Amelioration of Radiation-induced Liver Damage in Partially Hepatectomized Rats by Hepatocyte Transplantation

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Abstract

Hepatic tumors often recur in the liver after surgical resection. Postoperative radiotherapy (RT) could improve survival, but curative RT may induce delayed life-threatening radiation-induced liver damage. Because RT inhibits liver regeneration, we hypothesized that unirradiated, transplanted hepatocytes would proliferate preferentially in a partially resected and irradiated liver, providing metabolic support. We subjected F344 rats to hepatic RT and partial hepatectomy with/without a single intrasplenic, syngeneic hepatocyte transplantation. Hepatocyte transplantation ameliorated radiation-induced liver damage and improved survival of rats receiving RT after partial hepatectomy. We further demonstrated that transplanted hepatocytes extensively repopulate and function in a heavily irradiated rat liver.

Introduction

Novel therapeutic strategies are needed to decrease mortalities in patients with liver cancer. Despite apparently complete surgical resection, liver cancer frequently recurs (1). In many carcinomas, such as head and neck, uterus, and breast carcinoma, the combination of surgery and RT has been successful in improving the outcome. However, the major limitation in applying such a strategy to liver cancers is the induction of potentially lethal RILD, which may develop when more than 30–35 Gy of radiation are administered to the whole liver (2, 3). Moreover, the partially resected liver may be more susceptible to RILD because radiation is known to impair liver regeneration in this setting (4). HT has been shown to decrease the mortality in experimentally induced liver failure (5). HT into the liver also ameliorates pyrrolizidine alkaloid-induced liver disease (6), which is associated with veno-occlusive disease (7), similar to that seen in RILD (2). We hypothesized that radiation of partially resected liver will cause hepatocyte injury and suppress liver regeneration, during which transplanted hepatocytes with a normal regenerative potential would proliferate and repopulate the liver. The metabolic support provided by transplanted hepatocytes should ameliorate consequences and mortality associated with RILD. To test this hypothesis, rats were subjected to 68% hepatectomy followed by intraoperative radiation to the residual liver. Hepatocytes isolated from syngeneic donors were then transplanted into the liver by intrasplenic injection, which results in the translocation of a major fraction of the transplanted hepatocytes to the liver with permanent engraftment and function (8, 9). The results indicated that HT ameliorated RILD and significantly improved survival. In addition, there was extensive repopulation of the liver by the transplanted hepatocytes in animals receiving RT + PH.

Materials and Methods

Animals. Male F344 rats weighing 250–300 g were obtained commercially (Charles River Laboratories, NY) and housed in the Institute for Animal Studies at the Albert Einstein College of Medicine. The DPP IV-deficient (DPP-IV−) F344 rats were provided by the Special Animals Core of the Marion Bessin Liver Research Center. The animals were provided with pelleted chow and water ad libitum and were kept under 14-h light/10-h dark cycles. The animal protocols were approved by the institutional Animal Care and Use Committee.

Experimental Design. For survival experiments, 34 male rats were randomly assigned to two experimental groups (Table 1). Rats in one group (n = 17) underwent 68% PH followed immediately by intraoperative whole liver RT with 50 Gy. The dose was selected on the basis of a preliminary radiation dose escalation (15–50 Gy) experiment, which showed that a single 50-Gy RT dose to the liver after PH produced severe RILD and high mortality in F344 rats. Rats in a second group (n = 17) underwent 68% PH + RT as described above and subjected to HT 4 days later. For comparison, five rats received RT without PH, and five rats underwent PH without RT. To identify transplanted cells in the liver of recipients, DPP IV− F344 rats were used. DPP IV− rats underwent HT after PH + RT and were sacrificed at 12 weeks. For comparison, two additional groups of three DPP IV− rats each received HT with or without PH, without any liver RT. Animals from each of these groups were killed at 12 weeks for histological examination of the liver.

Surgical Procedures. Anesthesia was induced by isoflurane inhalation using a closed circuit. The abdomen was opened by a midline incision, and 68% hepatectomy was performed between 8 a.m. and 12 p.m. as described previously (10). During HT, hepatocytes were injected directly into the spleen of rats as described previously (9). Under ether anesthesia, the spleen was exposed, and 5 × 10⁶ hepatocytes suspended in 0.5 ml of RPMI 1640 were injected into the splenic pulp.

