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University of Alberta

Development of an Animal Model of Hepatitis C

by

David Frederick Mercer

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**

in

Experimental Surgery

Department of Surgery

Edmonton, Alberta

Spring 2000

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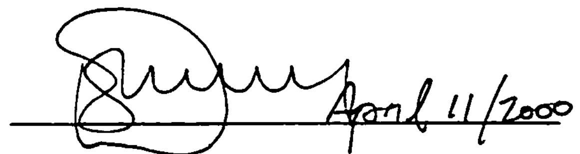

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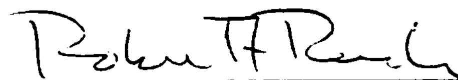

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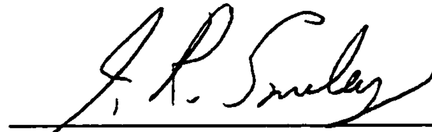

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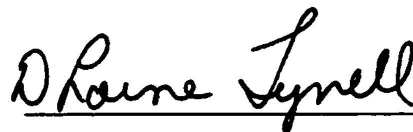

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Dedication

The completion of this body of study is a goal to which I have aspired for many years. There are three important people to whom I am indebted, and to whom I dedicate this thesis.

First, to my best friend and wife, Leann, who has always been so supportive of my annoying tendency to extend my education. The years I have spent at the University of Alberta have been the best years of my life, and the last four with you the best of all. We have grown into a solid team, and we both turn now with anticipation, and some trepidation, to the vast opportunities awaiting us. I am certain the upcoming years will be as wonderful as those we've shared already.

Finally, to my parents, Judy and Doug, whom I firmly believe are responsible for instilling in me the desire to always pursue knowledge and education in a steadfast manner. Without the moral framework and work ethic they provided me with growing up, I would never have gotten this far. Fundamentally, despite all the years of education and all the opportunities I have been fortunate enough to have, everything comes back to "One fish, two fish, red fish, blue fish".

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Abstract

The human hepatitis C virus (HCV) is an important cause of liver-related morbidity and mortality worldwide. A major impediment to developing antiviral therapies for HCV is the lack of an economical small animal model that supports viral replication. Our goal was to develop such a model, with the hypothesis that normal human hepatocytes transplanted into a suitable murine liver would be capable of supporting the replication of HCV. Using purified collagenase blends and a small volume perfusion circuit, viable human hepatocytes were reliably isolated from surgically obtained liver biopsies in high yield ($9.7 \pm 2.1 \times 10^6$ viable cell/gram tissue). Hepatocytes transplanted intrasplenically into the immunodeficient murine strain C.b17-SCID-beige translocated to and engrafted within the recipient liver, but revealed progressive loss of graft function over time (median survival 3 weeks). Addition of a 50% partial hepatectomy concurrent with transplantation extended graft function to a median of 6 weeks, confirming the capacity of human hepatocytes to respond in part to murine growth signals. Administration of human hepatocyte growth factor via implantable osmotic pumps extended graft function to a median of 8.2 weeks, showing species-specific growth factors can significantly enhance overall graft survival. Despite these improvements in graft survival, however, recipient mice were incapable of supporting detectable replication of the human hepatitis B virus, suggesting this system would be inadequate for supporting HCV replication. A novel strain of transgenic mouse was then developed, termed Alb-uPA/SCID-beige, which was shown through detection of human protein production and by immunohistochemical demonstration of human cell surface markers to have the capacity to stimulate rapid proliferation of human hepatocytes after transplantation, producing chimeric human-mouse livers which in some cases sustain hepatocyte function for prolonged (>30 weeks) periods of time. These chimeric animals were susceptible to infection both human hepatitis B and hepatitis C virus, and were capable of supporting replication of for extended periods (>18 weeks) at levels equivalent to those seen in humans. This represents the first robust, reproducible small animal model of human hepatitis C virus infection and replication, and with should significantly impact upon the ability to develop antiviral strategies against the hepatitis C virus.

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There are a number of people I have worked with over the past four years without whom I could not have completed my work, and to who I owe a debt of gratitude.

To the members of my committee I am especially indebted, foremost to Dr. Kneteman, for showing me how to keep pushing a project forward when everything seems to be going backwards, and for always directing my ideas into a focused plan of experimentation. I could not have had a better supervisor for such a multidisciplinary project. To Dr. Tyrrell, for making me more excited about research than I have ever been about almost anything in my whole life, and to Dr. Elliott for teaching me rigor of thought and how to prepare a clean and concise manuscript. And finally to Thomas, who I feel awkward calling Dr. Churchill as he has become a close friend, who always proved a willing recipient of my ideas and complaints, and who managed over 42 months to instill into me a healthy dose of skepticism about all things scientific.

I am grateful for the assistance of Drs. Greg Korbutt, Jonathan Lakey, and Anita Gainer who were always willing to provide advice, supplies, or assistance whenever I needed them, and for the coffee-shop counsel of Dr. Donna Douglas, whom I consider one of the finest scientific minds I encountered during my studies. I thank Dr. Peter Dickie and Karl Fischer for assistance with numerous molecular assays which were critical to the success of this project at many points. I thankfully acknowledge my surgical colleagues, Drs. Tina Mele, Ed Solano, Karim Alibhai, all of whom were excellent friends to have during graduate studies. Finally, I acknowledge with respect the assistance of Dr. Dan Schiller, to whom I entrust the ongoing maintenance of this project.

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His diligent work and love of Ho Ho Chinese Food were critical to permitting me to complete my thesis on schedule.

Finally, I would like to acknowledge the financial support behind my research, without which none of this would have been possible: The Alberta Heritage Foundation for Medical Research, for providing me with a three-year Research Fellowship, and the Department of Surgery at the University of Alberta, for providing additional salary; the Edmonton Civic Employees' Charitable Benefit Fund, The University of Alberta Liver Transplant Program, and the University Surgical Associates for providing operating funds.

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CHAPTER 1

GENERAL INTRODUCTION

> "We stand at the precipice of a grave threat to our public health... It affects people from all walks of life, in every state, in every country. And unless we do something about it soon, it will kill more people than AIDS."
>
> - Dr. C. Everett Koop, Former U.S. Surgeon General

INTRODUCTION TO CLINICAL PROBLEM

Human liver disease caused by the hepatitis C virus (HCV) has emerged over the past decade as one of the most difficult challenges facing the medical community. Long known under the guises of "non-A non-B hepatitis" [1-3] and "cryptogenic cirrhosis", the elucidation of the viral sequence in 1989 by Choo *et al.*[4] established the beginning of the era of concerted study of HCV. Increasingly accurate methods for detection of the virus, both directly and indirectly, have facilitated an appreciation of the enormous magnitude of the problem.

Currently, it is estimated that 1-2% of the world population is infected with HCV[5; 6]. Geographic variations in rates of *de novo* HCV infection result in disease prevalence ranging from 1-2% in North America and Europe to almost 15% in Egypt[6]. In Canada, prevalence of HCV infection is believed to reflect worldwide data, with an estimated 200-300 000 Canadians carrying the virus. In Alberta there are roughly 25 000 cases of HCV infection yielding an overall rate of 0.91%, in keeping with national estimates; perhaps due to variations in rates of risk behaviours such as intravenous drug use, there are approximately twofold more cases of infections in men (1.17% prevalence) compared to women (0.65% prevalence). Because only 5-25% of patients with acute HCV infection manifest symptoms sufficiently severe to warrant medical attention, incidence and prevalence of the infection is likely to be currently underreported[7]. With a high rate (80-85%) of progression to chronicity[8] hepatitis C is fast becoming a dominant source of liver-related morbidity and mortality worldwide.

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The previously unrecognized transmission of HCV through infected blood products has had a tremendous impact on the Canadian medical structure. Before routine screening of blood supplies for indirect evidence of donor infection with hepatitis C, the risk of transmission of the virus with each unit of blood was significant. During the period from 1983-85, it was estimated that the risk of seroconversion approached 0.3-0.5% per unit of blood transfused[9], and in the late 1970's the risk of transmission was higher still, with estimates ranging as high as 1-2 percent per unit[10]. This source of viral infection is responsible in large part for the epidemic being faced today. As a consequence of the infection of 27 000-45 000 Canadians with "tainted blood" between 1985 and 1989 and under the weight of a $5.0 billion law suit, the Canadian Red Cross was forced to file for bankruptcy in 1998[11]. Ultimately this led to the creation of the new Canadian Blood Service and altered the perception of the community, both medical and nonmedical, towards the used of blood products.

Hepatitis C has emerged as a significant source of infection-related morbidity and mortality within the field of surgery. In addition to the small but real risk to patients of transmission of the virus through blood products (~1/104 000), surgeons and operating room staff in particular are at risk for acquisition of the virus through needlestick-type injuries. The hepatitis C virus is transmitted through hollow-core needle injuries at a rate of up to 10%[12], making it intermediate between HIV (0.2-0.5%) and HBV (2-40%). Although the rate of infection through solid needle injuries is likely to be considerably less, the frequent occurrence of these injuries makes HCV transmission a significant concern. Additionally, because of the high rate of progression to cirrhosis (20-30%)[13] with the accompanying sequelae of portal hypertension and liver failure, as well as the recognized clinical association between chronic HCV and hepatocellular carcinoma[14], hepatitis C infection places a significant burden on liver transplantation programs. Chronic HCV infection is fast becoming the major indication for liver transplantation worldwide, comprising up to 35% of all transplants in published series[15-20]. Because of the chronic, indolent course of hepatitis C infection, it is anticipated that rates of end-stage liver disease will continue to increase rapidly over the next decade, placing an ever-increasing burden on the pool of donor organs available for transplant.

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Hepatitis C has had and will continue to have a significant impact on the population worldwide. Equally disturbing to the increasing realization of the widespread extent of infection is the fact that currently no good therapy is available for use in combating the progression of the disease. Progress towards developing a vaccine or antiviral therapy is severely hampered by the lack of a laboratory model of hepatitis C. As there is currently no robust *in vitro* or *in vivo* model of viral replication, knowledge of the molecular biology of the virus, as well as the development of antiviral strategies for use in human trials has advanced at a less-than-optimal pace. The creation of a small animal model of HCV replication would provide a vast leap forward in the capacity to develop novel approaches to combat the ever-increasing epidemic.

MOLECULAR BIOLOGY OF HCV

1. BASIC ORGANIZATION

Due in part to the lack of reproducible models of HCV, elucidating the structure and function of the virus has been difficult. The hepatitis C virus is a single positive-stranded RNA virus of approximately 9400 nucleotides in length, which is classified within the family *Flaviviridae*, which includes flaviviruses and pestiviruses. Although grouped within the family *Flaviviridae*, HCV exhibits a number of structural and processing features which are in some cases very distinct from other members in its family.

The organization of the HCV genome is outlined in Figure 1. The genome consists of a single large open reading frame, flanked by 5' and 3' untranslated regions (UTR), which codes for a polyprotein of approximately 3010-3030 amino acids[21]. The precursor polyprotein undergoes extensive post-translational processing by host- and viral-derived enzymes prior to assembly into the completed virus. Similar to related Flavi- and Pestiviruses, the nonstructural elements of the genome, especially the NS3 serine protease, the NS3 helicase and the NS5b RNA-dependent RNA polymerase are critical to the replication of HCV[22]. The internal ribosomal entry site (IRES) located within the 5' UTR also plays a key role in the initiation of translation of the viral polyprotein. The structural viral elements appear to be involved in the pathogenesis of clinical hepatitis C or act as targets for recognition by and/or evasion of the host immune

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response. Most of the viral proteins, nonstructural and structural, have potential as target sites for the development of antiviral therapies.

2. INTERNAL RIBOSOMAL ENTRY SITE (IRES)

The 5' UTR consists of a sequence of 341-344 nucleotides, which appears to be the most highly conserved region amongst HCV species[23]. Although this region is not translated into the viral polyprotein, it has been shown conclusively to contain an internal ribosomal entry site, which is critical to the initiation of translation on host cell ribosomes[24; 25]. The 5' UTR contains a highly ordered structure of stem-loops[26] which are crucial to the function of the region as an IRES[27; 28]. This higher-order structure is dependent on the entire 5' noncoding region[29], as well as the N-terminal (nt 1-123) basic portion of the core protein[30], for proper folding. A stable "pseudoknot" structure is postulated to form in the region immediately upstream of the initiating AUG codon[31]. The overall effect of this folding may be the stable presentation of an unpaired base sequence (nt 324-334, 5'-UCUCGUAGAC-3'), which has sequence complementarity with a conserved unpaired sequence at the 3'end of the eukaryotic 18s ribosomal RNA molecule[31].

The product of this seemingly complex folding of the 5'UTR appears to be a mechanism of initial ribosomal binding, involving eukaryotic 40S ribosomes, which does not require many of the eukaryotic initiation factor proteins (e.g. eIF4A, eIF4B, and eIF4F) that are absolute requirements for translation initiation in the vast majority of eukaryotic mRNAs[32]. While some cellular mRNAs encoding regulatory proteins do use an IRES-mediated mode of initiation, they also all appear to require the presence of these additional eIF proteins for formation of the 43S complex, and further progression to translation[33]. This process of ribosomal binding, then would seem to be unique to HCV (and closely related pestiviruses), distinct from host cell mechanisms, and potentially exploitable for blocking viral replication. Two separate studies have shown ~90% reduction in translation after use of antisense oligodeoxynucleotides[34] or small yeast-derived RNA oligonucleotides[35] to interfere with translation initiation. It is unclear how these approaches may function in an *in vivo* model, but at present they confirm at least

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proof-of-principle that blockade of IRES-mediated translation initiation may prove important in developing antiviral therapies.

3. CORE

The core (C) region of the genome codes a 22 kDa polyprotein, which may undergo post-translational processing by cleavage in its hydrophobic c-terminal sequence[36]. At present, little is known about *in vivo* structure or the role of core protein in viral assembly; it is known to be quite conserved amongst viral subtypes, and as such has been used as a target region for developing anti-HCV antibody assays. The core protein is also known to be a potent stimulator of both CD8+[37; 38] and CD4+[39] T-cells in vitro, and investigators have been able to determine the precise oligopeptide sequences responsible for the T-cell stimulation. Despite the presence of these stimulatory properties *in vitro*, no correlation was found between clearance of virus and presence of a strong *in vivo* cell-mediated anti-core effect. Recently, the presence of a strong CD4-mediated anti-core T-cell response has been shown to be highly correlated to the ability of interferon-α to induce a sustained (versus a transient or absent) antiviral response in HCV-infected patients[40]. In future, the viral core protein may prove to be an important target for the development of vaccination strategies designed to elicit a sustained antiviral T-cell response.

4. ENVELOPE

The viral envelope is produced from the E1 and E2/NS1 genes, which code for two glycoproteins, gp33 and gp72. The product of E2/NS1, the gp72 protein, contains a hydrophobic C-terminal end, which may serve as a membrane anchor while the N-terminal end is projected outside the cell membrane, subject to host immune surveillance[37; 41]. An approximately 30 n.t. sequence at this N-terminus of E2/NS1, the so-called "hypervariable region" (HVR1) of the viral genome, appears to play an important role in interacting with the host immune system. This region is highly subject to mutations, and there appears to be minimal sequence homology between viral species[42-44]. Mutation within HVR1 seems to result from immune pressures; support for this concept is based upon two major findings. First, incubating HCV-infectious serum with anti-HVR1 antibodies has been shown to abrogate its infectivity when inoculated

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into HCV-naïve chimpanzees, implying that HVR1 is a critical neutralization epitope[45]. Second, quasispecies variations within HVR1 occur more frequently and are more likely to be nonsynonymous mutations (i.e. mutations to a new amino acid) in patients who fail to clear acute infections[46; 47], while in immunocompromised patients, quasispecies evolution is curtailed[48]. The implication of these findings is that successful chronic infection involves mutations in HVR1 which render the host immune response ineffective, while reduction in immune selection is accompanied by a reduction in mutation frequency.

Convincing evidence has been recently published to indicate that a cell membrane-bound protein, designated CD81 in humans, binds avidly to the viral E2 protein[49]. This tetraspanin molecule is expressed on the surface of a number of cell types including hepatocytes and lymphocytes, and has considerable sequence homology between humans and chimpanzees in contrast to comparisons with other species. The major extracellular loop of this protein, termed EC2, appears to avidly bind to whole virus particles, and anti-CD81 antibodies prevent viral attachment to target cell lines[49]. This information may implicate E2 as a critical protein targeting the virus to susceptible host cells, and possibly to viral entry.

5. NS2

Little information is available about the NS2 protein product. It appears that the C-terminus of NS2, in concert with the N-terminus of NS3, codes for a Zn^{++}-requiring metalloproteinase, which is then involved in cleaving the junction between NS2 and NS3[50].

6. NS3

The NS3 domain of the HCV genome codes for a protease critical to the downstream post-translational processing required for production of a functional virus. Extending from nt. 3079-4971, it codes for a protein of ~631 amino acids[51]. A zinc-dependent metalloproteinase formed from the C-terminus of NS2 and the N-terminus of NS3 acts in *cis* to cleave the NS2/NS3 junction, thereby autoregulating its own activity[50; 52]. Sequencing and deletional analysis has shown the NS3 protease to be confined to the N-terminal 180 amino acids of the NS3 protein, and to contain the catalytic triad His

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1080, Asp 1107 and Ser 1165, characteristic of serine proteases[52-55]. NS3 cleaves in *cis* its own C-terminus, and then in *trans* all the remaining downstream protein junctions (NS4a/4b, NS4b/5a, NS5a/5b)[52]. Recently, the NS3 domain was crystallized, and visualized at 2.4A, both with and without its NS4a cofactor[56; 57]. It was shown to be highly similar in organization to trypsin-like proteases, with NS4a intimately associated into its structure.

The 450 amino acid C-terminus of the NS3 protein contains the viral helicase enzyme. Although the helicase activity has been known for a few years[58; 59], only more recently have the structure and function begun to be elucidated. The helicase domain of NS3 was shown to consist of two large C- and N-terminal domains, connected by a more flexible third domain[60]. A domain with NTPase activity is structurally coupled to the region between the two large terminal domains, suggesting that NTP cleavage causes conformational change in the helicase enzyme, likely related to its activity. From other studies, it has been determined that the helicase functions as a monomer[61], acts in 3'-5' direction[62], and is able to separate dsRNA, dsDNA, and RNA-DNA duplexes[58; 62]. Additionally, it can employ any NTP as an energy substrate, and appears to function equally as an isolated domain or as the C-terminus of the entire NS3 protein[63]. Currently there are no published studies exploring potential anti-helicase strategies in HCV or other systems. As new information arises and chemicals with putative antihelicase properties are developed, targeting the NS3 helicase may play a role in combination antiviral therapy.

