Hepatocyte Transplantation and Drug-Induced Perturbations in Liver Cell Compartments

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The potential for organ damage after using drugs or chemicals is a critical issue in medicine. To delineate mechanisms of drug-induced hepatic injury, we used transplanted cells as reporters in dipeptidyl peptidase IV–deficient mice. These mice were given phenytoin and rifampicin for 3 days, after which monocrotaline was given followed 1 day later by intrasplenic transplantation of healthy C57BL/6 mouse hepatocytes. We examined endothelial and hepatic damage by serologic or tissue studies and assessed changes in transplanted cell engraftment and liver repopulation by histochemical staining for dipeptidyl peptidase IV. Monocrotaline caused denudation of the hepatic sinusoidal endothelium and increased serum hyaluronic acid levels, along with superior transplanted cell engraftment. Together, phenytoin, rifampicin, and monocrotaline caused further endothelial damage, reflected by greater improvement in cell engraftment. Phenytoin, rifampicin, and monocrotaline produced injury in hepatocytes that was not apparent after conventional tissue studies. This led to transplanted cell proliferation and extensive liver repopulation over several weeks, which was more efficient in males compared with females, including greater induction by phenytoin and rifampicin of cytochrome P450 3A4 isoform that converts monocrotaline to toxic intermediates. Through this and other possible mechanisms, monocrotaline-induced injury in the endothelial compartment was retargeted to simultaneously involve hepatocytes over the long term. Moreover, after this hepatic injury, native liver cells were more susceptible to additional pro-oxidant injury through thyroid hormone, which accelerated the kinetics of liver repopulation. *Conclusion:* Transplanted reporter cells will be useful for obtaining insights into homeostatic mechanisms involving liver cell compartments, whereas targeted injury in hepatic endothelial and parenchymal cells with suitable drugs will also help advance liver cell therapy. (HEPATOLOGY 2008;47:279-287.)

To obtain fresh paradigms in tissue homeostasis after exposure to drugs or chemicals,[1] we considered that reporter cells will help elucidate perturbations in the liver, because genetically marked reporter cells can be inserted into liver compartments, including the parenchyma or hepatic endothelium.[2-4] It was noteworthy that transplanted cells did not proliferate in the normal liver, where cell turnover is minimal, although, depending on the extent of injury in native cells, proliferation in healthy transplanted cells was activated.[5,6] In this way, transplanted cells could repopulate the liver, where in specific situations the kinetics of liver repopulation reflected loss of native hepatocytes.[7-9] In healthy animals, genotoxic manipulations were particularly effective in promoting such liver repopulation with healthy cells. However, further insights are necessary to obtain pharmacologic approaches for repopulating the liver, particularly for clinical applications.

To develop cell compartment–specific hepatic perturbations, we used monocrotaline (MCT), a naturally occurring pyrrolizidine alkaloid with effects on the vascular endothelium,[10-12] in combination with phenytoin and rifampicin, which are widely used in humans. These drugs offered potential for unique interactions as phenytoin and rifampicin activate complex cellular events, including oxidative stress, DNA damage, and cytochrome P450

Abbreviations: ANOVA, analysis of variance; CCl_4, carbon tetrachloride; CYP, cytochrome P450; DPPIV, dipeptidyl peptidase IV enzyme; EM, electron microscopy; MCT, monocrotaline.

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(CYP) enzymes, e.g., CYP3A4, which converts MCT to reactive metabolites.[13-16] Previously, we developed suitable mechanisms in mutant rats lacking dipeptidyl peptidase IV enzyme activity (DPPIV− rats) to determine the fate of transplanted cells.[2,3,5-7] To determine whether transplanted cells could be used as reporters to identify changes in liver cell compartments, we used DPPIV− mice as recipients of healthy cells. Our studies established that transplanted reporter cells effectively identified perturbations in the hepatic endothelial and parenchymal cell compartments. Moreover, the combination of MCT, phenytoin, and rifampicin produced significant long-lasting changes in the liver that initially benefited transplanted cell engraftment and later resulted in the induction of proliferation in transplanted cells leading to extensive liver repopulation over time.

Materials and Methods

Drugs. Phenytoin and rifampicin (Sigma Chemical Co., St. Louis, MO) were dissolved in 20 M NaOH and MCT in normal saline with pH to 7.4. After dose-ranging studies, mice received 30 mg/kg phenytoin and 75 mg/kg rifampicin intraperitoneally daily for 3 days. MCT was given as a single 200-mg/kg dose intraperitoneally on the 4th day. Cells were transplanted 24 hours after MCT. Other drugs were 0.71% sodium valproate in drinking water, 0.5 mL/kg carbon tetrachloride (CCl_4) in olive oil (1:15, vol/vol) intraperitoneally, 8 mg/kg thyroxine in normal saline intraperitoneally (Sigma), and 0.3% cholic acid in rodent chow (Harlan Teklad Test Diets, Madison, WI).

Animals. DPPIV− mice in C57BL/6 background were 6 to 8 weeks old when used and were originally provided by Dr. D. Marguet (INSERM, Marseilles, France).[17] Cell donors were 8-week-old to 15-week-old C57BL/6 mice from the National Cancer Institute (Bethesda, MD). Hepatocytes were isolated by 2-step collagenase perfusion, and 2×10^6 viable cells were injected immediately in 0.2 mL RPMI 1640 medium into the splenic pulp as described.[4] The Animal Care and Use Committee at Albert Einstein College of Medicine approved protocols in compliance with National Research Council guidelines (Guide for the Care and Use of Laboratory Animals; United States Public Health Service publication, revised 1996).

