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REVIEW

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Hepatocyte transplantation: clinical and experimental application

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Abstract Hepatocyte transplantation has been proposed as an alternative to whole-organ transplantation to support many forms of hepatic insufficiency. Based on a significant body of work, the technique of hepatocyte transplantation has recently moved into the clinic in order to reestablish liver function without organ transplantation or to bridge the time between whole-organ liver transplantation. In addition, hepatocyte transplantation has also been proposed as a liver-directed gene therapy for a number of inherited hepatic disorders by transplanting either freshly isolated hepatocytes or genetically altered hepatocytes. To establish a research system based on the developing technology of hepatocyte transplantation, chimeric small animal models using human hepatocytes have recently been established, which would allow the study of human hepatocyte-specific functions, such as hepatitis viral infection and replication in vivo. Various aspects related to the recent progress and existing obstacles in the area of hepatocyte transplantation are summarized in this report.

Keywords Hepatocyte transplantation · Gene therapy · Hepatitis virus animal model · Retrovirus vector · Lentivirus vector

Abbreviations *AAT:* α_1-Antitrypsin · *FHF:* Fulminant hepatic failure · *HBV:* Hepatitis B virus · *HCV:* Hepatitis C virus · *HDV:* Hepatitis delta virus · *HGF:* Hepatocyte growth factor · *HIV:* Human immunodeficiency virus · *MLV:* Moloney-murine leukemia virus · *OLT:* Orthotopic liver transplantation · *OTC:* Ornithine transcarbamoylase

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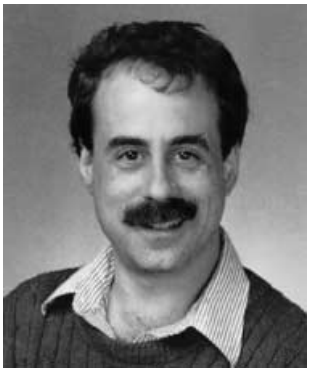

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Introduction

The liver is an important organ in the body where protein synthesis and metabolism of exogenous and endogenous substrates are performed. A number of inherited hepatic disorders and liver failures have been treated with orthotopic liver transplantation (OLT). Unfortunately, the number of patients who have benefited from OLT is very limited, because of the shortage of healthy donor livers. Therefore, techniques have been developed to isolate human hepatocytes for transplantation into recipient livers. Studies have been undertaken to improve the methods related to hepatocyte transplantation, which include hepatocyte isolation from donor livers, cell culture propagation of the hepatocytes, hepatocyte preservation, and genetic modification of the hepatocytes to provide liver-

specific functions. After many years of basic research, these approaches have moved into the clinics, where more than 50 patients have been treated by hepatocyte transplantation either with allogeneic [1, 2, 3, 4, 5, 6, 7] or autologous hepatocytes with or without genetic modification in vitro [8, 9]. For this reason, the transplantation of hepatocytes into recipient livers has the potential advantage for providing treatments of inherited liver disorders and liver failure regardless of the etiology [10, 11].

It has been widely believed that the optimal site for hepatocyte transplantation to treat hepatic diseases and/or disorders was the liver (orthotopic transplantation), and therefore the majority of the clinical trials that have been conducted were designed to transplant hepatocytes by direct administration into the liver via the portal or splenic circulation. However, others have found that ectopic transplantation of the hepatocytes (i.e., transplantation of hepatocytes to areas of the body other than the liver such as under the kidney capsule, subcutaneous space, and peritoneal cavity) can also have therapeutic efficacy [12, 13]. Furthermore, ectopic transplantation of hepatocytes have some advantage over the intraportal transplantation, because a large number of hepatocytes can be transplanted to most of the ectopic sites with minimally invasive techniques. The approach to transplant hepatocytes in an ectopic site may represent an alternative therapy if methods can be developed to prolong the survival and functional abilities of these transplanted hepatocytes. In a recent study, we have demonstrated as a proof-of-principle that hepatocytes can be engrafted long-term at ectopic sites, under the kidney capsule [14] and subcutaneous space [15]. Based on the success of ectopic hepatocyte transplantation, a chimeric mouse model system with human hepatocytes has been developed [14, 15], which allowed us to study various aspects of human hepatocyte-specific functions in vivo. These include mouse models that support human hepatitis viral infection and replication in vivo [14]. The experimental and clinical progress in the field of hepatocyte transplantation, which includes hepatocyte isolation, ex vivo manipulation of hepatocytes with retroviral and lentiviral vectors, and the development of small animal model systems using hepatocyte transplantation are the subject of this review.

Isolation and cell culture of hepatocytes

The technique used to isolate hepatocytes was originally proposed by Berry and Friend [16] using a two-step collagenase perfusion method in rodent livers. Modifications in the process of hepatocyte isolation in terms of collagenase concentration and perfusion rate based on the two-step collagenase perfusion method have been studied [17, 18] and used to isolate human hepatocytes for clinical application [1, 2, 3, 7, 8]. However, one major limitation for allogeneic transplantation with freshly isolated hepatocytes is the availability of donor livers at the necessary hepatocyte infusion time for the patient. It is relatively rare that a quality liver that is anatomically acceptable for transplantation is available for the isolation of hepatocytes [19]. Most nontransplanted livers are the result of traumatic tissue damage, high-grade macrosteatosis, or other disease states. In many clinical settings, the need for isolated hepatocytes is for acute situations, especially in cases of fulminant hepatic failure. Therefore, it remains an important issue to determine the factors that would enable primary cultured, hepatocyte progenitors and/or cryopreserved hepatocytes to maintain their biological functions at a level similar to freshly isolated cells.

Primary cultured hepatocyte

Although there have been recent advances in culturing of hepatocytes, hepatocytes in culture rapidly lose their hepatocyte-specific functions within several days [20]. The human hepatocytes isolated by the modified two-step collagenase perfusion method described above retain partial functionality for up to 4 days in cell culture as measured by microsomal enzyme expression [18]. We have recently confirmed that primary human hepatocytes can be cultured for at least 48 h and transplanted into mice without losing their in vivo hepatocyte-specific functions, protein expression, and regenerative capabilities following activation of c-Met after transplantation [14]. This period of time may be sufficient to provide the opportunity to ship and handle these cultured hepatocytes in order to perform emergency transplantation in any part of the world. This is substantiated by a recent and successful clinical use of cultured hepatocytes (for 48 h) for transplantation into a recipient liver as a bridge to whole-liver transplantation [4]. Another potential advantage would be the ability to transduce gene(s) of interest using viral or nonviral vector-based systems (see “Ex vivo gene therapy to hepatocytes”) into these hepatocytes prior to transplantation.

