver after colloidal injection demonstrated that the number of periportal Kupffer cells that engulfed carbon particles in HIR-treated animals (Fig 3F) was smaller than the sham irradiated controls (HIR, 24.1 ± 3.8 versus sham irradiated, 32.9 ± 3.3 , $p<0.05$) (Fig. 3E), indicating HIR suppressed the phagocytic function of periportal Kupffer cells.

HIR augments engraftment of transplanted hepatocytes in rat liver

To examine whether SEC disruption and suppression of the phagocytic function of Kupffer cells by HIR enhances donor hepatocyte engraftment, DPPIV-deficient F344 rats received HIR at various doses (sham/0, 5, 15 and 50 Gy), followed in one day by transplantation of DPPIV-proficient F344 donor hepatocytes. The number of engrafted hepatocytes was determined by histochemical staining for DPPIV activity 1, 3 and 7 days after HT. In sham-irradiated livers, 1–2 donor hepatocytes were engrafted in 20–30% of periportal areas, 7 days after HT (Fig. 4a). The number of engrafted hepatocytes increased significantly with frequent clustering of donor cells in 70–80% of the periportal regions in rats receiving HIR. The efficiency of donor cell engraftment increased in a dose-dependent fashion (Sham 0 Gy, 45.7 ± 6.0 cells/cm² versus HIR 5 Gy, 219.3 ± 14.5 cells/cm², $p = 0.0015$, Fig 4b–g). Thus, even at a 5Gy dose, donor hepatocyte engraftment increased 4–5-fold. At the highest tested dose (50 Gy), there was a >70-fold increase in the number of donor-derived hepatocytes (HIR 50 Gy, 3315 ± 37.5 , $p = 0.00009$). Furthermore, in contrast to sham-irradiated livers, donor cells were frequently seen in clusters in irradiated livers by 7 days (Fig 4d, e). Both the number and size of cell clusters increased with HIR dose, indicating that at higher doses HIR not only augmented the entry and engraftment of single or multiple adherent donor cells but also may have promoted selective donor cell proliferation (Fig 4g).

To assess whether the engrafted hepatocytes integrated in the liver parenchyma, liver sections were co-stained with DPPIV and ATPase enzyme histochemistry. Figure 4f demonstrates characteristic linear co-staining with DPPIV and bile canalicular ATPase of integrated hepatocytes in the HIR-treated liver. Although most donor cells entered in hepatic sinusoids or liver plates of HIR-treated animals, these cells did not integrate in the parenchyma on day 1 post-HT (data not shown). However by 7 days there was a significant increase in the number of integrated hepatocytes in HIR-treated animals, compared to sham controls.

HIR induces preferential proliferation of engrafted hepatocytes in response to hepatic mitogenic stimulus

Finally we wanted to examine whether the HIR-induced enhancement of donor cell engraftment results in a faster and preferential repopulation of the host liver by donor cells. To determine the liver repopulation capacity of engrafted hepatocytes, we provided a hepatic mitogenic stimulus by injecting a recombinant adenovirus expressing human HGF (Ad-HGF) intravenously, seven days after HT when engraftment and integration of donor cells in host liver parenchyma is complete. Three months after HT, liver repopulation was evaluated by DPPIV histochemical staining of the liver sections and by measuring DPPIV enzyme activity in liver homogenates. In animals treated with sham irradiation, donor cells failed to repopulate the liver. There was minimal proliferation of donor cells as indicated by small clusters (2–4 hepatocytes) in animals receiving 5 Gy HIR, indicating that lower doses of HIR increases cell engraftment without affording a selective growth advantage to the donor cells. However, in animals receiving 15 and 50 Gy HIR, donor cells preferentially proliferated resulting in extensive repopulation of the host liver (Fig 5A). Compared to sham-irradiated livers, there was a significant increase in DPPIV enzyme activity in animals receiving HIR with a gradual dose response (Fig 5B), indicating that higher doses of HIR enabled preferential proliferation of the engrafted hepatocytes.

