Monocrotaline Promotes Transplanted Cell Engraftment and Advances Liver Repopulation in Rats via Liver Conditioning

Brigid Joseph,[1] Vinay Kumaran,[1] Ekaterine Berishvili,[1] Kuldeep K. Bhargava,[2] Christopher J. Palestro,[2] and Sanjeev Gupta[1]

Disruption of the hepatic endothelial barrier or Kupffer cell function facilitates transplanted cell engraftment in the liver. To determine whether these mechanisms could be activated simultaneously, we studied the effects of monocrotaline, a pyrollizidine alkaloid, with reported toxicity in liver sinusoidal endothelial cells and Kupffer cells. The effects of monocrotaline in Fischer 344 rats were examined by tissue morphology, serum hyaluronic acid levels, and liver tests (endothelial and hepatocyte injury) or incorporation of carbon and ^{99m}Tc-sulfur colloid (Kupffer cell damage). To study changes in cell engraftment and liver repopulation, Fischer 344 rat hepatocytes were transplanted into syngeneic dipeptidyl peptidase IV–deficient rats followed by histological assays. We observed extensive endothelial injury without Kupffer cell or hepatocyte damage in monocrotaline-treated rats. Monocrotaline enhanced transplanted cell engraftment without changes in transplanted cell numbers or induction of proliferation in native hepatocytes over 3 months. In monocrotaline-treated rats, transplanted cells integrated into the liver parenchyma and survived in vascular spaces. To determine whether native hepatocytes suffered inapparent damage after monocrotaline, we introduced further liver injury with carbon tetrachloride subsequent to cell transplantation. Monocrotaline sensitized the liver to carbon tetrachloride–induced necrosis, which advanced transplanted cell proliferation, leading to significant liver repopulation. During this process, we observed proliferation of bile duct cells and small epithelial cells, although transplanted hepatocytes did not appear to reconstitute bile ducts. The studies showed that perturbation of multiple liver cell compartments by monocrotaline promoted transplanted cell engraftment and proliferation. In conclusion, development of drugs with monocrotaline-like effects will help advance liver cell therapy. (HEPATOLOGY **2006;44:1411-1420.**)

Many insights in mechanisms of transplanted cell engraftment and proliferation are necessary for improving results of liver-directed cell therapy. Recent studies established that cells engraft in the liver through complex mechanisms with roles in this process for hepatic sinusoidal vasomotor tone, as well as specific cell types, including liver sinusoidal endothelial cells (LSECs), Kupffer cells, and hepatic stellate cells.[1-6] Consistent with these mechanisms, manipulations aimed at sinusoidal vasodilatation, disruption of the hepatic endothelial barrier, modification of the extracellular matrix receptors in LSECs, and depletion of the Kupffer cell activity significantly improved transplanted cell engraftment. This has major effects on the kinetics of liver repopulation, which can be accomplished by various types of injury in native hepatocytes for conferring selective proliferation advantages to transplanted cells.[7-11]

It should be appropriate to consider whether simultaneous application of modifying influences would further improve transplanted cell engraftment and proliferation. For instance, previous studies have established that the plant-derived pyrrolizidine alkaloid, monocrotaline (MCT), causes widespread endothelial toxicity in the

Abbreviations: LSEC, liver sinusoidal endothelial cell; MCT, monocrotaline; DPPIV, dipeptidyl peptidase IV; CCl_4, carbon tetrachloride; ALT, alanine aminotransferase.

From the [1]Departments of Medicine and Pathology, Marion Bessin Liver Research Center, Cancer Research Center, Diabetes Center, and General Clinical Research Center, Jack and Pearl Resnick Campus, and the [2]Division of Nuclear Medicine, Long Island Jewish Medical Center Campus, Albert Einstein College of Medicine, Bronx, NY.

Received February 10, 2006; accepted August 22, 2006.

Supported in part by National Institutes of Health Grants R01 DK46952 and P30 DK41296.

Address reprint requests to: Sanjeev Gupta, M.D., Albert Einstein College of Medicine, Ullmann Building, Room 625, 1300 Morris Park Avenue, Bronx, NY 10461. E-mail: sanjvgupta@pol.net; fax: 718-430-8975.

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.21416

Potential conflict of interest: Nothing to report.

lung, liver, and kidney.[12] MCT reproduced changes associated with hepatic veno-occlusive disease in rat liver and additionally depleted Kupffer cells capable of reacting with ED2 antibody,[13] which recognizes the CD163 scavenger receptor antigen. This is noteworthy because gadolinium chloride also depleted ED2-positive Kupffer cells,[14] and such Kupffer cell depletion promoted engraftment of transplanted cells in the liver, consistent with an inhibitory role of Kupffer cells in this process.[3] In larger doses, MCT caused hepatocyte apoptosis,[15] whereas in lower doses, it promoted genotoxic DNA adduct formation in hepatocytes.[16] Hepatic genotoxicity after radiation and partial hepatectomy profoundly impaired the replication capacity of hepatocytes.[17] Similarly, the combination of partial hepatectomy and MCT promoted transplanted cell proliferation.[18] Another pyrrolizidine alkaloid, retrorsine, shares with MCT this property of inducing transplanted cell proliferation and has been useful for investigating liver repopulation mechanisms.[2,3,6,7] Therefore, we considered that MCT will be useful for defining the role of multiple cell compartment-specific perturbations in transplanted cell engraftment and proliferation. In this study, we addressed questions concerning the effect of MCT on LSECs, Kupffer cells, hepatocytes, and other cells in hepatocyte transplantation. We used the well-established rat hepatocyte transplantation system, in which transplanted cells are readily identified in mutant dipeptidyl peptidase IV–deficient (DPPIV−) F344 rats by morphological and molecular assays.[1-7]

Materials and Methods

Chemicals. MCT, carbon tetrachloride (CCl_4), mineral oil, and chemicals or reagents were obtained from Sigma Chemical Co. (St. Louis, MO). MCT was dissolved in normal saline and injected intravenously via the spleen in single doses of 160-200 mg/kg body weight. This route of administration was chosen to ensure first-pass delivery of the substance to the liver. Control animals received only saline. CCl_4 was diluted in mineral oil (1:1 v/v), and 1 mL of CCl_4 per kilogram was injected intramuscularly. Antibodies were against Ki67 (mouse monoclonal, 550609; BD PharMingen, San Diego, CA), OV-6 (mouse monoclonal, a kind gift from Dr. H. A. Dunsford), CK-19 (MO80 29M; Biodesign, Saco, ME), and CD45 (mouse monoclonal, 550566; BD PharMingen). Peroxidase-conjugated goat anti-mouse immunoglobulin G was obtained from Sigma Chemical Co. (Cat #3682). Diaminobenzidine color development was performed using a commercial kit (K3465; Dako Corp., Carpinteria, CA).

Animals. Rats 8-10 weeks of age weighing 120-150 g were used. Donor F344 rats were obtained from the Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). The Special Animal Core of Marion Bessin Liver Research Center provided DPPIV− F344 rats. Animals were housed under 14:10–hour light/dark cycles with unrestricted access to water and pelleted chow (PMI Nutrition International, Brentwood, MO). The Animal Care and Use Committee at the Albert Einstein College of Medicine approved animal protocols according to National Research Council guidelines (*Guide for the Care and Use of Laboratory Animals*, United States Public Health Services, revised 1996).

