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Chimeric Mice with Humanized Liver: Tools for the Study of Drug Metabolism, Excretion, and Toxicity

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Abstract

Recent developments in animal models have allowed the creation of mice with genetic alterations that cause hepatocyte damage that results, over time, in the loss of native hepatocytes. If donor, human hepatocytes are transplanted into these animals, they repopulate the host liver, frequently replacing over 70% of the native liver with human cells. Immunodeficient mice that overexpress urokinase-type plasminogen activator (uPA) and, alternatively, with a knockout of the fumarylacetoacetate hydrolase (Fah) genes are the two most common mouse models for these studies. These mice are called chimeric or “humanized” because the liver is now partially repopulated with human cells. In this report we will review the published work with respect to Phase I and Phase II metabolic pathways and the expression of hepatic transport proteins. While the studies are still at the descriptive stage, it is already clear that some humanized mice display high levels of repopulation with human hepatocytes, express basal and inducible human CYP450 genes, and human conjugation and hepatic transport pathways. When the strengths and weaknesses of these humanized mouse models are fully understood, they will likely be quite valuable for investigations of human liver-mediated metabolism and excretion of drugs and xenobiotics, drug–drug interactions, and for short- and long-term investigation of the toxicity of drugs or chemicals with significant human exposure.

Keywords

Human hepatocytes; cytochrome P450; conjugation; hepatic transport proteins; hepatotoxicity; drug–drug interactions

1. Introduction

By virtue of its expression of high levels of the enzymes involved in drug and xenobiotic metabolism and excretion, the liver has been the focus of much of the research related to drug metabolism and excretion. Also because of its critical role in xenobiotic and drug metabolism and excretion, the liver is a frequent target for the toxic manifestations of these agents. In addition to toxicity from chemical entities, hepatotropic viruses, and parasitic infections are responsible for massive amounts of human suffering and billions of dollars in health-care costs across the globe. Since there are large differences in their expression of the metabolic pathways involved in drug or xenobiotic metabolism and excretion or in the susceptibility of the liver to viral or parasitic infections between commonly used laboratory animals and humans, experiments with animals do not always faithfully predict or model what is observed in human subjects. To overcome some of these issues, mouse models have been created whereby the native mouse liver is replaced with human liver cells. Although this area of science is relatively new, there is sufficient data published to indicate that chimeric or “humanized mice” will be useful for investigations of metabolic pathways normally expressed in human liver. While humanized mice have been shown to be amenable to viral infections with hepatitis B or C and the hepatic stage of malaria, this chapter will

focus on the review of the literature on models of drug metabolism and excretory pathways in chimeric mice.

A number of approaches have been tried to get data relevant to humans, including replicating human cell lines, transgenic insertions of human genes, short- or long-term cultures of human hepatocytes and now also mice with humanized livers. Replicating cell lines have been used extensively to model human metabolism. However, there are no continuously replicating cell lines that provide normal liver levels of metabolic activity over a wide range of functions. Thus, they provide poor predictive value with respect to human liver metabolism. A second method that has proven useful is to humanize a mouse via a transgenic approach where the mouse ortholog is first knocked out and the human gene, such as a CYP gene, is inserted and expressed (1, 2). This approach has proven useful; however, there are a number of factors that may induce inappropriate expression of the transgene. The chromosome and the exact location where the BAC integrates can have a profound effect on transgene expression (3–5). The genetic background of the mouse strain also influences transgene expression presumably because of the presence or absence of modifier genes expressed concurrently in the mouse cells (6–8). The particular BAC containing the selected human gene may or may not contain all of the requisite *cis*- or *trans*-regulatory sites/sequences needed to observe normal expression of the transgene (9, 10). Also, even if the transgene is expressed appropriately in mouse hepatocytes, the remaining cellular components such as inhibitor and activator proteins and the cofactors for the reactions are all derived from the mouse and may not interact as efficiently or effectively with the human genes/gene products. Finally, drug metabolism and elimination does not rely on the expression of a single enzyme, but rather on the expression of whole pathways including Phase I and Phase II enzymes, hepatic transport proteins and the coordinated flow through these pathways during the metabolism and elimination process. Thus, one cannot create whole humanized metabolic networks inserting one gene at a time via the transgenic approach.

To overcome some of these limitations investigators have employed human hepatocytes in suspension or short- or long-term culture to investigate human-relevant drug metabolism processes (11–19). It is now clear that human hepatocytes provide the most useful and relevant data concerning the disposition of drugs by the human liver, but even these studies are limited to relatively short-term assays, the longest generally out to 30–40 days (20, 21). Recent efforts have focused on methods by which human liver metabolic pathways could be studied in long-term, *in vivo*, models. These models have had to be generated, not through cross-breeding of transgenic lines but in a brute-force approach whereby the liver of a suitable recipient animal is repopulated to high levels following the transplantation of human hepatocytes.