Tissue Analysis. Data were obtained from multiple mice in each group using 3 to 4 sections per animal. Formalin-fixed tissue sections of 10-μm to 15-μm thickness were stained with hematoxylin-eosin. To assess endothelial injury by electron microscopy (EM), liver was perfused through the portal vein from a 60-cm height with 3 to 4 mL 0.144 mol/L cacodylate buffer followed by 10 mL 1.5% glutaraldehyde in cacodylate buffer. After further incubation in glutaraldehyde, tissues were embedded in resin and stained with orcein. Ultrathin sections were examined under a JOEL electron microscope (Olympus, Tokyo, Japan). Endothelial integrity was graded under 1000× magnification in 50 consecutive sinusoids per mouse (n = 3) as follows: no injury (<25% sinusoid without endothelium); incomplete injury (25%-70% loss of endothelium); complete injury (no endothelial lining).

For apoptosis, terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling was used (ApopTag Peroxidase In Situ Apoptosis Detection Kit, Chemicon International Inc., Temecula, CA). Briefly, 5-μm cryosections were fixed in 1% paraformaldehyde in phosphate-buffered saline, pH 7.4 for 10 minutes and post-fixed in ethanol:acetic acid 2:1 (vol/vol) for 5 minutes at −20°C. Positive control tissues were incubated with 100 units/mL deoxyribonuclease (DNAse) (Worthington Biochemical Corp., Lakewood, NJ) for 10 minutes. Tissues were quenched in 3% H_2O_2 in phosphate-buffered saline for 5 minutes, incubated with terminal deoxynucleotidyl transferase for 1 hour at 37°C followed by anti-digoxigenin-peroxidase for 30 minutes and color development using diaminobenzidine (DAB+ kit, DAKO Cytomation, Carpinteria, CA).

For Ki67 staining, sections were fixed in paraformaldehyde, blocked with 3% goat serum in phosphate-buffered saline containing 0.1% Triton X-100 for 1 hour, followed by incubation with rabbit anti-Ki67 (1:1000, Novocastra, Newcastle-upon-Tyne, UK) and peroxidase-conjugated anti-rabbit immunoglobulin G (1:300, Sigma) for 1 hour each followed by DAB.

Assays for Hepatocyte Turnover. To identify DPPIV activity, 5-μm cryosections were fixed in chloroform-acetone and analyzed as described previously.[2] Transplanted cell numbers per liver lobule and per unit liver volume (mm^3) were quantitated as described with scoring under 400× of 100 consecutive lobules per liver lobe per animal using multiple sections.[18] The extent of liver repopulation with transplanted cells was measured by ImageJ software (NCI, Bethesda, MD) in images taken under 40× (Spot RT digital camera, Diagnostic Instrument Inc., Sterling Heights, MI).

CYP Expression. Tissue samples were dounce-homogenized in 0.25 M sucrose, 10 mM Tris hydrochloride, pH 7.5, and protease inhibitor cocktail (Calbiochem 539134, EMD Biosciences Inc., San Diego, CA). The lysate was centrifuged under 2000*g* for 25 minutes and then under 100,000*g* for 1 hour at 4°C. The pellet was sonicated, and 75 μg total proteins were separated in 10%

sodium dodecyl sulfate polyacrylamide gel electrophoresis. After transfer, nitrocellulose membranes were incubated for 1 hour with anti-CYP3A4 (1:4000, A4100; Xenotech, Lenexa, KA) and peroxidase-conjugated anti-rabbit immunoglobulin G (1:5000, 321804, Amersham Biosciences, Piscataway, NJ) for 1 hour with enzymatic chemiluminescence. Transblots were stained with Ponceau Red to visualize proteins. For immunostaining, 5-μm cryosections were fixed in 4% paraformaldehyde for 5 minutes at 4°C and blocked with 5% goat serum for 30 minutes. Anti-CYP3A4 (1:250) was applied for 1 hour, peroxidase-conjugated anti-rabbit immunoglobulin G (1:700, Sigma) was added for 30 minutes, and color was developed with DAB.

Serologic Assays. Alanine aminotransferase and total serum bilirubin were measured with an automated clinical system. Hyaluronic acid was measured with a commercial kit (Corgenix, Inc., Westminster, CO).[19]

Statistical Analysis. Data are presented as means ± standard deviation. The significances were analyzed by Student *t* test, Kruskall-Wallis analysis of variance (ANOVA), or ANOVA on ranks using Tukey test for pairwise comparisons of mean group ranks. $P < 0.05$ was taken as significant.

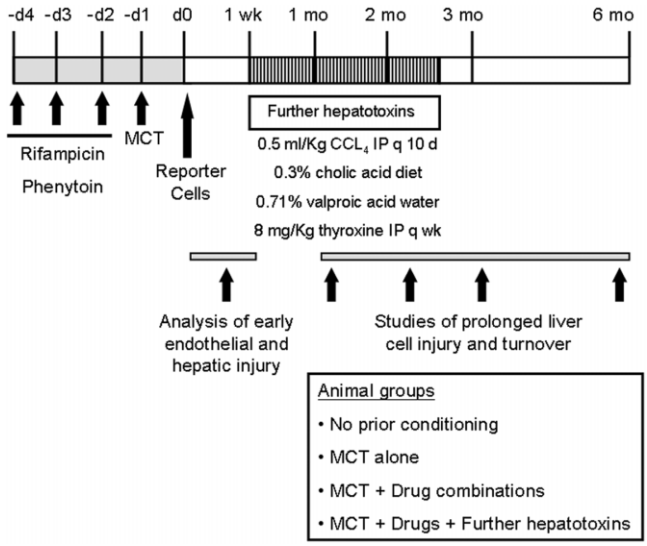

Fig. 1. Experimental design for changes in endothelial and hepatocyte compartments. To demonstrate the integrity of liver endothelium after perturbations, reporter cells were transplanted intrasplenically in animals primed over 4 days, as indicated, followed by analysis of cell engraftment over 1 week. Subsequently, changes in the hepatocyte compartment were analyzed through induction of proliferation in transplanted cells over up to 6 months. In additional animal groups, the effect of further liver injury with CCl_4 commencing 10 days after cell transplantation and cholic acid in diet, valproic acid in drinking water, or thyroxine commencing 1 week after cell transplantation for up to 8 weeks, was analyzed through changes in the extent of liver repopulation.