Assessment of Hepatic Endothelial Injury. Livers were perfused through the portal vein from a 60-cm height with 30-40 mL of 0.144 mol/L cacodylate buffer followed by 60 mL 1.5% glutaraldehyde in cacodylate buffer. Liver samples were further incubated in glutaraldehyde with resin embedding and orcein staining as previously described.[2-4] Ultrathin sections were examined under a JOEL transmission electron microscope (Olympus, Tokyo, Japan). Endothelial integrity was graded under ×1,000 magnification in 50 consecutive sinusoids per rat (n = 3) as (1) normal sinusoids (endothelial lining intact or <25% of the sinusoid without endothelium), (2) partial endothelial damage (25%-70% of the sinusoidal endothelium lost along with morphological damage in endothelial cells), or (3) total endothelial damage (endothelial cells lost completely).

Analysis of Kupffer Cell Function. To demonstrate phagocytosis of carbon, Pelican no. 17 India ink (Hannover, Germany) was centrifuged at 2,000*g* for 15 minutes and supernatant was mixed 1:5 (v/v) with normal saline containing 1% gelatin (Bio-Rad Labs, Richmond, CA), followed by intrasplenic injection of 0.1 mL (n = 3 each). After 30 minutes, animals were sacrificed and liver samples were frozen in methylbutane at −80°C for cryosections. Kupffer cells containing carbon in zone 1 (periportal) of 100 consecutive liver lobules per sample were graded as follows[3,4]: grade 1, minimal carbon incorporation; grade 2, carbon incorporated to the extent observed maximally in healthy rats; or grade 3, carbon incorporated more than the maximal extent observed in healthy rats. For assessing global Kupffer cell phagocytosis, we determined ^{99m}Tc-sulfur colloid incorporation with a commercial kit (Sulfur-colloid TechneScan, CIS-USA, Bedford, MA) as previously described.[3] Rats were given 100 μCi of ^{99m}Tc-sulfur colloid intrasplenically followed by gamma imaging for 30 minutes. Time-activity curves in hepatic regions of interest were then obtained.

Cell Isolation and Transplantation. Hepatocytes were isolated by standard two-step collagenase perfusion of the liver as previously described.[5] Cells were trans-

planted only when >80% excluded 0.2% trypan blue dye. For transplantation, 1×10^7 fresh hepatocytes were suspended in 0.5 mL serum-free RPMI 1640 medium and injected into splenic pulp over 10-15 seconds. Hemostasis was secured with a ligature around the lower pole of the spleen.

Identification of Transplanted Cells. Multiple liver lobes were sampled and frozen in methylbutane at −80°C. Cryosections of 5 μm thickness were fixed in chloroform acetone (1:1, vol/vol) at 4°C for 10 minutes, air-dried for 30 minutes at room temperature and subjected to DPPIV histochemistry as previously described.[1-6] Integration of transplanted cells in the liver parenchyma was analyzed by colocalization of bile canalicular DPPIV and ATPase activities, as described previously.[19] Transplanted cell numbers were determined by morphometry using multiple sections from various liver lobes per animal (n = 4-6 each). Typically, 100 fields centered on consecutive portal areas were scored under ×100 magnification. To analyze liver repopulation, sections were stained for DPPIV and microphotographs were obtained under ×40 magnification from multiple liver lobes per rat (n = 6) using a Spot RT digital camera (Diagnostic Instrument Inc., Sterling Heights, MI). The area occupied by transplanted cells was measured with ImageJ software (National Cancer Institute, Bethesda, MD).

Characterization of Liver Cells. Tissues were costained for DPPIV and ATPase activities to identify biliary cells expressing only ATPase. Expression of γ-glutamyltranspeptidase expression was demonstrated using previously described histochemical methods.[20] Tissue immunostaining was performed to localize CK-19 (primary antibody, 1:10; secondary antibody, 1:600), Ki67 (primary antibody, 1:500; secondary antibody, 1:150), OV-6 (primary antibody, 1:50; secondary antibody, 1:600), and CD45 (primary antibody, 1:10; secondary antibody, 1:600). For negative controls, primary antibody was omitted. For Ki67 staining, positive controls were from archival frozen tissue obtained 30 hours after two-thirds partial hepatectomy in F344 rats.

Serological Assay. Blood was collected from rats 6 hours, 1 day, and 2 days after MCT or saline treatment. Serum was separated and stored at −20°C. Hyaluronic acid content was measured with a commercial hyaluronic acid–binding protein sandwich assay (Corgenix, Inc., Westminster, CO) according to the manufacturer's instructions.[21] Serum alanine aminotransferase (ALT), alkaline phosphatase, and total bilirubin were measured using an automated clinical system.

Statistical Analysis. Data are expressed as the mean ± SD. Student *t* test and ANOVA with a Holm-Sidak test for pairwise comparisons of mean responses in different treatment groups were used for comparing data (SigmaStat 3.1; Jandel Scientific, San Rafael, CA). A *P* value of less than .05 was considered significant.

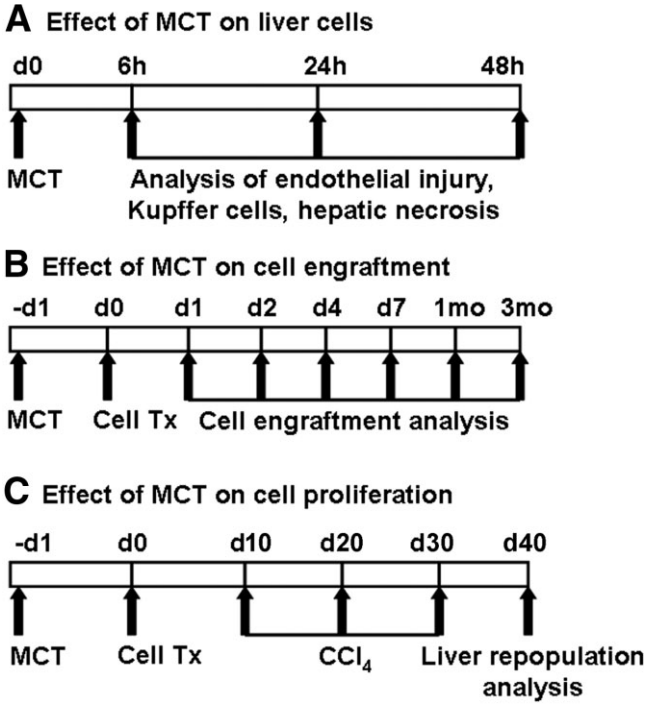

Fig. 1. General experimental design. (A) Depicts the strategy to administer MCT or saline to animals followed by analysis of perturbations in various cell types at intervals of up to 48 hours. (B) The effect of MCT on cell engraftment and proliferation was studied by transplanting cells 1 day after saline or MCT treatment followed by sacrificing animals at intervals shown for tissue analysis. (C) The effect of MCT on cell proliferation was further studied by giving CCl_4 thrice at 10-week intervals commencing 10 days after cell transplantation. Changes in transplanted cell proliferation were demonstrated by morphometric analysis. Control rats received saline alone. MCT, monocrotaline; Tx, transplantation; CCl_4, carbon tetrachloride.