Immortalized human hepatocytes

Present cell culture conditions have not allowed extensive proliferation of primary hepatocytes. Thus the establishment of an “immortal” hepatocyte cell line that could be continuously grown in unlimited quantities while retaining much of the characteristics of differentiated hepatocytes would be highly desirable. Cell immortalization could be generated by transferring specific oncogenes [21] into these hepatocytes. Immortalized human or murine hepatocyte cell lines have been primarily established by transfecting the simian virus 40 large T antigen [12, 22, 23, 24, 25, 26, 27, 28, 29] or culturing primary hepatocytes in a collagen gel sandwich [30]. These immortalized cell lines have been shown to retain the morphological and functional characteristics of differentiated hepatocytes, such as the expression of hepa-

tocyte-specific proteins, and drug-metabolizing enzymes through several early passages of these cells. Since primary nonimmortalized hepatocytes have been shown to demonstrate hepatocyte-specific functions only for a short duration, it appears that immortalized hepatocytes may be offered as a viable alternative for future use in hepatocyte transplantation.

To investigate the therapeutic efficacy of transplanted immortalized hepatocytes in vivo, several different animal models of hepatic insufficiency such as chemically induced hepatic encephalopathy [26], surgically induced acute liver failure [12], and inherited liver diseases [25] have been studied. When the immortalized hepatocytes were transplanted into the peritoneal cavity of rats with acute liver failure induced by 90% hepatectomy, more than 80% of the rats survived, whereas none of the rats without transplantation survived longer than 60 h [12]. Similarly, transplanting immortalized hepatocytes into the spleens protected the rats, which had undergone the surgical portacaval shunt procedure, from developing hepatic encephalopathy [26].

At the present time, the biosafety of transplanting immortalized hepatocytes into humans has yet to be fully appreciated. Although immortality is not sufficient for neoplastic transformation, most immortalized cells have an increased sensitivity for spontaneous, carcinogen-induced, or oncogene-induced neoplastic transformation [27], which would expose patients to an unacceptable tumorigenic risk after transplantation. This is one major problematic reason why it would be necessary to tightly regulate the growth of these immortalized cells and/or eliminate the factors resulting in immortalization before their transplantation. To avoid this potential problem, Fox et al. [25] have controlled the growth and degree of differentiation by immortalizing hepatocytes using thermolabile SV40 large T antigen. At the permissive temperature, the SV40 large T antigen is expressed in unlimited quantities, but once these cells are raised to physiological temperatures, the SV40 large T antigen levels rapidly begin to decline, resulting in growth arrest and cell differentiation. Another method to reverse the immortalization process was demonstrated in an elegant study by Kobayashi et al. [28] in which they transduced an immortalizing gene flanked by LoxP sites. After the immortalized hepatocytes were established, the genetically transferred immortalizing gene could be completely excised by transducing these cells with an adenovirus expressing Cre recombinase, which resulted in a precise site-specific excisional deletion of the DNA fragments flanking the gene responsible for immortalization. After the removal of the immortalizing gene, the hepatocytes showed more hepatocyte-specific functions in vitro than during the immortalization stage. The functionality of these cells in vivo was confirmed since rats with acute liver failure, which received cell transplants, showed prolonged survival [28]. To develop these immortalized cells for therapeutic applications, further studies are needed to determine how many passages they can undergo while still maintaining their original hepatocyte-specific functions.

Another potential application for immortalized human hepatocytes is in the development of bioartificial liver support devices. Such devices have previously been developed for the temporary support of individuals, who are undergoing liver failure, and are either awaiting for their own liver to recover or whole organ liver transplantation (reviewed in [31]). Due to the shortage of viable and healthy human hepatocytes, porcine hepatocytes have been used as an alternative source in the creation of bioartificial liver support devices, and these devices have been recently applied in the clinical setting [32]. However, the use of xenogenic tissues can potentially result in immunological problems and/or the transfer of xenotropic viruses to the host [31]. For these reasons the use of immortalized hepatocytes derived from human origin in bioartificial liver support devices would provide biological uniformity and greatly alleviate the immunological problems.

Cryopreservation of isolated hepatocytes

Since there is a severe limitation in the number of donor livers that can be utilized for the isolation of fresh hepatocytes, and there is a time-dependent viability of isolated hepatocytes, a need for the development of a cryopreservative system to allow the banking of hepatocytes for emergency transplantation exists. To achieve this goal, several factors in the cryopreservative process need to be optimized, including cryopreservative [33, 34, 35], serum concentration in the medium [33, 35], freezing and thawing rates [33, 34, 35, 36], and the cryopreservation period [34, 35]. In terms of cryopreservative agents, dimethylsulfoxide has been found to be the best cryopreservative relative to several other agents [33]. The dimethylsulfoxide concentration has been optimized by Chesne et al. [34], with 14% and 10–12% preservation achieved for rat and human hepatocytes, respectively. In addition, Dixit et al. [37] reported that microencapsulation of hepatocytes with a collagen matrix envelope by alginate polyL-lysine sodium alginate further protects the hepatocytes from cryopreservative damage. Moreover, the process of freezing and thawing hepatocytes has been found to be important to minimize cellular damage; hepatocytes should be frozen at a slow controlled rate at which the thawing of the hepatocytes should be performed quickly at 37°C [33, 35, 36]. There have been conflicting findings regarding hepatocyte viability following prolonged storage time. One study demonstrated unaltered viability and attachment rates on the culture dish preserved for up to 4 years [34], whereas another reported decreased viability after long-term cryopreservation [35, 38].

Nonetheless, cryopreserved murine hepatocytes in vitro continue to express drug-metabolizing systems, such as the inducible cytochrome P450, while in vivo these hepatocytes maintain their regenerative capacity after transplantation regardless of the cryopreservation period [35, 39, 40]. These particular findings using mu-

Table 1 Clinical trials related to human hepatocyte transplantation (*LC* liver cirrhosis, *CH* chronic hepatitis, *FHF* fulminant hepatic failure, *FH* homozygous familial hypercholesterolemia, *hAAT*, α_1-antitrypsin deficiency, *SE* sepsis; *OTC* ornithine transcarbamoylase deficiency, *CJ* Crigler-Najjar syndrome 1, *NA*, data not available)