2. Mouse Models

The mouse models used for repopulation studies have some common elements. The mice are severely immunodeficient so that they will accept the xenograft of human hepatocytes. The second common features are genetic alterations in genes expressed in the liver that result, over a period of time, in the destruction of native hepatocytes. The loss of hepatocytes in these models allows the donor-derived hepatocytes a niche for engraftment and the regenerative stimulus to promote proliferation of the donor cells in the mouse liver.

The earliest reports of substantial repopulation of a mouse liver with human hepatocytes were published in 2001 by Dandri et al. (22) and Mercer et al. (23). In the report by Dandri, mice were rendered immunodeficient by knocking out the recombinant activation gene-2 (RAG-2) while in the report by Mercer, the investigators used animals with severe combined

immunodeficiency (SCID). In both reports, the liver failure was induced by expression of an albumin-promoted urokinase-type plasminogen activator (uPA). A review of the background on previous studies with these types of animals is provided by Dr. Gilgenkrantz in **Chapter 26**. Cell damage in the uPA model is thought to be caused by the intracellular activation of plasminogen which in turn activates plasmin which induces proteolytic damage inside the hepatocytes, particularly in the endoplasmic reticulum. Since these initial studies were focused on propagation of hepatitis viruses, the low levels of repopulation (up to 15%) were sufficient for that application. More recently, through a combination of optimizing the protocol and selection of particularly useful donor hepatocytes, Tateno and coworkers report much more consistent and higher levels of repopulation with the Alb-promoted uPA/SCID (hereafter referred to as uPA/SCID) animals (24–28). Grompe and coworkers have used a different approach to generate humanized mice (29). They first generated mice in which fumarylacetoacetate hydrolase (Fah), a gene in the catabolic pathway for tyrosine, is deleted. Accumulation of the toxic metabolite fumarylacetoacetate induces chronic liver damage. The drug 2-(2-nitro-4-trifluoro-methylbenzoyl)1,3-cyclohexedione (NTBC) blocks the accumulation of the toxic metabolite and prevents liver damage, so that animals can be maintained in a healthy state while on the drug and selective pressure for repopulation of the liver with donor (Fah-proficient) cells can be applied by withdrawal of NTBC. This strategy has proven to induce a robust expansion of transplanted cells (30, 31). When the Fah(–/–) mice were crossed with Rag-2(–/–) and interleukin 2 receptor common gamma chain(–/–) mice, a triple mutant mouse was produced (FRG), which was immunodeficient at two loci and still retained the selective pressure provided by the Fah deficiency. When transplanted, the FRG mouse readily accepted the xenograft and mice are highly repopulated with human hepatocytes (29) (Fig. 27.1).

3. Properties of a Model Useful for Generating Chimeric Mice

A robust model for generating chimeric “humanized” mice would have some basic properties that are summarized in Table 27.1. When the uPA/SCID and FRGF models are compared, there are certain traits with the FRG model that are quite useful. The selective pressure to regenerate the liver is controllable in the FRG model but is lacking in the uPA/SCID animals. Since the FRG animals are maintained on NTBC prior to transplant, virtually no liver damage is observed in the animals. Selection can be initiated by withdrawal of NTBC at any desired time. Also since the animals are maintained in a healthy state on NTBC, the animals breed normally and because of this property homozygous Fah(–/–) mice can be used as breeders. There is high neonatal mortality with the Alb-uPA model and breeding is more difficult; heterozygous breeding pairs are normally maintained which lowers the yield of homozygous animals in any given litter that are useful for transplant studies. Also because of the overexpression of urokinase, the animals have a particularly severe bleeding problem which necessitates that the animals receive transplants within a short window of time prior to weaning. Since the selection cannot be controlled, the strong regenerative pressure put on the failing liver frequently results in the deletion of the uPA transgene in a small number of mouse hepatocytes that effectively compete with the transplanted human cells during liver repopulation. The FRG is a knockout and not a transgenic mouse model, so genotype reversion cannot occur, and this feature leads to perhaps one of the most important advantages of the FRG model, that is, serial transplantation. Because genotype reversion does not occur in the FRG model, the native mouse hepatocytes remain Fah(–/–) and cannot compete with Fah-proficient donor cells. Thus, human hepatocytes from one repopulated mouse can be recovered by collagenase perfusion and transplanted into additional mice, expanding the numbers of animals that can be repopulated from an original human donor. An example of serial transplants is presented in Fig. 27.2 that shows that hepatocytes from a single human donor generate which then can be used as donor cells for additional transplants. In the case shown, cells from one of the