Experimental Design. To identify effective MCT doses, we studied endothelial injury by electron microscopy in rats treated with 160, 180, and 200 mg/kg MCT versus saline-treated controls (n = 3 each). Subsequent studies incorporated a 200-mg/kg dose of MCT. The hepatotoxicity of MCT was studied in rats treated with saline (n = 9) or MCT (n = 12) after 6, 24, and 48 hours (n = 3 and 4, respectively) (Fig. 1A). These analyses used measures of endothelial injury, carbon incorporation in Kupffer cells, and identification of hepatocellular damage. ^{99m}Tc-sulfur colloid incorporation was assessed in additional saline- or MCT-treated rats after 24 and 48 hours, respectively (n = 3-4 each). To analyze cell engraftment, transplanted cell numbers and their location in liver parenchyma or intravascular spaces were determined in saline- or MCT-treated rats 1, 2, 4, and 7 days as well as 1 and 3 months after cell transplantation (n = 3-5 per

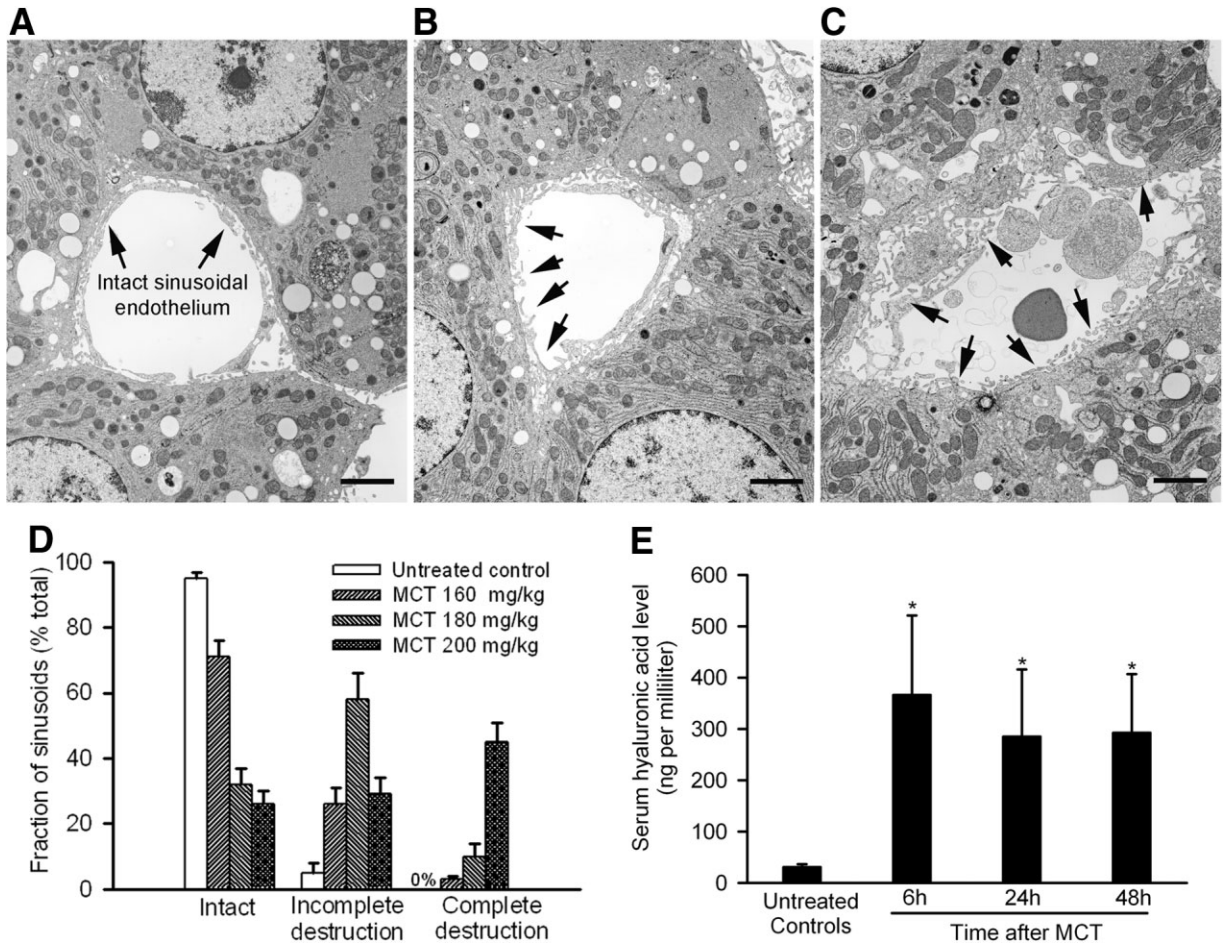

Fig. 2. MCT-induced liver sinusoidal endothelial injury. (A-C) Electron microphotographs show (A) control rat liver with intact sinusoidal endothelium, (B) rat liver 24 hours after 200 mg/kg MCT with partial endothelial loss and grade 2 injury (arrows), and (C) total loss of endothelium with grade 3 injury (arrows) (n = 3 each). (Original magnification ×2000; bar = 2 μm.) (D) Endothelial injury in control and MCT-treated rats after 48 hours. (E) Serum hyaluronic acid levels in control rats and rats treated with 200 mg/kg MCT (n = 3 each). *$P < .001$ versus controls. MCT, monocrotaline.

group per time) (Fig. 1B). In addition, transplanted cell proliferation was studied after 1 or 3 months. To determine whether MCT perturbed native hepatocytes in the long term, we examined the effect of CCl_4, which was administered at 10-day intervals 10 days after cell transplantation (Fig. 1C). Controls received saline alone followed by cell transplantation (n = 6 per experiment). Changes in transplanted cell numbers were analyzed 10 days after final CCl_4 administration. To demonstrate synergism between MCT and CCL_4-induced hepatotoxicity, we established groups of rats (n = 6 each) with saline or MCT treatment, followed 7 days later with CCl_4. In these animals, we assessed serum ALT and histological grading of hepatic inflammation as previously described[22] 1 day after CCl_4 administration. Except for studies lasting 3 months, experiments were repeated at least twice. Repeat experiments incorporated controls to permit data comparisons within each experiment, as well as across animal groups.

Results

MCT and Liver Sinusoidal Endothelial Cell Injury. Initial studies established that a 200-mg/kg dose of MCT was most effective at producing endothelial injury (Fig. 2A-D). For instance, within 24 hours after administration of 200 mg/kg MCT, endothelium was totally denuded (grade 3 injury) in 50% liver sinusoids compared with such injury in only 10% and 3% sinusoids after administration of 180 or 160 mg/kg MCT, respectively ($P < .001$, Student t test; n = 3 each). The pattern of endothelial injury was similar 48 hours after MCT administration at these doses. Serum hyaluronic acid measurements verified these findings and indicated that MCT-induced endothelial injury manifested rapidly—as early as 6 hours (Fig. 2E)—when hyaluronic acid levels were 12-fold greater than controls (367 ± 154 ng/mL vs. 31 ± 5 ng/mL; $P < .001$, Student t test). The serum hyaluronic acid levels were 9-fold above normal 24 and 48