Results

Experimental Design. Each animal group used 3 to 5 mice per time point. Initial studies using serum hyaluronic acid, light microscopy, EM, and liver tests indicated that 200 mg/kg MCT produced endothelial injury. After dose-ranging studies, we used 30 mg/kg phenytoin and 75 mg/kg rifampicin. To study combined drug effects, we used phenytoin and rifampicin for 3 days before MCT was given. Cells were transplanted 1 day after drugs (Fig. 1). We assessed cell engraftment after 7 days. To elucidate latent hepatic injury and its effects on liver cell turnover, we studied liver repopulation after 1, 2, 3, and 6 months. Typically, animals were treated with MCT alone, phenytoin or rifampicin plus MCT, or phenytoin plus rifampicin plus MCT. To establish drug dose–dependent changes, we used 50, 75, or 100 mg/kg rifampicin with fixed doses of phenytoin and MCT, followed by liver repopulation analysis. Gender-specific drug toxicity was defined in female mice. To reveal further perturbations, we used CCl_4, cholic acid, valproic acid, and thyroxine, as indicated.

Characterization of Early Changes. EM and serum hyaluronic acid levels showed that MCT rapidly damaged liver sinusoidal endothelial cells (Fig. 2). However, hepatocyte injury was not obvious in MCT-treated mice with light microscopy, EM, or blood tests (Table 1). Phenytoin plus rifampicin plus MCT produced only transient elevations in serum alanine aminotransferase, although liver histology and liver tests were normal over up to 2 months (Fig. 3).

Further studies showed limited hepatic apoptosis after phenytoin plus rifampicin plus MCT, with only 4 to 8 apoptotic cells per high-power field (×400) after 4 or 8 weeks (Fig. 4).

Similarly, only infrequent hepatocytes expressed the proliferation marker Ki67 (Fig. 5).

Early Liver Changes and Cell Engraftment. After MCT alone, engraftment of transplanted hepatocytes improved, reflecting superior entry of transplanted cells through disrupted endothelium into liver parenchyma (Fig. 6A,B). Phenytoin and rifampicin further improved cell engraftment, indicating additional endothelial injury (Fig. 6C-E). In drug-untreated mice, we observed 1.7 ± 0.1 transplanted cells per liver lobule and 753 ± 52 transplanted cells per cubic millimeter liver (Fig. 6F). In MCT-treated mice, 1.7-2-fold more transplanted cells were observed, $P < 0.001$, *t* tests. After phenytoin or rifampicin plus MCT, cell engraftment increased by up to 3-fold, $P < 0.05$, ANOVA.

Delayed Changes in Liver Cells. In mice treated

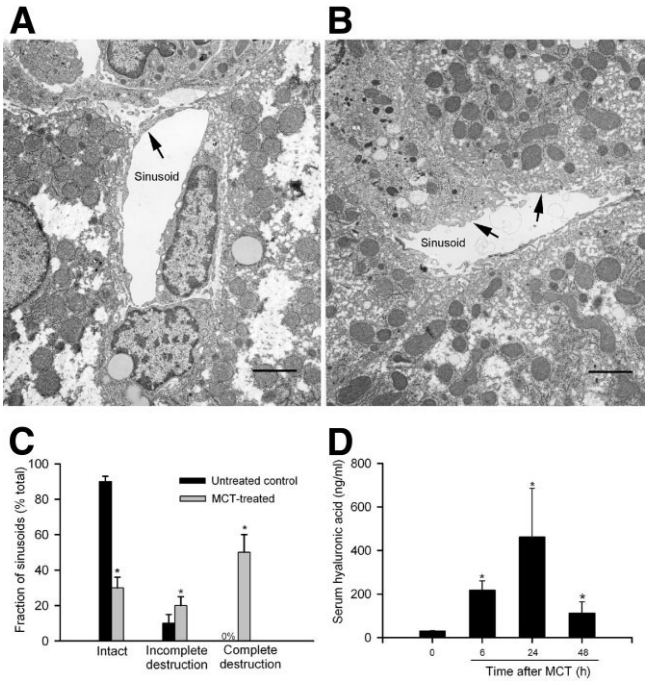

Fig. 2. Changes in liver sinusoidal endothelium after MCT. (A) EM showing typical appearance of normal liver sinusoidal endothelium forming a discontinuous layer (arrow). (B) Representative appearance of sinusoidal endothelium 24 hours after 200 mg/kg MCT. Note loss of endothelial cells and space of Disse (arrows). (C) Morphometric quantitation of sinusoidal integrity in healthy control mice and MCT-treated mice (n = 3 each). (D) Measurement of serum hyaluronic acid showing significant changes with 15-fold mean increase (30 ± 2 versus 461 ± 224 ng/mL) 24 hours after MCT. *$P < 0.05$. Bars in A and B, 2 μm.

with either no drugs or MCT alone, transplanted cell numbers were unchanged over 3 months (Fig. 7A), indicating that native hepatocytes were not damaged. By contrast, in phenytoin plus MCT–treated mice, small proliferating foci of transplanted cells appeared after 2 and 3 months (Fig. 7B). This became more pronounced in rifampicin plus MCT–treated mice (Fig. 7C). In mice treated with phenytoin plus rifampicin plus MCT, transplanted cell proliferation was accelerated with significant liver repopulation after 1 month (Fig. 7D), indicating that despite discrete exposures to drugs, native hepatocytes were perturbed in a long-lasting and profound manner.