recipient mice eventually gave rise to 15 additional humanized mice through four serial transplants. This means that even small numbers of mice receiving primary transplants can be used to generate larger numbers of humanized mice through serial transplants for large studies or that unique or precious human genotypes can be maintained and passed on to subsequent generations of mice long past the lifetime of the original recipient mouse. An added feature of the serial transplants is also shown in Fig. 27.1, that is, the numbers of animals successfully engrafted with human hepatocytes generally improve with serial transplantation, perhaps because of the high viability of cells recovered from highly repopulated mice or possibly because the human cells somehow adapt to the mouse environment in the initial mice and engraftment and repopulation proceeds more effectively upon serial transplantation.

Significant renal damage has been reported in Alb-uPA animals that are highly repopulated with human cells, whereas no renal damage was noted in highly repopulated FRG mice. A final advantage of the FRG model is its ability to accept xenografts from all human donors tested. In the initial study, successful engraftment was observed with hepatocytes from nine of nine different donor cases ranging in age from 1 to 64 years (29), while the reports with the Alb-uPA model generally rely on hepatocytes from a small number of young donor livers (24, 25, 27, 28, 32). It is important to point out, however, that high-level repopulation (>90%) with human hepatocytes can be obtained with both models, so that both approaches are highly effective when applied properly.

4. Methods for Generating Chimeric Humanized Mice in the FRG Model

The FRG mice are immune-deficient Fah knockout mice lacking the genes for *Rag-2* and the common gamma chain of the interleukin receptor. These animals breed normally while on NTBC. These FRG mice can be readily repopulated with human hepatocytes after pre-treatment with a vector expressing urokinase 1–3 days prior to transplantation.

The standard method involves injection of 250,000–1 million cells into the spleen of adult FRG mice. One to three days prior to transplantation, each mouse is given 1×10^9 pfu of an adenoviral vector expressing human urokinase (uPA). This manipulation significantly enhances initial cell engraftment. Any post-weaning animal can be transplanted and we prefer mice 4–6 weeks of age. Animals are given broad spectrum antibiotics prior to surgery and for 1 week after transplantation. NTBC is withdrawn on day 1 after transplantation to induce liver disease and graft selection. If a transplanted mouse loses >20% of its pre-transplant weight, NTBC is re-administered for 5 days. This break in selection permits the animal to recover before further selection. The level of engraftment and repopulation is monitored by measuring blood levels of human albumin monthly using an ELISA kit from Bethyl. Successful repopulation with mature human hepatocytes in chimeric mice is estimated by qRT-PCR for important markers of hepatocellular function, particularly drug metabolism. The expression of human hepatocyte-specific genes is normalized to human cyclophilin, beta-actin, and/or beta-2 microglobulin mRNA levels, and comparisons are made between the values obtained with chimeric mice and those observed in the donor tissue. The mRNA levels are also compared to human fetal liver. The panel of markers is informative to determine the maturity of the cells produced.

In general, the degree of humanization of the liver correlates with human albumin blood levels in that 1 mg/mL corresponds to ~20% human hepatocytes. In many animals very high levels of humanization can be achieved, as documented with immunohistochemistry for FAH or human albumin or cytokeratin expression, as well as FACS analysis of hepatocytes harvested from FRG mice stained with antibodies that react selectively with human or murine surface antigens (29).

In serial transplantation, the liver of a previously humanized mouse is perfused with collagenase and the isolated cells are re-transplanted into additional FRG mice. Since there is no possibility of genetic reversion in the FRG model, residual mouse cells in the transplant do not compete with the human cells for repopulation of the next generation mice (Fig. 27.2).

Although the initial studies were conducted by the transplantation of hepatocytes into the spleen of 4- to 6-week-old mice, using the same model (FRG mice), Bissig et al. reported that neonatal mice could receive direct injections of hepatocytes into the liver parenchyma, and levels of repopulation up to 7% were obtained (33). In separate studies, we reported nearly identical levels of repopulation (6–8%) in a mouse model of a metabolic liver disease following direct liver injection of hepatocytes into neonatal mice (34).

Although there is robust repopulation of the FRG mice with human hepatocytes, it is the result of integration and expansion of human cells, as there is no evidence of cell fusion in this model (29, 33). Also, the human cells are susceptible to transduction with lentivirus (33) or retroviruses (35) and express the transgene for at least 2 months, suggesting that the humanized models will also be useful for gene therapy protocols.