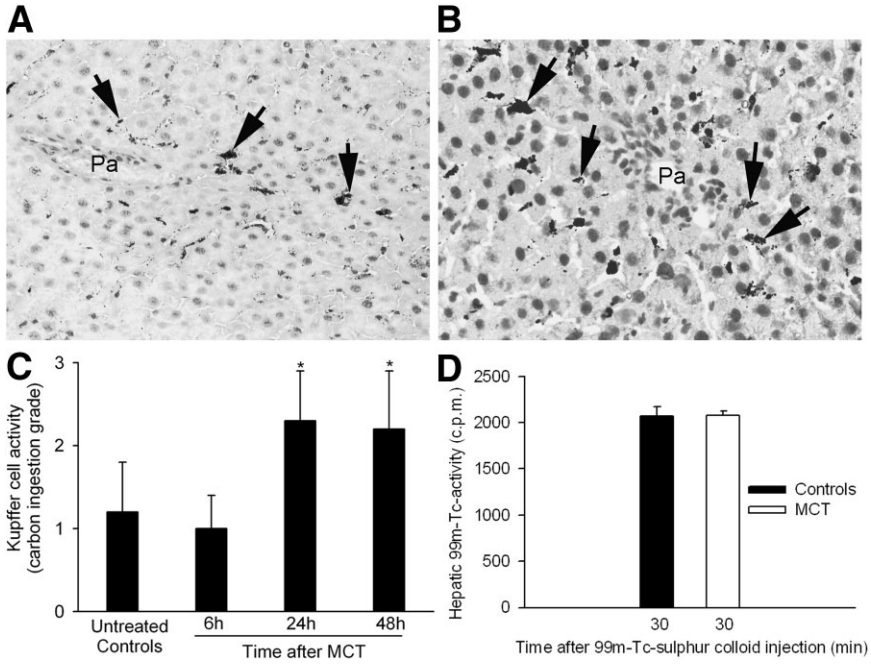

Fig. 3. Effect of 200 mg/kg MCT on Kupffer cells. (A,B) Typical appearance of Kupffer cells with carbon (arrows) adjacent to a portal area in (A) control rats treated with saline and (B) rats treated 24 hours earlier with MCT. (C) Morphometric analysis of carbon content in periportal Kupffer cells in saline-treated controls and MCT-treated rats (n = 3 each). *$P <$ .001 versus controls. (D) Incorporation of sulphur colloid in the liver of control rats and MCT-treated rats 24 hours after manipulations (n = 3 and 4 each, respectively). Pa, portal area; MCT, monocrotaline.

hours after MCT administration (286 ± 130 ng/mL and 293 ± 114 ng/mL, respectively; $P <$ .001, Student *t* test). Use of 200 mg/kg MCT did not produce mortality in rats.

MCT and Hepatocyte or Kupffer Cell Injury. In MCT-treated rats, morphological analysis of liver showed no obvious hepatocyte injury between 6 hours and 7 days after treatment, despite administration of up to 200 mg/kg MCT. This was verified by analysis of liver tests. For instance, 24 hours after administration of 200 mg/kg MCT, when endothelial injury was already pronounced, serum ALT levels in controls and MCT-treated rats were 31 ± 2 U/L and 53 ± 24 U/L, respectively (*P* value not significant), and total serum bilirubin was 0.9 ± 0.7 mg/dL and 0.5 ± 0.4 mg/dL, respectively (*P* value not significant).

Kupffer cell activity was unimpaired in MCT-treated rats, despite previous reports indicating depletion of ED2-reactive Kupffer cells after MCT administration.[13] Kupffer cells efficiently incorporated carbon in MCT-treated rats, with a higher grade of phagocytotic activity in periportal areas compared with control animals (Fig. 3A-C). Analysis of panhepatic Kupffer cell activity using ^{99m}Tc-sulphur colloid showed no differences in the hepatic accumulation of sulphur colloid in MCT-treated and control rats (Fig. 3D).

MCT Affects Engraftment of Transplanted Hepatocytes. In MCT-treated rats, more transplanted cells were observed in intravascular spaces, as well as within the liver parenchyma at all times (Fig. 4A-B). The increase in transplanted cell numbers was apparent after 1 day and also after 2, 4, or 7 days and 1 or 3 months after cell transplantation. In control rats, 1, 2, 4, and 7 days after transplantation, the number of transplanted cells in 50 consecutive liver lobules was within a steady range, with 101 ± 26, 108 ± 23, 98 ± 28, and 90 ± 9 transplanted cells, respectively (*P* value not significant; n = 4-6 rats each) (Fig. 4C). The corresponding transplanted cell number 1 month after transplantation was 129 ± 10, which was 1.3 ± 0.1–fold greater than at earlier times. In MCT-treated rats 1, 2, 4, and 7 days and 1 month following cell transplantation, we observed 7 ± 0.5–, 6 ± 0.2–, 8 ± 0.8–, 8 ± 0.7–, and 8 ± 0.1–fold more transplanted cells, respectively, in the liver parenchyma compared with corresponding controls ($P <$ .001, ANOVA with Holm-Sidak test; n = 4-6 rats). The transplanted cell number in the liver parenchyma in MCT-treated rats was 1.4 ± 0.2–fold greater after 1 month compared with the first 7 days after cell transplantation, although this was similar to that seen in control rats. In animals followed for up to 3 months after cell transplantation, transplanted cells did not show proliferation in either control or MCT-treated rats (Fig. 4A-B). The fraction of portal vein radicles containing transplanted cells increased in MCT-treated rats compared with control rats, on average by 1.7- to 2-fold during the course of 1 day to 7 days and 1 month after cell transplantation ($P <$ .001; ANOVA with Holm-Sidak test) (Fig. 4D). Similarly, more portal vein radicles contained transplanted cells in MCT-treated rats after 3 months (Fig. 4A-B). On the other hand, compared with the steady range and even some increase in transplanted cell numbers in the liver parenchyma, the fraction of portal vein radicles containing transplanted cells declined over time in controls, as well as in MCT-treated rats (Fig. 4D), suggesting the relative inadequacy of transplanted cell survival in this intravascular location. The