7. NS4a/4b

The NS4a protein product joins with the serine protease, and acts as an essential cofactor primarily in *trans* (although it can act in *cis* for its own cleavage) for the NS3 mediated cleavages at 3/4a, 4a/4b, and 4b/5a[64]. It markedly increases the conformational stability of the serine protease in SDS-containing solution[65], and together they form a detergent stable complex. Synthetic peptides comprising the central region (a.a. 21-34) of the NS4a protein have been produced, and have been demonstrated to exhibit the same cofactor activity as the entire protein[66]. As NS4a is an essential cofactor for three out of four cleavage sites, acts largely in *trans*, and because synthetic peptides can mimic its

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activity *in vitro*, it is a highly desirable target for the development of protease-inhibiting drugs. Little is known about the function of the NS4b protein product other than it appears to be highly immunogenic for CD4+ T-cells.

8. NS5a/5b

The NS51a region codes for a 56 kDa phosphoprotein which is seen to exist in normal and hyperphosphorylated forms[67]. It appears to function as a transcriptional activator; this activity seems to be contained within the C-terminal portion of the protein, and appears to be inhibited to some extent by its own N-terminal region[68]. Although its exact function is not known, it is known to contain a region termed the interferon sensitivity determining region (ISDR) which is somehow involved in the ability of the virus to evade the antiviral effects of interferon-α. This ISDR region is critical to the function of the NS5a protein, with mutations in this region away from the wild-type sequence appearing to enhance its transcriptional activity[69]. No specific sequence is associated with the interferon-insensitivity phenotype, although it does appear that the presence of a large variety of quasispecies at initiation of therapy may negatively impact on the antiviral activity of interferon[70]. The exact mechanism behind which the anti-interferon effect is mediated is unknown, although it has been shown that the NS5a protein can directly inhibit the activity of PKR, an antiviral effector mechanism stimulated by interferon-α[71].

Based upon the primary amino acid structure of the NS5b region, it was predicted to act as an RNA-dependent RNA-polymerase (RdRp)[72]. This prediction has subsequently been confirmed, and the properties of the polymerase have been partially elucidated[73]. Histologically, the viral polymerase is found within the cytoplasm, localized to areas around, but not within the host cell nucleus[74; 75]. The NS5b protein appears to exist as a complex with the NS3 and NS4a proteins which is targeted to the surface of the rough endoplasmic reticulum[76]; the C-terminal 21 amino acids of the polypeptide are irrelevant to the function of the polymerase but appear to be critical to this membrane association[77].

The NS5b polymerase is capable of initiating RNA transcription from a free 3' – OH molecule by employing a self-priming hairpin-turn in the template RNA, or when the

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3'-end of the template RNA is capped or blocked, by addition of a suitable oligonucleotides primer[73]. Although capable of initiating and completing replication of the HCV genome, NS5b appears to have no specific specificity for replicating HCV, and can employ any suitable RNA template[78]. This property would suggest that other intracellular factors may be involved in associating the viral RNA template with its polymerase, for efficient transcription. Although in future the viral polymerase may prove to be a target for antiviral therapy, at present knowledge of the actual process of viral replication is far too inadequate; the development of cell culture and small animal models will be absolutely critical for elucidating the molecular mechanisms of viral replication.

CLINICAL EFFECTS OF HCV INFECTION

1. MEDICAL EFFECTS

Acute infection with HCV is relatively mild, with clinical symptoms occurring in approximately 10% of patients. Symptoms of acute infection are quite nonspecific, with fatigue, mild abdominal pain and anorexia being most common; clinically evident jaundice occurs in approximately 15% of patients[79]. After infection, although HCV RNA is usually detectable within a few weeks, seroconversion generally does not occur for several months[5]. In contrast to other viruses such as hepatitis B, HCV rarely if ever causes fulminant hepatitis[80; 81]; rather, the majority of consequences are related to long-term infection.

Up to 85% of infected individuals will progress on to develop chronic hepatitis, of which approximately 60% develop chronic active hepatitis, and 20-30% cirrhosis[6]. In general, a period of 10-15 years elapses before symptoms of chronic active hepatitis become evident; liver cirrhosis occurs in ~20 years, and hepatocellular carcinoma at ~30 years after initial infection[79]. Although the symptoms of chronic hepatitis C are quite nonspecific and rarely are severe, the disease is generally slowly progressive within the liver. Histologically, the findings of chronic infection in the liver are characteristic but not absolutely specific for HCV[82]. Initially mild portal inflammation is seen, with lymphoid aggregates and mild periportal piecemeal necrosis. Fibrosis is initially inconspicuous, but with time extends to portal-portal and portal-central vein bridging. In

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later stages of disease, there are essentially no histologic features which allow differentiation of end-stage HCV infection from other causes of progressive liver failure.

Over the past decade since the discovery of the hepatitis C virus, the association between HCV infection and the development of hepatocellular carcinoma (HCC) has become increasingly clear. It appears that chronic infection with HCV now accounts for up to 50% of all cases of HCC in North America[14]. The risk of developing HCC increases with the severity of underlying liver disease; the annual risk is approximately 1% without cirrhosis, and 3-10% in patients with cirrhosis, depending on stage of the disease and other etiologic cofactors[83]. Coinfection with hepatitis B raises the yearly risk to considerably higher than either virus alone. Because HCV does not integrate into the host genome, the development of carcinoma may be related to interaction between the host cell and viral products: both NS3 protein and core protein have been shown to have potential transforming activities on host cells.[14]. It is the development of end-stage liver disease with the attendant complications of portal hypertension, liver failure and development of hepatocellular carcinoma that brings hepatitis C into the realm of the surgeon, where it has become an important source of both major hepatic resections and liver transplants.

2. LIVER TRANSPLANTATION AND HCV

In 1991, Martin et al published the first, albeit small, clinical series of HCV in liver transplantation[84]. Six patients anti-HCV positive by first generation ELISA underwent orthotopic liver transplantation (OLT), and five were found to be persistently positive for anti-HCV antibodies at one year post-transplant. Two patients with early return of anti-HCV status (<1 month) developed clinical infection in the post-transplant period and one of the six developed early recurrence of severe HCV infection necessitating retransplant at three months; the disease recurred in the second liver, and the patient died of septic complications after 9 months. While this was a small series of patients, it served to outline the significant clinical features of HCV infection in the liver transplant population: near universal recurrence in the transplanted organs, moderately-high incidence of early but generally clinically mild hepatitis with recurrence, and a small but significant portion of recipients with severe recurrence progressing to graft failure

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and death.

As chronic HCV infection is a major indication for liver transplantation worldwide, considerable investigation has been done into the recurrence of HCV viremia after transplantation. With widespread adoption of PCR techniques, high rates of persistent HCV infection have been found in post-transplant patients. These data are summarized in Table 1.

Clearly, one can see that recurrent HCV viremia post transplant is almost universal. That the post-transplant viremia is due to recurrence rather than de novo infection (e.g. from blood transplants or infected organs) was established in a study by Feray *et al*[85]. Sequence analysis of the hypervariable E2/NS1 sequence of the HCV genome showed >95% homology between pre- and post-transplant viral sequences, significantly greater than that from comparisons between different patients or published sequences of common American and Japanese strains; this high degree of homology would only exist if the pre- and post- sera contained the same virus. Infection of the new graft must therefore occur at the time of or shortly after the actual transplantation, possibly from other tissue sources of virus or from circulating HCV in the recipient's blood.

Although recurrent HCV viremia occurs with high frequency post-transplantation, the extent of viral load does not appear to correlate with clinical outcome. Levels of HCV RNA post-transplant are consistently elevated compared to pre-transplantation levels, presumably as a result of immunosuppression and secondary reduced antiviral activity[86]. Chazouilleres *et al.* quantified viremia pre- and post-OLT, showing that mean post-transplant levels of HCV RNA were 16-fold higher than pre- levels. A significant correlation between high pre-transplant RNA levels and high post-transplant levels was found, but no such relationship between post-OLT levels and liver damage was seen. In contrast, Feray *et al.* found HCV viremia to be significantly higher in those with post-transplant hepatitis compared to those without. Presently there is no clear relationship established between elevated HCV RNA levels post-transplant and the development of clinical hepatitis.

While the relationship between levels of HCV RNA and histologic damage to liver grafts is obscure, what is clear is a significant occurrence, usually 40-60%, of

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recurrent clinical hepatitis C in patients transplanted for HCV-related liver disease. In the majority of studies, despite this high rate of clinical recurrence the disease seems initially to take a mild course, with little effect on graft survival[18; 87-90]. The initial data on early graft function and patient survival suggested that overall there was little difference in outcome between patients with HCV recurrence and noninfected controls. A series by Feray *et al.* reported 1-, 2- and 5-year survivals of approximately 95%, 90% and 80%[91], which was similar to the 94%, 89% and 87% 1-, 2- and 3-year survivals presented by Ascher[17]

Although the majority of published reports support a benign course of recurrent HCV infection in the short term post-transplant, some series suggest a less benign course, with earlier development of active hepatitis not only histologically but also clinically[16; 92]. More recent reports have been considerably less optimistic than earlier authors with respect to patient and graft survival, suggesting that HCV-infected patients trend towards poorer outcome with longer follow-up[92; 93]. In the nontransplant setting, hepatitis C is a disease of chronic duration; it is anticipated that with longer follow-up, studies will reveal a significant detrimental effects of HCV recurrence on graft function.

In Martin's original series of six patients[84], one patient was seen to develop early recurrence of severe hepatitis C, which caused graft failure, and prompted a second transplantation. The disease again recurred in the second liver, causing severe compromise and death. Histologic examination post-mortem showed recurrence of end-stage cirrhosis nine months after the second transplant. This disturbing occurrence of severe early recurrent disease has been seen in other studies[92; 94], and poses a small but significant clinical risk to the transplant population. Almost all require retransplantation, and examination of the explanted liver generally shows advanced cirrhosis or confluent necrosis, with no histologic evidence of rejection. The reason for this small but significant rate of severe recurrence has not yet been established.

3. CURRENT THERAPY

There is no universally efficacious therapy currently available for HCV infection. Interferon-α was first used as therapy for NANB hepatitis in 1986[95], and has subsequently become the predominant antiviral therapy in use for hepatitis C. A meta-

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analysis of trials using interferon α-2B showed significant improvements at end of treatment in liver enzyme levels (47% vs. 4% normalization of ALT), histologic response (73% vs. 38% histologic improvement), and viral response (29% vs. 5% viral RNA undetectability) in patients treated with interferon versus untreated patients[96]. However, as is generally seen with all interferon trials, a six-month sustained response to therapy occurs considerably less frequently (normalization of ALT 23% vs. 2%; absence of viremia 8% vs. 1%). Interferon-α is generally administered at a dose of 3 million units 3 times per week for a 6 month period; the use of higher doses (e.g. 6 m.u. vs. 3 m.u. t.i.weekly) or longer dosing intervals (12 months vs. 6 months) has a relatively mild effect on improving end-of-treatment response, and a slightly more pronounced effect on long-term response (increased dose 28% sustained viral response vs. 19% standard dose; increased duration 27% versus 14% standard duration). Similar data is seen in trials using interferon alfa-2a[97], interferon alfa-n1 (lymphoblastoid interferon)[98] and consensus interferon[99]. A major limiting factor in the use of interferon as an antiviral agent is the high incidence of side effects. Virtually all patients will experience some form of side effects, ranging from mild flu-like symptoms in the majority of patients to major neuropsychiatric disorders, autoimmune diseases, and significant hematologic, cardiac and hepatic compromise in a small minority (<1%)[100].

Ribavirin, a guanosine analog, initially showed some promise in treating HCV in nontransplant populations[101]. Subsequent investigation has shown that although ribavirin can reduce liver enzyme levels to normal it has little or no impact on viral levels, and liver enzymes rapidly rebound after discontinuation of the drug[102]. For these reasons, ribavirin was discarded as an antiviral monotherapy; it has, however, found a role in combination therapy with interferon-α. In three major multicenter randomized trials, therapy with a combination of ribavirin and interferon has been shown to be superior to interferon alone at producing a sustained antiviral response (31-43% vs. 6-19%)[103-105]. Based upon these studies, at present the gold standard for HCV antiviral therapy is interferon-α2b 3 m.u. three times weekly for 12 months, in combination with 1000-1200 mg ribavirin daily. The major side effect of ribavirin therapy is hemolysis which necessitates dose reduction in 10-15% of patients[102], and somewhat more when used in combination therapy.

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CELL CULTURE MODELS OF HCV

To facilitate dissection of the processes of HCV replication and determine potential targets for antiviral therapy, the development of an in vitro culture system is necessary. The ideal in vitro culture model would be easily obtainable and straightforward to maintain, susceptible to infection with a variety of HCV strains, and support active viral replication for prolonged or indefinite periods of time at meaningful levels of detection; at present, no such model exists. The plethora of publications supporting disparate cell lines as appropriate culture models of HCV is in itself testament to the lack of superiority of any one system. Species-restriction of the hepatitis C virus, coupled with primarily hepatocyte-specific replication and inherent difficulties in culturing hepatocytes has led to relatively few systems with even a potential for supporting any viral replication, and none with widely accepted promise as a robust model of HCV replication.

1. NONTRANSFORMED HEPATOCYTE CULTURES

Prior to the discovery of the hepatitis C virus, attempts at culturing non-A non-B hepatitis (NANBH) focused on the use of chimpanzee hepatocytes cultures, as the chimpanzee was an established in vivo animal model of NANBH. Prince *et al.*[106] attempted infection of primary chimpanzee hepatocyte cultures with sera infectious for NANBH, and found what was then considered evidence of active infection in the form of tubular cytoplasmic structures felt to be associated specifically with NANB infection. The specificity of the observed tubular structures for viral infection was disputed shortly thereafter however[107], when similar structures were demonstrated in noninfected hepatocytes in culture. Using a chimp-derived antibody specific for a NANB-associated antigen, Jacob et al.[108] presented possible immunohistochemical evidence of infection in cultures of hepatocytes isolated from chimpanzees known to carry NANBH. Unfortunately the degree of staining was limited to less than 10% of the cultured cells, and the hepatocytes persistently degenerated after 3-4 weeks in culture. In an attempt to provide further supporting but indirect evidence of the production of infectious viral particles in culture, media taken from cultures between 3 and 28 days post-inoculation

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was pooled and concentrated, and used to inoculate a hepatitis B-immune chimpanzee. Although an episode of hepatitis characteristic of NANB infection was established in the recipient animal, this could be explained by infectious particles from the initial chimpanzee inoculum retained in the early pooled media samples. At this point, no direct evidence of viral replication was apparent; attempts at producing such evidence would await the elucidation of the viral sequence and development of suitable viral RNA assays.

The best evidence for short-term viral replication in a chimpanzee hepatocyte culture system was later published by Lanford et al.[109], from the same group which presented the aforementioned chimpanzee data. Using RT-PCR, positive stranded viral RNA was detected to the termination of experimentation at 25 days. Parallel analysis of baboon hepatocyte cultures (which are not susceptible to HCV infection) allowed the effects of "carryover" of HCV RNA from the initial infectious inoculum to be characterized, demonstrating amplification of residual RNA up to 11 days after inoculation. This result must be carefully considered when viewing the results of other HCV culture studies. The development of a novel RT-PCR protocol employing tagged DNA primers allowed for sensitive discrimination of negative and positive stranded RNA, the former considered a marker for active viral replication. Using this technique, (-)-stranded HCV RNA was detected similarly to 25 days post-inoculation, providing more conclusive albeit limited evidence for viral replication within a chimpanzee hepatocyte culture system.

Although the demonstration of short-term viral replication was a significant advance, this paper was perhaps more important for its thorough exploration of many of the problems associated with RT-PCR detection of HCV RNA in culture systems. As mentioned above, this study quantified the effects of viral carryover, bringing into question any studies which employ (+)-strand RNA detection alone as a marker of early viral replication. Second, it pointed out the potential lack of specificity inherent in some protocols for strand-specific RT PCR, citing false priming of the incorrect strand by cDNA primers, self-priming of RNA strands, and random priming by nucleotide contaminants as potential confounders. Again, this prompts the reader to carefully consider the techniques of RT PCR employed in individual studies prior to accepting the data as reliable. Third, the levels of HCV released into the culture medium were

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considerably lower than those remaining cell-associated, making serial passage of an in vitro HCV infection difficult. Finally, the authors recognized that not all hepatocytes cultures were susceptible to infection, nor were all chronically-infected donor sera infectious under these conditions. This latter observation is likely to have important implications in all in vitro systems, as well as potentially relevant application to the in vivo infection of an animal model.

There are few published attempts at utilizing primary human hepatocyte cultures to support viral replication, and none with sufficiently high impact to be regarded as a reproducible model. Ito *et al.* presented data to suggest replication was occurring in cultured human hepatocytes at up to 56 days post-inoculation[110]. Of seven cultures initiated, four were terminated after two weeks, and one at four weeks; the remaining two persisted to eight weeks. It is somewhat surprising that cultures were maintained up to 8 weeks, or even four weeks, without any of the special culture conditions typical of other successful human hepatocyte culture systems, such as collagen-coated tissue plates, collagen-sandwiching, or co-culturing with fibroblasts - as the authors fail to provide any information about differentiated function of hepatocytes (e.g. hepatocyte specific protein synthesis or metabolism), one cannot be confident that these sustained cultures are actually differentiated hepatocytes. Using a competitive RT PCR system in an attempt to quantify viral production in cultures, HCV RNA levels appeared to increase 10 000-fold over the initial 4-5 days, and remained at around this level to the termination of cultures; additionally, the RNA detectable in the culture medium was within one log of that detectable in cultured cells. This pattern of consistent high RNA titer in culture media has not been reproduced in other systems, and is in direct contradiction with that of Lanford et al.[109], who found no detectable RNA in culture supernatant over 25 days. Based upon the inconsistencies within the paper by Ito et al., as well as the lack of subsequent reproducibility by other groups, one must conservatively conclude that these results likely do not represent a valid in vitro human hepatocyte model.

2. T-CELL-BASED CULTURE SYSTEMS

Based upon an earlier report of transmission of HCV to a chimpanzee through infusion of leukocytes, Shimizu et al. attempted to initiate HCV infection in a human T-

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cell line, MOLT-4, with or without coinfection by another unrelated murine leukemia virus[111]. Viral RNA was detected from 3-7 days, and then sporadically up to 3 weeks. Further attempts at characterization of the infection using immunohistochemistry and in situ RT PCR hybridization revealed staining in approximately 1% of total cultured cells. Although the viral RNA is described by the authors as intracellular, it is difficult to appreciate this from the published figures, and appears equally likely to be located on the surface of the cells in question, and thus may represent adsorbed virus.