In male mice, the extent of liver repopulation 3 months after cell transplantation was as follows: MCT alone, <1%, phenytoin plus MCT, 9% ± 2%, rifampicin plus MCT, 21% ± 5%, and phenytoin plus rifampicin plus MCT, 46% ± 12%, $P < 0.05$, ANOVA (Fig. 8A). After 6 months, liver repopulation in phenytoin plus rifampicin plus MCT–treated mice increased to 62% ± 4%. Plotting of data using linear regression showed that liver repopulation to 50% took approximately 4 months in phenytoin plus rifampicin plus MCT–treated mice, reflecting equivalent losses of native cells during that period (Fig. 8B). This liver repopulation kinetics was 2-fold greater than that of rifampicin plus MCT–treated mice and 5-fold greater than that of phenytoin plus MCT–treated mice, reflecting higher attrition rates in native cells.

To demonstrate the effect of larger amounts of rifampicin, since this drug induced greater transplanted cell proliferation than phenytoin, we used 50, 75, and 100 mg/kg rifampicin with fixed doses of 30 mg/kg phenytoin and 200 mg/kg MCT. Recipients of 100 mg/kg rifampicin exhibited 50% to 60% mortality even before cell transplantation, indicating greater acute toxicity. Rifampicin produced dose-dependent changes in liver repopulation after 2 months, which was more with 75 mg/kg (23% ± 7%) or 100 mg/kg rifampicin (23% ± 3%) compared with 50 mg/kg rifampicin (11% ± 7%), $P < 0.05$, ANOVA.

To demonstrate sex-specific mechanisms in hepatotoxicity, we treated female mice with 75 or 100 mg/kg rifampicin plus 30 mg/kg phenytoin plus 200 mg/kg MCT. Female mice were less susceptible to drug toxicity with no mortality despite 100 mg/kg rifampicin combination. This was verified by less liver repopulation in female mice (Fig. 8C); 18-fold less, compared with male mice treated with 75 mg/kg rifampicin after 3 months, $P < 0.001$, t test (Fig. 8A). In recipients of phenytoin plus rifampicin, hepatic CYP3A4 expression was induced as demonstrated by immunostaining and western blotting (Fig. 8D-G).

Drug-Treated Liver Was Susceptible to Further Perturbations. Treatment first with phenytoin plus rifampicin plus MCT and then CCl_4 produced injury in native, as well as transplanted cells in middle and perivenous zones of the liver lobule (Fig. 9B). Consequently, liver repopulation decreased to only 14% after 2 months, approximately 50% of that in CCl_4-untreated control mice. Also, the toxic bile salt cholic acid produced injury in native and transplanted cells, leading to decreased liver repopulation of only 7% after 2 months.

Table 1. Changes in Liver Tests

	Serum ALT (U/L)		Total Serum Bilirubin (mg/dL)	
Time After Treatments	MCT only	Phen+ Rif+ MCT	MCT only	Phen+ Rif+ MCT
1 day	41 ± 0.3	998 ± 462*	0.47 ± 0.12	0.47 ± 0.07
2 day	39 ± 3	351 ± 270	0.40 ± 0.06	0.47 ± 0.07
3 day	69 ± 28	208 ± 100	0.47 ± 0.22	0.57 ± 0.12
4 day	40 ± 2	93 ± 11	0.40 ± 0.06	0.33 ± 0.03
7 day	64 ± 14	126 ± 13	0.80 ± 0.20	0.80 ± 0.15

Normal control mice: serum ALT, 43 ± 2 U/L; total serum bilirubin, 0.30 ± 0.06 mg/dL.

*P value <0.05 versus normal control mice, ANOVA on ranks with Tukey Test.

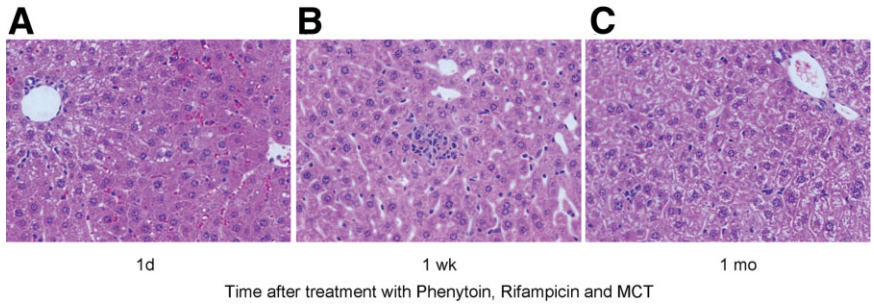

Fig. 3. Liver after drug treatments. Representative images from mice treated with 30 mg/kg phenytoin and 75 mg/kg rifampicin for 3 days followed by 200 mg/kg MCT. The liver histology was normal, except for infrequent areas of focal necrosis and mononuclear cell infiltration 1 week after treatments. These results were verified in 3 repeat studies. Original magnification ×400; hematoxylin-eosin stain.

Similarly, liver repopulation declined to 5% after valproic acid after 2 months (Fig. 9C). However, treatment with thyroxine significantly enhanced transplanted cell proliferation, suggesting selective oxidative injury in native cells previously exposed to phenytoin, rifampicin, and MCT (Fig. 9D,E).

Discussion

These studies established that transplanted reporter cells effectively elicited damage in hepatic endothelial and parenchymal compartments. Improvement in transplanted cell engraftment verified onset of endothelial damage, as this benefits entry of transplanted cells into the liver parenchyma.[20] Analysis of transplanted cell proliferation, which requires loss of native cells, showed that the liver had undergone changes, which were not demonstrated by conventional tissue analysis. Recently, unexpected drug toxicity was encountered after use of even single drugs, for example, liver failure with the antiviral drug fialuridine,[21] the anti-diabetic drug troglitazone, and the anti-thrombotic drug, ximelagatran.[22] Therefore, despite innovative applications of toxicogenomic, proteomic, metabolonomic, and *in vitro* assays for assessing mechanisms of drug toxicity, further approaches are needed.