5. Expression and Induction of CYP450 Genes, Proteins, and Metabolic Activities in Humanized Mice

The first report of the investigation of human CYP450 gene expression in highly humanized uPA/SCID mice was by Tateno et al. in 2004 (24). She reported high levels of repopulation of many animals with human cells and indicated that nearly one-third of their transplants resulted in animals with a level of repopulation with human hepatocytes estimated to be greater than 70%. These investigators calculated what they termed the replacement index (RI), which was determined as the percent of the area occupied by human hepatocytes in representative tissue sections each made of six or seven liver lobes. Human cells were determined by immunohistochemistry with antibodies that specifically react with human but not mouse cytokeratin 8/18 or by in situ hybridization studies with hDNA probes. The RI as calculated from hDNA correlated well with the RI calculated by immunohistochemistry and both correlated well with the amount of hAlb in the mouse plasma or serum. In future studies, the RI calculated by immunohistochemistry and circulating hALB would be used to estimate the level of replacement of the mouse liver with human cells. Animals with 50% repopulation, or greater, suffered multisystem damage, particularly necrosis and atrophy of the kidney, that had to be treated with anti-complement drugs. It was suggested that the secretion of human complement factors by hepatocytes resulted in severe proteolytic damage to host tissues. Administration of Futhan, an anti-complement drug, reversed the symptoms and allowed higher level repopulation with human hepatocytes. These investigators could identify human-specific mRNA for CYPs 1A1, 1A2, 2C9, 2D6, and 3A4 and reported a profile of gene expression that was similar to the original donor liver. Protein expression of CYP2C9 was confirmed by Western blots. Microsomes isolated from chimeric mice showed diclofenac 4-hydroxylase activity that was significantly greater than the mouse control animals. These investigators reported that the level of many of the CYP genes was actually higher in chimeric mice with highly repopulated livers than in the original donor liver tissue. This observation was confirmed in subsequent reports.

Expression of CYP enzymes, in vivo, was investigated in greater detail by Katoh et al. (25). These investigators measured the mRNA levels by quantitative RT PCR, protein expression by Western blots, and metabolic activity for specific human CYPs based on drug metabolism studies with microsomal proteins isolated from the chimeric mice. They reported the expression of all of the major human CYPs and like Tateno et al., previously,

showed that the level of expression of the human CYP in the mouse liver correlated well with the levels of circulating hAlbumin (hAlb) detected in the serum of chimeric mice. Thus, the levels of repopulation of the chimeric mice can be assessed and estimated simply by monitoring the levels of circulating hALB. When compared to control, non-transplanted animals, microsomes from chimeric mice demonstrated significant metabolic activity generally attributed to specific CYPs including CYP2C9-mediated diclofenac-4-hydroxylase (DICOH), 3A4-mediated dexamethasone 6-hydroxylase (DEXOH), 2A6-mediated coumarin 7-hydroxylase (COH), 2C8-mediated paclitaxel 6-hydroxylase (PTXOH), 2C9-mediated *S*-mephenytoin 4-hydroxylase (MPOH), and 2D6-mediated debrisoquine 4-hydroxylase (DBOH). These authors also observed that protein and activity levels measured in highly repopulated mice were frequently slightly higher than that observed in a sample obtained from the donor liver; however, in general they concluded that the mice maintained the genotype and phenotype of the donor.

The expression of mature human liver genes in the FRG model was reported by Azuma et al. (29). In these studies the expression of the genes in the chimeric mouse liver was compared to adult liver ($N=8$ donors) and also to the levels of expression observed in fetal human liver tissue. The expression of the mature liver genes Alb, CYP1A2, and CYP3A4 was found to be expressed at the same levels as observed in the donor livers. The expression of alpha fetoprotein (Afp) was detected in all tissues, but the levels on the FRG mice were the same as those observed in mature human liver and much lower than those observed in fetal human liver. These data indicate that human hepatocytes maintain a mature phenotype following transplantation and engraftment into FRG mice. Similar studies of fetal versus the adult phenotype were not reported with the uPA/SCID animals.