Reference	Cause of liver disease	Type of transplanted hepatocytes	Number of hepatocytes transplanted	Route of transplantation
Mito et al. [8]	LC (*n*=9), CH (*n*=1)	Freshly isolated autologous hepatocytes	1.7×10^7–6.0×10^8	Intraportal
Habibullah et al. [7]	FHF (*n*=7)	Pooled blood group-matched human fetal hepatocytes	6×10^7/kg	Intrasplenic
Grossman et al. [9], Raper et al. [61]	FH (*n*=5)	Hepatocytes transfected with human LDL receptor cDNA using retroviral vectors in vitro before transplantation	1×10^9–3×10^9	Intraperitoneal
Strom et al. [1, 4]	hAAT (*n*=1), LC (*n*=1), SE (*n*=1), FHF (*n*=3), OTC (*n*=1)	Hepatocytes cryopreserved for 1.5 weeks–8 months (*n*=5) or 48-h cultured hepatocytes (n=1)	7.5×10^6–1.7×10^8	Intraportal
Soriano et al. [5]	FHF (*n*=3)	Cryopreserved (duration was not described) hepatocytes	4×10^7–4×10^9	Intrasplenic
Bilir et al. [6, 41]	FHF (*n*=5), LC (*n*=3)	Hepatocytes cryopreserved for 1– 8 months (*n*=5; NA *n=3*)	3×10^9 (1) NA (7)	Intraportal
Strom et al. [2]	FHF (*n*=7), LC (*n*=2), hAAT (*n*=1)	–[a]	NA	Intraportal
Fox et al. [58]	CJ (*n*=1)	Freshly isolated hepatocytes	7.5×10^9	Intraportal
Fisher et al. [3]	FHF (*n*=1)	–	8.8×10^8	Intraportal

[a] Hepatocyte transplantation clinical cases reviewed by Strom et al. but not previously been reported in the literature are shown

rine hepatocytes are important for the potential application of cryopreserved hepatocytes for human transplantation in the clinical setting, but further studies are required to determine whether cryopreserved human hepatocytes can retain their proliferative abilities compared to their murine counterparts. At the present time, cryopreserved hepatocyte transplantations have been performed in 16 clinical cases with subacute or acute liver failure (Table 1) [4, 5, 6, 41]. In a report with four clinical cases by Strom et al. [4], all the four patients maintained cerebral perfusion and cardiac stability, and two of the four successfully bridged to subsequent whole-liver transplantation. It is also important to note that no major complications have arisen from this procedure [4].

Intraportal (splenic) hepatocyte transplantation

Hepatocyte transplantation has the potential to become an established therapy as long as several criteria can be achieved, specifically the isolation of functional cells that have the ability to survive for a long time (i.e., years), and an effective method of delivery to a particular site. Previous studies have demonstrated therapeutic efficacy of hepatocyte transplantation through a variety of liver disease models, which include hereditary tyrosinemia type I, Wilson disease, α_1-antitrypsin (AAT) deficiency, apolipoprotein deficiencies, familial hypercholesterolemia, and liver failure due to the extensive liver removal or end-stage liver cirrhosis [28, 42, 43, 44, 45, 46, 47]. Because the host liver represents an ideal "home" for the transplanted hepatocytes in terms of the unique hepatic organization and interactions with nonparenchymal liver cells [48], all of these experiments were conducted by infusing cells to the liver through intraportal or intrasplenic routes. For transplanted hepatocytes to engraft into the host liver and remain functionally viable over the long term, the most important criterion was for these cells to translocate from the portal pedicle into the liver microenvironment, as described by several groups [49, 50, 51]. These investigators showed that shortly after hepatocyte infusion, the transplanted cells become stacked at the portal vein radicles, which results in some of the cells being deposited at the hepatic sinusoid. Although the majority of the hepatocytes are cleared from these areas, a portion of the cells start to translocate into the space of Disse by disrupting the sinusoidal endothelium and finally join adjacent host hepatocytes; this process takes approx. 20 h to occur [49]. In the next few days, bile canaliculi between the transplants and host cells are also reconstituted [49], and it is this ability to reconstitute new functional biliary systems that allows this method of hepatocyte transplantation to be applied towards diseases that have biliary secretion defects [52]. The spleen is also a viable target tissue for transplantation of hepatocytes since it offers the ability to form differentiated chord structures and reform nearly normal hepatic architectures [4, 53, 54].

Complications related to the intraportal hepatocyte transplantation

One of the major limiting factors in utilizing either the intraportal or intrasplenic approach is the number of viable cells that can be engrafted without causing complications. It is believed that the maximal percentage of engraftment that can be achieved is in the range of 2–5% of the host hepatocytes. This was determined by a number of transplantation studies described below [9, 43, 55]. In terms of intraportal transplantation, the major complication has been found to be portal vein thrombosis, which resulted in liver failure and severe portal hypertension, hemorrhage, and migration of cells to the lungs leading to pulmonary embolism [56]. Portal hypertension is associated with a high probability of intrapulmonary deposition of hepatocytes, as shown in a previous study in the rat [57]. Clinical trials of intraportal hepatocyte transplantation have confirmed subsequent portal hypertension and/or pulmonary dysfunction albeit for a transient period of time [1, 6, 9, 58]. Intrasplenic transplantation has also resulted in similar complications, with a large proportion of hepatocytes (approx. 80%) migrating out of the spleen into the portal circulation [54, 57, 59]. In one clinical trial, an attempt to treat ornithine transcarbamylase (OTC) deficient patients who received 10^9 hepatocytes via portal vein injection, showed an increase of approx. 70% in portal pressure [1]. Moreover, Bilir et al. [6] reported a transient increase in oxygen requirement due to the cell migration into the lungs after transplantation. To minimize these potential complications, investigators have either ligated the hepatic artery to decrease the sinusoidal blood flow prior to hepatocyte infusion or prolonged the time period for hepatocyte injection into the portal vein [58, 60]. The latter method was used in a clinical trial attempting to treat Crigler-Najjar syndrome in which 7.5×10^9 hepatocytes (5% of the original liver mass) were successfully transplanted through the portal vein over a longer period of time (15 h) [58]. Despite these possible complications, clinical trials of hepatocyte transplantation have been performed without causing any reported fatalities.

Clinical trials of hepatocyte transplantation

Acute or chronic liver failures

The reported clinical trials related to hepatocyte transplantation are summarized in Table 1. The first reported clinical experience using hepatocyte transplantation for liver failure was performed by Mito et al. [8], who transplanted autologous hepatocytes into ten patients with either liver cirrhosis or chronic hepatitis. The hepatocytes were isolated from a surgically resected piece of the lateral liver lobe and were transplanted through the intrasplenic route. The presence of the transplanted hepatocytes, which is also called "splenic hepatization" [53], was confirmed by radioisotope imaging at the injected sites and was found in eight of nine cases, but longer survival (>10 months) of the hepatocyte engraftment was observed in only one patient. In this study, all of the procedures were performed without severe complications to the patients. Unfortunately, this trial of transplanting autologous hepatocytes for the treatment of cirrhotic livers was reported to be clinically ineffective. However, it is important to note that this initial study pioneered the path for more than 40 clinical hepatocyte transplantation trials to be conducted, mainly in the United States [1, 2, 3, 4, 5, 6, 7, 9, 41, 58, 61].