Whole Liver Radiation. Immediately after PH, animals were positioned on a specially constructed polystyrene (aquaplast) platform. A jig was aligned on aquaplast and separated into two compartments through which a longitudinal port (5.0 × 7.0 cm) is accessible for irradiation. Two 3 × 4-cm lead shields (2 mm thick) were wedged beneath the liver and overlaying the stomach and intestines without compressing the hepatic and aortic vessels. A 320 MGC Philips orthovoltage unit operating at 320 kVP, 10 mA, and 0.5 mm copper filtration was used (320 cGy/min hepatic exposure to the midline at a 2-cm depth within the jig at a 35 cm-source to-surface distance). Thermoluminescence dosimetry was used for a liver phantom within the jig as the basis for all corrected dose calculations. After irradiation, animals were examined daily. Rats with severe cachexia or moribund condition were euthanized and recorded as non-survivors.

Hepatocyte Isolation and Perfusion. Cells were isolated with a modified collagenase perfusion method using male F344 rats, as originally described by Berry and Friend (11). After liver dissociation, cells were filtered through an
80-μm Dacron mesh and washed twice at 50 × g for 1 min each. Cell viability was determined by trypan blue dye exclusion. Hepatocytes with >90% viability were used for transplantation.

**Histological Analysis.** Liver was embedded in OCT, frozen in liquid nitrogen, and stored at −70°C or fixed in formalin for paraffin embedding and standard H&E staining. Reticulin and trichrome stains were performed in a standard histopathology laboratory. To analyze DPP IV activity *in situ*, 5-μm-thick cryostat sections were fixed in chloroform and acetone (1:1, v/v) for 10 min at 40°C, as described previously (12). After air drying, sections were incubated for 30 min at room temperature in a solution containing 0.4 mg glycyl-L-proline-4-methoxy-2-naphthylamide, along with 1 mg Fast Blue B salt in PBS (pH 7.4). The reaction was terminated by washing with water and sections were counterstained with hematoxylin.

**Statistical Analysis.** Operative mortality was regarded as any death occurring within the first 4 days of PH and radiation, and these animals were excluded from survival analysis. Time-adjusted survival of animals was analyzed by the Kaplan-Meier method (13). The significance of differences was analyzed by the log-rank test, with *P* < 0.05 considered as significant.

**Results and Discussion**

Intraoperative RT has been used to improve local control for a variety of gastrointestinal cancers and could be used to deliver a high dose of radiation to the tumor resection bed and adjacent liver tissues harboring micrometastasis with shielding of other abdominal organs. Unfortunately, in therapeutically effective doses, hepatic irradiation causes severe RILD, often resulting in death after 3–4 months. The purpose of this study was to begin to examine whether HT could modify this process. Because RILD usually manifests itself within 4 months after irradiation, we observed irradiated animals for at least 120 days or until they became moribund.

**Survival.** During the 120 days of observation, none of the rats subjected to PH alone or to RT alone died. Seventy percent of rats (12 of 17) receiving RT + PH died in 12 weeks. In the RT + PH + HT group, the mortality was reduced to 35% (6 of 17; Fig. 1). The median survival in the RT + PH group was 65 days (8 of 17 died during the first 6 weeks) and >120 days (*P* = 0.02) in the RT + PH + HT group (only 1 of 17 rats died during the first 6 weeks).

**Histopathological Changes.** Examination of paraffin sections of formalin-fixed liver biopsy specimens showed minimal changes in the animals subjected to RT without PH, including occasional foci of inflammatory cells and mild microvesicular steatosis. In animals that received RT + PH and died within the first 6 weeks, severe histopathological changes of RILD, including extensive loss of hepatocytes (Fig. 2A), and various degrees of micro- and macrovesicular steatosis in centrilobular areas (Fig. 2B) were seen. The predominantly centrilobular steatosis was reminiscent of other forms of liver injury, including oxidative stress as in alcoholic or toxic hepatitis (14) and the perivenous hepatocellular loss that has been reported in humans (2). In animals in the PH + RT group that survived beyond 12 weeks, the steatosis was less pronounced, but focal hepatic necrosis and various degrees of portal fibrosis and bile duct proliferation were seen (Fig. 2, D and E). Hepatocytes with multiple nuclei, nuclear pleomorphism, and megalonuclei were observed. These findings indicated the accumulation of postmitotic hepatocytes (15), suggesting a radiation-induced cell cycle block.

In the PH + RT + HT group, only 1 animal died during the first
6 weeks. Therefore, to examine histological changes at early time points, six additional rats were subjected to PH + RT + HT, of which 3 were sacrificed 1 and 3 weeks later, respectively. Hepatic histopathological changes were much less prominent (Fig. 2C) in this group than in rats undergoing PH + RT. There was no steatosis, although two rats showed minimal loss of centrizonal hepatocytes. Transplant recipients in the PH + RT + HT group, which survived more than 12 weeks, did not develop the late histopathological changes characteristic of RILD, such as bile duct proliferation and fibrosis (Fig. 2F). In contrast, livers of rats in the PH + RT + HT group that died after 7 weeks manifest features of RILD including centrizonal steatosis, cell loss, and bile duct proliferation.