Absence of transplanted cell proliferation after MCT alone indicated that phenytoin and rifampicin contributed to hepatic injury. Interactions between MCT, phenytoin, and rifampicin were at multiple levels, including CYP3A4 regulation by phenytoin and rifampicin, as shown here, through regulation of other CYP isoforms by phenytoin and rifampicin, and also by MCT itself, as pyrrolizidine alkaloids regulate CYP2E1 and other CYPs.[13,14,23] Also, sex-specific mechanisms altered drug toxicity in this model, with phenytoin and rifampicin inducing CYP3A4 less in female mice. However, CYP3A4 induction was less than 2-fold different between male and female mice, whereas liver repopulation (or native liver cell turnover) was 18-fold different. Therefore, additional mechanisms, such as direct genotoxicity, pro-

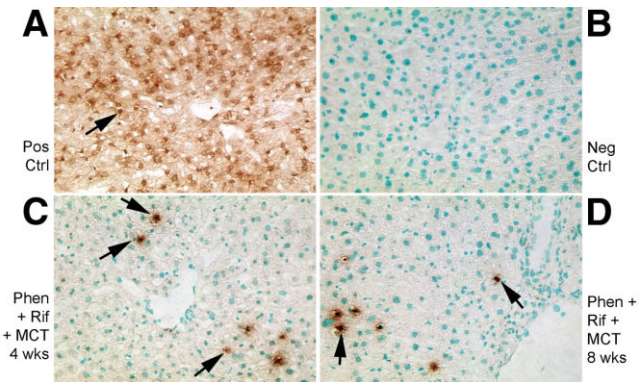

Fig. 4. Terminal deoxynucleotidyl transferase-mediated nick-end labeling for apoptosis. (A) Mouse liver pretreated with deoxyribonuclease showing extensive terminal deoxynucleotidyl transferase-mediated nick-end labeling–positive cells (arrow). (B) Negative control mouse liver with no terminal deoxynucleotidyl transferase showing no reaction product. (C and D) Liver from mice treated with 30 mg/kg phenytoin and 75 mg/kg rifampicin for 3 days followed by 200 mg/kg MCT 4 weeks (C) or 8 weeks before the analysis. Note some apoptotic cells (arrows) under these conditions. The data were verified in two independent studies. Original magnification ×400; methyl green counterstain, panels B, C, and D.

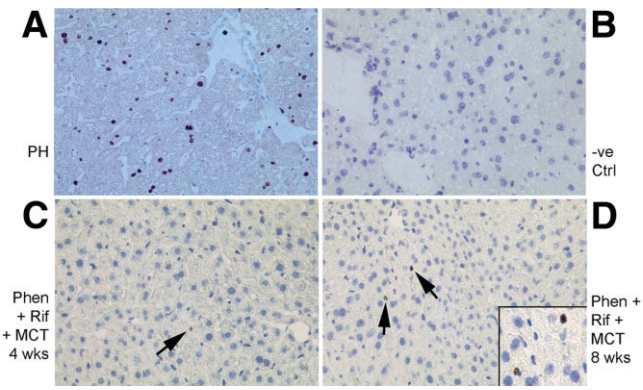

Fig. 5. Hepatic Ki67 expression. (A) Liver from mouse 40 hours after two-thirds partial hepatectomy with Ki67 expression in a significant fraction of hepatocytes. (B) No Ki67 staining in normal mouse liver. (C,D) Liver from mouse treated with 30 mg/kg phenytoin and 75 mg/kg rifampicin for 3 days followed by 200 mg/kg MCT 4 weeks (C) or 8 weeks before sacrifice. In these animals, hepatocytes rarely expressed Ki67 (arrows and inset), essentially similar to normal mouse liver. These studies were repeated twice. Original magnification ×400; toluidine blue counterstain.

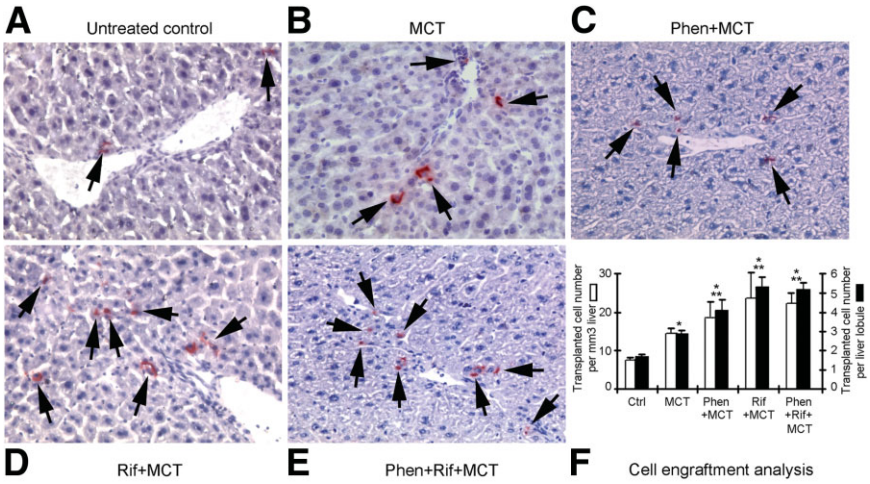

Fig. 6. Transplanted cell engraftment in DPPIV− mice. Shown are transplanted cells in periportal areas in untreated mice and mice treated with 200 mg/kg MCT alone, or MCT after 3 days of 30 mg/kg phenytoin or 75 mg/kg rifampicin as indicated. Panels A through E show transplanted cells with DPPIV histochemistry (red color, arrows). Panel F shows morphometric analysis of transplanted cell numbers per mm^3 liver and per liver lobule. $*P <$ 0.05 versus drug-untreated control group; $**P <$ 0.05 versus MCT-treated group. The data are from 3 replicate studies. Original magnification ×400, toluidine blue counterstain.