Recognizing the limitations of this cell line in supporting viral replication, the authors examined a different human T-cell-based line, designated HPB-Ma, as a potential host for HCV[112]. Positive-stranded HCV RNA was detected consistently for 17 days after inoculation of cultures, and then intermittently for variable periods up to a maximum of 76 days in selected cases. While these results were an improvement over those seen with MOLT-4, the observed replication remained intermittent, with no quantification of viral production offered. Additionally, the data at later timepoints (after 40 days) must be viewed with extreme caution, as the authors combined PCR primers for both positive- and negative-stranded RNA (i.e. sense and antisense primers) in an attempt to amplify either species of RNA if present: the potential for false priming of the newly generated antisense (or sense) strands, yielding false positive results is significant. In a subsequent publication, infected HPB-Ma cells were co-cultured with non-infected HPB-Ma cells which has been transfected with antibiotic resistance genes[113]. Using antibiotic selection to separate the "recipient" from the "donor" cells, transfer of infection to the non-infected cells was demonstrated. This was a rather ingenious way of circumventing the problem of extremely low HCV yield associated with T-cell based culture systems, and proved that the cells were capable of producing infectious viral particles. Despite this interesting finding however, this system was clearly limited by the low levels of viral replication observed, ultimately restricting its utility as an in vitro model. Similar results have been presented by other investigators using an alternate T-cell line, termed MT-2[114-117]. It appears that T-cell based culture systems do not support replication at either a level or duration adequate for widespread use in viral studies. The initial premise that HCV infection could be transmitted through infected leukocytes may have been flawed, with the observed infectivity possibly related to adsorbed virus or serum contamination of

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leukocyte preparations. Interestingly this suggestion is being borne out in clinical literature, where peripheral mononuclear cells are increasingly seen to be if anything only a very minor reservoir of HCV infection[118; 119].

3. TRANSFORMED HEPATOCYTE CULTURES

Much of the impediment to developing in vitro HCV models has stemmed from difficulty maintaining human hepatocytes in a differentiated form within a culture system. To circumvent this problem, attempts have been made to support HCV replication in immortalized hepatocyte-based cell lines. The rationale underlying this approach is that such lines might retain sufficient differentiated hepatocyte function to uptake and support replication of HCV, while avoiding the dedifferentiation to a fibroblast-like morphology commonly seen with primary hepatocyte cultures.

The PH5CH cell line is derived from normal hepatocytes immortalized by transfection with simian virus 40 (SV40) large T antigen expression vector, and expresses numerous markers of differentiated hepatocyte function. In a paper by Kato et al.[120], positive-stranded RNA was detected by RT PCR in cultured PH5CH cells up to 12 days with one infectious inoculum, and up to 30 days with another derived from a different HCV-infected donor. The authors presented data on quantification of RNA, although at most timepoints the RNA titer was very close to or below what would be considered a reliable level for viral quantification. (i.e. 10^1-10^2). Unfortunately no analysis of negative-stranded RNA was presented, which is definitely suboptimal given the relatively recent date of publication (1996). Although there may be some evidence that PH5CH cells permit limited HCV replication, it is at best inconsistent and not sufficiently reproducible to be accepted as a reasonable model. Similar unimpressive results were seen with attempts to replicate HCV in a human embryonic hepatocyte line (WRL68) and a common hepatoblastoma line (Hep G2)[121]. What is more interesting from the paper by Kato and two subsequent follow-up studies[114; 122] however is the recurrent finding that some virally-infected human inocula are infectious for PH5CH cells and others are not, a finding that corroborates similar observations made by Lanford et al. in the aforementioned chimpanzee studies. As suggested by Kato, this may be related to

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genotype profiles in differing human sera, and perhaps to variation in infectivity related to changes within the HVR1 hypervariable sequence.

In a well described series of experiments published by Yoo et al.[123], some improvement in reproducibility of HCV RNA production was obtained by transfection of the Huh7 hepatoma line with in vitro transcribed HCV RNA. Transcribed RNA covering the full length HCV genome, as well as a second transcript truncated at nt. 154 of the 5' UTR (shown in vitro to be a more efficient template for translation of the viral polyprotein) were transfected into Huh7 cells, which were cultured under very stringent conditions of alternating serum-rich and serum-free media cycles. Cells were seen to proliferate in serum-rich conditions, but transcription of HCV RNA progressively decreased, as indicated by a loss of RNA detection; in serum-poor conditions transcription would recover, but at the expense of cell viability. In this fashion, episodic HCV RNA production could be produced to 35 days post-transfection, although again only at relatively low titers. While not strictly a system of HCV infection, this approach showed promise as a potential in vitro model of viral replication.

Very recently this approach was improved upon to produce what appears to represent the first robust long-term *in vitro* model of HCV replication[78]. Based upon total RNA isolated from the liver of a patient with chronic hepatitis C infection, the authors generated a consensus sequence (from the various viral quasispecies present within that individual's liver) of the entire viral open reading frame (ORF), with separately amplified 5' and 3' non-translated regions (NTR). When various cell lines and primary human hepatocytes were transfected with these full-length transcripts, no evidence of significant replication was seen. In an attempt to improve on these early disappointing results, selectable replicons were generated, which would confer neomycin resistance to cell replicating the HCV genome (bicistronic constructs, composed of the HCV IRES, the neomycin phosphotransferase gene (*neo*), an IRES from encephalomyocarditis virus, and the HCV viral sequence from NS2 to the terminus of the 3' UTR – the structural viral proteins were excluded from the construct). When these constructs were transfected into the Huh-7 hepatocyte line, a few clones were generated that appeared to be replicating the HCV sequence along with the *neo* gene. Further analysis confirmed that replication of the nonstructural genes was occurring, and appropriately functional viral nonstructural

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proteins were translated. This system should allow for investigation of some of the processes of viral replication, and should prove suitable for *in vitro* analysis of the efficacy of potential antiviral compounds (targeted to the nonstructural proteins). Although this is the most significant advance to date in developing an *in vitro* model of HCV, it is not yet an ideal model for viral study. It is limited to replication of the transfected sequence only rather than being applicable to a wider variety of viral genotypes, and as such can provide no information about infectivity or potential mechanisms of cell entry. Furthermore, because the viral structural proteins are not included in the transfected construct, no information about viral packaging processes or the generation of infectious particles is possible.

At present, the perfect *in vitro* HCV viral replication system does not exist. Systems employing nontransformed hepatocytes in culture seem to have a reasonable potential for supporting viral replication, but are severely limited by an inability to maintain cultures long-term. Lymphocyte-based cell lines do not appear to support consistent viral replication at levels sufficiently high to be useful for study. Transforming hepatocytes to render them easier to culture does not at present appear to provide an adequate host environment for viral replication, with measured viral production being both weak and intermittent. The best success to date has come from transfecting hepatocyte-based cell lines with subgenomic viral RNA, to produce a robust model of partial viral replication. Defining the minimum host cell requirements necessary to support viral uptake and replication will be critical in designing a more ideal *in vitro* model of HCV infection.

ANIMAL MODELS OF HCV

To adequately explore both the biology of the hepatitis C virus and the potential for developing pharmacologic and immunologic therapies, a widely available robust animal model of HCV replication is required. As defined by the National Academy of Sciences Institute of Laboratory Animal Resources, "An animal model is a living organism in which normative biology or behavior can be studied, or in which a spontaneous or induced pathological process can be studied, or in which a spontaneous or induced pathological process can be investigated, and in which the phenomenon in one or

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more respects resembles the same phenomenon in humans or other species of animal"[124]. The ideal animal model should exhibit a number of important characteristics, as first described by Leader and Padgett[125], and later outlined by Held. It should:

1. Accurately reproduce the disease or lesion under study
2. Be available to multiple investigators
3. Be exportable
4. If genetic, the species should be polytocous (producing multiple offspring)
5. Allow for multiple biopsies/samples
6. Fit into the animal facilities of most laboratories
7. Be easily handled by most investigators
8. When possible, be available in multiple species
9. Survive long enough to be usable.

At present, there is no accepted animal model of hepatitis C which satisfies all of these criteria, or even the majority of them. A wide variety of approaches have been employed in attempting to solve this problem, ranging from primates to non-primates and transgenic mice to transplanted mice, each with individualized strengths and weaknesses. An examination of these strengths and weaknesses will assist in establishing the parameters for development of a widely accepted animal model of HCV.

1. PRIMATE MODELS

In 1979, in a letter to the Lancet, Tsiquaye and Zuckerman described the first use of the chimpanzee (*pan troglodytes*) as a model for studying the transmission of the non-A non-B hepatitis (NANBH) virus.[3]. Shortly thereafter a number of other publications exploring both acute and chronic infections confirmed the utility of the chimpanzee model in studying NANBH,[126-129]; almost all subsequent in vivo work in studying transmission of HCV has made use of the chimpanzee model. The susceptibility of chimpanzees to infection with human HCV does not appear to extend to all primates, however. Investigators have been unable to establish infections in Cynomologous monkeys, Rhesus monkeys, green monkeys, Japanese monkeys, Doguera baboons, and cottontop tamarins[130; 131]. Of all primates tested, only the marmoset monkey appears to

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have some limited sensitivity to infection, which is at quite low levels of replication, and offers no significant advantage over the chimpanzee model[126; 132]

The chimpanzee model of hepatitis C is analogous in many respects to the disease as seen in humans[133]. Chimpanzees appear to be susceptible to the same viral strains and infectious inocula as are humans, and develop chronic infections at about the same rate of 70-85%. The timing of serum HCV RNA positivity, as well as its course over time and relation to liver enzyme levels appears also to be similar between both species. There is an important difference between the model and the disease in humans, however: chimpanzees do not appear to develop chronic active hepatitis, with progression to cirrhosis and hepatocellular carcinoma as is seen in approximately 30% of human subjects[82]. Over time, the animals appear to develop considerably milder histologic features more typical of chronic persistent hepatitis in humans, and rarely if ever develop more severe pathologic changes. Thus while the chimpanzee system is excellent at modeling the features of viral replication typical of human disease, it is less suitable for correlating these viral changes with clinical outcomes.

Although the chimpanzee model has been invaluable in determining the basic nature of a hepatitis C infection and will likely continue to be the system closest in nature to the human disease, it is not an ideal choice as a model for study of potential antiviral therapies. There are few laboratory facilities suitably designed to allow chronic study of infected chimpanzees, which severely limits the number of investigators with access to the model. The maintenance of such a model is extremely costly, and as such studies to date have been limited to very small numbers of animals; such small numbers are not well suited to screening of antiviral compounds or immunization strategies. Finally, there are ethical implications associated with the use of an animal species so close phylogenetically to our own for exploratory research. Developing a small animal model of hepatitis C infection would alleviate these concerns, and would likely prove much more useful to the worldwide research community.

2. SMALL ANIMAL MODELS

Because of the difficulties inherent to the use of chimpanzees to study disease, researchers have searched for an alternate animal model of HCV infection. A small

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animal model would be employable in a wide variety of laboratory situations, and would be particularly suited to the development of novel antiviral therapies. With the stringent species specificity of the virus, there are no readily available animal species susceptible to infection with human HCV. Likewise, there are no known viral analogues to HCV in the animal world which might allow for potential generalization to the human situation, such as the duck hepatitis B virus and groundsquirrel hepatitis B viruses as human hepadnavirus models.

Recently, a tree shrew indigenous to Southeast Asia, *tupaia belangeri chinensis*, has been suggested as a potentially susceptible small animal host of human HCV[134]. When examining the experimental data closely, of 26 animals infected with serum pooled from infected Chinese patients, only 8 animals developed any indication of infection, as evidenced by detection of positive-stranded RNA in serum samples. Of these 8 animals, each sampled 11 times, only one animal was positive at more than two time points (positive at weeks 2,5,7,14 and 27); the remainder were positive at only one or two timepoints over the 47 week course of the experiment, with no apparent pattern emerging from the timing of test positivity. A second group of 18 animals infected with pooled serum from Spanish donors revealed limited evidence of infection in two animals (positive RNA at a single timepoint) and sporadic positivity in two others. While touted by the authors as a possible small animal model of HCV, it is difficult to believe that any meaningful information could be derived from a model where serum HCV RNA-positivity is so sporadic and at best at low levels of replication. Coupled with the fact that wild tree shrews are frequently infected with a tree shrew herpes virus known to cause hepatitis, this limited replication severely restricts the utility of *tupaia* as a model of human hepatitis C.

With the lightning-fast advances in molecular technology, investigators have turned to the development of transgenic mice to explore component function of the hepatitis C virus in a small animal system. While not truly models of infection, they do provide some valuable insights into the pathogenesis of hepatitis C. Mice transgenic for HCV envelope genes were first produced in 1995 by Koike et al.[135]; despite high levels of envelope protein production, these animals showed no evidence of hepatic pathology. Further examination of these mice revealed however that they do develop a progressive

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exocrinopathy in salivary and lacrimal glands, pathologically typical of Sjogren's syndrome[136]. This type of sialadenitits is a known extrahepatic manifestation of HCV infection in certain patients. More recently, interesting insight into the mechanism of hepatic carcinogenesis associated with HCV infection has been presented using a murine model transgenic for the HCV core protein[137]. Initially, these animals showed hepatic steatosis, characteristic of HCV infection, but with no evidence of inflammatory infiltrate. At later timepoints, however, approximately 25% of male animals developed hepatocellular adenomas, frequently containing nodular foci of carcinoma. The core protein was immunohistochemically localized to the nucleus, which supports the notion that the core protein is intimately involved in HCV-associated carcinogenesis. An attempt was made by Matsuda et al. to develop a mouse carrying the full-length cDNA sequence of the hepatitis C virus as a transgene[138]. Of 9 transgenic lines carrying the entire HCV genome under various controlling promoters, only one line showed evidence of detectable mRNA and core protein in liver, and even then the expression of the transgene was at extremely low levels. This approach currently appears inadequate for viral study. While these various transgenic systems do allow some assessment of the interaction of viral proteins with host tissues, they do not represent models of HCV infection. They are limited in the spectrum of proteins produced, express transgenic proteins in tissues where they might not normally be expressed in a natural infection, and may produce viral proteins at nonphysiologic levels. All of these factors can combine to obscure the relationship between the models and the natural viral infection.

With the relative dearth of natural animal models of HCV infection, investigators have been forced to explore new avenues to establishing potential viral models. Human hepatitis viruses, and especially the hepatitis C virus, appear to be restricted largely to replicating within fully differentiated human cells. A potential approach to circumventing the species-specificity of viral infection is to establish a long-term xenograft of human hepatocytes within a permissive murine host. Theoretically this tissue, if sustained sufficiently well within a foreign microenvironment, should possess the capacity to support viral replication in vivo. There are a limited number of approaches published within the body of scientific literature that attempt to create such a system.

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The earliest attempt at such a transplant system was published in 1995 by Galun et al[139]; the host for transplanted human liver fragments was a BNX (beige-nude-X-linked immunodeficient) mouse. To render the recipient mice more receptive to human grafts each animal was preconditioned with lethal irradiation to destroy any existing immune progenitor cells, followed by reconstitution of bone marrow from a cb17/SCID donor (which in itself lacks functional B- and T-cells). Small fragments of human liver tissue were then implanted under the kidney capsule. The hepatitis C virus was introduced into the system through use of liver biopsies from HCV-positive donors, ex vivo incubation of non-infected human liver tissue with HCV-infected serum prior to implantation, and injection of HCV-infected serum into mice following transplantation of normal, non-infected human liver tissue. Graft success appears to have been determined only histologically; of animals followed for 12 weeks, 15 of 33 technically successful transplants (60 attempted transplants total) had histologic evidence of persistent engraftment. The authors described “partial regression in graft size . . . observed with time”, but then shortly thereafter stated “transplants developed major histologic changes”. As no functional data was presented, it is difficult to determine the actual rate of successful engraftment in these experiments, raising immediate challenges to the fundamental basis of this model.

The data for HCV replication is similarly obscure. Using liver biopsies taken from HCV-infected donors, 19 of 31 animals with “successful engraftment” were identified as HCV RNA positive, “. . .first observed 2 weeks after transplantation and continued to be detected intermittently for ~2 months after transplantation.” Each animal was positive at only one timepoint, and no consistent propagated infections were reported. When non-infected liver fragments were incubated with infectious serum prior to implantation, similarly 15 of 28 animals were positive at a single timepoint. In a final experiment, which the authors erroneously describe as a “control”, noninfected tissue was transplanted followed by inoculation with high-titer HCV-positive serum. Of 12 successful engraftments, no animals exhibited HCV positivity at any timepoint. This is in fact the most telling data from the entire experiment, demonstrating that the engrafted tissue is either incapable of infection with HCV, or of insufficient mass to produce replicating virus at detectable levels. It is a mistake to describe this experimental group

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as a control, for if the model truly maintains differentiated human hepatocytes capable of supporting viral replication, it should be infectable with high-titer infectious human serum. It appears that while the "trimeric" mouse model shows limited evidence of supporting HCV replication at low levels for a limited period of time, it is far too inconsistent in viral production to be considered as a useful model of HCV infection. Furthermore, the inability to infect normal tissue with HCV-positive serum seriously limits the utility of the model in exploring mechanisms of viral infection and entry, as well as any potential vaccine strategies. At present, this system requires considerable improvement before being accepted as a robust small animal model of HCV infection.

Despite a wide variety of approaches, investigators in the field of hepatitis C have be unable to develop a widely accepted animal model of HCV infection which is useful to the majority of laboratories. Although a natural host for human HCV, the chimpanzee is impractical for use in most settings. No small animal model appears to harbour the virus naturally, excepting perhaps the tree shrew; even in this case however, the utility of the model is rather circumspect due to widely inconstant viral detection. The only attempt at developing a model of HCV through human tissue transplantation has had only very limited success. For investigation into an antiviral therapy for HCV to continue, it is imperative that this obstruction to progress be overcome, through the development of a robust small animal model of hepatitis C.

PRINCIPLES OF HEPATOCYTE TRANSPLANTATION

The use of hepatocyte transplantation as a method for the treatment of inborn errors of metabolism has been proposed for more than two decades[140]. Experiments in murine[141], rodent[142], and larger animal models[143] have shown promise, with prolonged function of hepatocyte grafts in some cases.

Early hepatocyte transplantation studies in rats by Mito et al suggested the spleen as a suitable spot for implantation of hepatocytes[144], demonstrating that both autografted and isografted hepatocytes retained relatively normal histologic structure by light and electron microscopy. This "hepatized" spleen was shown to have higher basal levels of cellular proliferation than normal liver tissue[145], and to respond to appropriately to liver

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regenerative signals resulting from performance of a 70% partial hepatectomy[142; 146]. Supportive results were published by other groups in rat models[147; 148], larger mammals[149] and humans[150]. Based upon these studies, it was believed that after intrasplenic transplantation foreign hepatocytes tended to remain within the splenic parenchyma as the final site of engraftment.