HIR augmented engraftment of porcine hepatocytes in cynomolgus monkeys

Encouraged by our findings that low-dose HIR enhanced hepatocyte engraftment in rats, we wanted to develop a clinically feasible protocol of preparative HIR for HT. To validate the HIR effect in non-human primates, in preliminary studies we infused porcine hepatocytes into the portal vein of cynomolgus monkeys after right portal vein embolization (PVE), low-dose HIR (10 Gy), or a combination of HIR and right PVE. PVE was performed to stimulate compensatory hypertrophy of the unoccluded lobes, since portal vein branch occlusion was shown to induce atrophy of occluded hepatic lobes, thereby providing mitotic stimulation (29). All animals received identical immune suppression regimen, which included thymoglobulin, methylprednisolone, and anti-CD25 at the time of HT, and Cyclosporine A, FTY720, RAD, and methylprednisolone, thereafter. The *in vivo* function of transplanted hepatocytes was determined by measuring serum porcine albumin, which was used as a surrogate marker for initial engraftment of donor hepatocytes. As demonstrated in Fig 6, porcine albumin levels in monkeys treated with PVE only increased slightly after HT in a pattern identical with that seen following transplantation in naïve monkeys (data not shown), indicating that PVE does not, by itself, enhance initial engraftment of donor hepatocytes. Preparative HIR, followed by transplantation of porcine hepatocytes, 1 day post-HIR, resulted in a large increase in porcine albumin levels even when the transplanted cells were preserved in cold storage prior to transplantation. PVE and HIR resulted in a 30-fold increase in porcine albumin levels after HT using freshly isolated hepatocytes, confirming the extent to which preparative HIR augments initial engraftment of donor hepatocytes in monkeys, and indicating that engraftment may be further amplified by selective proliferation of engrafted cells induced by PVE.

Discussion

Our results show that, at low doses, preparative HIR of recipient rats enhances the overall efficiency of donor hepatocyte engraftment. HIR disrupted the hepatic sinusoidal endothelial barrier and suppressed the phagocytic function of Kupffer cells transiently, which was, nevertheless, sufficient for facilitating the engraftment of transplanted hepatocytes and integration into liver cords. Enhancement of initial engraftment occurred at low HIR doses (5–10 GY), whereas higher doses were required for subsequent proliferation of the

individual engrafted cells, supporting our hypothesis that the mechanisms of these two effects were different.

Induction of apoptosis of hepatic SECs by low doses of HIR could have facilitated the entry of the donor hepatocytes into the perisinusoidal space of Disse and their migration into the liver plate. Similar effects were reported using agents that induce sinusoidal endothelial cell damage, such as, cyclophosphamide (8) or doxorubicin (10). However, there are some important differences with HIR. First, drugs act upon the whole liver, while the effect of HIR can be spatially conformed to parts of the liver, thereby, augmenting engraftment only in the irradiated liver lobes. Instead of using an agent that induces sinusoidal cell injury in the whole liver, partial liver irradiation increases the safety of the preparative regimen by leaving portions of the normal liver intact. Second, drugs have systemic toxicity, such as, bone marrow toxicity, while local liver irradiation fails to exhibit systemic toxicity. Finally, agents that augment engraftment usually do not induce proliferation of hepatocytes. At lower doses, up to 10 Gy, HIR augments donor cell engraftment. At higher doses, in addition to enhancing engraftment, HIR also reduces the proliferative capacity of host hepatocytes, thereby providing a competitive proliferative advantage to the engrafted, donor cells. Therefore, the two effects of HIR should act synergistically to result in rapid and massive repopulation of the liver, making HIR highly desirable as a preparative regimen for HT.

Although the fenestrated hepatic sinusoidal endothelium allows regulated access of macromolecules to the space of Disse, it is an important physical barrier to the engraftment of transplanted hepatocytes. HIR induced apoptosis of SEC within 6 hours as demonstrated by TUNEL staining. This effect was comparable with that seen in the small intestinal microvascular endothelium, which underwent apoptosis within 4 hours of whole body irradiation in C57Bl/6 mice (30). TEM of rat livers demonstrated that the disruption of the hepatic SEC was first evident by 6 hours after HIR and peaked in 24 – 48 hours. We, therefore, transplanted hepatocytes 1–2 days after HIR. We also determined serum HA levels as a marker of hepatic SEC integrity. HA is a straight-chain glycosaminoglycan polymer, present in the extracellular matrix. More than 90 % of circulating HA is removed by hepatic SECs, via specific HA receptor-mediated endocytosis (31). Increasing levels of circulating HA serves as a biomarker of dysfunction of hepatic SEC following liver injury (27). We found that serum HA levels reached peak values within 24 hours of HIR and HA returned to baseline within 3 – 7 days of HIR, probably due to the restoration of the sinusoidal endothelial barrier by endothelial cell proliferation. It is possible reduced clearance by SECs was compensated over time by enhanced renal excretion, which is the second major mechanism of removal of circulating HA (32).