oxidant damage, or even protection by estrogens, may have been involved. [12]

The ability of transplanted cells to proliferate in mice treated with MCT, phenytoin, and rifampicin indicated sparing of these cells from secondary processes, such as recycling of toxic metabolites released by damaged or dying cells. The ability of transplanted cells to retain drug metabolic activity will be significant given that greater expression of specific CYPs makes perivenous hepatocytes more susceptible to toxins.[6,24] As transplanted cells engraft in periportal areas of the liver lobule, in contrast to perivenous areas, this protects transplanted cells from perivenous toxins. However, during liver repopulation, when transplanted cells enter midzonal or perivenous areas of the lobule, gene expression patterns switch to match that of adjacent native hepatocytes. Therefore, CCl_4-induced loss of transplanted cells should not be surprising in view of its metabolism in perivenous and midzonal areas of the liver. Similarly, toxic bile salts potentiate hepatic apoptosis and accelerate liver repopulation in the setting of abnormal biliary transport.[25,26] In our studies, decreased liver repopulation in mice treated with cholic acid

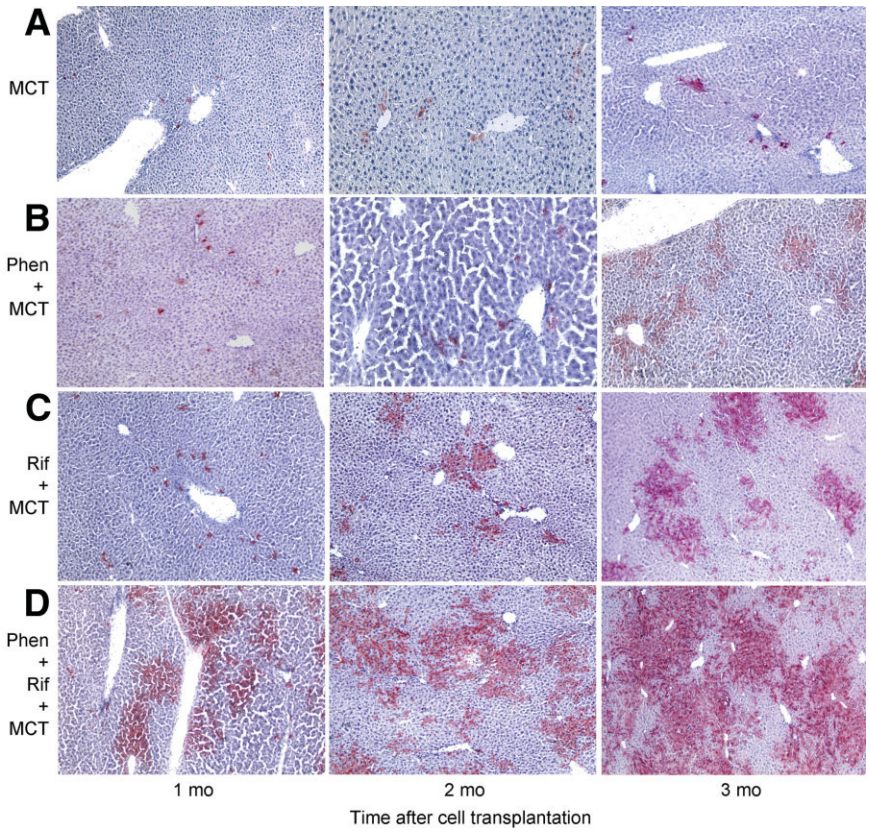

Fig. 7. Long-term fate of transplanted cells. Shown is transplanted cell proliferation in DPPIV− mice treated with MCT alone (A), phenytoin and MCT (B), rifampicin and MCT (C), and phenytoin, rifampicin, and MCT (D) before cell transplantation. Transplanted cells were identified by DPPIV histochemistry (red color). In mice treated with MCT alone, transplanted cells did not proliferate. These data were verified in a second repeat study. Original magnification ×100; toluidine blue counterstain.