As systems to more accurately locate transplanted hepatocytes developed, the final fate of intrasplenically-transplanted hepatocytes became more clear. Initially using a donor mouse strain transgenic for hepatitis B surface antigen (HBsAg)[151], and later with strains transgenic for human α-1 antitrypsin (hAAT) and *Escherichia coli β-*galactosidase (β-Gal)[141], hepatocytes transplanted into the spleen were shown to translocate to the liver and engraft within the host liver parenchyma. When splenectomy was performed one month after transplantation, no reduction in serum levels of the transgene products (HBsAg or hAAT) was observed, suggesting a paucity of cells within the spleen; histologic analysis further confirmed presence of transplanted cells within recipient liver parenchyma but absence within the spleen. After labeling donor hepatocytes with ^{111}In, scintigraphy and gamma counting was used to quantitated the distribution of transplanted cells: within 2 hours of transplantation, 55% of transplanted cells had migrated to the liver, with only 15% of radioactivity remaining within the spleen[151]. These experiments firmly established the liver as the final site of engraftment for the majority of viable transplanted hepatocytes. This fact combined with the considerably reduced mortality associated with intrasplenic injection in mice versus intraportal injection[141] renders the spleen the optimal sit for implantation.

Much of the mechanics of engraftment of hepatocytes has been worked out by Gupta and colleagues, using a dipeptidyl peptidase IV (DDPIV)-deficient rat model[152]. The expression of DDPIV localizes on the cell membrane to the bile canalicular domains, allowing for histologic assessment of the integration of transplanted cells into host liver plates. At two hours after transplantation, significant numbers of hepatocytes were seen within liver sinusoids as well as in portal areas. Between 16 and 20 hours, sinusoidally-located cells transmigrate across the perisinusoidal space of Disse, to enter into the surrounding host liver plate; by 24 hours after transplant no cells are seen within the liver sinusoids[153]. Similarly, by 24 hours no intraportal cells are seen, but in contrast they do

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not appear to engraft into surrounding parenchyma, and rather appear to be permanently lost. The bile canalicular domains of the transplanted cells begin to align appropriately by 24 hours after transplantation, and by 7 days normal gap junctions are seen to form between donor and recipient hepatocytes suggesting full integration. Although the actual mechanism of movement from the sinusoidal space to the surrounding liver plate is not yet known, the physical location of donor cells within host sinusoids appears critical to successful engraftment. This localization in turn appears to be dependent on size relationships between hepatocytes and portal vein radicles, with larger vessels permitting more distal movement of hepatocytes into sinusoids[154]. As only about 20% of transplanted cells will engraft initially[155], enhancing the movement of hepatocytes into liver sinusoids might improve overall engraftment.

Once transplanted hepatocytes are engrafted into host liver parenchyma, it appears that there is very little graft cell proliferation[153]. The total number of hepatocytes which may be transplanted into the liver at one time under optimal conditions appears to be limited to a maximum of approximately 20% of the total hepatocyte mass[154]. Because a significant number of transplanted cells fail to engraft successfully after implantation (see above), methods to enhance proliferation of transplanted cells are necessary to achieve a final graft cell volume sufficient to have a biological impact on the host.

One of the commonest methods to stimulate early graft proliferation is performance of a 50-70% partial hepatectomy (PH) concurrent with or shortly before the implantation of hepatocytes. The liver is unique as an organ in its ability to "regenerate" after partial resection. Within minutes after PH, urokinase receptors translocate to the hepatocyte membrane, and urokinase activity increases[156]. By 1 hour post-PH, along with other immediate-early genes HGF levels are seen to rise 20-fold[156]. After approximately 8 hours DNA synthesis is initiated, and by 24-36 hours post-PH hepatocytes begin actively replicating to restore the "preset" liver volume[157]. Addition of PH to intrasplenic transplantation has been shown in rat models to have a positive proliferative impact on engrafted cells, generally increasing graft cell numbers to 2-3 times that of control animals with no PH[142; 158; 159]. A similar proliferative effect has been demonstrated by the administration of carbon tetrachloride, a potent acute hepatotoxin, prior to the implantation of donor cells[160]. Although effective at stimulating an early

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proliferative response in transplanted cells, the duration of stimulation is limited to the period of regeneration of the native liver; as such the graft cells are generally induced to divide only once or twice prior to completion of regeneration. To produce host livers which contain a majority of transplanted cells, sustained stimulation to graft proliferation appears to be needed.

ANIMAL MODELS OF HEPATOCYTE PROLIFERATION

1. NONTRANSGENIC MODELS

The regenerative signaling elaborated from the recipient liver is the product of the response to liver injury; as described above, this may take the form of a surgical or a chemical injury. To effect a sustained proliferative response, systems have been developed to produce a prolonged sublethal injury within the liver. Vrancken-Peeters *et al.* produced an adenoviral vector which contained the coding sequence for a modified non-secreted form of murine urokinase-type plasminogen activator (termed Ad.PGK-muPA), and infused 5 x 10^9 plaque forming units of this vector into the portal vein of prospective recipient mice[161]. As the viral vector was taken up into the recipient livers, the production of intracellular urokinase induced liver injury which was followed by approximately 10 days of strong regenerative signaling. By transplanting 2 x 10^6 non-adenovirally infected congeneic cells (roughly 2% of the liver hepatocyte mass) into the portal vein, a final engraftment of ~8% of host liver mass was achieved, implying 3-4 graft cell divisions occurred during the period of transient adenoviral infection. While this approach is clearly not the final answer to stimulating graft cell proliferation, it does support the concept of a more sustained injury stimulating enhanced graft cell mass.

In addition to inducing a sublethal liver injury, the above adenoviral model also introduced the concept of providing donor cells with a selective proliferative advantage. The noninfected donor cells were able to respond normally to regenerative signals, while the urokinase-producing host cells were presumably compromised in their ability to replicate normally. This selective advantage is in contrast to the proliferative signals produced by performance of a partial hepatectomy, wherein both donor and recipient cells are stimulated equally by the upregulation of stimulatory proteins. A different use

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of this principle of selective expansion was employed by Mignon *et al*[162]. A mouse strain was developed carrying the human transgene Bcl-2, which afforded the mouse protection against Fas/CD95- (Fas ligand) mediated apoptotic liver failure. Recipient wild-type mice were transplanted with 1 x 10^6 transgenic hepatocytes, followed by a course of weekly treatment with sublethal doses of an anti-Fas antibody, Jo-2, for 8 to 12 weeks (a single treatment with Jo-2 induced apoptotic cell death in approximately 30% of recipient hepatocytes). Selective proliferation of the transgenic hepatocytes was seen within the recipient livers, with an estimated final engraftment of up to 15% in optimal cases. Although impractical for use in a clinical situation of hepatocyte transplantation, this method again supports the two critical concepts in sustained hepatocyte proliferation: prolonged regenerative signaling through repeated or sustained liver injury, and selective proliferative advantage of transplanted hepatocytes over native liver cells.

An ingenious approach to providing a selective proliferative advantage was published by Laconi *et al*[163]. Rather than transplanting normal cells into an abnormal liver, as is common amongst the above-described models, the authors blocked the ability of an otherwise normal liver to proliferate in response to injury. The drug retrorsine is a pyrrolizidine alkaloid commonly found in plants such as hogwort and ragwort, and known to cause liver disease in cattle and sheep. The drug is taken up and rapidly metabolized within host hepatocytes to a toxic derivative which effectively blocks the hepatocyte cell cycle at the late synthetic or early gap2 phase; this metabolic block prevent cells from undergoing normal mitosis for a period of 1-2 months. Recipient rats were given two doses of retrorsine at two week intervals, followed four weeks later by transplantation of normal congeneic hepatocytes (the DPPIV +/- rat system was employed to distinguish donor and recipient hepatocytes, as described above) with concurrent 66% PH. Engraftment of livers with up to 99% donor derived cells in some cases was observed, suggesting that this elegant system is capable of producing recipient livers which are comprised of almost totally donor-derived hepatocytes. In this model performance of PH initiates the stimulus to regeneration, but in this case recipient hepatocytes are blocked from responding to the regenerative signals thus producing a sustained proliferative environment. Transplanted donor cells are not affected by the blockade of mitosis, and as such have the requisite proliferative advantage needed for

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high level engraftment. This system may provide an excellent pharmacological adjunct to enhancing the engraftment of donor cells seen in other genetically-based hepatocyte proliferation models, such as the Alb-uPA model.

2. FAH-DEFICIENT TRANSGENIC MOUSE

There are two major models of hepatocyte proliferation commonly cited in the literature which are based upon transgenic or knockout mouse models. The first is the Alb-uPA model, which is the subject of much of this thesis and will be described extensively following. The second model is the fumaryloacetoacetate hydrolase (FAH)-deficient mouse, which was generated as a model of human hereditary tyrosinemia type I, a disease caused by an enzyme deficiency within the pathway of tyrosine catabolism[164]. If left alone, mice homozygous for this gene knockout die of hepatic dysfunction during the early neonatal period, suffering from severely deranged patterns of mRNA expression, especially in genes responsive to cAMP. A drug exists however, 2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3-cyclohexanedione (NTBC), which block tyrosine catabolism upstream of FAH, thus preventing buildup of hepatotoxic metabolites. This drug is capable of preventing the hepatic failure phenotype associated with FAH deficiency, allowing prolonged survival of homozygous mice.

When four to eight week-old mice were transplanted with normal FAH (+) syngeneic hepatocytes, followed by cessation of therapy with NTBC, selective proliferation of the donor cells was seen, with final engraftments of up to 80% in some cases as assessed by immunohistochemistry. In contrast, when NTBC was provided daily after transplantation, proliferation of graft cells was considerably reduced, to ~5% or less of total liver mass. Interestingly, this period of regeneration occurred over 6-8 weeks, a feature in common with the Alb-uPA model, as perhaps a reflection of the number of cycles of proliferation needed to achieve high level engraftment in recipient livers. This data clearly demonstrates that FAH-deficiency, with its resultant accumulation of toxic tyrosine metabolites within the liver, produces the sustained proliferative signals in recipient mice needed to induce rapid proliferation of normal donor cells. The authors went further in their study to demonstrate that significant final engraftment (~50%) of normal donor cells could be achieved with as few as 1000 donor cells – testament to the

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vast replicative potential present with each individual hepatocyte. This model would appear to be similar to the Alb-uPA model in its utility as a model for hepatocyte proliferation, with advantages in that the use of NTBC allows recipients to be transplanted at older ages, and the development of FAH activity represents a gain-of-function which can be assayed to reflect successful engraftment, a property which is absent in the Alb-uPA model. However, its utility has only been demonstrated in a congeneic system and it may not be as useful in a xenogeneic system. Further, no reports of rendering the mouse strain immunocompromised to accept xenogeneic grafts have been published, thus the robustness of the strain when crossed with a phenotypically weaker strain, such as a nude-athymic or SCID-beige mouse, has not been established.

3. Alb-uPA TRANSGENIC MOUSE

The Alb-uPA transgenic strain was developed in 1990 by Heckel *et al.* for studying neonatal hypofibrinogenemia and the pathophysiology of plasminogen hyperactivation[165]. The transgene consisted of the complete coding sequence of the murine urokinase gene including flanking untranslated sequences, which was placed under the control of a murine albumin enhancer/promoter sequence. A portion of the human growth hormone gene (from its 3' untranslated region) was added to the 3'-end of the transgene, to allow discrimination of endogenous and transgenic urokinase mRNA. As albumin is produced exclusively within the liver, this targeted the production of high levels of urokinase to the liver parenchyma. Neonatal mice were shown to have a severe bleeding diathesis, which resulted in lethal hemorrhage in approximately 50% of homozygous offspring within 48 hours of birth. In those homozygotes that survived the initial 48 hours of life, 80% would progress on to liver failure and death within 2-3 weeks; the remaining 20% survived this period, and over time slowly corrected their coagulation abnormalities.

When surviving offspring were euthanized and their livers examined grossly, they appeared to be composed of multiple confluent nodules of relatively normal-looking hepatic tissue. This was in contrast to the pale, shrunken livers observed in mice dying of liver failure. In a follow-up study by the same group, the nodules were examined histologically and by Southern blot analysis[166]. It was determined that each nodule was

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likely derived from a single clonal progenitor cell which had spontaneously deleted the transgene, presumably through random intrachromosomal rearrangement. It appeared that while high levels of expression of the transgene were toxic to the transgenic hepatocytes, after spontaneous deletion, transgene-deficient cells had a high capacity for proliferation and could effectively repopulate the liver with normal cells.

The capacity for cell proliferation was exploited in two further studies, published separately in 1994[167] and 1995[168]. Initially, hepatocytes isolated from congeneic donors (which lacked the Alb-uPA transgene, but carried a second transgene, MT-*lacZ*) were transplanted intrasplenically into recipients heterozygous for the Alb-uPA transgene. The transplanted cells were demonstrated to selectively proliferate within the recipient livers, similar to cells which had spontaneously deleted the transgene, with up to 80% replacement of diseased liver. By determining the final diameters of regenerated nodules and comparing them to the diameter of individual cells, it was estimated that each nodule was the product of approximately 12 divisions from a single progenitor. In the second series of experiments, the Alb-uPA transgenic strain was crossed with a Swiss athymic nude mouse strain, to render the transgenic mice T-cell deficient. Donor hepatocytes isolated from rat livers were implanted intrasplenically, and again the transplanted cells demonstrated a significant proliferative advantage. The overall extent of engraftment (estimated by the percent of rat DNA and rat-derived transferrin mRNA production from selected liver samples) was determined, and appeared to correlate with the zygosity of the transgene, i.e. homozygous animals had a considerably higher overall engraftment that heterozygous animals. This final set of experiments established the capacity of the model to stimulate the replication of xenogeneic (albeit phylogenetically closely related) hepatocytes, resulting in significant final liver engraftment.

Although the Alb-uPA transgenic model was shown to be highly effective at inducing rapid expansion of transplanted nontransgenic cells, the actual mechanism underlying the stimulatory effect remained unknown. Recent studies have shed considerable light on the molecular mechanisms underlying this effect. Histologic examination of the regenerating nodules using markers of cellular proliferation has shown that maximal cell labeling occurs at around 7 days of age, and that the majority (i.e. 60-80%) of cells within a regenerating nodule are actively dividing during the first 4-

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6 weeks[169]. In an effort to explain the proliferative effect, Locaputo *et al.* compared mRNA expression of various growth factors between the diseased liver and regenerating nodules. The overall level of growth factor mRNA expression (HGF, HGFL, IGF-1, aFGF and TGF-α) within the liver (both diseased and regenerative) at one month after birth was equivalent to that seen in normal livers at 36 hours after treatment with carbon tetrachloride (a potent hepatotoxin). Levels of HGF mRNA within the regenerating nodules themselves were similar to those in normal liver, but were significantly increased to five time normal in the diseased liver compartment. The implication is the diseased liver is attempting to stimulate regeneration, but is unable to respond to its own stimulatory signals; the reason for the failure to respond is unclear. In addition to an increase in stimulatory signaling, the diseased liver compartment was shown by Northern blot to express elevated levels of the growth inhibitory molecules TGF-β_1, activin A, and p53. Further examination of the diseased compartment by TUNEL assay showed evidence of increased cellular apoptosis, paralleling the increases in inhibitory molecules. Unfortunately the analysis of stimulatory signaling changes was limited to levels of mRNA expression, and changes in the translational products were not addressed; however, it would appear that the transgenic liver responds to autotoxicity of the transgene by producing elevated levels of HGF. As urokinase-type plasminogen activator is the primary enzyme involved in converting HGF to its active dimeric form, the transgenic environment might provide a further supranormal stimulus to hepatocyte regeneration. These mechanistic questions await further analysis, and will be addressed in concluding statements at the completion of this thesis.

Building upon the earlier work on expansion of transplanted xenogeneic hepatocytes by Rhim *et al.*[168], the Alb-uPA model has been shown to have utility as a model for viral diseases. By transplanting woodchuck hepatocytes into transgenic recipients, Petersen *et al.* demonstrated that a chimeric woodchuck/mouse liver could be developed[170]. These livers were capable of sustaining replication of woodchuck hepatitis virus (WHV), a hepadnavirus virus analog to human hepatitis B, either by transplantation of chronically WHV-infected cells or by secondary infection of transplanted normal woodchuck hepatocytes. Sustained viremia was demonstrated for up to 10 months, as was responsiveness to treatment with corticosteroids (increased viremia) and interferon-α

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(decreased viremia). The authors claimed to have up to 90% repopulation of recipient livers with woodchuck hepatocytes; this figure is quite misleading. The authors actually demonstrated that the amount of woodchuck derived DNA in five separate biopsies from a single animal appeared qualitatively to be up to 90% in one biopsy, and was considerably less in others. While the data on viral replication appears valid, the actual extent of engraftment must be viewed with caution. Very recently, data showing replication of human hepatitis B virus in Alb-uPA mice with chimeric human/mouse livers was presented in abstract form[171]. The authors reported reconstitution of mouse livers of up to 10% with human cells, with demonstration of HBV core protein in liver sections by immunohistochemistry. As this work has not yet been published, no further analysis of the experimental results is possible.

From these two studies, two important points can be derived. First, it would appear that in principle the Alb-Upa model holds promise as a system for supporting replication of HCV, although demonstrating this in a WHV, or even a human HBV system, has little generalizability to the considerably more difficult HCV system. Second, it appears that producing high-level human chimerism may prove to be considerably more difficult in this model than it has been with the more closely related rat or woodchuck species. In summary, it appears that the Alb-Upa mouse, if rendered suitably immunodeficient, should be capable of sustaining human hepatocyte chimerism. If sufficient levels of engraftment can be achieved, this environment should prove hospitable to infection by and replication of the human hepatitis C virus.

THESIS AIMS

1. GENERAL GOALS AND CENTRAL HYPOTHESIS

At present there is neither a vaccination nor a widely efficacious therapy available for the prevention or treatment of human hepatitis C infection. One of the most significant impediments to progress has been the lack of a model of the viral life cycle, either *in vitro* or *in vivo*, with which investigators can explore antiviral strategies. The goal of this series of experiments was to develop a novel small animal model of hepatitis C infection which would be widely useful to investigators studying both basic viral molecular biology and antiviral pharmacology/immunology.

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Based upon the knowledge that hepatitis C infectivity appears to be restricted to humans and chimpanzees and that replication of HCV in transformed hepatocytes, at least *in* vitro, is significantly impaired, we felt the optimal strategy would be to attempt to infect nontransformed human hepatocytes. To this end, we hypothesized that normal human hepatocytes, once transplanted into a murine liver, would be capable of infection by and support of replication of HCV. This would result in a mouse which carried the hepatitis C virus within its own liver, without actual infection of native murine hepatocytes. The development of this model followed a step-wise progression of experiments beginning with basic isolation of hepatocytes from human liver biopsies and leading to the detection of active replication of HCV within chimeric mouse livers; each step in the process was dependent largely upon the successes (and failures) of the antecedent experiments.