The extensive bile duct proliferation seen in animals undergoing PH + RT without HT may have arisen from preexisting bile duct epithelium or may represent the proliferation and differentiation of putative periportal stem cells. After PH, the lost liver mass is normally replaced by the proliferation of mature hepatocytes, but when the mature hepatocytes lose their proliferative capacity, such as after 2-acetylaminofluorane treatment (16), proliferation of periportal stem cells comes into play. Radiation could induce cell cycle block and thereby inhibit hepatocyte regeneration. The increased cell loss seen in RILD over a prolonged time period could be the result of the postmitotic cell death of injured hepatocytes. It is possible that the failure of the irradiated hepatocytes to undergo compensatory regeneration results in the expansion of the periportal stem cells, leading to bile duct proliferation. With continuing hepatocellular loss in animals receiving PH + RT, proliferative stimuli remain in the host liver, which allows nonirradiated transplanted hepatocytes to eventually proliferate and extensively repopulate the host liver. In long-term survivors after HT, the lost liver mass is replenished by proliferation of the transplanted hepatocytes, which may have prevented the compensatory expansion of periportal stem cells and consequent bile duct proliferation. In contrast, the presence of bile duct proliferation in animals that died after receiving PH + RT and HT could be due to failure of engraftment and/or repopulation of the transplanted hepatocytes in these animals. Repeated HT has been shown to enhance liver repopulation (17) and might further improve the survival rate in irradiated rats.

**Engraftment and Liver Repopulation by the Transplanted Cells.** For histochemical identification of the engrafted hepatocytes within the host liver, hepatocytes obtained from normal F344 rats were transplanted into congenic DPP IV − F344 recipients (12). DPP IV staining of cryostat sections of DPP IV − host livers will only stain DPP IV + donor hepatocytes (red staining of plasma membranes). Liver sections from recipients subjected to PH without RT showed only isolated donor cells scattered within the liver cords (Fig. 3, A and B). In contrast, in the RT + PH + HT group, by 12 weeks, there was a massive proliferation of the donor hepatocytes, repopulating most of the liver (Fig. 3, C and D). For biochemical evaluation of the extent of liver repopulation by...
the DPP IV+ donor hepatocytes, DPP IV activity was assayed in liver homogenates. Untreated DPP IV− rat livers showed no DPP IV activity. The fraction of the hepatocyte mass replaced by the progeny of transplanted hepatocytes was estimated by comparing hepatic DPP IV activities in recipients with those in the DPP IV+ donors. Donor hepatocytes constituted 76.9 ± 3.9% by 12 weeks in rats in the RT + PH + HT group. In contrast, among the rats that received PH without RT, the donor cells accounted for only 12.7 ± 1.8% of the recipient liver hepatocytes after 12 weeks. The difference between the irradiated and the nonirradiated groups was significant (P < 0.004). These results show that irradiation is required for inducing preferential proliferation of the transplanted hepatocytes because PH alone did not result in hepatocyte repopulation of the host liver. Continued loss of the irradiated hepatocytes over a prolonged period provides sustained proliferative stimulus to the transplanted cells, which does not occur after PH alone, because in the latter case, the regeneration is complete in less than 1 week.

In conclusion, we demonstrate that the combination of PH and whole liver irradiation leads to extensive RILD in rats, which should allow systematic analysis of mechanisms involved in RILD. Furthermore, we demonstrate that HT has the potential to ameliorate the consequences of RILD with improved survival of animals. The capability of transplanted hepatocytes to divide repeatedly and repopulate the host liver has been shown in inherited disorders that cause the death of mature hepatocytes, such as in Alb-uPA transgenic mice (18), in Long Evans Cinnamon rats that have hepatocellular copper overload (19), and in fumarylactoacetate hydrolase-deficient mice (20). In this study, we show that such massive proliferation and repopulation of the liver can also occur in RILD after PH + RT. Our rat model exhibits many characteristic features of human RILD, including centrilobular hepatocellular injury and atrophy. However, events associated with veno-occlusive disease, such as the formation of platelet lakes and the fibrin deposits that are seen in human RILD, were not found in this model. The ability of transplanted hepatocytes to proliferate, repopulate, and function in a heavily irradiated rat liver warrants investigation into whether such a strategy could ameliorate RILD and benefit patients requiring hepatic resection and adjutant RT for intrahepatic malignancies. Whether targeted ablation of the liver with radiation could help increase liver repopulation with hepatocyte transplantation in noncancer settings is another possibility worth considering.

References
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