As the largest population of resident macrophages in the body, Kupffer cells are also reported to play an important role in eliminating transplanted cells shortly after hepatocyte transplantation (7). In contrast to the effect of HIR on SECs, we found that the number of Kupffer cells was not affected by HIR. However, HIR suppressed the phagocytic function of the Kupffer cells resulting in a decrease in colloidal carbon clearance. HIR-induced suppression of the phagocytic activity of Kupffer cells could contribute to improved survival of the transplanted hepatocytes by allowing them more time to engraft and integrate into the hepatic architecture. Since HIR can disrupt endothelial cells and inhibit kupffer cell phagocytosis at the same time, it is difficult to separate the effect of the two events. However, previous studies have demonstrated that elimination of Kupffer cells with systemic administration of gadolinium chloride can, at best, augment hepatocyte engraftment by 2 – 4 fold (7). In contrast HIR can improve hepatocyte engraftment by 5 – 70-fold in a dose-dependent manner, suggesting that in addition to its effect on Kupffer cell

function, HIR enhances engraftment by additional mechanisms, such as, disruption of hepatic sinusoidal endothelium.

Our results demonstrate that at HIR doses as low as 5 Gy, there is significant enhancement of engraftment of the donor hepatocytes in rat liver. Although our rodent studies have demonstrated convincingly the role of HIR in augmenting engraftment and subsequent proliferation of donor hepatocytes, primate studies represent an absolutely necessary step toward the application of preparative hepatic irradiation in humans. This is particularly true because the irradiation effects in the liver vary in different classes of species. Our results demonstrate that as in the case of rodents, a single dose of 10 Gy preparative HIR was sufficient to increase significantly the engraftment of donor porcine hepatocytes in cynomolgus monkeys, as shown by the secretion of porcine albumin in the plasma. Additionally, immunosuppressive effects of the local irradiation could contribute to the improved survival of allogeneic hepatocytes or porcine xenograft. In clinical situations, radiation-induced liver damage occurs after irradiation of the whole liver at doses of 35 Gy or higher. Thus, the ability of low doses of HIR to increase the engraftment and integration of transplanted hepatocytes in rodents and in pilot studies in monkeys is highly significant in terms of potential translation of this strategy to clinical application. Furthermore, the technical feasibility of delivering HIR to isolated regions of the liver, without inducing RILD, makes HIR an attractive preparative regimen for HT-based treatment of inherited liver diseases.

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References

1. Smets F, Najimi M, Sokal EM. Cell transplantation in the treatment of liver diseases. *Pediatr Transplant*. 2008; 12:6–13. [PubMed: 18186884]
2. Ohashi K, Park F, Kay MA. Hepatocyte transplantation: clinical and experimental application. *J Mol Med*. 2001; 79:617–630. [PubMed: 11715065]
3. Fox IJ, Chowdhury JR, Kaufman SS, Goertzen TC, Chowdhury NR, Warkentin PI, Dorko K, et al. Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. *N Engl J Med*. 1998; 338:1422–1426. [PubMed: 9580649]
4. Grompe M. Therapeutic liver repopulation for the treatment of metabolic liver diseases. *Hum Cell*. 1999; 12:171–180. [PubMed: 10834103]
5. Gupta S, Bhargava KK, Novikoff PM. Mechanisms of cell engraftment during liver repopulation with hepatocyte transplantation. *Semin Liver Dis*. 1999; 19:15–26. [PubMed: 10349680]
6. Guha C, Parashar B, Deb NJ, Sharma A, Gorla GR, Alfieri A, Roy-Chowdhury N, et al. Liver irradiation: a potential preparative regimen for hepatocyte transplantation. *Int J Radiat Oncol Biol Phys*. 2001; 49:451–457. [PubMed: 11173140]
7. Joseph B, Malhi H, Bhargava KK, Palestro CJ, McCuskey RS, Gupta S. Kupffer cells participate in early clearance of syngeneic hepatocytes transplanted in the rat liver. *Gastroenterology*. 2002; 123:1677–1685. [PubMed: 12404242]
8. Malhi H, Annamaneni P, Slehria S, Joseph B, Bhargava KK, Palestro CJ, Novikoff PM, et al. Cyclophosphamide disrupts hepatic sinusoidal endothelium and improves transplanted cell engraftment in rat liver. *Hepatology*. 2002; 36:112–121. [PubMed: 12085355]
9. Smets FN, Chen Y, Wang LJ, Soriano HE. Loss of cell anchorage triggers apoptosis (anoikis) in primary mouse hepatocytes. *Mol Genet Metab*. 2002; 75:344–352. [PubMed: 12051966]