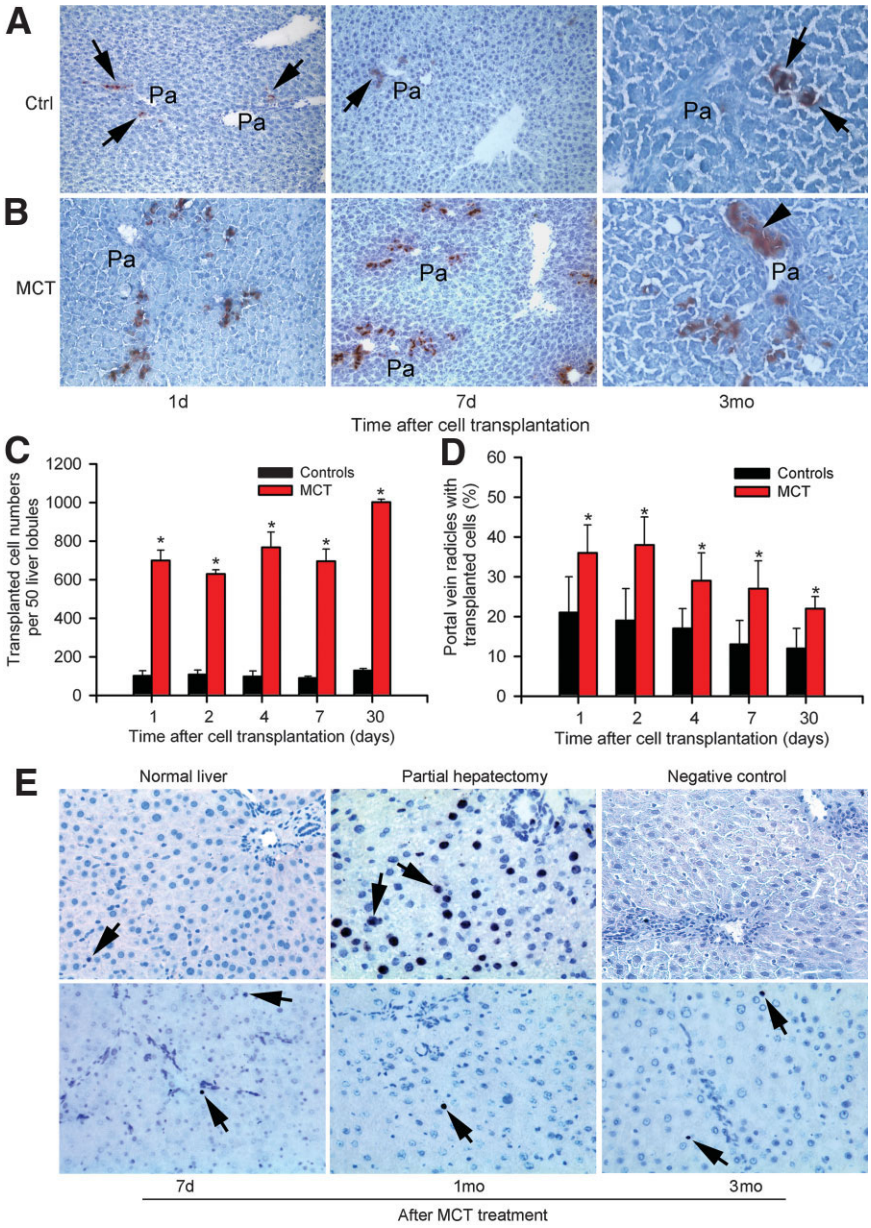

Fig. 4. Changes in cell engraftment following 200 mg/kg MCT. (A,B) Transplanted cells with DPPIV histochemistry in saline-treated and MCT-treated rats. Arrows indicate the periportal location of transplanted cells; the arrowhead indicates transplanted cells in the portal vein radicle after 3 months. (Original magnification ×200; far right panels, ×400 [toluidine blue counterstain].) (C) Morphometric analysis showing several-fold more transplanted cells in the liver parenchyma of MCT-treated rats. (D) Morphometric analysis of the fraction of portal vein radicles containing transplanted cells. (E) Absence of increase in Ki67-expressing cells (arrows) in rats treated with MCT followed by cell transplantation. In contrast with rat liver after partial hepatectomy, only rare cells expressed Ki67 in normal rat liver and MCT-treated rat liver. *$P < .001$ versus corresponding controls. (Original magnification ×400 [toluidine blue counterstain].) Ctrl, control; MCT, monocrotaline; Pa, portal area.

absence of transplanted cell proliferation in MCT-treated cell recipients suggested a lack of increased hepatocyte turnover in animals. This was verified by Ki67 expression in tissues (Fig. 4E).

Because disruption of the hepatic endothelial barrier promotes cell engraftment in the liver,[2,4] we studied the kinetics of transplanted cell engraftment. Histochemical staining for DPPIV and ATPase activities permitted identification of bile canalicular domains in transplanted and native hepatocytes, respectively, to determine whether plasma membrane structures were promptly reconstituted and transplanted cells integrated sooner in the liver in MCT-treated rats. Transplanted cells showed reconstitution of bile canaliculi more often after 1 or 2 days in MCT-treated rats (62 ± 8%) compared with control rats (28 ± 5%) ($P < .001$, Student *t* test) (Fig. 5A-C). Transplanted cells within intravascular spaces showed reconstitution of bile canaliculi without joining the bile canalicular network in the native liver (Fig. 5D), as would be expected.

Effect of MCT Pretreatment and Kinetics of Liver Repopulation. In view of the potential for hepatic genotoxicity of MCT,[16] we determined whether improved cell engraftment in MCT-treated animals could be amplified with additional liver injury. CCl_4 was useful for this, in line with previous studies that have established that transplanted cells in zone 1 of the liver lobule were spared from CCl_4 toxicity, which was restricted to the perivenous areas due to metabolic activity of hepatocytes in this region.[23] In control rats treated with three cycles of CCl_4, proliferation in transplanted cells was observed to a relatively

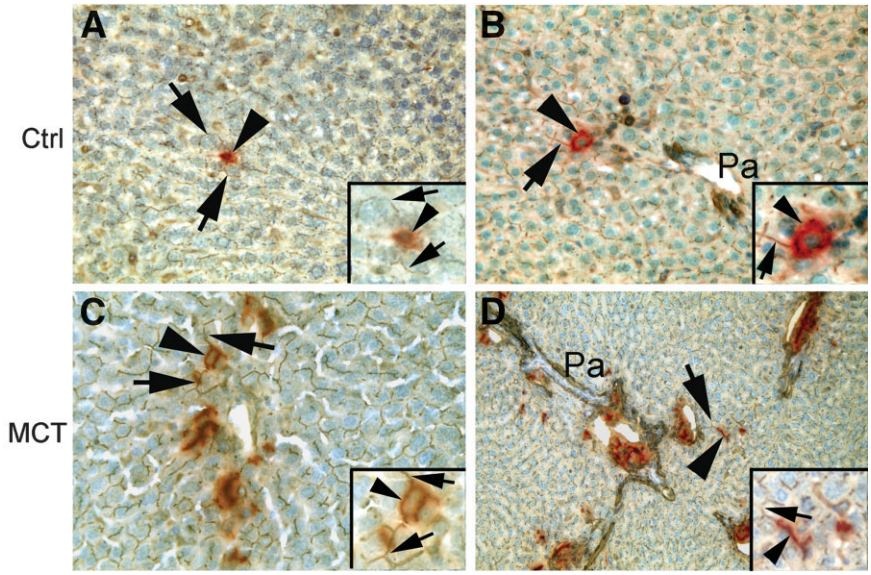

Fig. 5. DPPIV and ATPase histochemistry showing reconstitution of bile canaliculi. (A-B) Control and MCT-treated rats (C-D) 2 days or (B,D) 7 days after cell transplantation. DPPIV activity in transplanted cells is shown in red (arrowheads) and ATPase activity in native hepatocytes is shown in brown (arrows). Diffuse DPPIV activity in panel A indicates absence of bile canalicular reconstitution, while linear DPPIV staining in panel B indicates restoration of bile canaliculi. Panel C shows completion of bile canalicular reconstitution in most transplanted cells in MCT-treated liver after only 2 days. Panel D shows restoration of bile canaliculi in transplanted cells situated in portal vein radicles, although cells were separated from native hepatocytes. Insets show magnified views of the areas described. (Original magnification ×400; panel D, ×100; [methylgreen counterstain].) Ctrl, control; MCT, monocrotaline; Pa, portal area.

limited extent (Fig. 6A-B), and morphometric analysis showed that not more than 1.6 ± 0.6% of the liver was replaced by transplanted cells (n = 6). On the other hand, liver repopulation in recipients of 200 mg/kg MCT and CCl_4 increased to 48.3 ± 7.5% ($P < .001$, Student t test; n = 6) (Fig. 6C). In recipients of 200 mg/kg MCT before cell transplantation, liver repopulation after 14 days immediately before the second CCl_4 dose, after 21 days immediately before the third CCl_4 dose, and after 1 month following three CCl_4 doses was 12 ± 2%, 27 ± 5%, and 48 ± 8%, respectively ($P < .001$, ANOVA with Holm-Sidak test; n=5-6 each). We further observed biliary proliferation in rats treated with MCT and CCl_4, which was not observed in recipients of MCT alone (Fig. 6D), suggesting that the biliary compartment had not been an original target of MCT-induced genotoxicity. The proliferating bile duct compartment and additional nonparenchymal epithelial cells showed differences in the nuclear morphology and overall organization into acinar or other arrangements. Furthermore, whereas mature bile duct cells expressed ATPase intensely (Fig. 6D), ATPase expression was often limited or absent in proliferating nonparenchymal epithelial cells. Similarly, we observed CK-19 expression or OV-6 immunostaining in mature bile duct cells, but not in this population of nonparenchymal cells (Fig. 6E-F). On the other hand, some of the nonparenchymal epithelial cells expressed γ-glutamyltranspeptidase, similar to the expression of ATPase activity in some of these cells (Fig. 6G). Immunostaining for the CD45 marker showed that these cells were not blood-derived inflammatory cells (Fig. 6H).