At present, it is believed that at least 20% of the normal hepatocyte mass is required to allow the liver to function within normal physiological parameters (reviewed in [10, 62]). Unfortunately, the number of hepatocytes that can be realistically transplanted in patients has been limited to less than 5% of the recipient original liver mass, largely due to the potential problems as described in previous section.

Regardless of this limitation, several clinical trials have been subsequently performed to treat fulminant hepatic failure (FHF), which was caused by either hepatitis virus, acute toxic hepatitis, extensive liver surgery or alcohol-induced liver cirrhosis. In a study by Bilir et al. [6] five FHF patients who were not candidates for OLT received hepatocyte transplantation, and three of the five survived substantially longer (>10 additional days) than expected based on clinical experience. Although whole-liver transplantation is an established therapy for FHF, the limited organ donor supply requires a bridging technique to sustain patients with FHF until a donor liver becomes available. For this reason, we discuss several clinical trials which have been conducted primarily to determine whether hepatocyte transplantation would provide a temporary hepatic functional assist to bridge the time of hepatocyte to OLT. Fourteen FHF candidates who were awaiting OLT received hepatocyte transplantation at different institutions [1, 2, 3, 4, 5]. Six of the 14 successfully bridged to OLT, which was performed 1–10 days after the hepatocyte transplantation [2]. Moreover, two of the 14 FHF patients "spontaneously" recovered following hepatocyte transplantation such that they regained their normal physiological hepatic function without having to undergo OLT [2, 3]. The successful details of spontaneous recovery of the liver have been reported by Fisher et al. [3] in a 37-year-old woman with FHF who was infused with 8.8×10^8 allogeneic hepatocytes into the liver through a catheter placed into the portal vein. Remarkably, this patient fully recovered, with a rapid fall in serum ammonia levels, and was discharged from the hospital by week 2. Similar results of spontaneous recovery have been reported by Habibullah et al. [7], who found both of the two FHF patients with grade 3 encephalopathy to recover without requiring subsequent liver transplantation. However, in control FHF patients with similar grades of encephalopathy who did not receive hepatocyte transplantation no spontaneous recovery was observed in a trial by Strom et al. [4], whereas 50% of patients recovered in a trial by Habibul-

lah et al. [7]. These studies demonstrate the important role that hepatocyte transplantation plays during the critical dysfunctional phase of FHF by either allowing the original liver to recover "spontaneously" their normal function or to act as a temporal bridge to OLT.

Although positive results have been suggested with the procedure utilizing hepatocyte transplantation, one must be careful in evaluating the clinical efficacy using this approach because the clinical outcome of FHF varies with the cause of the liver disease [63]. In addition, the number of patients in these studies were insufficient and were not randomly assigned to treatment groups, and it therefore appears premature to conclude any clinical efficacy casually related to the transplanted hepatocytes. However, some other encouraging clinical effects using hepatocyte transplantation, even in patients who could not be bridged to OLT, have resulted in significant reductions in blood ammonia levels, an important functional marker for liver dysfunction, and improvements in hyperammonemic encephalopathy [1, 4, 5, 6, 7, 41]. Regardless, further clinical investigations are necessary in order to determine several important factors about the transplantation of hepatocytes into patients with failing livers: (a) the optimal number of hepatocytes, which are necessary to support the critical status of the liver, (b) the route of transplantation that would allow more cells to be infused. and (c) the appropriate time frame for hepatocyte transplantation depending upon the cause and severity of the liver disease.

Inherited liver disorders

Hepatocyte transplantation has also been used clinically to treat inherited liver disorders. To date there have been four such reported cases: OTC deficiency, AAT deficiency, and Crigler-Najjar syndrome type 1 [1, 4, 58]. Strom et al. [1] infused hepatocytes through the portal vein into the liver of a patient with OTC deficiency. Two separate infusions were performed 30 days apart, but this procedure was not sufficient to produce a life-long cure. This was not unexpected because a larger number of genetically normal hepatocytes are likely to be required in order to fully correct this disease. In a separate study to treat human AAT deficiency, a small number of hepatocytes (2.2×10^7 viable cells) were transplanted into the spleen of the patient. The long-term beneficial effects of hepatocyte transplantation for this and other AAT cases cannot be fully understood since OLT was performed 2–4 days following the hepatocyte infusion [2, 4].

The most encouraging results came from a transplantation of hepatocytes to treat the genetic disorder known as Crigler-Najjar syndrome type 1 [58]. This disease results from the lack of uridine diphosphoglucuronate glucuronosyltransferase activity in the liver. This enzyme is essential for the conjugation and the excretion of bilirubin, which in its absence causes individuals to be at risk for kernicterus and brain damage due to the accumulation of unconjugated bilirubin. For these patients to survive the initial treatment must involve the use of high-intensity phototherapy to reduce serum bilirubin levels. However, phototherapy alone is commonly ineffective, resulting in the need for OLT for survival [64]. It remains to be determined whether hepatocyte transplantation can provide additional survival time for patients who are awaiting whole organ liver transplantation. A 10 year-old woman with Crigler-Najjar syndrome type 1 received 7.5×10^9 allogeneic hepatocytes through a portal vein catheter in combination with phenobarbital therapy. This resulted in a 14-fold increase in the hepatic uridine diphosphoglucuronate glucuronosyltransferase activity leading to a substantial reduction in the serum bilirubin level from 25 mg/dl (pretransplantation) to 10–15 mg/dl, even though the daily phototherapy was decreased by half, from 12 h (pretransplantation) to an average of 6 h per day. This finding that there was a reduction in the number of phototherapy hours as a result of the hepatocyte transplantation was important since it helped provide additional quality time. Another important finding was the long-term persistence of the transplanted hepatocytes for over 9 months (length of period followed in the report) [58]. However, future studies will need to be performed to determine whether subsequent hepatocyte transplantation procedures would improve efficacy for these and/or other genetic hepatodeficiency states.

Ectopic hepatocyte transplantation

Ectopic hepatocyte transplantation, defined in this report as a transplantation site for the hepatocytes other than the liver or spleen, can potentially provide more space to transplant a greater number of donor hepatocytes. Moreover, it has been shown to produce fewer complications than transplantation through the intraportal route. Future applications that would be important for ectopic hepatocyte transplantation may be for inherited liver disorders and modifying the outcomes of hepatic failure as long as techniques can be optimized to allow long-term survival of the transplanted donor cells. Another advantage for ectopic transplantation from an experimental standpoint is that donor cells can be readily distinguished from the host, which makes it easy to evaluate the status of the cells in biopsy specimens [23] (Fig. 1). On the other hand, transplanted cells administered through the intraportal route are difficult to ascertain, and considerable effort is required to determine the transplants by detecting specific proteins or chromosomes found only in the transplanted cells [42, 45, 65, 66].