10. Kim KS, Joseph B, Inada M, Gupta S. Regulation of hepatocyte engraftment and proliferation after cytotoxic drug-induced perturbation of the rat liver. *Transplantation*. 2005; 80:653–659. [PubMed: 16177641]
11. Yuan RH, Chen HL, Hsu MK, Lee PH, Chang MH. Attenuation of Kupffer cell function in acute on chronic liver injury enhanced engraftment of transplanted hepatocytes. *World J Surg*. 2007; 31:1270–1277. [PubMed: 17437157]
12. Fausto N. Liver regeneration. *J Hepatol*. 2000; 32:19–31. [PubMed: 10728791]
13. Michalopoulos GK, DeFrances MC. Liver regeneration. *Science*. 1997; 276:60–66. [PubMed: 9082986]
14. Milan M. Survival of the fittest. Cell competition in the *Drosophila* wing. *EMBO Rep*. 2002; 3:724–725. [PubMed: 12151328]
15. Simpson P. Parameters of cell competition in the compartments of the wing disc of *Drosophila*. *Dev Biol*. 1979; 69:182–193. [PubMed: 446891]
16. Oertel M, Menthena A, Dabeva MD, Shafritz DA. Cell competition leads to a high level of normal liver reconstitution by transplanted fetal liver stem/progenitor cells. *Gastroenterology*. 2006; 130:507–520. [PubMed: 16472603]
17. Laconi E, Oren R, Mukhopadhyay DK, Hurston E, Laconi S, Pani P, Dabeva MD, et al. Long-term, near-total liver replacement by transplantation of isolated hepatocytes in rats treated with retrorsine. *Am J Pathol*. 1998; 153:319–329. [PubMed: 9665494]
18. Guha C, Sharma A, Gupta S, Alfieri A, Gorla GR, Gagandeep S, Sokhi R, et al. Amelioration of radiation-induced liver damage in partially hepatectomized rats by hepatocyte transplantation. *Cancer Res*. 1999; 59:5871–5874. [PubMed: 10606225]
19. Guha C, Parashar B, Deb NJ, Garg M, Gorla GR, Singh A, Roy-Chowdhury N, et al. Normal hepatocytes correct serum bilirubin after repopulation of Gunn rat liver subjected to irradiation/partial resection. *Hepatology*. 2002; 36:354–362. [PubMed: 12143043]
20. Deb NJ, Kawashita Y, Shah SJ, Roy-Chowdhury N, Liu L, Gorla GR, Vikram B, et al. Selective hepatic lobar repopulation by transplanted hepatocytes after ligation of a portal vein branch and preparative hepatic irradiation. *Hepatology*. 2001; 34:153A.
21. Takahashi M, Deb NJ, Kawashita Y, Lee SW, Furgueil J, Okuyama T, Roy-Chowdhury N, et al. A novel strategy for in vivo expansion of transplanted hepatocytes using preparative hepatic irradiation and FasL-induced hepatocellular apoptosis. *Gene Ther*. 2003; 10:304–313. [PubMed: 12595889]
22. Guha C, Yamanouchi K, Jiang J, Wang X, Roy Chowdhury N, Santana A, Shapiro LJ, et al. Feasibility of Hepatocyte Transplantation-Based Therapies for Primary Hyperoxalurias. *Am J Nephrol*. 2005; 25:161–170. [PubMed: 15849463]
23. Landis CS, Yamanouchi K, Zhou H, Mohan S, Roy-Chowdhury N, Shafritz DA, Koretsky A, et al. Noninvasive evaluation of liver repopulation by transplanted hepatocytes using ³¹P MRS imaging in mice. *Hepatology*. 2006; 44:1250–1258. [PubMed: 17058269]
24. Berry MN, Friend DS. High-yield preparation of isolated rat liver parenchymal cells: a biochemical and fine structural study. *J Cell Biol*. 1969; 43:506–520. [PubMed: 4900611]
25. Nagata H, Nishitai R, Shirota C, Zhang JL, Koch CA, Cai J, Awwad M, et al. Prolonged survival of porcine hepatocytes in cynomolgus monkeys. *Gastroenterology*. 2007; 132:321–329. [PubMed: 17241882]
26. Grinko I, Geerts A, Wisse E. Experimental biliary fibrosis correlates with increased numbers of fat-storing and Kupffer cells, and portal endotoxemia. *J Hepatol*. 1995; 23:449–458. [PubMed: 8655963]
27. Ikeda T, Yanaga K, Kishikawa K, Kakizoe S, Shimada M, Sugimachi K. Ischemic injury in liver transplantation: difference in injury sites between warm and cold ischemia in rats. *Hepatology*. 1992; 16:454–461. [PubMed: 1639355]
28. Saba TM. Physiology and physiopathology of the reticuloendothelial system. *Arch Intern Med*. 1970; 126:1031–1052. [PubMed: 4921754]
29. Ilan Y, Roy-Chowdhury N, Prakash R, Jona V, Attavar P, Guha C, Tada K, et al. Massive repopulation of rat liver by transplantation of hepatocytes into specific lobes of the liver and