2. GENERAL THESIS OUTLINE

The first chapter of this thesis reviews the significant body of knowledge underlying the formulation of this series of experiments. The basic biology of hepatitis C is reviewed, both viral molecular structure and clinical pathophysiology, with additional focus on the aspects of hepatitis C infection most relevant to the general surgical community. A summary of proposed *in vitro* HCV models is presented, as well as potentially competing animal models, both to provide insight into alternate approaches to model development and to clearly highlight the advantages of the model developed herein over previous published attempts. Finally, the basic principles of hepatocyte transplantation are reviewed along with strategies to enhance hepatocyte graft proliferation. This information, especially the detailed history of the Alb-uPA transgenic mouse model, is central to the approach to model development employed in this thesis. It is hoped that this summary chapter will provide the reader with the requisite background to place this series of experiments within an appropriate clinical context, and to facilitate further understanding of the technical complexities involved in developing a chimeric mouse liver capable of supporting human HCV.

The goal of the second chapter is to outline the development of a technique to reproducibly isolate significant numbers of viable hepatocytes from surgically-obtained

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biopsies. This critical first step was necessary before any further experimentation could proceed. It is important in any system employing human tissue that the yield from each piece of human tissue be maximized.

The third chapter outlines a series of experiments performed to determine two specific purposes: to determine the responsiveness of human cells to growth signals, and to assess the effects of human derived growth factors on the longevity of human hepatocyte grafts. One of the key features requisite for any transplantation-based model of HCV would be prolonged duration of human cell engraftment. These experiments were performed to determine if adequate engraftment of human cells might be produced within a SCID-beige recipient mouse liver, as a prelude to attempting infection with a human hepatotrophic virus.

The fourth chapter presents an attempt to establish a viral infection in a series of human hepatocyte grafts placed into immunodeficient murine recipients. Using HBV as a surrogate for hepatitis C infection, we investigated the ultimate utility of the SCID-beige model as a host for a human viral infection. The results of these findings would help to elaborate which factors in a hepatocyte transplantation model are likely to be critical in developing a successful viral model, and prompted a change in animal models from a SCID-beige to a SCID-bg/Alb-uPA recipient.

The fifth chapter present a significant body of work performed in developing a novel strain of mouse, the SCID-bg/Alb-uPA, with the capacity to stimulate vigorous proliferation of human hepatocyte grafts. The utility of this strain as a recipient of human hepatocyte xenografts is confirmed, followed by a series of experiments with human viruses which establish this system as a stable model of human viral liver disease. This chapter represents the most significant body of experimentation within the thesis, culminating in the production of the first viable model of human HCV

The sixth and final chapter summarizes the progression in thought from initial hypothesis to final development of the HCV model. The advantages and disadvantages of the model are critically appraised, especially with respect to other models proposed by competing groups. Finally, a number of directions for future research are proposed, to both optimize the efficiency of the model and to expand its utility in the study of the

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pathophysiology of HCV infection, as well as the development of new therapies and vaccination strategies.

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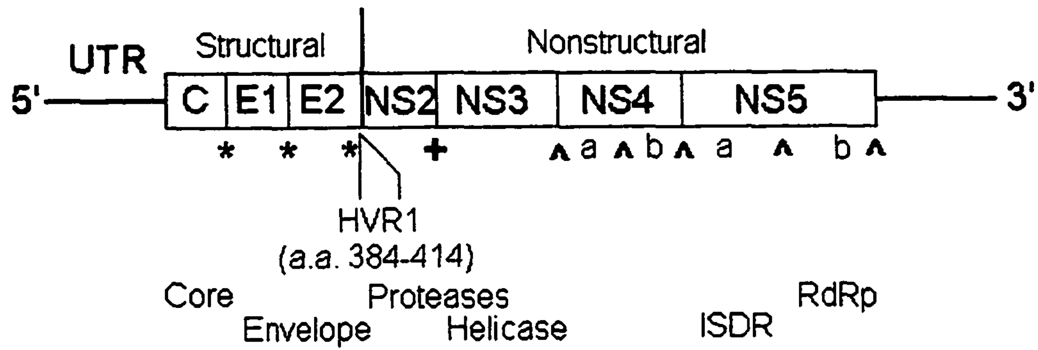

Figure 1. Organization of HCV genome. Polyprotein cleaved by: * - host signal peptidases, + - NS2/NS3 metalloproteinase; ^ – NS3/4a serine protease

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Study Author, Year	Method	Number	Recurrence (%)
Konig, 1992[18]	PCR	15	87
Sallie, 1992[16]	PCR	12	83
Wright, 1992[88]	PCR	89	95
Ascher, 1994[17]	ELISA	162	>95
Feray, 1994[91]	PCR	62	80
Hsu, 1994[172]	PCR	14	100
Lumbreras, 1994[173]	RIBA/PCR	14	100
Male, 1994[174]	PCR	8	86%
Wreghitt, 1994[175]	ELISA/PCR	14	93%

Table 1. Recurrence of HCV infection post-liver transplantation.

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CHAPTER 2

USE OF HIGH-PURITY ENZYME BLENDS IMPROVES ISOLATION OF HUMAN HEPATOCYTES FROM SURGICAL LIVER BIOPSIES

INTRODUCTION

Transplantation of hepatocytes has been proposed as a method for providing temporary or permanent liver support in cases of acute and chronic liver failure, as well as a strategy for correcting inborn errors of metabolism[1]. Isolated hepatocytes also hold promise for use in bioartificial liver-assist devices[2]. The critical first step in any of these clinical applications is the ability to initially isolate hepatocytes from donor livers or donor liver segments; the relative scarcity of donor organs emphasizes the need for reliable high-yield preparations of viable liver cells.

The majority of publications in the field of human hepatocyte isolation present techniques for isolating cells from whole livers, generally obtained from donor organs rejected as unsuitable for transplantation[3]. Many of these techniques require the construction of a large-scale dedicated isolation apparatus, and significant expenditure in the form of reagent costs such as collagenase. Additionally, with improvement in techniques of organ procurement, storage, and transplantation, the increasing use of "marginal" donor organs has further reduced the supply of organs available for experimentation. These factors combined prevent many investigators from undertaking experiments with human hepatocytes.

Concurrent with advances in transplantation techniques has been improvement in the ability to perform extended hepatic resections for conditions such as hepatocellular carcinoma and metastatic colorectal cancer; this potentially increases the supply of smaller segments of human tissue which are ideal for laboratory use. The number of publications addressing techniques for isolation of human hepatocytes from liver biopsies is relatively limited, however[4], and the methods themselves are often plagued by inconsistency of yield. Furthermore, there are no studies which address the effects of different collagenase preparations on the success of hepatocyte isolations.

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Recently, the use of purified collagenase isoform blends has been shown to improve the success of recovery of functional islets of Langerhans from both human[5] and dog pancreata[6]. We hypothesized that these purified blends would enhance the dissociation of human liver tissue, and thereby improve upon the inconsistency and poor hepatocyte yield from surgical biopsies which has characterized other studies. We report herein our evaluation of two purified enzyme blends [Liberase RH (Rodent Hepatocyte); Liberase CI (Canine Islet)] in dissociating viable human hepatocytes from surgically-obtained liver biopsies, and compare them with a third unpurified crude enzyme preparation (Sigma Type IV). Additionally, we describe a simplified apparatus for isolation assembled from readily available commercial products, and a cost-effective method for reproducible high-viability yields of human hepatocytes from small surgical biopsies.

METHODS

Procurement of Human Liver Tissue

Segments of human liver were obtained at laparotomy from areas which would normally have been discarded after pathological examination. In all cases, reasonable attempts were made to excise samples shortly after occlusion of segmental vascular inflow to minimize the warm ischemic time of the sample. Data on patient age, sex, indication for operation, total warm and total cold ischemic times and gross pathologic liver appearance was recorded for each case. Informed consent was obtained from all patients prior to the procurement of tissue, and ethical approval for the use of human tissue was obtained from the University of Alberta Faculty of Medicine Human Research Ethics Board.

Preparation of Low-Volume Tissue Perfusion Circuit

The circuit used for perfusion of human liver segment in shown in Figure 2. Disposable intravenous extension sets (product #2C5625, Baxter Healthcare Corp., Deerfield, IL) and disposable three-way stopcocks (product #01-041210002, COBE Canada Ltd., Scarborough, ON) were used for circuit construction . A Graham-type glass condenser is used as a heat exchanger, and the remainder of the apparatus is assembled from standard laboratory glassware; short lengths of polyethylene tubing are used to adapt the

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condenser to the extension tubing. All non-disposable equipment is fully steam autoclavable. A peristaltic pump capable of a rate of 50 ml per minute and a 37°C water bath with a simple submersible pump completes the apparatus.

Isolation of Human Hepatocytes

After excision, the tissue sample was moved to a back table, and using an 18-gauge intravenous catheter, perfusing vessels on the cut surface were flushed repeatedly with ice-cold Dulbecco's phosphate-buffered saline without calcium and magnesium (DPBS CMF) (ICN Pharmaceuticals, Costa Mesa, CA) containing 0.5mM Na_2EDTA. After the majority of blood was flushed out and the tissue was sufficiently cooled (approximately 100-150 ml total volume), it was placed in an additional 50ml of DPBS CMF/EDTA solution within a sterile specimen container, and immediately transported to the lab on ice.

Within a sterile hood appropriately-sized intravenous catheters (16- or 18-gauge) were cut to 1cm length, inserted into one or two of the largest inflow vessels, and sutured in place with a 5-0 prolene suture (Ethicon, Peterburough, ON). The cannulated liver section was placed on a low-volume perfusion circuit, and perfused at 100cm H_2O pressure in non-recirculating fashion with an additional 250ml of DPBS CMF/EDTA solution warmed to 37°C (5-10 minutes). Excess electrolyte solution was purged, and the circuit was switched to recirculating mode. The entire recirculating circuit was then primed with 60 ml of buffer solution (67 mmol/l NaCl, 6.7 mmol/l KCl, 4.8 mmol/l $CaCl_2 \cdot 2H_2O$, 100 mmol/l HEPES, final pH 7.6) containing enzyme (concentration and type specified below) to remove any air bubbles, and the tissue was perfused at 50ml/min and 37°C for 30-35 minutes, in a modification of the procedure first described by Seglen[7]. After enzymatic perfusion, digested tissue fragments were removed to a sterile glass plate, and gently teased apart with cotton swabs. Hepatocytes were separated from surrounding collagen matrix into a crude suspension by gentle manipulation with cotton swabs, avoiding areas of poor digestion. Aliquots of the collagenase-containing solution from the circuit (5-10 ml) were used to dilute the crude suspension to a final volume of 50-60 ml.

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The crude suspension plus enzyme was placed into a 37°C water bath for 25 minutes with periodic gentle agitation to allow secondary enzymatic digestion of small tissue clumps, followed by rapid cooling in an ice bath for 5 minutes. The cooled suspension was filtered twice through sterile stainless steel mesh (200μm and 80μm mesh sizes), and 10ml aliquots of the resulting filtrate were layered over a 10ml Percoll gradient (s.g. 1.04; Sigma, St. Louis, MO), and spun at 400 *g* for 5 minutes. The layered supernatant was discarded, and cell pellets were resuspended in 5ml aliquots of ice-cold Hank's Balanced Salt Solution (HBSS; ICN Pharmaceuticals) and subsequently combined into a single 50ml centrifuge tube. Cells were washed twice with 50ml aliquots of HBSS by centrifuging at 200 *g* for 3 minutes to further remove red blood cell contamination and dead cells and the final cell pellet was resuspended in ice-cold Belzer-University of Wisconsin Cold Storage Solution (UW-CSS; DuPont Pharmaceuticals, Scarborough, Ontario). Duplicate aliquots of the final suspension were counted using a hemocytometer, and viability determined in standard fashion by trypan blue exclusion.

Three different enzyme preparations were utilized separately in digestions: Sigma Type IV (Sigma, St. Louis, MO) 500 mg/l; Liberase RH (Roche-Boehringer Mannheim, Indianapolis, Indiana) 1000 mg/l; and Liberase CI (Roche-Boehringer Mannheim) 380 mg/l. The three groups were not randomized but were performed in sequential fashion, using all donors presenting over a period of time regardless of donor characteristics. Primary outcomes measured were total cells isolated, percent viability of final suspension and total number of viable cells isolated; in later isolations tissue weight pre- and post-digestion was recorded to normalize for variability in perfusion quality. Data was further analyzed *post hoc* to explore potential relationships between donor (age, sex, indication for operation) and procedural (warm ischemic time, time to collagenase perfusion, quality of perfusion) variables and primary outcomes.

Statistical Tests

Categorical variables were compared with a Chi-square test, and continuous variables were compared using a Student's t-test, ANOVA, or Pearson bivariate correlation as appropriate. All tests of significance were two-tailed, with significance defined as $p<0.05$. Statistics were performed using SPSS v. 9.0 (SPSS Inc., Chicago, IL).

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RESULTS

A total of 28 isolations were performed on tissue biopsies ranging from 10-25 cm^3 in size; donor and procedural characteristics for each collagenase type are summarized in Table 2-1. The three experimental groups were similar in donor characteristics (age, sex, indication for operation) and isolation variables (warm ischemic time, quality of peripheral tissue perfusion, total isolation time). Poor overall tissue perfusion was noted in some cases, due either to perfusion of only a small subsegment of a tissue sample, or to the presence of multiple large vascular channels which prevented development of adequate peripheral perfusion pressure by providing a low-resistance outflow path. As the number of poor perfusions was similar in each group, and variation in perfusion quality is a practical reality in human hepatocyte isolations, the data was analyzed with and without poor perfusions in overall analysis.

A summary of the primary outcomes of interest is presented in Table 2-2. A significant improvement in final viability, irrespective of perfusion quality, was seen in the Liberase RH group compared with the other groups. When cases with poor perfusion were excluded, the viability in the Liberase RH and Liberase CI groups was increased (90.9 to 93.4% and 80.8 to 81.1%, respectively) whereas the Sigma IV group was slightly decreased (80.1 to 79.4). No difference was seen between groups in comparing total cells isolated or total viable cells isolated. Tissue wet weight before and after collagenase perfusion was available for most isolations in the Liberase CI group: data from these isolations is summarized in Table 2-3. The calculated mean total viable cells per gram tissue was $24.2 \pm 5.6 \times 10^6$ (mean ± SEM).

Exploratory *post hoc* data analysis revealed an association between donor sex and final viability, with female donors having a slightly higher final viability than males (87.3 vs. 80.6, $p=0.041$). Although close to the limit of significance, donor age also appeared to have a correlation with total cells isolated ($p=0.053$) and total viable cells isolated ($p=0.048$), with higher donor age being associated with increased yield of cells (correlation coefficients 0.370 and 0.377, respectively). Subjective perfusion efficiency was the only procedural variable associated with improved viable cell yield (137.5 ± 30.8

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x 10^6 vs. 71.7 ±25.4 x 10^6, p=0.052). No correlation between primary outcomes and indication for operation, warm ischemic time, or total isolation time was observed.

Although primary outcomes reported were based on per cent cell recovery and dye exclusion techniques, cells were shown in subsequent experiments to maintain normal protein synthetic function, respond appropriately to mitotic signals, and tolerate freeze-thaw cycles (data not shown).

DISCUSSION

Hepatocyte transplantation hold immediate promise as a modality for treating hereditary disorders of metabolism, and future promise as a potential alternative to whole-organ orthotopic liver transplantation. Because of a limited supply of human tissue, the majority of experimental work in the past has focused on rat, mouse, and porcine hepatocytes to elaborate the fundamentals of hepatocytes isolation and the mechanisms of hepatocyte engraftment within host tissues[8-12]. As such, there are relatively few studies specifically addressing the techniques of human hepatocyte isolation, and none which examine the effects of different enzyme preparations on the critical endpoints of cell viability and total viable cell yield.

Collagenase, the main enzyme employed in digesting liver samples for isolation of hepatocytes, is a fermentation product of the bacterium *Clostridium histolyticum*. Crude collagenase preparations which are typically used in hepatocyte isolations are often characterized by wide variation in collagenase activity between lots and contamination with multiple enzymatic and non-enzymatic peptides, endotoxin, and other cellular debris. These differences in turn lead to variations in effectiveness of isolations, as well as degradation of collagenase activity over time[13]. Recently, researchers at Roche-Boehringer Mannheim have separated the key enzymatic components used in enzymatic dissociation of cells and tissues. This purified enzyme blend has eliminated the lot-to-lot variability experienced with other preparations of crude enzymes. When these purified enzyme blends were evaluated in human and canine pancreatic islet isolation, significant improvements in islet recovery resulted[5;6]. We felt these improvements in enzyme purity might also translate into benefits in viability and viable cell yield when applied to isolation of human hepatocytes.

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Of the two purified enzyme blends used for human hepatocyte isolation, the Liberase RH blend, specifically designed for rodent hepatocyte isolation, showed a significant increase in final hepatocyte viability as compared to the crude Sigma blend and Liberase CI blends. The second purified blend, Liberase CI (designed for canine islet isolation), showed only a marginal benefit by comparison. No significant overall difference was seen between enzyme groups in the other primary outcomes of total cells isolated and total viable cells isolated. This statistical comparison was dominated by a wide variation in cell numbers seen in the Sigma IV group, and to a lesser extent by variation within the other two groups.

The initial dispersion of the liver segment into individual cells and cell clusters is dependent upon the delivery of enzyme into the tissue by even distension with enzyme-containing buffer solution. The quality of distension in turn is dependent on characteristics of the tissue in question such as a single dominant perfusing vessel and few low-resistance outflow channels effecting higher tissue perfusion pressures. To eliminate the effects of perfusion quality as a potential confounder of the study outcomes, cases subjectively graded as "poor perfusion" at the time of isolation were filtered out, and the outcome data reanalyzed by ANOVA. When only cases of "good perfusion" were analyzed, Liberase RH was superior in all categories to the other collagenase blends, although again statistical significance was not achieved in total cell number and total viable cell number due to the relatively high variation within the three groups. In both the overall analysis and the analysis by perfusion quality, the purified enzyme blends had considerably reduced variation from isolation-to-isolation as compared to the crude enzyme preparation group. The elimination of impurities in the collagenase preparation may account for such difference, and suggests that the purified blends are superior in providing consistency of isolation over a period of time. However, the fact that reasonably high variation in isolated cell numbers still exists within the two purified enzyme groups further suggests that other factors, including perfusion quality, have an important effect on the success of hepatocyte isolations.