These findings were consistent with the activation of a heterogeneous cell population during this process of MCT plus CCl_4-induced injury and suggested that MCT and CCl_4 induce synergistic damage in native hepatocytes. To verify this possibility, we performed additional studies in which rats were treated with MCT or saline, followed 7 days later by CCl_4. In comparison with saline treatment, prior treatment with MCT resulted in greater CCl_4-induced liver injury (Fig. 7).

Discussion

Targeting of multiple liver cell compartments improved engraftment and proliferation of transplanted cells. Disruption of the hepatic endothelial barrier by MCT had profound effects on transplanted cell engraftment. Judging from the extensive loss of endothelial integrity, in addition to elevated levels of serum hyaluronic acid, which is cleared by LSECs through an avid receptor-dependent process,[21] it is most likely that endothelial disruption was directly responsible for improved cell engraftment in rats treated with 200 mg/kg MCT. Additional CCl_4-induced synergistic damage accelerated transplanted cell proliferation, leading to significant liver repopulation within 1 month, suggesting an unmasking of genotoxic damage in hepatocytes exposed to MCT and a possible combination of this mechanism with enhanced susceptibility to reactive CCl_4 metabolites, perhaps with modulation of specific P450 isoforms, similar to the induction of CYP2E1 by retrorsine.[20]

On the other hand, we were unable to establish an effect on cell engraftment of combined endothelial and Kupffer cell injury, because MCT did not impair Kupffer cell function in our studies. MCT was previously found to alter the balance of ED1- and ED2-immunoreactive Kupffer cells, with depletion of the latter subgroup.[13,14] However, we studied phagocytic function in Kupffer cells and demonstrated

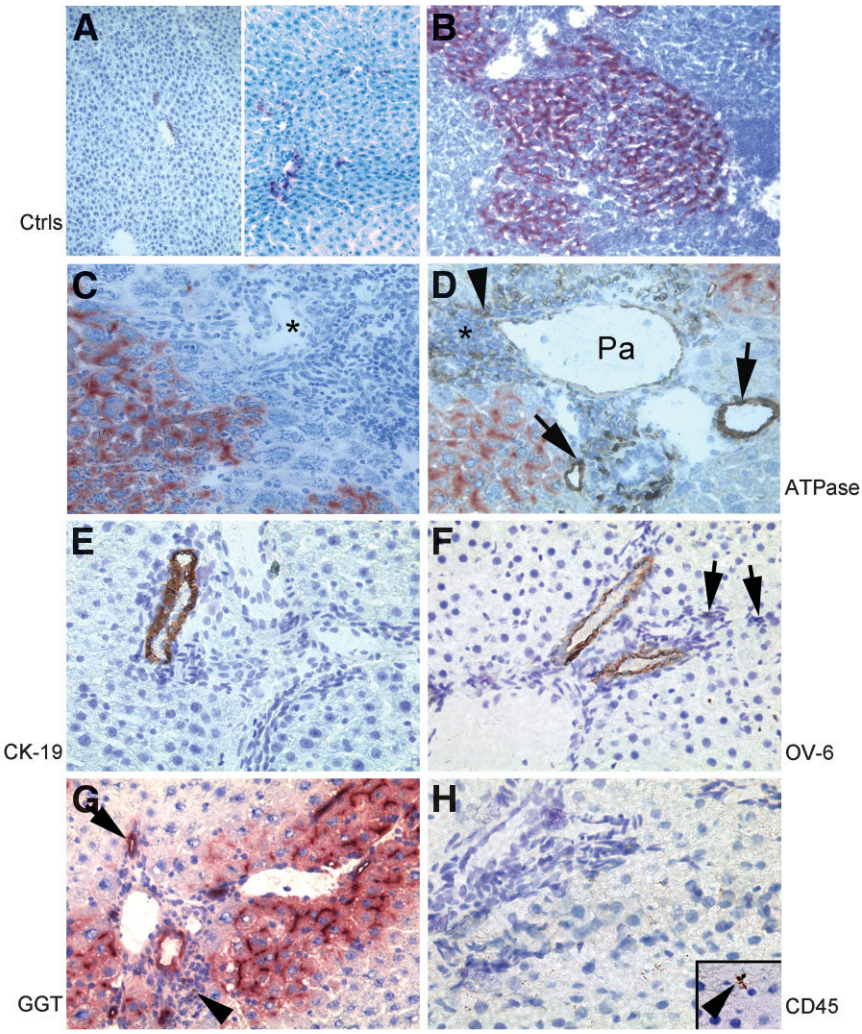

Fig. 6. Liver repopulation and changes in rat liver. (A) Occasional transplanted cells in periportal area in a saline-treated control rat (left) and more transplanted cells following treatment with three cycles of CCl_4 (right). (B) Rat treated with MCT plus three cycles of CCl_4 with significant liver repopulation. (C) Biliary and nonparenchymal epithelial cell proliferation (asterisk) in a rat treated with MCT and CCl_4. (D) Bile ducts with intense ATPase expression (arrows) interspersed with cells showing weaker ATPase (arrowhead) or no ATPase activity (asterisk). (E) Restriction of CK-19 expression in bile duct cells. (F) OV-6 antibody staining was observed in bile duct cells and occasionally in small cells in periportal areas (arrows). (G) γ-Glutamyltranspeptidase staining in bile ducts (arrow) and in some nonparenchymal epithelial cells (arrowhead). (H) CD45 staining in only sinusoidal cells (inset) and not in epithelial liver cells. All tissues shown were from recipients of cells. (Original magnification ×200 [panel A], ×40 [panel B], ×400 [panels C-H]. Panels A-C, DPPIV stain; panel D, DPPIV plus ATPase stain; panels A-H, toluidine blue counterstain.) Ctrls, controls; GGT, γ-glutamyltranspeptidase; Pa, portal area; ATPase, adenosine triphosphatase.

that this Kupffer cell property was intact, despite MCT-induced endothelial injury. Therefore, other manipulations will be necessary to investigate the effect of combined interference with LSECs and Kupffer cells in transplanted cell engraftment and liver repopulation.

The findings shown here should be particularly helpful in developing manipulations that could be applied in the clinical situation. Although endothelial disruption using cyclophosphamide or doxorubicin improved transplanted cell engraftment in DPPIV− rats,[2,4] MCT was far more efficient in this respect. Of course, systemic toxicities of cyclophosphamide or doxorubicin make these drugs relatively less desirable, although in the cell transplant setting, use of such drugs on only a single occasion should decrease their potential for systemic toxicity. Nonetheless, superior integration of transplanted cells in the liver parenchyma in MCT-treated rats was in agreement with the quicker passage of cells through the space of Disse and into the liver plate,[24] similar to cyclophosphamide-induced endothelial disruption.[2] Also, delaying cell transplantation to 48 hours after MCT improved cell engraftment (not shown), in agreement with prolonged endothelial disruption following MCT. Doxorubicin was also effective in disrupting endothelial disruption for several days, thereby providing a long window for superior engraftment of transplanted cells in rats.[4] Of course, greater survival of transplanted hepatocytes within portal vein radicles indicated that MCT promoted engraftment of cells in larger venous structures. However, transplanted cells located in intravascular spaces did not associate with native hepatocytes, and the biliary apparatus was not restored in these cells. Therefore, bile produced by these cells must be secreted into the blood for clearance by either native or transplanted hepatocytes in the liver parenchyma, which should be possible.