Previous reports have transplanted hepatocytes at several different ectopic sites, including the intraperitoneal cavity [67], pancreas [68], mesenteric leaves [69, 70], intrapulmonary artery [71], lung parenchyma [72], under the kidney capsule [73], interscapular fat pads [74], and subcutaneous space [75, 76]. Irrespective of the ectopic site chosen by these investigators, the transplanted donor cells resulted in very short-term survival, which is due largely to the inadequate initial donor cell engraftment

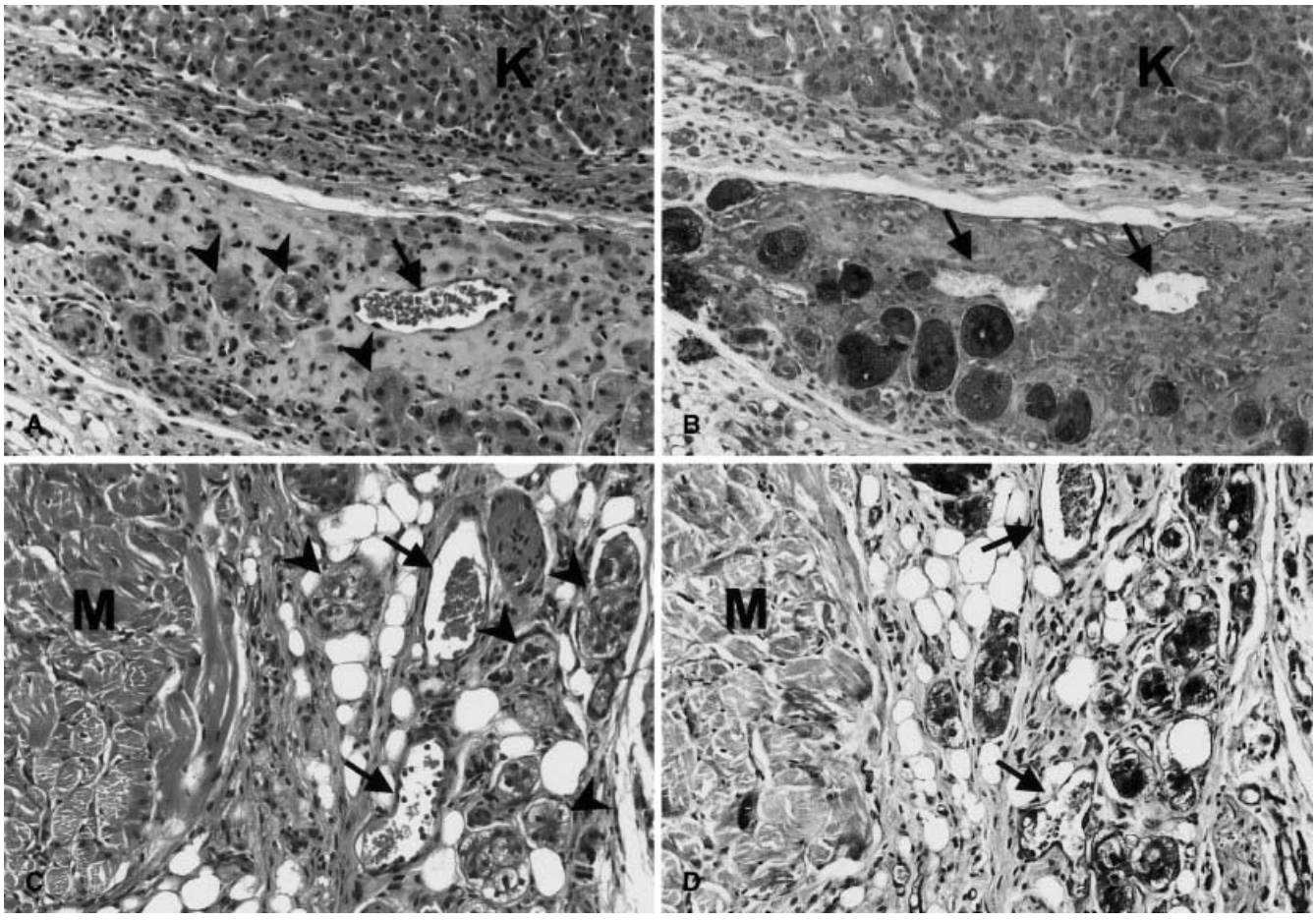

Fig. 1A–D Histological characteristics of human hepatocytes transplanted at ectopic sites of mice. Hepatocytes were isolated from human liver pieces and transplanted under the kidney capsule (**A**,**B**) or subcutaneous space on the back (**C**,**D**) of NOD/SCID mice. Hepatocytes were cotransplanted with the extracellular matrix gel known as Matrigel. c-Met antibodies were injected intravenously every 2 weeks for the first 57 days. Mice were killed 24 weeks after the transplantation, and histological analyses were performed for hematoxylin and eosin staining (**A**,**C**) and human α_1-antitrypsin immunohistochemical staining (**B**,**D**). Note transplanted human hepatocytes retain their characteristic morphology (*arrowheads*) and showed strong signal for α_1-antitrypsin. Vessels are newly formed within the transplants (*arrows*). Original magnification, ×200. *K* Kidney tissue; *M* skeletal muscle

and lack of neovascularlization to support their survival [56]. In order to support successful initial donor cell engraftment at the ectopic site, several modifications have been performed to provide an extracellular matrix component to act as an anchor for these donor cells and would aid in prolonging hepatocyte survival and differentiation [14, 67]. Demetriou et al. [67] have shown that rat hepatocytes attached to the dextran microcarrier coated with collagen type I and transplanted into the intraperitoneal cavity result in a therapeutic function in Gunn rats, which is a rat model for Crigler-Najjar syndrome for up to 6 days. Recently, we have found that cotransplantation of the hepatocytes with an extracellular matrix gel showed significantly better survival in hepatocytes transplanted under the kidney capsule as well as the subcutaneous space [14]. Both of these experiments using an extracellular matrix component have successfully recruited new blood vessels within or close to the transplants, which might have contributed to longer persistence and viability of the transplants [14, 15] (Fig. 1A, C). Establishment of vascular network is likely required for the growth and function of the transplanted hepatocytes. Ajioka et al. [77] have transfected the vascular endothelial growth factor gene (*VEGF*) into the primary hepatocytes and transplanted them into the abdominal cavity. *VEGF*-transfected hepatocytes formed large colonies with vascular networks around the transplants.