The yield of viable cells per gram of tissue is an important outcome parameter in comparing techniques of isolation between studies, providing some compensation for the inherent differences in perfusion quality between samples. Data on tissue weights pre-

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and post-perfusion with collagenase solution were obtained in the Liberase CI group, allowing calculation of the yield of viable cells per gram tissue weight. The mean yield of $9.7 \pm 2.1 \times 10^6$ viable cells per gram of tissue is similar to other published results (Table 2-4). The reduced variability in our data may be in part due to improvements in consistency of isolation associated with the use of purified collagenase blends. However, despite a reduction in variability, the mean yield per gram is not different from that presented by Ballet[4] or Ryan[14], both of whom used crude collagenase preparations for isolation. Again, this supports the concept that other factors such as perfusion quality may be critical in determining overall outcome. The dispersed tissue weight is the difference in tissue mass before and after perfusion with collagenase, and corrects for differences in perfusion quality between samples. When this correction is factored in, the mean viable cell yield per gram of dispersed tissue is increased considerably to $24.2 \pm 5.6 \times 10^6$. This value is not available in other studies for comparison.

A second important factor in the improved consistency of our results is an extremely short warm ischemic time (mean 5 ± 1 minute), as compared to other published studies (Table 2-4). A longer duration of warm ischemia has been associated with reduced cell yields[4]; by close communication between the surgical and isolation teams, we are able to procure samples minutes after occlusion of major vascular inflow. Interestingly, the two studies reporting results similar to ours also obtained surgical samples within minutes of clamping of vascular inflow[4]. In pilot studies we have shown that freshly isolated hepatocytes may be maintained at 4°C in University of Wisconsin (UW) solution with little decrease in viability for up to 48 hours, after which viability slowly declines at a rate of approximately 20% per day (data not shown). Because of limits in the number of centers which will be able to secure access to human tissue, we propose that cells should be isolated at the originating center as shortly as possible after occlusion of vascular inflow, after which they may be safely stored short-term in UW solution and shipped to other centers for use in experimentation. In this respect, we differ from Caruana *et al.*[15], who suggest that tissue be flushed with UW and cold-stored for shipment prior to isolation of hepatocytes; our improved results support our approach.

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Exploratory *post hoc* analysis of the experimental data confirmed a significant association between perfusion quality and overall outcome ($p<0.01$). The quality of an individual perfusion appears to be dependent on characteristics of each liver sample, such as one or two dominant vessels perfusing most of the isolated segment. We have found that selecting for cannulation one or two vessels which appear to supply flow to the bulk of the liver biopsy provides reasonable perfusion quality for the majority of samples. Ryan *et al.* took a different approach in cannulating up to seven vessels per segment to ensure wide distribution of flow[14]; their excellent results support equally well this approach to cannulation and perfusion. The highly significant difference attributed to perfusion quality is likely to be a true association, despite the *post hoc* nature of the analysis. In contrast, the associations between outcome and female sex or increasing age are closer to the statistical limit of $p=0.05$. We can find no support in the literature for improvements in isolation quality attributable to female sex or increasing age; in fact, analysis by Dorko *et al.* found increasing age to negatively influence isolation outcome[16]. While these associations do appear evident from our data, care must be taken in overinterpreting their validity because of the exploratory nature of the statistical tests used.

The hepatocyte isolations in this series of experiments were performed using a small-volume perfusion circuit, which was prepared from readily available commercial materials, all of which are either disposable or easily autoclavable (Figure 2). With the addition of a peristaltic pump and a warm water bath this system can be easily assembled on the benchtop of most laboratories, and provide consistent high quality hepatocyte isolations. Because the total circuit volume is only 40 ml, in general 60-70 ml of collagenase perfusate is more than adequate to perfuse a 15-25 cm^3 liver biopsy, significantly reducing the amount of enzyme required per isolation and thus helping contain the major cost of the procedure.

In summary, we have utilized a low-volume perfusion circuit combined with high-purity enzymes and short cold ischemia times to produce high-yield isolation of human hepatocytes from surgical liver biopsies. The use of high-purity collagenase preparations subjectively enhances the initial dispersion of liver tissue, and improves the final viability of cell preparations. However, differences in perfusion quality between

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liver samples appears to play at least as important a role in overall outcome as enzyme type. Hepatocytes isolated by these methods have been used in a number of subsequent experiments, and have been shown to function normally and respond appropriately to mitotic signals. This method will provide any laboratory with access to human liver tissue, even in relatively small amounts, the ability to isolate hepatocytes with reproducibly high yield and viability, at a reasonable cost per isolation.

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		Collagenase		
		Sigma IV	Liberase RH	Liberase CI
No. Isolations		8	10	10
Age (y)		59.1 ± 6.5	60.2 ± 5.2	52.2 ± 3.8
Sex (M:F)		2:6	4:6	7:3
Diagnosis	HCC	2	2	2
	Mets	4	3	4
	Other	2*	5†	4‡
Warm Ischemia (min)		12 ± 4	12 ± 4	5 ± 1
Perfusion	Good	7	5	8
	Poor	1	5	2
Total Isolation Time (min)		145 ± 14	137 ± 3	136 ± 3

Table 2-1. Characteristics of donor population and isolation variables. Differences between groups are all non-significant. HCC – hepatocellular carcinoma; Mets – metastases to liver (9 colorectal, 1 breast). * liver donor cutdown, cholangiocarcinoma; † hemangioma, focal nodular hyperplasia (2), cholangiocarcinoma, benign biliary tract obstruction; ‡ neurosarcoma, hepatic adenoma, focal nodular hyperplasia, hemangioma.

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		Total Cells Isolated (x 10^6)	**Viability (%)**	**Viable Cells Isolated** (x 10^6)
All Cases	**Sigma IV**	236 ± 77	80.1 ± 3.9	197 ± 63
	Liberase RH	161 ± 44	90.9 ± 1*	149 ± 41
	Liberase CI	211 ± 37	80.8 ± 2.0	173 ± 31
Good Perfusion Only	**Sigma IV**	230 ± 89	79.4 ± 4.4	190 ± 72
	Liberase RH	255 ± 60	93.4 ± 1.3*	239 ± 57
	Liberase CI	235 ± 41	81.1 ± 2.2	192 ± 35

Table 2-2. Comparison of primary outcomes between collagenase groups. Viability determined by trypan blue exclusion. NC- not calculable; * $p < 0.05$ compared to other groups (ANOVA with Student-Neumann-Keuls *post hoc* analysis)

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Case	Tissue Weight (g)	Total Cells (x 10^6)	Viability (%)	Total Viable Cells (x 10^6)	Viable Cells per Gram (x10^6)
1	17.0	319	83.8	267	15.7
2	-	307	86.5	266	-
3	13.9	190	74.6	142	10.2
4	10.7	171	86.3	148	13.8
5	21.8	60	72.6	44	2.0
6	19.5	453	81.8	370	19.0
7	19.2	137	89.6	122	6.4
8	21.7	156	85.2	133	6.1
9	19.6	111	73.5	82	4.2
10	-	205	73.9	152	-
Mean± SEM	17.9 ± 1.4	211 ± 37	80.8 ± 2.0	172 ± 31	9.7 ± 2.1

Table 2-3. Outcome data from hepatocyte isolations with Liberase CI.

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Author, year	N	Cold Ischemia (h)	Viability (%)	Total Viable Cells per Gram (x 10^6)
Ballet *et al.*, 1984[4]	8	NS	80.6 ± 4.3	12.3 ± 5.6
Hsu *et al.*, 1985[17]	5	1	70-90	1-3
Ryan *et al.*, 1993[14]	58	NS	74 ± 2.2	11.0
Dorko *et al.*, 1994[16]	8*	15.5	90.3 ± 1.2*	16.6 ± 3.6*
Hewitt *et al.*, 1997[18]	5	8	68.0 ± 2.0	1.9 ± 0.7
Caruana *et al.*, 1999[15]	9	3.7	94.9 ± 0.6	0.25 ± 0.03
Present study	28	0.8	80.8 ± 2.0	9.7 ± 2.1

Table 2-4. Summary of human hepatocyte isolation data from published reports. Means and SEM calculated when possible from experimental data. NS – not specified. * Only able to isolate cells in 8 of 23 attempts.

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Misc/hepatocyte isolation system.cdr

Figure 2. Small-volume perfusion apparatus for isolation of human hepatocytes

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CHAPTER 3

EFFECTS OF MURINE- AND HUMAN-DERIVED REGENERATIVE SIGNALS ON SURVIVAL OF HUMAN HEPATOCYTES IN A XENOGENEIC ENVIRONMENT

INTRODUCTION

The development of a long term support system for human hepatocytes would facilitate experimentation into the pathophysiology of human liver disease. Unfortunately, long-term maintenance of human hepatocytes in primary culture has proven difficult, and the durability of human cultures is highly variable[1-3]. Currently, hepatocyte culture systems are inadequate for the study of viral hepatitides which are of a chronic nature and appear to replicate only at low levels within *in vitro* systems[4; 5]. Transformed hepatocyte cell lines such as HepG2[6], PH5CH[7-9] and HUH-7[10] overcome the problems associated with maintenance of long-term cultures, but are significantly different from nontransformed hepatocytes in certain respects, and as such do not appear to be effective in supporting HCV replication.

Isolated hepatocytes have been shown to engraft within host liver parenchyma when introduced into the portal venous system in congeneic models[11; 12]. Similar results have been obtained in allogeneic and xenogeneic models when hepatocyte transplantation is accompanied by abrogation of the host immune response through immunosuppression[13; 14] or immunodeficiency[15]. Transplantation of human liver tissue into a murine host would permit the study of human hepatocytes in a structural microenvironment closely simulating their own. Once transplanted hepatocytes are engrafted into host liver parenchyma, however, it appears that there is very little graft cell proliferation[16]. Because a significant number of transplanted cells fail to engraft successfully after implantation[16], methods to enhance proliferation of transplanted cells are necessary to achieve a final graft cell volume sufficient to have a biological impact on the host. While experimental interventions such as partial hepatectomy concurrent with cell transplantation have been shown to enhance graft longevity[17-19], these experiments have been limited to syngeneic or allogeneic systems. The development of models of hepatocyte proliferation, such as the Alb-uPA transgenic[20; 21] or FAH-deficient mouse[22]

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have provided exciting possibilities for early expansion of transplanted hepatocytes, but again experiments have been limited to congeneic or closely related xenogeneic (rat) hepatocyte donors. Human hepatocytes within a murine liver would be surrounded by a xenogeneic extracellular milieu, and may not be able to respond appropriately to murine cytokines and growth factors. This may have important implications not only for the survival of human hepatocytes grafts within a mouse, but also by extension to any other situation where xenogeneic tissue transplants are contemplated for treatment of disease, such as bioengineered porcine islets for the treatment of human type I diabetes mellitus[23, 24].

In this series of experiments, we demonstrate that human hepatocytes can be successfully transplanted into immunodeficient cb17/SCID-bg mice, but that their survival in the absence of any stimulatory signals is reduced. Proliferative stimulation of graft cells through performance of a 50% partial hepatectomy at the time of transplantation enhances graft survival, although overall it remains considerably foreshortened. Supplementation of the cellular microenvironment with human-specific hepatocyte growth factor (hHGF) has an effect on graft survival superior to partial hepatectomy. These findings suggest that the provision of species-specific growth factors may be important in optimizing the function of tissues transplanted across xenogeneic barriers.

METHODS

Animal Care

25-30 g male cb17/SCID-bg mice (Taconic Farms, Germantown, NY) were used as recipients. All animals were kept in virus/antigen free conditions, and were stored and manipulated in accordance with guidelines established by the Canadian Council on Animal Care (1993). Ethical approval for use of human tissue was obtained from the University of Alberta Faculty of Medicine Research Ethics Board, and approval for animal experimentation was obtained from the University of Alberta Animal Welfare Committee.

Isolation of Hepatocytes

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The procedure for hepatocyte isolation is outline in detail in Chapter 2. Briefly, human tissue was obtained at laparotomy by a wedge biopsy of normal tissue, which would otherwise have been discarded after hepatic resection, the majority of operations performed were for primary or metastatic liver tumours. Samples of liver tissue (10-25cm^3) were removed as soon as possible after clamping of vascular inflow, to minimize warm ischemic time, and were promptly flushed with 100-150 mL of ice-cold Dulbecco's phosphate buffered saline Ca^{++}/Mg^{++} free (DPBS-CMF) solution (ICN Biomedicals, Aurora, OH). Tissue was placed on ice, and immediately transported to the lab for further processing. Under sterile conditions, 16-18 gauge intravenous catheters were cut to 1 cm lengths, introduced into 1-2 of the largest perfusing vessels of a tissue wedge, and sutured in place. Other moderate-sized vessels were suture-ligated to reduce outflow from the liver specimen and enhance peripheral perfusion.

A two-step collagenase perfusion was performed using a modification of the procedure established by Seglen[25]. Tissue was initially perfused with 250 ml of DPBS-CMF solution at 37°C, in non-recirculating fashion, at 30-50ml/min. This was followed by a recirculating perfusion with 70 ml of a buffered salt solution (67 mmol/l NaCl, 6.7 mmol/l KCl, 4.8 mmol/l $CaCl_2 \cdot 2H_2O$, 100 mmol/l HEPES, final pH 7.60) containing 0.1% Sigma Type IV collagenase (Sigma, St. Louis, MO) or Liberase RH (Boehringer Mannheim, Indianapolis, IN), at 37°C for 30 min. Tissue was then gently fractured with cotton swabs, washed with aliquots of the collagenase perfusate, and the crude hepatocyte suspension was placed in a 37°C water bath for 25 min. After cooling in an ice bath for 5 minutes, the crude suspension was filtered through 100 μm stainless steel mesh and purified by density-gradient centrifugation over Percoll (density 1.04 g/mL, Sigma) at 400 g for 4 min. The cell pellet was washed twice in ice-cold Hank's Balanced Salt Solution (HBSS) and gently suspended in 0°C Belzer University of Wisconsin Cold Storage Solution (UW-CSS; DuPont Pharmaceuticals, Scarborough, Ontario) for short-term storage prior to use. Cell counts were performed immediately with a hemocytometer using trypan blue exclusion to assay viability of final cell preparations. All cells were used for transplantation within 4 h of final suspension in UW-CSS.

Transplantation of Hepatocytes

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The concentration of stored cells was adjusted to 5×10^7 cells/ml prior to transplantation. Aliquots of 50 µl (2.5×10^6 cells) or 100 µl (5.0×10^6 cells) of cell suspension were washed with cold 0.9% saline solution immediately before transplantation, and drawn up into a 27-gauge butterfly injector (Abbott; Sligo, R. Ireland) attached to a 1-mL syringe. In a sterile operating hood, recipient mice were anaesthetized with a Halothane/O_2 mixture (2-3% for induction/1-1.5% for maintenance), the abdomen was shaved and prepared with Betadine solution and a 1-2 cm midline laparotomy incision was made. The inferior pole of the spleen was grasped lightly between curved forceps and the needle tip was introduced 3-4 mm into the splenic pulp (in some cases, a 50% partial hepatectomy was performed immediately prior to implantation of cells; see below). The cell plug was slowly injected into the inferior pole over a one-minute period. The needle was removed and a small titanium ligating clip was placed over the injection site to ensure hemostasis and prevent backflow of injected cells. After confirming hemostasis, the spleen was returned to its native position and abdomen was closed in two layers.

Fluorescent Labeling of Hepatocytes with CFSE

In early experiments, some samples of hepatocytes were labeled with 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes Inc., Eugene, OR) prior to implantation. Immediately preceding re-suspension of human hepatocytes in UW-CSS, approximately 5×10^7 cells were separated out and suspended in 3.0 ml HBSS. CFSE (0.42 µg) was added to the suspension and the volume adjusted to 5ml (final concentration of 0.15 µM CFSE). The cell suspension was placed in a water bath at 37°C for 15 min, washed twice in ice-cold HBSS, and finally re-suspended in cold Belzer UW-CSS. Cell fluorescence was confirmed prior to transplantation using fluorescence microscopy.

Fluorescence Microscopy

At pre-determined points after transplantation (1,2,3,4,6 and 8 weeks), randomly selected animals had their livers and spleens removed for histologic analysis. Samples were blocked in paraffin and sectioned for microscopy. Microscopy was performed

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using a Transmitted-Light Photomicroscope III with an Epifluorescent Condenser IIIRS (Carl Zeiss, West Germany).

Partial Hepatectomy

Prior to transplantation, animals were randomly allocated to receive 5 x 10^6 human hepatocytes intrasplenically (Control) or 2.5 x 10^6 cells immediately following 50% partial hepatectomy (PH) In the latter group, once anaesthetized the left lobe of the liver was rotated medially into the incision and a 2-0 silk ligature was placed around its base, ligating the inflow vessels to the left hepatic lobe *en masse*. The left lobe was carefully excised and after ensuring hemostasis, the liver was returned to its native position. The subsequent transplantation procedure was performed as described above.

Detection of Human Albumin in Mouse Serum by Immunoprecipitation and Western Blot

Samples of mouse serum were obtained by jugular venous puncture at weekly intervals to a maximum of 12 weeks. Aliquots of mouse serum (20 μl) were incubated with an anti-human albumin monoclonal antibody (Clone HSA-9; Sigma), and antigen-antibody complexes were precipitated with protein G-agarose (Boehringer-Mannheim). Immunoprecipitates were then heated for 5 minutes at 98°C in SDS buffer containing 0.2 M dithiothreitol, separated on a 7.5%SDS-polyacrylamide gel by electrophoresis and transferred to nitrocellulose. Western blots were prepared in standard fashion[26] using a second anti-human albumin monoclonal antibody (Clone HSA-11; Sigma) conjugated to biotin as the primary recognition antibody. A streptavidin-horseradish peroxidase conjugate (Pierce, Rockford, IL) was employed as the secondary reading antibody, and photodetection of immune complexes was accomplished by addition of enhanced chemiluminescence reagents (Pierce). Chemiluminescent bands on autoradiographs were scanned with a Umax Astra 1200S scanner and VistaScan DA v. 1.2.2 imaging software (UMAX Corp., Fremont CA). Quantification of HSA peaks was performed using NIH Image 1.60/fat software (National Institute of Health, Bethesda MD).