The activation of biliary cells and small nonparenchymal epithelial cells during CCl_4-induced clearance of hepatocytes exposed to MCT suggests that these cells were spared from MCT toxicity. Because MCT must be converted to toxic metabolites before DNA adducts can form,[16] this will be in agreement with the absence of MCT utilization in proliferating biliary or nonparenchymal epithelial cells due to the lack of relevant P450 expression (*e.g.*, the 3A4 isoform) for metabolizing MCT.[25]

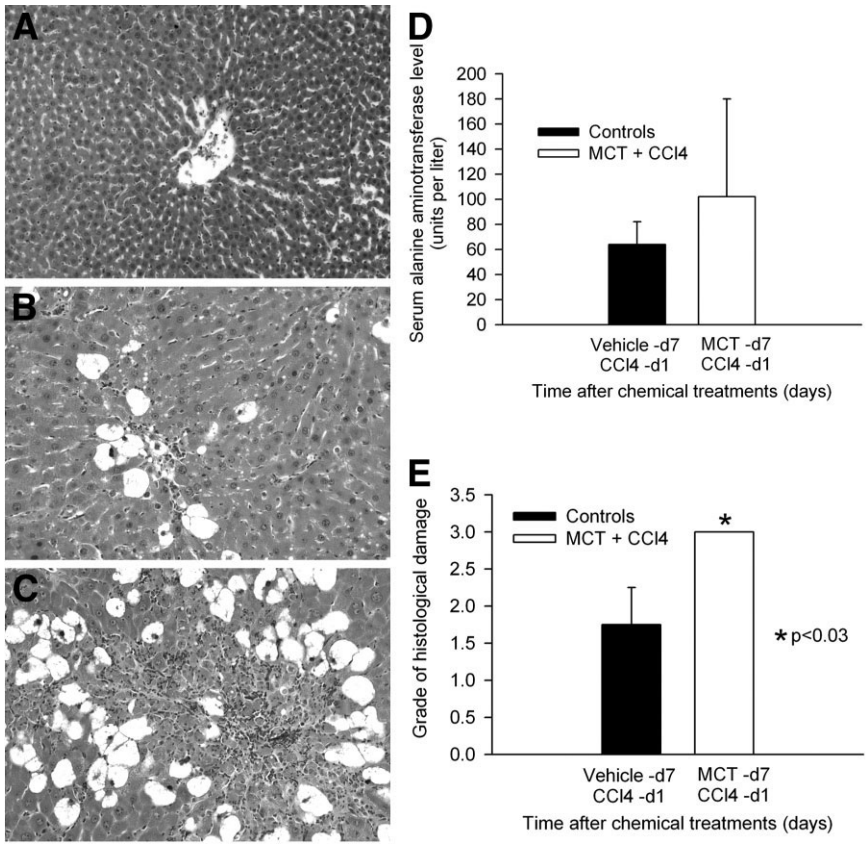

Fig. 7. Synergistic hepatotoxicity of MCT and CCl_4. (A) Normal rat liver without injury. (B) Rat liver after saline treatment followed by CCl_4 administration with limited perivenous necrosis. (C) Rat liver after MCT treatment followed by CCl_4 administration showing more perivenous injury. (D) Increased serum ALT levels with a trend toward higher ALT levels in rats treated with MCT plus CCl_4. The difference did not reach statistical significance. (E) Histological grading of liver injury, which was significantly greater in MCT plus CCl_4–treated rats, as shown by ANOVA with Holm-Sidak test. MCT, monocrotaline; CCl4, carbon tetrachloride. Original magnification, ×100.

The emergence of such new cell populations following extensive hepatic injury has the potential to develop additional insights into stem/progenitor cell compartments in the liver (*e.g.*, in comparison with the "small cell" population originating in the liver of retrorsine-treated rats)[26] as well as the oval cell population, which arises from within the liver itself.[27,28] Initial characterization of proliferating cells in MCT and CCl_4-treated rats suggested that these cells lacked markers of mature hepatocytes (*e.g.*, DPPIV-positive bile canalicular domains) and of mature bile duct cells (*e.g.*, CK-19), as well as of transitional cells (*e.g.*, OV-6). However, some of the proliferating cells did express ATPase and γ-glutamyltranspeptidase, which can be expressed by both hepatocytes and bile ducts. The small hepatocyte-like progenitor cells demonstrated previously in rats treated with retrorsine and partial hepatectomy showed a different kinetics of evolution, because those cells resembled fully differentiated hepatocytes by 14 days after partial hepatectomy.[26] On the other hand, cells emanating in retrorsine/partial hepatectomy–treated rats were negative for OV-6, which was similar to the proliferating cells in MCT- and CCl_4-treated rats shown here, suggesting involvement of parenchymal epithelial rather than ductal cell compartments in their origin. Analysis of the potential of small hepatocyte-like cells isolated from retrorsine/partial hepatectomy–treated rats followed by transplantation studies demonstrated the capacity of these cells to produce mature hepatocytes. In the future, such studies should be helpful in characterizing the potential of small cells identified in our studies.[29]

Although MCT effectively synergized with CCl_4 in activating liver repopulation, neither MCT nor CCl_4 is a candidate for clinical use in view of their systemic toxicities, including the oncogenic potential of MCT.[30] However, it is clear that in MCT-treated rats, CCl_4 considerably amplified further liver injury. In previous studies, MCT induced apoptosis in hepatocytes, and inflammatory cell infiltrates were not responsible for hepatic MCT toxicity,[15,31] which was verified in our studies. Proapoptotic mechanisms have certainly been effective in liver repopulation.[10] Perhaps new pharmacological approaches should be developed to reproduce MCT-like effects on LSECs and/or hepatocytes without incurring systemic toxicities. It is possible to identify suitable genotoxic manipulations for inducing proliferation in transplanted cells. For instance, radiation-based genotoxicity in combination with ischemia/reperfusion–induced oxidative stress was effective for liver repopulation in DPPIV− rats, a finding that should be clinically relevant.[11]

In conclusion, use of the MCT/CCl_4 regimen to promote transplanted cell engraftment and proliferation in animals should be useful for experimental studies. It was

clear in our studies that MCT alone did not induce liver cell proliferation, as demonstrated by Ki67 expression and lack of transplanted cell proliferation, which was different from retrorsine-induced liver injury, because retrorsine was sufficient by itself for inducing liver repopulation.[32] However, in these studies, liver repopulation was slower, and 2 months were needed for an average of 40% liver repopulation and 5 months for 70% liver repopulation. In contrast, the kinetics of liver repopulation elicited by the MCT/CCl_4 combination was comparable in our experience to that in male DPPIV− rats conditioned with retrorsine and two-thirds partial hepatectomy, where $52 \pm 8\%$ of the liver was repopulated in 4 weeks after cell transplantation.[33] However, an advantage of the MCT and CCl_4 regimen was that the waiting period of several weeks needed for priming rats with retrorsine or MCT followed by two-thirds partial hepatectomy could be avoided.[7,18] Therefore, the MCT/CCl_4 system can be used at relatively short notice for cell transplantation studies without the imposition of additional intra-abdominal surgery and its attendant morbidity or mortality.