Despite these numerous modifications at the ectopic transplantation site, long-term survival has yet to be unequivocally demonstrated. In addition, for successful ectopic hepatocyte transplantation to occur, it is important to maintain the supply of hepatotropic stimulation to achieve long-term survival and cell specific functions. The regenerative stimulus induced by performing partial hepatectomy results in a very minor effect on the survival of the transplants [70]. Since the portal blood contains rich hormonal factors for hepatotropic stimulation, while the general circulation does not [78], Sano et al. [69] and Kaufmann et al. [70] surgically created a portacaval shunt in order to shunt blood and hormonal factors from

the portal system to the general circulation. This surgical procedure allowed the delivery of hepatotropic factors to the ectopically transplanted hepatocytes, which resulted in higher survival rates for a limited period of time[69, 70], but the portacaval shunting procedure would not be readily applicable for clinical use. For this reason, to prolong the survival of the transplanted hepatocytes, recent studies in our laboratory have been performed to address this problem.

We hypothesized that the limited survival is due in part to the lack of an extracellular matrix and/or the absence of essential growth signals from hepatocyte growth factor (HGF). As described above, cotransplantation of the hepatocytes with an extracellular matrix gel aided the initial cellular attachment to the ectopic tissue. In vivo delivery of HGF to recipients would limit its use for clinical application because of its very short half-life (in the order of minutes) and extremely high cost [79]. To develop a clinically more applicable strategy to supply essential growth signals to the transplanted hepatocytes we have used a monoclonal agonistic antibody against human c-Met, which is the receptor for HGF and has been shown to have properties analogous to HGF. This antibody was developed by Schwall et al. (Genetech, California, USA) and can recognize the extracellular domain of human but not murine c-Met. These investigators have found that the c-Met antibody can stimulate the induction of DNA synthesis in mink lung cells in vitro at similar levels as HGF. In addition, they have shown long-term residence of c-Met antibody in ferret serum (>50 h) following intravenous administration in vivo (Hillan and Schwall et al., unpublished data). Because the c-Met antibody demonstrated specificity for the HGF receptor while maintaining similar biological activity as HGF, the authors administered c-Met intravenously and interestingly, showed persistent survival of the transplanted human hepatocytes in immunodeficient mice under the kidney capsule as well as the subcutaneous space [14, 15]. The maintenance and function of the hepatocytes were histologically confirmed by the persistence of serum reporter molecules as well as by histological examination on samples at 24 weeks after transplantation (Fig. 1). This effect may be due to the multifunctional signaling known to occur through HGF/c-Met signal transduction that affects tropic, proliferative, and cytoprotective response on hepatocytes [80]. By looking at the cellular proliferation after transplantation, the authors have found that 20–35% of the transplanted hepatocytes proliferated during the 2-week period [15]. This study confirmed that cellular turnover is indeed one of the key factors for stabilizing hepatocellular function. Further studies need to be performed to optimize the delivery route and timing of delivery for the c-Met antibody in future clinical applications.

Ex vivo gene therapy to hepatocytes

Gene therapy techniques have been extensively investigated for more than 10 years [81]. In order to overcome several limitations of the allogeneic hepatocyte transplantation, such as difficulties in obtaining cells at the appropriate timing and the need for permanent immunosuppression therapies, researchers have attempted to develop hepatocyte-directed ex vivo gene therapy using autologous hepatocytes. Genetic modification of autologous hepatocytes allows reconstituting genetic function that is absence in specific pathological processes. Ex vivo gene transfer includes isolation of autologous hepatocytes from a piece of the host liver followed by cell culture propagation and gene transduction in vitro. These genetically modified hepatocytes are then subsequently transplanted back into the host individual. Inherited liver disorders are, in theory, good targets for hepatocyte-directed ex vivo gene therapy [82]. Among several currently available gene transfer systems, retroviral vectors would be an ideal tool because of their ability to integrate proviruses into the host genome, which potentially would allow long-term, sustained transgene expression [81, 83]. Another potential application of the ex vivo gene therapy is for the graft modulation achieved by transfecting “protective” genes leading the immune tolerance or specific unresponsiveness in the host. This could be applied for the xenogenic hepatocyte transplantation in the future (reviewed in [84]). For this reason, we reviewed ex vivo hepatocyte-directed gene therapy techniques using two distinct retroviral vector systems, specifically Moloney-murine leukemia virus (MLV) and human immunodeficiency virus (HIV) based lentiviral vectors.

Moloney murine leukemia based retroviral vectors for hepatic gene transduction

Oncogenic retroviruses such as MLV have been one of the most widely used vectors for hepatocyte gene transduction in vitro [43, 83, 85, 86, 87]. For MLV-mediated gene transduction, MLV mobilization into the nuclei of cells requires nuclear membrane dissolution (i.e., cells must be undergoing cell division); otherwise chromosomal integration cannot occur. Because hepatocytes in culture do not continuously cycle, researchers have struggled to develop methods to transduce a larger proportion of cells. To achieve higher transduction efficiency, several factors in culture and transduction process have been optimized, including plating density of the hepatocytes and the timing of vector infection after plating the cells on the dish. In terms of plating density of hepatocytes, $2–4\times10^6$ of the hepatocytes per 10-cm dish appears to be the optimal density for MLV-mediated transduction [43, 87]. Susceptibility to infection by MLV vectors is maximal when isolated hepatocytes have been cultured 36–72 h in vitro [43, 85]. Under such conditions, early studies showed that 10–30% transduction ef-

ficiency can be achieved [43, 85, 87, 88]. Addition of growth factor(s), such as epidermal growth factor and HGF to the culture medium, has been shown to promote hepatocyte proliferation and increase transduction efficiency [89]. In addition, the ability to obtain higher titers of MLV by pseudotyping the envelope with vesicular stomatitis virus G glycoprotein has also allowed improved transduction of primary hepatocytes in culture [90].

Ex vivo hepatocyte-directed gene therapy was first experimentally applied to treat inherited liver disorders using the Watanabe rabbit model for familial hypercholesterolemia by Chowdhury et al. [88]. This approach offered long-term genetic reconstitution of the low-density lipoprotein receptor, albeit at low levels (2–4% of normal), yet this level of expression resulted in the reduction in serum cholesterol by 25–45% [88]. Other experimental trials using ex vivo hepatocyte-directed gene therapy have succeeded in the partial correction of disease phenotypes, such as hAAT deficiency and hereditary tyrosinemia type 1 [43, 45]. In addition, this ex vivo approach was attempted in five compound heterozygous familial hypercholesterolemia patients [9]. Although this small study resulted in limited, if any, therapeutic effects, there were no observable short-term and/or long-term complications, which demonstrates the safety of ex vivo hepatocyte-directed transplantation [61]. It is important to note that other investigators have reported that transplanting hepatocytes that have been cultured for 6 days following transient MLV transduction resulted in inefficient stability of the hepatocyte transplanted at the target site [83]. Although these studies demonstrate the potential for ex vivo gene therapy using cultured hepatocytes, significant improvements in hepatocyte stability along with the development of newer vector systems that allow more efficient and rapid transduction of isolated hepatocytes, and improvements in large scale cultures (e.g., roller bottles) would be highly beneficial to promote future clinical trial applications.