A more detailed investigation of CYP2D6-mediated metabolism of debrisoquin, *in vivo*, in chimeric mice was reported (36). Here the authors repeat the observations made previously that the metabolic activity associated with CYP2D6 correlates with the level of repopulation of the mouse liver with human hepatocytes, which in turn was indirectly estimated by measuring the levels of circulating hAlb. When debrisoquine (DB) is administered to non-transplanted control mice, metabolism of DB occurs, although the production of the specific metabolite, 4-hydroxy-DB (4-OHDB), was not observed. They also report that the production of 4-OHDB was significantly inhibited by prior administration of quinidine (100 mg/kg/day for 3 days), a known inhibitor of CYP2D6 in humans. Prior administration of paroxetine (30 mg/kg/day for 3 days), a selective serotonin reuptake inhibitor, and a mechanism-based inactivator of human CYP2D6 also caused a significant decrease in the area under the curve for the production of 4OHDB. It was reported that quinidine and paroxetine selectively inhibited the human hepatocyte-mediated production of 4-OHDB, but did not inhibit the background, mouse-mediated metabolism of DB, supporting the hypothesis that the 4-OHDB metabolite is human specific. Although intermediate levels of repopulation were not investigated, the specific production of 4OHDB in chimeric mice was observed in animals that were repopulated to a level of 70% or more with human hepatocytes, while mice with 10% or less repopulation with human hepatocytes were indistinguishable from control, non-transplanted animals. The level of repopulation required to observe metabolism attributable to the presence of human hepatocytes will likely differ between test drugs and will depend on the specificity of metabolite to human metabolism and the level of metabolism observed in non-transplanted mice for those metabolites that are not specific to human hepatocytes.

The expression of CYP2A6 and coumarin hydroxylase activity was investigated in chimeric mice with an intention of determining if the level of repopulation of the liver could be estimated from the level of humanization of CYP2A6-mediated metabolism (37). Microsomes were prepared from chimeric animals with different levels of repopulation

ranging from 0 to 84% as estimated by circulating hAlb levels and cytokeratin immunohistochemistry (24). The human liver expresses CYP2A6, and the mouse expresses Cyp2A5; however, there is significant overlap in substrate specificity. Since there was significant COH activity even in non-transplanted uPA/SCID animals, specific inhibitors of mouse and human COH activity were identified. Benzaldehyde and undecanoic-lactone (100 μ M each) were identified as specific inhibitors of human (CYP2A6) or mouse (Cyp2A5)-mediated COH activity. As in the earlier papers, these authors attempted to correlate the RI with serum hAlb levels. In these studies, using large numbers of chimeric animals ($N = 17$) with a wide range of levels of repopulation, they did not see a good correlation between RI and hAlb levels until estimated levels of repopulation reached 50%. Thereafter there was a steep rise in hAlb as RI increased and a better correlation of RI and hAlb. This trend was not only observed with hAlb levels, as the estimated RI or hAlb levels did not correlate well with the COH activity until the level of repopulation reached or exceeded 50%. Thus, with respect to COH activity and hAlb secretion the mice did not display the humanized phenotype until a RI of 50% or more was obtained. These observations are unfortunate, because only 9 of the 17 mice used in these studies displayed RI of 50% or greater and would have provided reliable estimates of human-mediated metabolism.

In addition to the basal levels, the induction of CYP enzymes, *in vivo*, in uPA/SCID chimeric mice was also investigated. Tateno et al. reported that when chimeric mice were administered prototypical CYP450-inducing agents such as rifampicin (Rif) or 3-methylcholanthrene (3-MC), *in vivo*, increased expression of mRNA for CYP3A4 and CYP1A1 and 1A2 was observed. Interesting, however, was that no increase in the expression of CYP2C9 or 19 was noted in these experiments following Rif administration.

Katoh et al. reported that prior treatment of the mice for 4 days with rifampicin (50 mg/kg/day) induced the expression (RNA and protein) of CYP3A4 and DEXOH activity, CYP2A6 (RNA and protein) and COH activity, CYP2C9 (RNA and protein) and DICOH activity, and CYP2C19 expression (RNA) and MPOH activity (38). Except for CYP3A4, the level of induction of other genes was relatively small, and a subsequent report using this mouse model failed to demonstrate a significant induction of CYP2A6, 2C8, 2C9, or 2C19 following rifampicin treatment (32), so a certain amount of variability is to be expected in the system. Rifampicin treatment also increased the levels of mouse Cyp 3A11 expression and testosterone 6-beta-hydroxylase activity, which would confound *in vivo* metabolic studies of the metabolism of CYP3A substrates; however, prior induction with Rifabutin, a compound structurally similar to rifampicin, failed to induce mouse 3A expression of enzymatic activity, so rifabutin might be a useful and selective human CYP3A-inducing agent for *in vivo* studies with humanized mice.