References

1. Slehria S, Rajvanshi P, Ito Y, Sokhi R, Bhargava KK, Palestro CJ, et al. Hepatic sinusoidal vasodilators improve transplanted cell engraftment and ameliorate microcirculatory perturbations in the liver. HEPATOLOGY 2002; 35:1320-1328.
2. Malhi H, Annamaneni P, Slehria S, Joseph B, Bhargava KK, Palestro CJ, et al. Cyclophosphamide disrupts hepatic sinusoidal endothelium and improves transplanted cell engraftment in rat liver. HEPATOLOGY 2002;36: 112-121.
3. 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.
4. 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.
5. Benten D, Kumaran V, Joseph B, Gupta S. Hepatocyte transplantation activates hepatic stellate cells with beneficial modulation of cell engraftment. HEPATOLOGY 2005;42:1072-1081.
6. Kumaran V, Joseph B, Benten D, Gupta S. Integrin and extracellular matrix interactions regulate engraftment of transplanted hepatocytes in the rat liver. Gastroenterology 2005;129:1643-1653.
7. Laconi E, Oren R, Mukhopahyay DK, Hurston E, Laconi S, Pani P, 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.
8. Guha C, Sharma A, Gupta S, Alfieri A, Gorla GR, Gagandeep S, et al. Amelioration of radiation-induced liver damage in partially hepatectomized rats by hepatocyte transplantation. Cancer Res 1999;59:5871-5874.
9. De Vree JM, Ottenhoff R, Bosma PJ, Smith AJ, Aten J, Oude Elferink RP. Correction of liver disease by hepatocyte transplantation in a mouse model of progressive familial intrahepatic cholestasis. Gastroenterology 2000; 119:1720-1730.
10. Mallet VO, Mitchell C, Guidotti JE, Jaffray P, Fabre M, Spencer D, et al. Conditional cell ablation by tight control of caspase-3 dimerization in transgenic mice. Nat Biotechnol 2002;20:1234-1239.
11. Malhi H, Gorla GR, Irani AN, Annamaneni P, Gupta S. Cell transplantation after oxidative hepatic preconditioning with radiation and ischemia-reperfusion leads to extensive liver repopulation. Proc Natl Acad Sci U S A 2002;99:13114-13119.
12. Shah M, Patel K, Sehgal PB. Monocrotaline pyrrole-induced endothelial cell megalocytosis involves a Golgi blockade mechanism. Am J Physiol Cell Physiol 2005;288:C850-C862.
13. DeLeve LD, McCuskey RS, Wang X, Hu L, McCuskey MK, Epstein RB, et al. Characterization of a reproducible rat model of hepatic veno-occlusive disease. HEPATOLOGY 1999;29:1779-1791.
14. Kono H, Fujii H, Asakawa M, Yamamoto M, Maki A, Matsuda M, et al. Functional heterogeneity of the kupffer cell population is involved in the mechanism of gadolinium chloride in rats administered endotoxin. J Surg Res 2002;106:179-187.
15. Copple BL, Rondelli CM, Maddox JF, Hoglen NC, Ganey PE, Roth RA. Modes of cell death in rat liver after monocrotaline exposure. Toxicol Sci 2004;77:172-182.
16. Wang YP, Yan J, Beger RD, Fu PP, Chou MW. Metabolic activation of the tumorigenic pyrrolizidine alkaloid, monocrotaline, leading to DNA adduct formation in vivo. Cancer Lett 2005;226:27-35.
17. Gorla GR, Malhi H, Gupta S. Polyploidy associated with oxidative DNA injury attenuates proliferative potential of cells. J Cell Sci 2001;114:2943-2951.
18. Witek RP, Fisher SH, Petersen BE. Monocrotaline, an alternative to retrorsine-based hepatocyte transplantation in rodents. Cell Transplant 2005; 14:41-47.
19. Gupta S, Rajvanshi P, Lee C-D. Integration of transplanted hepatocytes in host liver plates demonstrated with dipeptidyl peptidase IV deficient rats. Proc Natl Acad Sci USA 1995;92:5860-5864.
20. Gupta S, Rajvanshi P, Malhi H, Sokhi RP, Slehria S, Vasa SRG, et al. Cell transplantation causes loss of gap junctions and activates GGT expression permanently in host liver. Am J Physiol Gastroint Liver Physiol 2000;279: G815-G826.
21. Deaciuc IV, Bagby GJ, Lang CH, Spitzer JJ. Hyaluronic acid uptake by the isolated, perfused rat liver: an index of hepatic sinusoidal endothelial cell function. HEPATOLOGY 1992;17:262-272.
22. Joseph B, Bhargava KK, Trunco G, Kumaran V, Palestro CJ, Gupta S. Regulation of hepatobiliary transport activity and noninvasive identification of cytokine-dependent liver inflammation. J Nucl Med 2005;46:146-152.
23. Gupta S, Rajvanshi P, Aragona E, Yerneni PR, Lee C-D, Burk RD. Transplanted hepatocytes proliferate differently after CCl4 treatment and hepatocyte growth factor infusion. Am J Physiol 1999;276:G629-G638.
24. Gupta S, Rajvanshi P, Sokhi RP, Slehria S, Yam A, Kerr A, et al. Entry and integration of transplanted hepatocytes in liver plates occur by disruption of hepatic sinusoidal endothelium. HEPATOLOGY 1999;29:509-519.
25. Reid MJ, Lame MW, Morin D, Wilson DW, Segall HJ. Involvement of cytochrome P450 3A in the metabolism and covalent binding of 14C-monocrotaline in rat liver microsomes. J Biochem Mol Toxicol 1998;12:157-166.
26. Gordon GJ, Coleman WB, Grisham JW. Temporal analysis of hepatocyte differentiation by small hepatocyte-like progenitor cells during liver regeneration in retrorsine-exposed rats. Am J Pathol 2000;157:771-786.
27. Wang X, Foster M, Al-Dhalimy M, Lagasse E, Finegold M, Grompe M. The origin and liver repopulating capacity of murine oval cells. Proc Natl Acad Sci U S A 2003;100(Suppl 1):11881-11888.
28. Menthena A, Deb N, Oertel M, Grozdanov PN, Sandhu J, Shah S, et al. Bone marrow progenitors are not the source of expanding oval cells in injured liver. Stem Cells 2004;22:1049-1061.
29. Gordon GJ, Butts GM, Grisham JW, Coleman WB. Isolation, short-term culture, and transplantation of small hepatocyte-like progenitor cells from retrorsine-exposed rats. Transplantation 2002;73:1236-1243.
30. Shumaker RC, Robertson KA, Hsu IC, Allen JR. Neoplastic transformation in tissues of rats exposed to monocrotaline or dehydroretronecine. J Natl Cancer Inst 1976;56:787-790.
31. Copple BL, Ganey PE, Roth RA. Liver inflammation during monocrotaline hepatotoxicity. Toxicology 2003;190:155-169.
32. Laconi S, Pillai S, Porcu PP, Shafritz DA, Pani P, Laconi E. Massive liver replacement by transplanted hepatocytes in the absence of exogenous growth stimuli in rats treated with retrorsine. Am J Pathol 2001;158:771-777.
33. Wu YM, Joseph B, Gupta S. Immunosuppression using the mTOR inhibition mechanism affects replacement of the rat liver with transplanted cells. HEPATOLOGY 2006;44:410-419.