Lentiviral vectors for hepatic gene transduction

To overcome some of the limitations with the MLV vectors, lentiviral vectors derived from HIV-1 have recently been developed [91], which have the ability to infect and transduce both actively dividing and nondividing cells. Nonprimate-derived lentiviral vectors are also available. It is believed to be capable of transporting the preintegration complex into the nucleus with the aid of nuclear localization signals found within viral protein components and *cis*-acting sequences in the HIV genome (reviewed in [81]). To expand the tropism, the lentiviral vector has been pseudotyped with vesicular stomatitis virus G glycoprotein [91], which virtually allows transduction in a variety of cell types in vitro and in vivo.

The vesicular stomatitis virus G glycoprotein pseudotyped HIV-1 based lentiviral vectors have successfully been delivered in vivo to several tissues, which are normally quiescent in the cell cycle, such as the neurons in the brain [91], skeletal muscle [92], photoreceptors in the retina [93], airway epithelia [94], and liver [92, 95, 96].

Hepatocyte transduction in vivo with lentiviral vectors was found to be significantly higher than MLV-based vectors in mouse livers [95]. Moreover, in regenerating livers in which the hepatocytes are in cycle, lentiviral vectors gave significantly higher hepatocyte transduction than MLV-based vectors [95, 96]. In terms of the hepatocytes in cell culture, we performed studies using isolated primary mouse hepatocytes. Lentiviral vector transduction with a phosphoglycerate kinase promoter driving the expression of nuclear localized lacZ lead to significantly higher (five- to tenfold) transduction in the primary mouse hepatocytes than MLV-based vectors (Ohashi et al., unpublished data). High transduction efficiency could also be achieved on cultured human fetal hepatocytes by lentiviral vector as reported by Zahler et al. [97]. The ability for lentiviral vectors to produce higher levels of transduction in vitro as well as in vivo provides a potential important application for future liver-directed gene therapy [96].

Hepatocyte repopulation in vivo

Investigators have recently shown that native hepatocytes have the capability to repopulate within diseased livers in vivo under conditions in which there are large selective advantages [98, 99, 100, 101, 102]. In the human liver, Kvittingen et al. [99] found newly repopulated hepatocytes originating from a single hepatocyte, which had spontaneously reverted to a normal phenotype in the livers of patients with hereditary tyrosemia type 1. By determining the size of the nodule, the single hepatocyte had undergone 20 rounds of cell division [99]. In this case, the normal hepatocyte had a selective advantage over the mutant cells that have a cell autonomous lethal defect. In a separate study, Ohashi et al. [100, 102] provided in vivo evidence that hepatocytes underwent active cell division in patients suffering from hepatitis virus infection. By comparing the differences in the telomere length in the hepatocytes between hepatitis virus-infected versus noninfected patients, human hepatocytes infected with hepatitis virus have been shown to have on average more than 40 cell divisions compared to noninfected individuals [100].

Taken together, there is growing evidence suggesting the ability of these transplanted hepatocytes to proliferate and repopulate within the recipient liver under conditions in which donor cells have a selective advantage [42, 45, 65, 66, 103, 104, 105]. In 1994 Rhim et al. [103] showed repopulation in the liver of albumin-urokinase transgenic mice of up to 80% by transplanting syngeneic wild-type hepatocytes. In this model, the urokinase expressing host hepatocytes are destroyed while the normal donor cells have the selective advantage. In another study, Overturf et al. [45] showed highly active prolifera-

tion of the transplanted hepatocytes in a murine model of hereditary tyrosinemia type 1. Following transplantation of hepatocytes, which approximated about 1% of the total liver mass, these hepatocytes repopulated nearly 80% of the diseased liver over an 8-week period. In the same study, therapeutic efficacy was also achieved by transplanting as few as 1000 donor hepatocytes [45]. Interestingly, significant hepatocyte proliferation in vivo of more than 69 divisions was possible by serial transplantation of the repopulated hepatocytes into other recipient mice with hereditary tyrosinemia type 1 [104]. In addition, hematopoietic stem cells have been shown to represent an alternative approach for hepatocyte repopulation. Lagasse et al. [105] recently showed that hematopoietic stem cells, which were transplanted into mouse livers, had the potential to undergo the differentiation process of hepatocyte repopulation in vivo. However, the efficacy with this approach without selective pressure is likely to be extremely small.

To simulate the varying states of proliferation that are observed in diseased livers, several studies have been designed to promote proliferation in naive mice in order to induce repopulation of the transplanted hepatocytes [42, 65, 66]. One study by Vranken-Peeters et al. [65] produced liver injury to the recipient liver with an adenoviral-mediated transfer of a modified urokinase-type plasminogen activator gene prior to hepatocyte transplantation. This method of proliferation led to the repopulation of 8.6% of the transplanted hepatocytes within the liver. Similar results have been reported by Mignon et al. [66], who found proliferative stimuli to be generated by inducing fas-mediated apoptosis selectively to the recipient liver after transplanting transgenic hepatocytes overexpressing the *bcl-2* gene, which protects the cells from the fas-mediated apoptotic challenge. The therapeutic efficacy using this hepatocyte repopulation strategy has recently been shown using apolipoprotein E knockout mice animal model systems. The study showed that the hepatocytes transplanted into apolipoprotein E knockout mouse liver repopulated approx. 16% and produced approx. 30% of normal serum apolipoprotein E level [42]. One recent study showed that mice with hypercholesterolemia achieved approx. 17% of normal serum apolipoprotein E levels following hepatocyte repopulation by the transplants [42].

In spite of the reported therapeutic efficacy of hepatocyte repopulation in animal models of inherited liver disorders, Braun et al. [106] recently showed that repopulation is less efficacious when the transplant is targeted to livers that have underwent acute liver failure. These investigators concluded that a certain period of time is necessary for the establishment of fully functional parenchyma with the repopulated hepatocytes. Further research needs to be performed to elucidate the importance of the method of hepatocyte repopulation for different liver diseases and disorders, and also whether ectopic transplantation of hepatocytes can retain the capacity for therapeutic repopulation.