Basal and induced CYP1A family genes were also investigated in chimeric mice by Uno et al. (2). Because they are easier to use, many investigators prefer to use continuously growing cell lines or humanized transgenic animals carrying a single or only a few human CYP genes. In this chapter the authors compared the results of their investigation of CYP1A1 and CYP1A2 expression and metabolic activity between the chimeric humanized mice, a transgenic mouse strain carrying on a bacterial artificial chromosome (BAC) the human CYP1A1_CYP1A2 locus and lacking the mouse Cyp1a1 and Cyp1a2 orthologs and continuously growing cell lines Hepa-1c1c7 and HepG2. They reported that the transgenic humanized mouse strain expressed far lower CYP1A1-mediated metabolic activity than expected based on the RNA levels and what was observed in wild-type mice. In contrast hCYP1A2 appeared to function nearly as well as the mCyp1a2 in the wild-type mouse liver. These authors conclude that there are significant differences between the different models: chimeric mice, transgenic humanized mouse strains, and replicating cells lines. Depending on the application each can be used if one carefully characterizes the system and

understands the limits of response. The results clearly indicated that the RNA expression and metabolic activity observed in the replicating cell lines were not reliable indicators of those processes, in vivo, and that the cell lines were no substitutes for authentic mouse or human liver.

Since chimeric mice are quite rare, one does not necessarily want to have to terminate the animal and harvest liver tissue to conduct an analysis or to determine if CYP enzymes were induced by a specific treatment. A non-invasive method was reported for the examination of CYP3A4 metabolic activity in chimeric mice (39). When challenged with dexamethasone, human hepatocytes preferentially produce 6-*b*-hydroxydexamethasone while mouse hepatocytes preferentially produce 6-hydroxy-9 α -fluoro-androsta-14-diene-11 β -hydroxy-16 α -methyl-3,17-dione. Thus, human-specific metabolism of dexamethasone can be identified even when there is a significant background of metabolism contributed by the mouse liver. Emoto et al. (39) used these differences in metabolism to develop a non-invasive method to detect induction of CYP2A4 in chimeric mice with humanized liver. These investigators examined the change in the ratio of 6 β -hydroxy DEX to DEX in the urine of animals before and after administration of rifampicin and determined that an increase in this ratio correlated with the induction of CYP3A4 in the human cells of chimeric mice. The authors also indicated that CYP3A5 was also able to metabolize Dex to the 6-hydroxy dexamethasone; however, the intrinsic clearance by CYP3A4 was approximately 50-fold higher than that of CYP3A5, so under ordinary circumstances the majority of DEXOH produced will be the result of CYP3A4-mediated metabolism. This technique works when one already understands the metabolic profile of the test drug when administered to mice and human subjects. A significant contribution of the mouse liver to the disposition of a drug in the uPA-SCID chimeric mice certainly confounds the analysis of drug metabolism and disposition using this model.

Members of the CYP1A family were also induced by prior treatment of mice with 3-methyl cholanthrene (3-MC, 20 mg/kg/day) for 4 days. Steady state RNA levels for CYP1A1 and 1A2 were induced by 3-MC, and increases in CYP2A2 protein could be detected by Western blot. These investigators could not identify a metabolic assay for CYP1A activity because of high background activity in control mice. As expected, neither rifampicin nor 3-MC treatment increased CYP2D6 RNA, protein, or metabolic activity.

In later studies, Nishimura et al. investigated the induction of CYP expression, ex vivo, in hepatocytes isolated from chimeric mice (27). In these studies hepatocytes were isolated and cultured from the liver of humanized mice. As expected, exposure of these cultured cells to prototypical inducers, beta-naphthoflavone (BNF) or rifampicin, resulted in significant increased expression (RNA) of CYP1A2 (2–6-fold) and CYP3A4 (2–8-fold), respectively. The authors did not report on the analysis of drug metabolic activity associated with these CYPs, perhaps due to excessive background metabolism by native mouse hepatocytes. This hypothesis is supported by the studies of Uno et al., where the authors analyzed microsomal proteins for CYP1A-mediated metabolism using four different assays (2). Ethoxyresorufin *O*-deethylase (EROD) and benzo(*a*)pyrene hydroxylase are general assays for CYP1A activity, but with a tendency to be more representative of CYP1A1 activity, while methoxyresorufin *O*-deethylase and acetanilide 4-hydroxylase are relatively more specific for CYP1A2 activity. The background activity in all four assays in control animals, that is, the non-transplanted uPA/SCID animals, was equal to or greater than the metabolic activity observed in human chimeric animals with repopulation indices of 50–70%. This was true of both the basal levels and the activity measured following maximal induction of CYP1A activity by prior exposure to TCDD. Like the CYP2A6-mediated metabolism of coumarin cited above, the background activity in the mouse interferes with the analysis of human-specific metabolism.