- ligation of portal vein branches to other lobes. Transplantation. 1997; 64:8–13. [PubMed: 9233693]
30. Paris F, Fuks Z, Kang A, Capodieci P, Juan G, Ehleiter D, Haimovitz-Friedman A, et al. Endothelial apoptosis as the primary lesion initiating intestinal radiation damage in mice. Science. 2001; 293:293–297. [PubMed: 11452123]
31. Fraser JR, Alcorn D, Laurent TC, Robinson AD, Ryan GB. Uptake of circulating hyaluronic acid by the rat liver. Cellular localization in situ. Cell Tissue Res. 1985; 242:505–510. [PubMed: 4075374]
32. George J, Stern R. Serum hyaluronan and hyaluronidase: very early markers of toxic liver injury. Clin Chim Acta. 2004; 348:189–197. [PubMed: 15369754]

Fig. 1. HIR induces apoptosis in sinusoidal endothelial cells

Tunel staining of rat livers (n=2 in each group) demonstrates few apoptotic cells in unirradiated liver (A). At 6 hours after HIR, scattered apoptotic cells (black arrow) appeared (B) in the liver with frequent TUNEL-stained sinusoidal endothelial cells (C) and occasional TUNEL-stained hepatocytes (D, white arrow). By 24 hours post-HIR the number of apoptotic cells decreased in the liver (E) (Original magnification: A, and E, $\times 100$; B, $\times 400$; C and D, $\times 1000$). F, Bar graph demonstrating TUNEL-stained apoptotic SECs per high power field, counted in 20 high power fields (HPF, $\times 400$) from each slide.

Fig. 2.

Transmission electron micrographs of the liver after HIR, 0 Gy (A) and 50 Gy (B). Unirradiator liver had intact sinusoidal endothelial cell lining with characteristic fenestrae (arrow, A). Twenty four hours after HIR, TEM demonstrates disruption of endothelial cell lining with exfoliation of endothelial cells in irradiated livers (arrow, B). (C) Changes in serum alanine transaminase (ALT) and hyaluronic acid (HA) levels after HIR.

Fig. 3.