Continuous Administration of Human Hepatocyte Growth Factor

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A solution containing human HGF [21.4 mg NaCl, 25 µl of 10 000 i.u./ml Heparin (Hepalean; Organon Teknika, Toronto, Canada), 5 µg HGF (Sigma), 1195 µl double-distilled H_2O] was prepared; a control solution was prepared in similar fashion containing all components except HGF. A series of implantable microosmotic pumps (Alzet model 1002: 100µl capacity, 0.25µl/hr rate for 14 days; Alza Corporation, Palo Alto, CA) were filled with either control or HGF-containing solution 12-18 hours prior to isolation and transplantation of hepatocytes, and were placed in sterile 0.9% saline solution at 37°C to ensure pump activity at time of implantation. A 1.5-2.0 cm length of polyethylene tubing (PE-20; Intramedic, Parsipanny, NJ) was cut and prepared for attachment to each pump as per manufacturers instructions. Immediately prior to transplantation of hepatocytes, an extended midline laparotomy was performed, and using microsurgical magnification the distal end of the polyethylene cannula was placed into the pyloric branch of the portal vein and secured in place with a 10-0 nylon suture. The cannula was tunneled through the mesentery of the colon, and the attached pump was placed intraperitoneally within the pelvis, taking care to ensure the cannula did not occlude the portal vein at implantation or after the pump was placed in its final position. Animals were then transplanted with 5×10^6 human hepatocytes intrasplenically as described above. At the time of experimentation, animals were randomly selected to receive cells plus an implantable pump containing HGF (Group HGF), cells plus a pump containing control solution (Pump), or cells alone and a sham operation (Sham).

Statistical Analysis

Differences in graft survival between experimental groups were compared using a nonparametric two-tailed Mann-Whitney rank-sum test or Kruskal-Wallis test as appropriate, with significance defined at the $p<0.05$ level. Graft failure was defined as intensity of the HSA band in a sample below the signal background of nontransplanted SCID mouse serum from a western blot, as determined by computerized densitometry.

RESULTS

Hepatocyte Isolation and Transplantation

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The median hepatocyte donor age was 67y (range 17-89). Mean warm ischemic time and total preparation time were 8 ± 2 min (mean ± SEM) and 116 ± 12 min respectively. Average final percent viability, as assessed by trypan blue assay was 86.8 ± 1.9%, with a mean yield of 1.89 ± 0.59 x 10^8 viable cells per sample.

Preliminary experiments were performed to optimize the number of human hepatocytes used in transplantations. Optimal results were seen with transplantation of 5 x 10^6 human cells without or 2.5 x 10^6 with partial hepatectomy (data not shown). After the initial recovery from surgery, mice behaved normally and no long-term adverse sequelae from transplantation were observed.

Fluorescence Microscopy

To verify that transplanted human hepatocytes could translocate from the spleen to the liver and incorporate into the host liver parenchyma, cells were labeled with CFSE prior to transplantation to permit detection of donor cells in sections of recipient liver by fluorescence microscopy. Animals were euthanized periodically at points up to 12 weeks post-transplant, and livers and spleens were harvested for histologic analysis.

1 week: Fluorescent cells labeled with CFSE were detected within the liver parenchyma and generally were localized around portal venous structures (Figure 3-1a). Hepatic parenchyma maintained normal histologic appearance with hematoxylin-eosin (H&E) staining and the fluorescent cells were indistinguishable from the background of mouse hepatocytes by normal light microscopy (not shown). Occasional clusters of cells were noted within splenic parenchyma.

2-4 weeks: Hepatocytes maintaining strong fluorescence were clearly detectable, and were found in more peripheral locations, as opposed to periportal areas (Figure 3-1b). Fluorescent cells were generally found in clusters of 10-20, although single and double cells were also observed. Liver parenchyma appeared normal with H&E staining (not shown). At four weeks, only occasional fluorescent cells were detected in splenic sections.

6-12 weeks: Isolated clusters of fluorescent cells were detected in sections taken up to 12 weeks post-transplant, although both the number of cells observed and the strength of fluorescence had diminished considerably. By 12 weeks only rare cell

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clusters were observed, located in both peripheral and periportal locations. No fluorescent cells were detected in any splenic sections by 12 weeks.

Effect of Partial Hepatectomy on Graft Function

As a nonlethal method of assaying human hepatocyte graft function, weekly serum samples from graft recipients were assessed by detection of human albumin (HA) production using immunoprecipitation and western blotting; a representative blot is shown in Figure 3-2. Graft function was considered to have ceased when the HA signal from a serum sample fell below the background level of the assay for two consecutive weeks, with duration of graft function measured at the point of last detectable signal.

In the group that received 5 x 10^6 cells and no partial hepatectomy (Control, n=7), the median length of albumin production was 3 weeks (range 2-8 weeks). In contrast, the group that received 2.5 x 10^6 cells immediately after 50% partial hepatectomy (Experimental, n=7) had a higher median graft function of 6 weeks (range 4-10 weeks). The difference between groups was statistically significant ($p=0.019$, Mann-Whitney U test); results of this experiment are summarized in Table 3-1.

Effect of Human Hepatocyte Growth Factor on Graft Function

Initially, attempts were made to insert silastic cannulas directly into the portal vein for delivery of HGF or control solutions; universal mortality was encountered in the early post-operative period secondary to acute portal occlusion. To circumvent this problem, the pyloric branch of the portal vein was used for cannulation, leaving the main portal vein free from direct occlusion. The pyloric branch was successfully cannulated and implantable microosmotic pumps placed in 12 animals, 6 each in control and HGF groups, followed by transplantation of 5 x 10^6 human hepatocytes intrasplenically. Again, significant (50%) mortality was seen in each group in the first post-operative week; in all cases the cause of death at necropsy was portal vein thrombosis, with venous infarction of the entire small and proximal large bowel. All sham experiments (n=4) were performed without incident.

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The remaining 3 animals in each group, as well as the sham control group were followed to 12 weeks with weekly serum sampling and assay for human albumin production. Surviving animals carrying implantable pumps appeared outwardly normal throughout the period of observation, as did all sham animals. The results of this experiment are summarized in Table 3-2. There is a statistically significant advantage to duration of graft function in the group receiving HGF compared with the sham and control pump groups ($p=0.05$, Kruskal Wallis test), with graft survival being increased to 59 days in two animals and 80 days in the third.

DISCUSSION

The use of hepatocyte transplantation as a method for the treatment of inborn errors of metabolism has been proposed for more than two decades[27]. Experiments in murine[12], rodent[18], and larger animal models[28] have shown promise, with prolonged function of hepatocyte grafts in some cases. Based on these and other studies, we believed the possibility existed to transplant human hepatocytes in a similar fashion into a murine host. The mouse liver would simulate the normal structural microenvironment of the human hepatocytes, which might prolonging their survival and perhaps allowing them to be used as a model for replication of human hepatitis viruses *in vivo*.

While direct intraportal injection of cells has been used successfully in rats[29], in pilot studies we found the mortality associated with intraportal injection in the murine model to be considerably higher than with intrasplenic injection, as has been noted by others [12]. Use of the spleen for hepatocyte transplantation allows for careful injection of hepatocytes under direct vision and ease of hemostasis after cannula removal. It has been determined that the majority of hepatocytes transplanted into the spleen rapidly translocate to the liver, and a portion enter into venous sinusoids and subsequently engraft within the host liver parenchyma[11; 16]. This fact combined with the considerably reduced mortality associated with intrasplenic injection made the spleen the optimal sit for implantation. Pilot experiments were then performed to determine the optimal number of cells to be used in later studies. Graft size was optimized at $5x10^6$ cells without and $2.5x10^6$ with partial hepatectomy. Using larger graft sizes ($1x10^7$ cells), the majority of animals died during the first post-operative week; post-mortem examination

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of the livers revealed generalized pallor and diffuse punctate hemorrhages, with microscopic evidence of generalized hepatic necrosis, findings also observed by others[30]. After optimization of the transplantation procedures, perioperative mortality was reduced to below 10%.

Once engrafted, human hepatocytes are indistinguishable from host hepatocytes by standard histological techniques. To detect engraftment of human hepatocytes, cells were labeled with CFSE, an intracellular fluorescent dye, prior to implantation. CFSE has been shown to be useful in locating transplanted donor hepatocytes within recipient livers[31]. The dye crosses the cell membrane and is esterified within the cytoplasm, rendering it fluorescent; this chemical conversion prevents the dye from subsequently diffusing back out of the labeled cells into surrounding tissues, even after cell death[31].

Using fluorescence microscopy, labeled cells were demonstrated throughout the majority of histologic sections taken in the first few weeks after transplantation, and in decreasing numbers at up to 12 weeks post-transplant. These cells had the typical histological appearance of hepatocytes, and were not distinguishable from surrounding parenchymal cells by conventional light microscopy. Cells were seen primarily in periportal regions in earlier section, and at later timepoints were also seen more peripherally as described by others[28]. The decrease in fluorescent cell numbers over time likely represented progressive graft loss due to senescence of donor hepatocytes, however it may also have reflected degradation of fluorescence, or even dilution of fluorescence secondary to cell division[31], although the latter possibility is highly unlikely as unstimulated hepatocyte grafts rarely proliferate[16].

Although useful for short-term localization of hepatocytes, there was less confidence in the specificity of the intracellular fluorescent label beyond 4 weeks. Additionally, the use of histologic sectioning to follow graft progression necessitated recipient sacrifice, or at least multiple laparotomies and biopsies, to obtain reliable data. An assay was needed which would allow grafts to be followed serially in individual animals with minimal morbidity or mortality. The production of albumin is an exclusive function of hepatocytes, and albumin molecules are sufficiently structurally distinct between species to be specifically recognized by monoclonal antibodies. By separating human albumin from mouse serum samples using immunoprecipitation and Western

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blotting, a functional assay for the detection of differentiated human hepatocyte graft function was designed.

One of the commonest methods to stimulate early graft proliferation is performance of a 50-70% partial hepatectomy (PH) concurrent with or shortly before the implantation of hepatocytes, exploiting the unique ability of the liver to regenerate after partial resection. Addition of PH to intrasplenic transplantation has been shown in rat models to have a positive proliferative impact on engrafted cells, generally increasing graft cell numbers to 2-3 times that of control animals with no PH[17-19]. It was unclear whether the same effect would be observed in a xenogeneic system wherein significant structural differences might exist between host cytokines and donor cell receptors.

A statistically significant increase in graft survival was produced by the addition of PH, from a median of 3 weeks in controls to a median of 6 weeks in the experimental group. This advantage occurred despite the fact that animals in this group received only one half (2.5×10^6) of the number of human cells transplanted into the non-PH (5×10^6) group (number of cells reduced in the former to maintain a constant ratio of cell number to cross-sectional portal venous area). Within minutes after liver resection, urokinase receptors translocate to the membranes of residual hepatocytes and urokinase activity increases[32]. By 1 hour post-PH, along with other immediate-early genes hepatocyte growth factor (HGF) levels are seen to rise 20-fold[32],and by 8 hours DNA synthesis is initiated as a precursor to active replication of hepatocytes to restore the "preset" liver volume[33]. The finding of an increased median duration of graft function suggests that human hepatocytes are able to respond in part to the regenerative signals elaborated by the residual murine liver after partial hepatectomy. This in turn implies a sufficient degree of structural homology between human and murine cytokines to permit at least partial agonism on human receptors *in vivo*. Despite this improvement however, the median survival of human hepatocytes was still considerably reduced compared to that within the native human environment.

It was noted in histologic sections from earlier timepoints post-transplantation that human cells appeared to co-localize in small aggregates within the surrounding murine parenchyma. This raised the possibilities of recognition of uniquely-human receptors on the cell surface which are recognized by other human hepatocytes, or production of

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human cytokines and/or growth factors which act in a paracrine fashion to encourage aggregation of human cells. From this, we hypothesized that provision of a species-specific growth factor might have a pronounced effect on the survival of hepatocytes that are bathed continuously in a foreign cytokine milieu.

Hepatocyte growth factor (HGF) is a pleiotrophic mammalian growth factor originally isolated in 1984 from both rat platelets and post-hepatectomy serum[34; 35] and has been described as the most potent hepatocyte mitogen[36]. Growing knowledge about the central role of HGF in regeneration has lead to attempts to directly influence hepatocellular function by administration of HGF. Through intravenous administration into the peripheral venous system, HGF has been shown to influence hepatocyte graft function in a rat model[37], and enhance hepatocyte proliferation after partial hepatectomy in mice[38]. Based on these factors, we selected recombinant human HGF as the growth factor most likely to have a positive impact on human graft survival.

Intraportal infusion of recombinant human HGF was associated with an increase in graft survival over both pump controls and sham controls, increasing median graft survival to 59 days (8.4 weeks). In comparison, implantation of control pumps was associated with a decrease in median graft function, although the small numbers in each group mandates caution in overinterpreting smaller inter-group differences. The improvement in graft survival was greater than that observed with performance of a 50% PH (Figure 3-3), after which human hepatocytes are exposed to a cascade of host-derived regenerative signals, including murine HGF. This data suggests that human hepatocyte grafts within a xenogeneic host can have a pronounced response to supplementing the microenvironment with human growth factors, superior to the response to an increase in host-derived murine growth factors.

A study by Gupta *et al.* examined the effects of administering human HGF on the rate of DNA synthesis and cell proliferation in congeneic rat and mouse models[37]. They found an increase in DNA synthesis similar in magnitude to that accompanying performance of a 35% PH, but no overall increase in the number of transplanted cells. The implication was that HGF administration upregulated hepatocyte synthetic function, but did not stimulate proliferation. It is important to remember however that this study and others examining the effects of HGF on hepatocyte proliferation[38; 39] are using human

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HGF to stimulate proliferation of murine or rodent hepatocytes. This situation is distinctly different from the intent of the present study, which was to address the effects of human growth factors on human cell grafts. Human HGF has been shown *in vitro* to be stimulatory to murine *c*-met receptors, but not *vice versa*, i.e. murine HGF does not appear to act strongly on human *c*-met receptors[40]; further, hepatocyte growth factor shares only 90% protein sequence homology between human and murine forms[41]. As such, although human HGF will stimulate murine hepatocyte, it is likely to have a more profound effect on its native target, the human hepatocyte *c*-met receptor. HGF was administered in the present experiment at a dose of 1μg/kg/day for a total of 14-16 days, a regimen longer in duration but considerably reduced in overall dose than the study by Gupta *et al* (2.4 mg/kg/day x 7 days). The improvement in graft function seen after HGF administration at a significantly lower dose supports the assertion that the effect of human growth factor on a human receptor is considerably stronger than on a murine receptor.

Despite the improvement in graft survival, however, the overall duration still remains short of the normal lifespan of a human hepatocyte within its native environment. Furthermore, the progressive loss of graft function with time suggests transplanted cells are not sustaining replication leading to permanent engraftment, which would be most desirable in a murine model for long-term viral studies. Although murine models of liver proliferation such as the Alb-uPA[20; 42] or FAH-deficient mouse[22] appear to have the capacity to stimulate initial proliferation of transplanted hepatocytes, this proliferative signal lasts for only 6-8 weeks; a more sustained human-specific signal may be required to maintain human grafts on an extended or permanent basis. The high mortality and additional operative time associated with permanent indwelling cannulation of the portal system makes the use of implantable microosmotic pumps impractical. Co-transplantation of an HGF-secreting cell line[43], or development of a murine strain transgenic for human HGF might serve to confer the benefits of a species-specific growth factor without the additional mortality.

In summary, we have demonstrated that human hepatocytes can translocate to and successfully engraft within murine liver parenchyma. Stimulation of graft proliferation with a cascade of host-derived cytokines, effected through performance of a 50% partial

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hepatectomy performed at the time of transplantation, appeared to double the median length of graft function. However, the addition of a single species-specific growth factor had a pronounced effect of graft survival superior to that of 50% PH. These findings suggest that species-specific growth factors and cytokines within the hepatocyte microenvironment may have an important trophic effect on overall human graft survival, and that supplementation with human-derived growth factors may play an important role in achieving long-term or permanent engraftment of human tissues within murine hosts. This may have implications for any situation where xenogeneic tissue transplants are considered for disease therapy.

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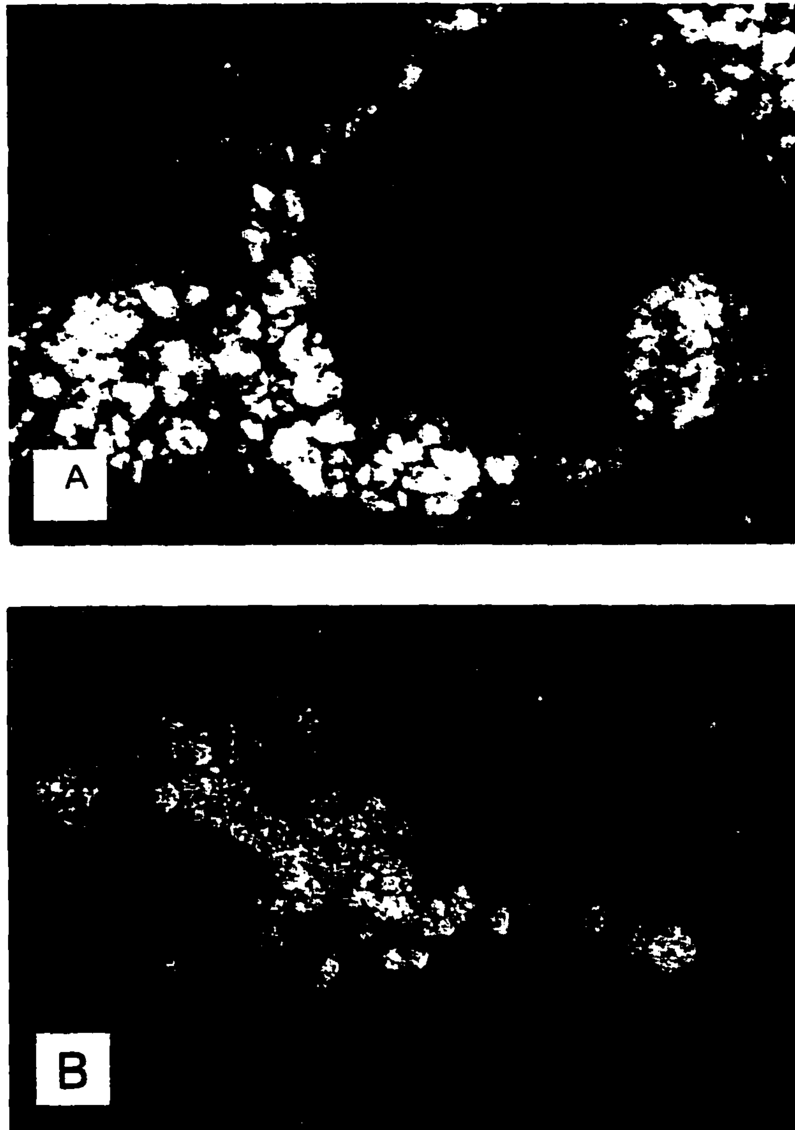

Figure 3-1. Fluorescence microscopy of CFSE-labeled human hepatocytes at (A) one week post-transplantation and (B) four weeks post-transplantation.