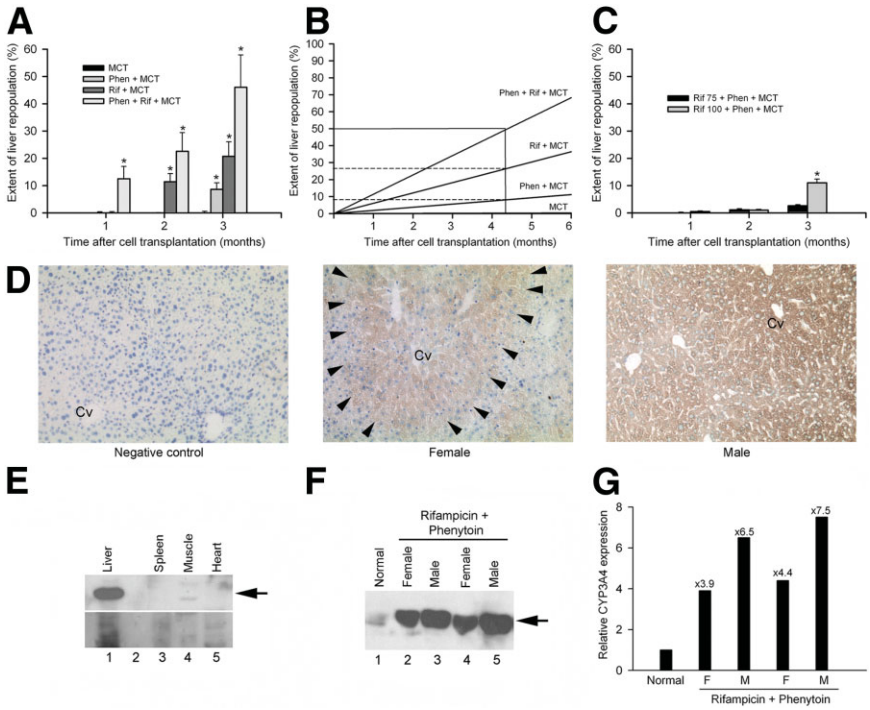

Fig. 8. Analysis of liver cell turnover. (A) Morphometric analysis showing progressive transplanted cell proliferation in drug-treated male mice, particularly after phenytoin, rifampicin, and MCT. (B) Analysis of liver repopulation kinetics indicating 50% liver was repopulated after 4 months in phenytoin, rifampicin, and MCT-treated mice. This liver repopulation rate was substantially higher than that for mice treated with only rifampicin and MCT or phenytoin and MCT. (C) Showing less liver repopulation in female DPPIV− mice treated with 75 or 100 mg/kg rifampicin, 30 mg/kg phenytoin, and 200 mg/kg MCT. (D) Immunostaining for CYP3A4 with negative control liver showing no staining, similar to drug-untreated normal male or female mice. After 30 mg/kg phenytoin and 75 mg/kg rifampicin for 3 days, CYP3A4 was stained in perivenous areas (Cv) of female liver (middle panel, arrows) and more extensively in the male liver (panel on right). (E) Western blot showing CYP3A4 expression in the liver (lane 1) and not in spleen (lane 3), skeletal muscle (lane 4), or heart (lane 5) of a mouse (top panel, arrow indicates expected 3A4 band; bottom panel, Ponceau Red staining of transblot). Lane 2 was empty. (F) Western blot analysis of CYP3A4 expression in mouse livers after induction using rifampicin and phenytoin for 3 days. (G) Densitometric scanning of bands in panel F.

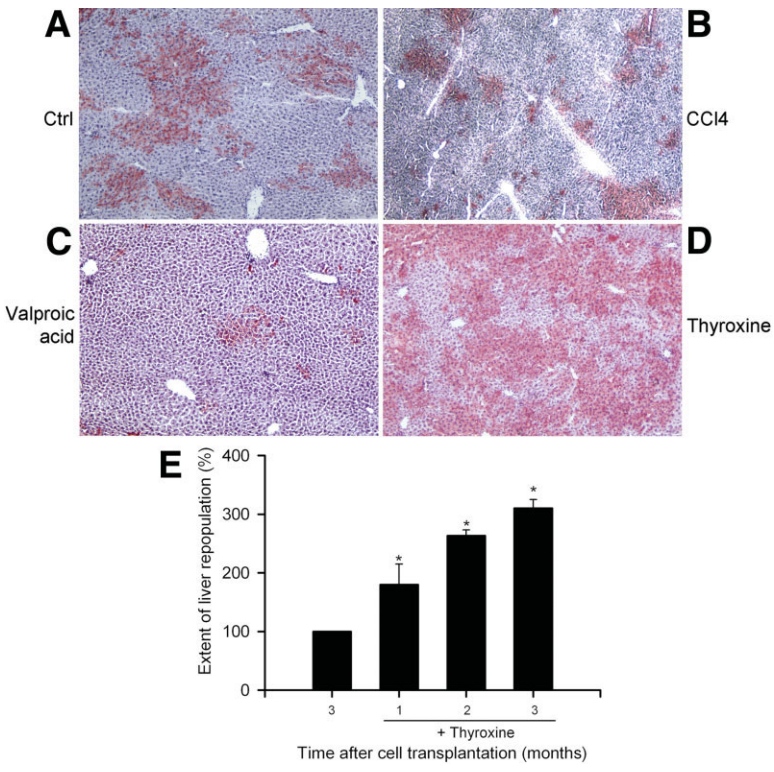

Fig. 9. Effect of additional drugs on hepatocytes in phenytoin-treated, rifampicin-treated, and MCT-treated mice. Shown is liver repopulation in male DPPIV− mice approximately 2 months after cell transplantation followed by additional treatments. (A) Control mice with phenytoin, rifampicin, and MCT treatment before cell transplantation. (B) Treatment with additional CCl_4 showing loss of transplanted cells from zones 2 and 3 of the liver. (C) Treatment with additional valproic acid showing loss of transplanted cells throughout the liver lobule. (D and E) Treatment with additional thyroxine showing more extensive liver repopulation compared with control mice. These findings were verified in 2 separate studies. Original magnification, ×100; toluidine blue counterstain.

indicated that both native and transplanted hepatocytes were affected. Similarly, nonselective damage in native and transplanted hepatocytes likely accounted for decreased liver repopulation after valproic acid, which causes cell injury through pro-oxidant and other mechanisms.[27] Conversely, thyroxine administration to mice treated with phenytoin, rifampicin, and MCT accelerated liver repopulation, which was in agreement with the ability of thyroid hormone to stimulate hepatic DNA synthesis while promoting apoptosis in cells with genotoxic damage.[28,29]

Taken together, these findings indicated that in mice exposed to phenytoin, rifampicin, and MCT, liver injury was age-dependent and gender-dependent, as shown here with 6-week-old to 8-week-old male mice. We used cell donors of 8 weeks' to 15 weeks' age for convenience; their age should have been immaterial unless aging-associated genetic lesions were to alter replication in healthy donor cells, which has not been shown. We consider that the central mechanism responsible for liver repopulation in MCT-treated mice involves loss of native cells, thereby requiring healthy transplanted cells to proliferate. Although cell transplantation in the liver transiently activates Kupffer cells, stellate cells and endothelial cells with hepatic expression of trophic, for example, hepatocyte growth factor, vascular endothelial growth factor, matrix-remodeling, metalloproteinases, and other genes that affect cell engraftment,[2,3,30,31] whether transplanted cell proliferation during liver repopulation is regulated by specific factors released by liver cells is unknown. Nonetheless, because transplanted cells retain physiologically regulated function after liver repopulation,[6,7] including in animal models of disease,[9,26,32] our findings should offer impetus for drug-based strategies to damage the native liver before cell transplantation. This should facilitate studies of cell and gene therapy, stem/progenitor cells, as well as animal model development.