This problem of high levels of background activity is not a characteristic of chimeric mice in general. In the FRG mice, animals with levels of repopulation with human hepatocytes estimated to be 10%, 30%, and 60% all showed basal EROD levels significantly higher than the non-transplanted mouse controls and also showed robust (8- to 40-fold) induction of EROD activity following a 2-day exposure to beta-naphthoflavone (29). As described above, the differences between these models most likely result from the spontaneous deletion of the transgene in the uPA/SCID model and competitive repopulation of the mouse liver with mouse cells concurrently with human hepatocyte repopulation. The mouse cells that have successfully deleted the transgene are robust and healthy and metabolize drugs in a manner characteristic for that mouse. Remnant mouse cells in the FRG mice are still deficient in *Fah* and are not robust or healthy when the animals are off of NTBC, thus they contribute little to the overall metabolic activity that can be measured in this model. Similar results were observed with CYP3A4-mediated metabolism of testosterone to the 6-beta-hydroxy metabolite, where the background activity with cultures of FRG hepatocytes alone contributed negligible amounts of product to that observed in control, rifampicin, or phenobarbital-induced cells from chimeric mice. The lack of competition and metabolic activity from the remnant mouse cells may be an important difference between the FRG and the uPA/SCID models especially when in vivo or ex vivo drug metabolism studies are planned.

6. Expression of Phase II Enzymes in Chimeric Mice

Chimeric humanized mice also express human Phase II enzymes, although the lack of specific substrates and useful specific antibodies prevented a detailed examination of some Phase II pathways (26). The expression (RNA) of UDP-glucuronyltransferase (UGT) 1A1, 1A9, and 2B7 was measured in chimeric mice, and the metabolic activity attributed to UGT2B7, morphine 6-glucuronyltransferase, was readily detected in assays of microsomal proteins. Troglitazone sulfotransferase activity attributed to sulfotransferase (SULT) 1A1 and estrone 3-sulfotransferase activity attributed to SULT1E1 were analyzed in assays of subcellular fractions (microsomes and/or cytosolic proteins). Only RNA levels for SULT1B1 were analyzed because of a lack of suitable and specific metabolic assays and antibodies for Western blots. Sulfamethazine *N*-acetyl transferase attributed to *N*-acetyltransferase-2 (NAT2) activity was also measured in sub-cellular preparations derived from chimeric mice. As with SULT and NAT expression and metabolic activity, the expression of glutathione transferases (GST) genes 1A1, 1A2, and T1 correlated well with the levels of circulating hALB in chimeric mice. The authors concluded that the chimeric mice express functional Phase II genes and enzymatic activities and that the mice should be useful for investigations of these pathways in a manner relevant to humans.

Elimination of drugs or xenobiotics from the body is dependent on the coordinated action of several pathways and normally includes metabolism by Phase I enzymes, conjugation of the parent drug and/or the metabolites, followed by transport out of the liver back into the circulation for possible urinary elimination or transport into the canicular space between hepatocytes which leads to biliary elimination in the feces. Since chimeric mice have humanized many parts of these pathways one might expect that following the administration of drugs, the mice would exhibit a human profile of drug elimination. This hypothesis was examined by Okumura et al. following administration of cefmetazole (CMZ), an antibiotic in the cephalosporin family (40). This drug is normally eliminated unchanged in rodents and humans, but by different routes. In humans, CMZ is eliminated predominately into the urine, while in rats and mice, the biliary pathway predominates. When CMZ was administered to uPA/SCID mice 23% of the dose was recovered in the urine while 59% of the dose was recovered in the feces. In chimeric mice with humanized livers, 81% of the dose was recovered in the urine over a 24-h period, while only 6% was recovered in the feces. These

data indicate that when the liver of mice is repopulated with human hepatocytes, the preferred route of elimination of this drug changes from a rodent-type elimination to the general profile normally observed in humans. These data suggest that chimeric mice will be useful for investigating the pathways by which drugs or xenobiotics are excreted. Evidence of connections (immunohistochemical) between human hepatocytes and the mouse biliary tree was also reported by Meuleman et al., although drug elimination was not studied (41).

7. Toxicology Studies with Chimeric Mice with Humanized Liver

A long-sought goal of toxicology is the prediction of human toxicity using model systems (42). Chimeric mice offer the possibility of conducting toxicology studies with a compound toward human hepatocytes in vivo. Sato et al. reported the administration of acetaminophen to ICR mice and to uPA/SCID chimeric animals (43). Acetaminophen caused severe centrilobular necrosis in the ICR mice at doses of 400 or 1,400 mg/kg, while chimeric mice showed evidence of only mild vacuolation of hepatocytes, few TUNEL-positive cells, and a mild decrease in CYP2E1 expression as determined by immunohistochemical methods. At 24 h, all ICR mice were dead, while all chimeric animals were alive. Thus, selective APAP toxicity could be demonstrated between control and chimeric mice; however, in this case chimeric animals were much less sensitive to APAP toxicity as compared to control animals. The chimeric animals used for these studies showed high levels of repopulation with human cells (RI of 68–95%). Despite these high levels of repopulation with human cells, the animals still showed evidence of ongoing regeneration and proliferation in the human regions of the liver with a PCNA-labeling index ranging from 22 to 68%. The authors suggested that since the areas of liver containing human hepatocytes also showed evidence of continued replication, the human hepatocytes in the uPA/SCID animals may be functionally immature, and the resistance of the human cells to APAP toxicity might have resulted from the inability of the human cells to fully activate the toxicant. Clearly additional work needs to be done to determine the mechanism(s) responsible for the selective toxicity to mouse hepatocytes in this model.