Chimeric small animal models using human hepatocytes

At the present time, research on specific human hepatocyte-related diseases and disorders is greatly hampered due to the inadequate availability of in vitro and in vivo hepatocyte model systems. To further complicate these studies, primary cultured hepatocytes de-differentiate following a short period of time resulting in potentially large differences between their in vivo and in vitro properties [20]. Currently available human hepatocyte cell culture systems offer extremely limited use as a research tool for human hepatitis viruses such as hepatitis B (HBV) and C virus (HCV) because of their low infection and replication efficiency [107, 108]. Because of the narrow host range of these viruses, the existing animal models are limited to chimpanzees, which are expensive and in short supply. At present, only the liver of the tupaia can support HBV replication.

To overcome these limitations, it would be ideal to establish a chimeric mouse model using human hepatocytes in order to study the human hepatocyte-specific functions in vivo. Several attempts to transplant the human hepatocytes in various ectopic sites of immunodeficient mice or rats have failed to achieve sustained survival [15, 107, 109, 110, 111]. Tumor cells that have originated from human hepatocytes have been adopted for the sustained survival in small animals [112], but they often showed different characteristics than their normal human hepatocytes. We hypothesized that the loss of the transplanted human hepatocytes may be due in part to the lack of an extracellular matrix along with the absence of essential growth signals such as HGF. For this reason, we found that cotransplantation of the hepatocytes with an extracellular matrix gel helped to support the initial hepatocellular attachment to the ectopic tissue. For the latter problem, we provided human c-Met (receptor for HGF) antibody, which phospholylates human but not murine c-Met. By applying the two modifications described above (see “Ectopic hepatocyte transplantation”), chimeric reconstitution with human hepatocytes (transplanted under the kidney capsule) have been achieved for over than 150 days in immunodeficient SCID/NOD mice [14]. More importantly, the transplanted human hepatocytes retained their characteristic morphology during the experimental period (Fig. 1). This chimeric status was also achieved when human hepatocytes were transplanted into the subcutaneous space of mouse [15].

In order to establish whether this chimeric mouse could serve as an in vivo model for infection with human viruses, human HBV-positive serum was infused 3 days after human hepatocyte transplantation. HBV infection and replication occurred within the transplants in vivo, and these mice developed HBV viremic status approximately 2 weeks after viral infusion, which remained for over 150 days (Fig. 2). These HBV viremic mice were further inoculated with HDV to determine whether they could produce a superinfection model for hepatitis delta

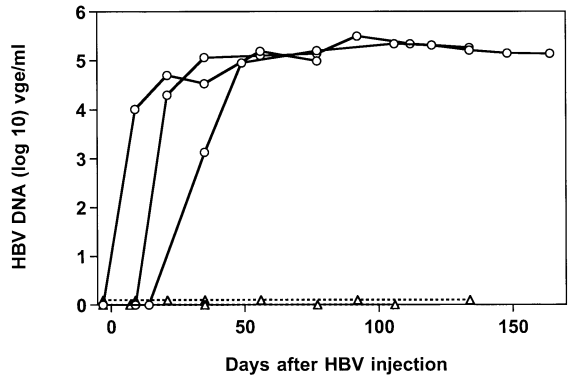

Fig. 2 A mouse model for in vivo infection with hepatitis B virus (*HBV*). Mice were transplanted with human hepatocytes on day –3, and c-Met antibody were injected on days –2, 12, 26, 40, and 54. Mice were inoculated with 200 µl HBV-infected human serum. HBV DNA in the mouse serum was quantified by HBV DNA polymerase chain reaction enzyme-linked immunosorbent assay [15]. *Circles* Mice that received human hepatocytes and HBV; *triangles* control mice that received HBV but no human hepatocytes

virus (HDV). HDV is a particularly interesting virus because it requires coinfection of HBV or similar hepadnavirus for being enveloped [113]. The HBV-positive chimeric mice developed HDV infection and viral replication as determined by the viremic state of the animals. These findings indicated that this chimeric mouse system supports the entire life cycle of these viruses in vivo from the initial viral infection to viral replication and release of the viruses back into the serum. These essential aspects of the HBV and HDV life cycle in small animals should provide an excellent opportunity in developing antiviral strategies. It would also be possible to study the hepatitis virology from the immunological aspects in vivo by reconstituting the SCID mouse as well as the Trimera mouse (described below) with functional human lymphocytes [114, 115]. Furthermore, the established chimeric mouse system would be a good alternative to study numerous other aspects of chronic human hepatocyte-specific functions in vivo.

Several different approaches have also been reported toward the establishment of a chimeric mouse system with human hepatocytes. Ilan et al. [111] have transplanted human liver fragments into the mice preconditioned by lethal irradiation and radioprotected with SCID mouse bone marrow, which was called the "Trimera mouse." Brown et al. [29] have reported long-term graft survival in the liver of Rag-2 immunodeficient mice in which immortalized human hepatocytes established by transfecting the SV40 T-antigen gene, which doubled in number over a 72-h period in cell culture, were transplanted instead of primary isolated hepatocytes. In these two experiments, HBV infection to liver fragments or immortalized human hepatocytes were established by infecting HBV or transfecting the HBV genome in vitro before transplantation. By transplanting these tissues into the mice, both of the mouse systems resulted in HBV viremia, while natural infection in vivo could not be observed [29, 111]. The Trimera mouse supported the viremic status for a limited period (40–50 days), while the Rag-2 mice maintained viremia for up to 5 months. Nonetheless, it is important to note that therapeutic efficacy of several anti-HBV agents has been demonstrated in the Trimera mouse system [111, 116]. Similarly, Galun et al. [117] have developed a HCV viremic mouse by transplanting HCV-infected human liver tissue using the Trimera mouse system. Taken together, we now have chimeric small animal model systems reconstituted with human hepatocytes, which allows the elucidation of hepatitis virus functions in vivo.

Future prospects

The considerable efforts that basic and clinical researchers have undertaken in the past several years have laid the foundation for the better understanding regarding the methods needed for hepatocyte transplantation, such as hepatocellular isolation, cell culture propagation and viability, immunosuppression, and hepatic gene transduction, for the utilization in therapeutic protocols. At the present time, however, this approach is still in the early stages of development to be used as a clinical procedure for liver disorders. Nevertheless, there have been several successful clinical reports discussing the beneficial effects using this approach in both environmentally and genetically affected individuals with either FHF with encephalopathy stage 3 or Crigler-Najjar syndrome. It is important to note that the hepatocyte transplantation procedure is much less invasive and less expensive for patients than OLT. With further modifications, we and others believe that hepatocyte transplantation could offer beneficial effects to a number of patients with liver diseases, who are awaiting OLT, as a viable alternative therapy. Finally, the chimeric small animal with transplanted human hepatocytes would serve as an animal model to understand a variety of human liver diseases which is currently not available, and we hope that research based on this system would contribute to the development of new therapeutic options.

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