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References

1. Tafazoli S, Spehar DD, O'Brien PJ. Oxidative stress mediated idiosyncratic drug toxicity. Drug Metab Rev 2005;37:311-325.
2. 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.
3. Wu YM, Joseph B, Gupta S. Immunosuppression using the mTOR inhibition mechanism affects replacement of the rat liver with transplanted cells. HEPATOLOGY 2006;44:410-9.
4. Benten D, Follenzi A, Bhargava KK, Kumaran V, Palestro CJ, Gupta S. Organ-specific targeting of transplanted endothelial cells in intact mice. HEPATOLOGY 2005;42:140-148.
5. Sokhi RP, Rajvanshi P, Gupta S. Transplanted reporter cells help in defining onset of hepatocyte proliferation during the life of F344 rats. Am J Physiol Gastrointest Liver Physiol 2000; 279:G631-G640.
6. Gupta S, Rajvanshi P, Sokhi R, Vaidya S, Irani AN, Gorla GR. Position-specific gene expression in the liver lobule is directed by the microenvironment and not by the previous cell differentiation state. J Biol Chem 1999; 274:2157-2165.
7. 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.
8. Guo D, Fu T, Nelson JA, Superina RA, Soriano HE. Liver repopulation after cell transplantation in mice treated with retrosine and carbon tetrachloride. Transplantation 2002;73:1818-1824.
9. Guha C, Yamanouchi K, Jiang J, Wang X, Roy Chowdhury N, Santana A, et al. Feasibility of hepatocyte transplantation-based therapies for primary hyperoxalurias. Am J Nephrol 2005;25:161-170.
10. Kasahara Y, Kiyatake K, Tatsumi K, Sugito K, Kakusaka I, Yamagata S, et al. Bioactivation of monocrotaline by P-450 3A in rat liver. J Cardiovasc Pharmacol 1997;30:124-129.
11. DeLeve LD, Ito Y, Bethea NW, McCuskey MK, Wang X, McCuskey RS. Embolization by sinusoidal lining cells obstructs the microcirculation in rat sinusoidal obstruction syndrome. Am J Physiol Gastrointest Liver Physiol 2003;284:G1045-G1052.
12. Tofovic SP, Salah EM, Mady HH, Jackson EK, Melhem MF. Estradiol metabolites attenuate monocrotaline-induced pulmonary hypertension in rats. J Cardiovasc Pharmacol 2005;46:430-437.
13. Shimada H, Furuno H, Hirai K, Koyama J, Ariyama J, Simamura E. Paraquat detoxicative system in the mouse liver postmitochondrial fraction. Arch Biochem Biophys 2002;402:149-157.
14. Bhuller Y, Jeng W, Wells PG. Variable in vivo embryoprotective role for ataxia-telangiectasia-mutated against constitutive and phenytoin-enhanced oxidative stress in Atm knockout mice. Toxicol Sci 2006;93:146-155.
15. Chowdhury A, Santra A, Bhattacharjee K, Ghatak S, Saha DR, Dhali GK. Mitochondrial oxidative stress and permeability transition in isoniazid and rifampicin induced liver injury in mice. J Hepatol 2006;45:117-126.
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. Guieu R, Fenouillet E, Devaux C, Fajloun Z, Carrega L, Sabatier JM, et al. CD26 modulates nociception in mice via its dipeptidyl-peptidase IV activity. Behav Brain Res 2006;166:230-235.
18. Rajvanshi P, Kerr A, Bhargava KK, Burk RD, Gupta S. Efficacy and safety of repeated hepatocyte transplantation for significant liver repopulation in rodents. Gastroenterology 1996;111:1092-1102.
19. 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.
20. 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.
21. McKenzie R, Fried MW, Sallie R, Conjeevaram H, Di Bisceglie AM, Park Y, et al. Hepatic failure and lactic acidosis due to fialuridine (FIAU), an investigational nucleoside analogue for chronic hepatitis B. N Engl J Med 1995;333:1099-105.
22. Kaplowitz N. Idiosyncratic drug hepatotoxicity. Nat Rev Drug Discov 2005;4:489-499.
23. 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.
24. 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.

25. Sokol RJ, Straka MS, Dahl R, Devereaux MW, Yerushalmi B, Gumpricht E, et al. Role of oxidant stress in the permeability transition induced in rat hepatic mitochondria by hydrophobic bile acids. Pediatr Res 2001;49:519-531.
26. 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.
27. Raza M, al-Shabanah OA, al-Bekairi AM, Qureshi S. Pathomorphological changes in mouse liver and kidney during prolonged valproate administration. Int J Tissue React 2000;12:15-22
28. Oren R, Dabeva MD, Karnezis AN, Petkov PM, Rosencrantz R, Sandhu JP, et al. Role of thyroid hormone in stimulating liver repopulation in the rat by transplanted hepatocytes. HEPATOLOGY 1999;30:903-913.
29. Malik R, Mellor N, Selden C, Hodgson H. Triiodothyronine enhances the regenerative capacity of the liver following partial hepatectomy. HEPATOLOGY 2003;37:79-86.
30. Joseph B, Malhi H, Bhargava KK, Palestro CJ, McCuskey RS, et al. Kupffer cells participate in early clearance of syngeneic hepatocytes transplanted in the rat liver. Gastroenterology 2002;123: 1677-1685.
31. 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.
32. Oren R, Dabeva MD, Petkov PM, Hurston E, Laconi E, Shafritz DA. Restoration of serum albumin levels in nagase analbuminemic rats by hepatocyte transplantation. HEPATOLOGY 1999;29:75-81.