A–D. HIR does not affect the number and distribution of Kupffer cells in rat livers. The Kupffer cells were visualized by immunostaining of rat livers (n=2 in each group) using the ED2 anti-macrophage antibody. Livers were harvested one day after treatment with sham irradiation (A), HIR / 50 Gy (B, pericentral and C, periportal) and GdCl₃ injection (D). E–H. HIR decreases the number of carbon-engulfed macrophages. Hematoxylin-eosin stained liver sections demonstrate enlarged carbon-loaded Kupffer cells in sham irradiated (E), HIR-treated (periportal, F and pericentral, G) and GdCl₃-treated (H) livers. Compared to the sham irradiated controls, the number of carbon-loaded Kupffer cells was significantly decreased ($P < 0.05$) in HIR-treated livers, primarily around periportal areas.

I. Bar graph demonstrating the number of Kupffer cells per high power field ($\times 400$) in rat livers. J – K. HIR suppressed the phagocytic function of Kupffer cells. J. Colloidal carbon clearance test. Twenty-four hours after HIR or sham irradiation, India ink was injected into the inferior vena cava and blood sample was drawn at various time points (0.5 – 20 min) to determine serum colloidal carbon content using spectrophotometry. Relative concentration of colloidal carbon in blood, at various time points was measured and expressed as a ratio to the concentration of blood carbon at 0.5 minutes after intravenous injection. **K. Phagocytic index.** The carbon clearance reflects the phagocytic function of Kupffer cells. Compared to sham irradiated controls, HIR suppressed the phagocytic index of Kupffer cells ($p = 0.027$). In each liver section, the number of Kupffer cells and cells containing cytoplasmic carbon particles were counted in 20 consecutive periportal or pericentral areas under a HPF ($\times 400$).

Figure 4. HIR augments the engraftment of transplanted hepatocytes
DPPIV enzyme histochemistry, 7 days post-HT, demonstrates red staining of donor cells in rat livers (n=2 in each group) treated with sham irradiation (a), 5 (b), 15 (c), 30 (d) and 50 Gy (e). Note a dose-dependent increase in the number of engrafted donor hepatocytes in rat liver. At 7 days, transplanted hepatocytes were integrated with host hepatocytes, having linear red color co-localizing bile canaliculae at ATPase positive sites (f). (g) Dose response of HIR on hepatocyte engraftment and repopulation. Transplanted DPPIV-positive hepatocytes were quantitated in recipient livers and engraftment was scored as total of isolated single cells and number of donor cell clusters (>3 hepatocytes). Each donor cell cluster was nominally scored as a single engraftment from a donor hepatocyte. Note that at low doses, HIR promotes donor cell engraftment. However, at doses > 15 Gy, HIR also induces repopulation. In each liver section, the number of DPPIV positive transplanted cells was counted in 10 low power fields (LPF, 100×) and in 40 – 80 consecutive periportal areas in HPF (×400) and expressed as the number of transplanted cell foci per 100 cm² of liver.

Fig. 5. HIR provides a selective growth advantage to the engrafted donor cells in response to a hepatic mitogenic stimulus

F344 rats (n=2 in each group) were treated with either sham irradiation, or increasing doses of HIR (5, 15 and 50 Gy), followed by HT 1 day after HIR. A recombinant adenovirus expressing human HGF was injected intravenously 7 days after HT. DPPIV enzyme histochemistry (A) and enzyme activity (B) demonstrating progressive repopulation of the host liver by the engrafted donor cells 3 months after HT.

Porcine Hepatocyte Graft Survival In Cynomolgus Monkeys

Fig 6.

Serum porcine albumin levels in cynomolgus monkeys following transplantation of porcine hepatocytes in monkeys treated with either PVE only (_ _), HIR (____), or HIR and PVE (----). Animals treated with PVE only or treated with HIR and PVE were transplanted with freshly isolated porcine hepatocytes, while animals with HIR alone received 24hr cold preserved porcine hepatocytes. Porcine albumin levels were not significantly increased by PVE alone, but HIR improved initial engraftment even when the donor hepatocytes transplanted were disadvantaged by cold preservation, resulting in an increase in porcine albumin levels in monkeys treated with HIR, or HIR and PVE.