8. Summary and Future Directions

It is clear that the liver of mice can be humanized by the transplantation of human hepatocytes if specific genetic alterations in the mice allow the acceptance of the xenograft and also provide for a selective regenerative stimulus to the donor cells. It is also clear that virtually all of the human CYP or Phase II conjugation pathways and transport proteins examined are expressed in the chimeric animals. What is not entirely clear in many of the studies is how the levels of expression of these pathways in the chimeric mice compare to the expression of the same pathways in the donor liver. RNA levels or metabolic activity corresponding to CYP1A2 and 3A4 (24, 29) or 2C9 and 19 or 2A6 (24, 37) were reported, and animals with highly repopulated animals showed profiles that corresponded well with those observed in the donor tissue; however, additional CYPs, Phase II, and transport pathways need to be examined and quantified to get a clearer picture of the level of maturation of these pathways in chimeric mice. Since primers to quantify specific human genes can be devised, there is much more data available on the levels of RNA for the human genes than there is on protein levels or metabolic activity specific to the human hepatocytes. Thus, antibodies and assays that can discriminate between human and mouse cells are also needed. The presence of remnant mouse hepatocytes even in highly repopulated animals continues to be a significant problem, especially with the uPA/SCID animals (2, 27, 37), whereas the background mouse hepatocytes do not seem to contribute much metabolic activity in the FGR model (29).

Although the drug-metabolizing genes are relatively well studied in these animals, additional basic studies are still needed to determine how the architecture of the remodeling/regenerating chimeric human liver might affect drug metabolism and excretion and overall hepatic metabolism. Relatively little is known of the level of interaction or connections between the human hepatocytes within the regenerative nodules. Careful studies will also be needed to identify the interaction of sinusoidal endothelial cells (mouse or human) with hepatocytes or to quantify the number of tight junctions between hepatocytes, the relative proportion of hepatocytes that form bile canicular structures, and the level of integration of the human hepatocytes with mouse or human components of the biliary tree. Sato et al. reported that in chimeric mice the human hepatocytes from trabecular cord-like structures and sinusoid-like structures were observed, that were lined with endothelial cells, although Kupffer cells were not apparent in the humanized areas (43). Since normal hepatic function relies on the proper interaction of hepatocytes with endothelial, Kupffer, stellate, and biliary components of the liver, these questions are not merely academic. Finally, other aspects of human liver metabolism can now be investigated. What are the levels and what types of bile acids are produced in chimeric mice? How functional is the enterohepatic circulation? What are the levels of cholesterol, HDL, LDL, and lipoproteins in chimeric mice? Can we induce fibrosis in the human portions of the mouse liver? Can we create other models of metabolic liver disease using this system? Will long-term toxicology studies with chimeric animals be able to predict the potential human toxicity of new drug candidates? Can we use chimeric mice to propagate or scale up unique genotypes, such as cells from specific ethnic groups, therefore addressing the concern about genetic diversity of the human population? Will long-term toxicology studies with chimeric animals be able to provide an earlier and more sensitive prediction of drug-induced liver injury (DILI) in human patients? Can we use the mice as continuous and real bio-reactors that could provide human hepatocytes on demand from our vivariums? Each of these questions is important and will surely be addressed in the near future.

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Fig. 27.1.

Humanized mouse liver model (FRG). A deficiency in Fah activity leads to the accumulation of metabolites of tyrosine that are toxic to native hepatocytes. Transplants of Fah-proficient hepatocytes lead to replacement of the native cells and repopulation of the liver with donor cells. Stem cells from various sources may be useful sources of hepatocytes in the future.

Fig. 27.2.
Serial transplantation of human hepatocytes.

Table 27.1

Properties useful for the generation of chimeric mice

	Albumin-uPA	FRG
Extensive liver humanization	+	+
Controllable selection	–	+
Genotype reversion	+	–
Age of transplant	Only young (pre-weaning)	Any age
Breeding efficiency	Low	High
Bleeding	+	–
Renal damage after repopulation	+	–
Serial transplantation	–	+