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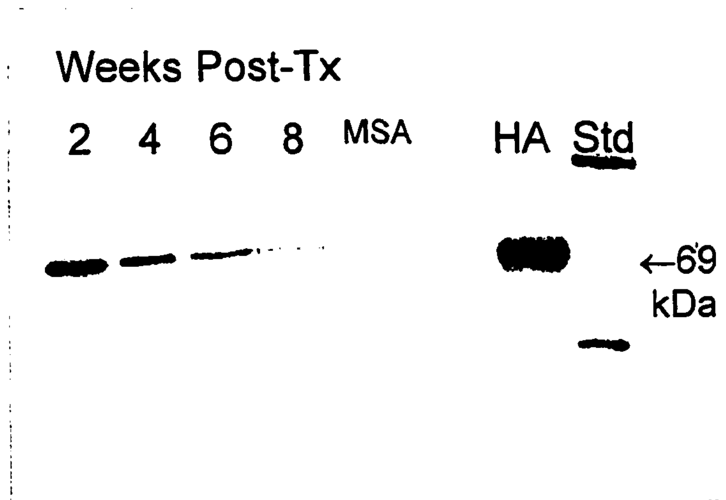

Figure 3-2. Detection of human albumin production post-transplantation by immunoprecipitation and Western blotting. HA – purified human albumin (positive control); MSA – mouse serum albumin (negative control); Std – molecular weight standards.

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Group	Duration of Graft Function (weeks)	Mean Rank	Sum of Ranks
Control (5 x 10^6 cells, no PH)	2,2,2,3,3,4,8	4.93	34.50*
Experimental (2.5 x 10^6 cells + 50% PH)	4,4,6,6,6,8,10	10.07	70.50*

Table 3-1. Effect of 50% partial hepatectomy (PH) on duration of human hepatocyte graft function. *Groups are significantly different by nonparametric statistical analysis ($p=0.019$, Mann-Whitney U test).

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Group	Duration of Graft Function (days)	Mean Rank
Sham + 5 x 10^6 cells	14, 28, 35, 71	5.75
Pump (control) + 5 x 10^6 cells	7, 12, 18	2.33
Pump (HGF) + 5 x 10^6 cells	59, 59, 80	8.33*

Table 3-2. Effect of human HGF on duration of human hepatocyte graft function. * p=0.05 by Kruskal Wallis test

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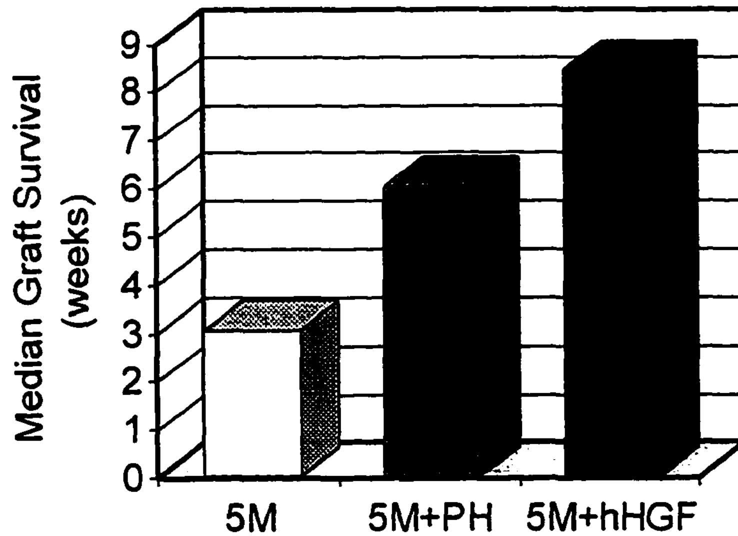

Figure 3-3. Effect of murine- and human-derived proliferative signals on graft survival. 5M – 5 x 10^6 human cells alone; 5M+PH – 5 x 10^6 human cells plus 50% partial hepatectomy; 5M+hHGF – 5 x 10^6 human cells plus human hepatocyte growth factor.

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CHAPTER 4

HUMAN HEPATOCYTES XENOGRAFTED INTO C.B17-*scid-bg* MICE FAIL TO SUPPORT PRODUCTIVE INFECTION WITH HUMAN HEPATITIS B VIRUS

INTRODUCTION

The development of therapies for viral hepatitis in humans is hampered by difficulties in establishing a laboratory model of functioning human hepatocytes. Studies of hepatitis B virus (HBV) are limited primarily to the use of animal viruses analogous to those in humans, such as hepatitis B viruses in the California ground squirrel[1] or Peking duck[2]. While much of the information gathered from these systems may be generalizable to human disease, there are some differences in pathophysiology which may only be resolved by direct experimentation on human viruses. The development of a long term support system for human hepatocytes would facilitate exploration of antiviral therapies by allowing mass screening of potentially efficacious compounds directly against human viral pathogens.

Hepatocellular transplantation was initially proposed as a therapy for enzyme deficiencies in 1976 by Matas et al[3], and its utility has been investigated in rodent models[4], larger mammals[5], and humans[6; 7]. Hepatocyte grafts can be maintained within an allogeneic or xenogeneic environment for prolonged periods of time when the host rejection response is abrogated through immunosuppression [8-10] or immunodeficiency[11]. Transplantation of human liver tissue into an immunodeficient murine host would permit the study of human hepatocytes in a microenvironment closely simulating their own. Theoretically, recipient mice could then be infected with human viral pathogens, thereby permitting the study of viral replication and antiviral therapies in a small animal system.

This paper documents the establishment of human hepatocyte grafts in a series of immunodeficient cb17-SCID-beige mice, followed by inoculation with HBV-positive human serum. Evidence of active infection and viral replication was not seen, despite sustained differentiated graft function. This failure may have been due to a lack of graft proliferation after transplantation with progressive loss of graft cell mass. Unless additional methods to stimulate hepatocyte proliferation are employed to enhance total

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graft size, it is unlikely that this SCID model will be adequate for supporting human viral hepatitis viruses *in vivo*

METHODS

Animal Care and Ethics

25-30 g male cb17/SCID-bg mice (Taconic Farms, Germantown, NY) were used as recipients. All animals were kept in virus/antigen free conditions, and were stored and manipulated in accordance with guidelines established by the Canadian Council on Animal Care (1993). Ethical approval for use of human tissue was obtained from the University of Alberta Faculty of Medicine Research Ethics Board, and approval for animal experimentation was obtained from the University of Alberta Animal Welfare Committee.

Human Hepatocyte Isolation

Human tissue was obtained at laparotomy by wedge biopsy of a segment of normal tissue which would otherwise have been discarded after hepatic resection. The detailed procedure for isolation of human hepatocytes is outlined in Chapter 2. Basically, a two-step enzymatic perfusion was performed in a modification of the procedure established by Seglen[12], using a purified enzyme preparation (Liberase RH; Roche-Boehringer Mannheim, Indianapolis, IN) for tissue dissociation After enriching viability of the crude hepatocyte suspension by density gradient centrifugation over Percoll (density 1.04 g/mL; Sigma, St. Louis, MO) at 400 g for 4 min, the cell pellet was washed twice in ice-cold Hank's Balanced Salt Solution (HBSS, ICN Biomedicals, Aurora, OH) and then gently suspended in 0°C Belzer University of Wisconsin Cold Storage Solution (UW-CSS; DuPont Pharmaceuticals, Scarborough, Ontario) for storage prior to us. Duplicate cell counts were immediately performed, using trypan blue exclusion to assay viability of final cell preparations. All cells were used for transplantation within 4 h of final suspension in UW-CSS.

Partial Hepatectomy(PH) and Transplantation of Hepatocytes

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In a sterile operating hood, recipient mice were anaesthetized with 1-2% Halothane/O_2, and a 1-1.5 cm midline laparotomy incision was made. The left lobe of the liver was rotated medially into the incision, a 2-0 silk ligature placed around its base, and it was carefully excised. After ensuring hemostasis, the liver was returned to its native position. Aliquots of 100 µl (5.0×10^6 viable cells) of final cell suspension were washed with cold 0.9% saline solution immediately before transplantation, and drawn up into a 27-gauge butterfly injector (Abbott, Sligo, R. Ireland) attached to a 1-mL syringe. The inferior pole of the spleen was grasped lightly between curved forceps and the needle tip was introduced 3-4 mm into the splenic pulp. The cell plug was slowly injected into the inferior pole over a one-minute period, the needle was removed and a single titanium clip was placed over the injection site to ensure hemostasis. After confirming hemostasis, the spleen was returned to its native position and abdomen was closed in two layers. Transplants were performed in two groups of 5 mice each (Groups I and II) separated by a one week interval.

Inoculation with HBV-Positive Serum

Serum obtained from a patient with high-titer chronic active HBV infection was stored on ice and inoculated into mice within 2 hours of procurement. Group I received 0.25 ml of undiluted serum intravenously at 10 days after transplantation via the internal jugular vein; Group II received 0.25 ml undiluted serum in similar fashion at 3 days after transplantation. Animals were then followed with serum sampling by jugular venous puncture at weekly intervals to termination of the experiment at 80 days.

Detection of Graft Function

Aliquots of mouse serum (20 µl) were incubated with an anti-human albumin monoclonal antibody (Clone HSA-9; Sigma), and antigen-antibody complexes were precipitated with protein G-agarose (Boehringer-Mannheim). Immunoprecipitates were then heated for 5 minutes at 98°C in SDS buffer containing 0.2 M dithiothreitol, separated on a 7.5%SDS-polyacrylamide gel by electrophoresis and transferred to nitrocellulose. Western blots were prepared in standard fashion[13] using a second anti-human albumin monoclonal antibody (Clone HSA-11; Sigma) conjugated to biotin as the

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primary. A streptavidin-horseradish peroxidase conjugate (Pierce, Rockford, IL) was employed as the secondary, and photodetection of immune complexes was accomplished by addition of enhanced chemiluminescence reagents (Pierce).

Detection of HBV Infection

Aliquots of serum (20 μl) taken at selected timepoints were tested for presence of HBsAg using a sandwich ELISA kit (Heprofile HBsAg; ADI Diagnostics) with plate analysis performed using a Dynatech MRX microplate spectrophotometer (Dynex; Middlesex, England). Essentially this is a sandwich ELISA system using a monoclonal anti-HBsAg antibody for capture and a polyclonal chimpanzee anti-HBsAg:peroxidase conjugate as a reading antibody. Both positive and negative human serum controls, as well as negative murine serum controls were included in all assays. Detection of HBV DNA in serum was performed by polymerase chain reactions (PCR) on DNA isolated from 12.5 μl of mouse serum, using primers specific to highly conserved regions of the HBV genome (5'-AGACCACCAAATGCCCCTAT-3' and 5'-GGCGTTCACGGTGGTCTCC-3'), with DNA isolation and PCR amplification conditions as previously described[14]. All analyses were performed in blinded fashion, and included negative and positive controls.

Statistical Analysis

Mean graft survival and mean absorbances at each timepoint were compared between groups using a two-tailed Student's t-test, with a significance level of $p<0.05$.

RESULTS

Isolation and Transplantation of Hepatocytes

Hepatocytes used in transplantations were isolated on two separate occasions from different donors. The first tissue donor (transplanted into Group I) was a 61-year old male with metastases to the liver from a colonic adenocarcinoma, and normal underlying liver function. Final cell suspension viability was 93.9%, and a total of 3.93×10^8 viable hepatocytes were isolated from the liver segment. The second tissue donor (transplanted into group II) was a 43-year old female with a benign giant hemangioma,

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again with normal underlying liver function. The final cell suspension viability was 88.8%, but because the donor segment was considerably smaller than normal and the initial dispersion was poor, viable cell yield was reduced to 3.8×10^7. In each case, after performance of a 50% PH, 5×10^6 viable human hepatocytes were implanted into the inferior poles of the spleens of 5 recipient animals. No perioperative mortality occurred, and all animals recovered uneventfully from surgery and appeared normal throughout the experiment.

Human Hepatocyte Graft Survival

Differentiated function of hepatocyte grafts was assayed by detection of human albumin in mouse serum using immunoprecipitation and Western blotting. Grafts were considered to have failed when the human albumin signal from a sample was equivalent to the background signal of blank mouse serum, and the total length of graft function was measured at the time of last positive signal. In group I graft survival was 7.4 ± 0.4 weeks (mean ± SEM) whereas in group II graft survival was 5.0 ± 0.8 weeks; this difference was statistically significant ($p=0.038$, Student's t-test). Stronger signals were seen initially in all cases, but progressively weakened over time; representative Western blots are shown in Figure 4-1.

Detection of Hepatitis B Infection

As a serum marker of infection, weekly serum samples were assayed for HBsAg by sandwich ELISA. Result of analysis are represented graphically in Figure 4-2. After an initial peak in HBsAg level at 4 days post-inoculation, sample absorbances returned to near background levels in both groups. In Group I, absorbances remained at the level of background throughout the remainder of the time course. Mean Group II readings were higher than Group I at all timepoints, reaching statistical significance at 33, 40 and 54 days after inoculation ($p<0.05$, Student's t-test). The Group II absorbance means were persistently close to the upper limit of the 95% confidence interval around the mean, crossing marginally beyond at 33 and 40 days. Positive control human serum run at the same time had a mean absorbance of 0.789 (n=2). Samples drawn at 4, 11, and 17 days

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post-inoculation were tested for HBV DNA by PCR; all samples were negative for HBV DNA. Donor serum tested concurrently was strongly positive for HBV DNA.

DISCUSSION

While there are some natural animal hosts for hepadnaviruses analogous to human HBV, such as the Pekin duck[2] and the California ground squirrel[1], which are adaptable to use in most laboratory settings, the same cannot be said for the hepatitis C virus. The chimpanzee, while a natural host for hepatitis C[15], is impractical for use by most investigators due to relative scarcity, difficulty in breeding, and exorbitant costs associated with acquisition and maintenance; additionally, ethical concerns about testing on animal species phylogenetically closely related to humans further limits their experimental utility. A murine model of hepatitis C infection would be ideal to allow investigators to explore the basic biology of HCV, and screen promising candidate antiviral compounds for efficacy against HCV *in vivo*. We proposed to circumvent the inherent resistance of murine liver to HCV infection by establishing a graft of human liver tissue within an immunodeficient murine host, and then attempting to infect the human cells *in vivo* with the natural human virus. In this manner the human cells are placed in a structural microenvironment which mimics their own natural environment, a feature which has been shown to be important in enhancing the longevity of *in vitro* cultures[16; 17].

To avoid a xenogeneic rejection response to the human hepatocyte grafts, cells were transplanted into cb17-SCID-beige recipients. The *scid* mutation, originally identified by Bosma *et al.*[18], is a deficiency in a DNA-dependent protein kinase catalytic subunit which prevents VDJ recombination in immunoglobulin and T-cell receptor genes[19], rendering animals homozygous for the defect deficient in cells from both B- and T-cell lineages; the addition of the *beige* mutation makes animals additionally natural killer (NK) cell deficient[20]. Animals carrying these mutations have been shown to be receptive to other forms of human tissue graft[21; 22], with little or no evidence of rejection.

To establish the potential utility of the SCID-beige system as a model for supporting replication of hepatotrophic human viruses, infection with human HBV was attempted. HBV is a DNA virus of the *Hepadnaviridiae* family, which causes both

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asymptomatic and symptomatic forms of hepatitis in human recipients failing to mount an adequate antiviral immune response[23]. Although the ultimate intent of experimentation was to establish a model useful for supporting all human hepatitis viruses and especially the hepatitis C virus, HBV was chosen as a cost-effective starting point, realizing the major differences that exist between the two viral species. In the clinical setting, HBV is considered more infectious than HCV, as evidenced by the differences in acquisition rates in hospital personnel after needlestick injuries: HBV infections occur in 2-40% of HBV-contaminated needlestick events, while HCV infections occur in only 3-10% of HCV-contaminated needlestick events. These observations suggest that HCV is about three to four times less infectious than HBV[24]. Additionally, HBV replicates at a higher rate than HCV with levels ranging from 10^5 to 10^9 infectious particles per mL in chronic HBV infections[25] compared to 10^2 to 10^8 particles per mL in HCV infections[26]. Both of these features support the notion that infection of human hepatocyte grafts with HBV should be easier than with HCV. While the positive result of establishing an active HBV infection would not necessarily imply the same success with HCV, a negative result would make the possibility of establishing a productive HCV infection considerably more remote.

Hepatocyte grafts were successfully established in all 10 experimental animals, concurrent with the performance of a 50% partial hepatectomy (PH). Performance of a PH at the time of hepatocyte transplantation has been shown by others in congeneic and allogeneic rodent systems [4; 9], and by our own laboratory in the xenogeneic human-to-mouse system employed herein (see Chapter 3) to have a positive influence on graft survival. Although a difference in overall graft survival as measured by duration of graft albumin production was seen between the two groups, the overall mean graft survival (6.2 ± 0.6 weeks, mean ± SEM) was statistically indistinguishable from previous data (see Chapter 3). The additional advantage in graft survival of Group I over Group II may have been related to a more difficult hepatocyte isolation in the latter case (a tenfold-reduction of viable cell yield in Group II compared with Group I) which may have produced sublethal damage to the donor cells not detected by the trypan blue assay.

Hepatitis B surface antigen (HBsAg) is the earliest identifiable marker of serum infection, but may not necessarily be conclusive evidence of active viral replication[27].

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The initial peak in HBsAg levels observed at four days after inoculation almost certainly reflects the load of viral protein present in the initial human serum inoculum. After this initial period, HBsAg signal declined to a level compatible with background signals in mouse serum. Although detectable HBsAg levels were higher in Group I throughout the experiment, and in some cases were marginally above the 95% confidence interval around the background mean, it is unlikely they represent real evidence of infection. The higher mean absorbances noted in Group II are noted throughout the experiment up to completion at 10.7 weeks, despite the fact that the overall mean duration of graft survival was only 5.0 ± 0.8 weeks. As murine cells are naturally resistant to infection by HBV, there could be no translation of the viral genome after the human grafts are extinguished. The possibility that residual human hepatocyte graft might exist at levels below the limits of detection of the human albumin assay, which in turn might support limited viral replication, would favour persistence of viremia in Group I with a longer mean graft survival. In this group however, HBsAg levels were consistently lower than in Group II, making this possibility unlikely. Analysis of HBV DNA production in serum samples was negative at the timepoints tested, suggesting that active viral replication was not occurring. Although it is conceded that the latency period of viral infection may certainly be beyond this initial period, it is highly unlikely in the face of declining graft function and persistently undetectable HBsAg levels that further investigation would prove fruitful.

The results of this experiment lead to the overall conclusion that transplantation of human hepatocytes into a SCID-beige host is unlikely in its present form to represent a viable model for supporting replication of human hepatitis viruses. Even with the survival advantage accrued to the human grafts by performing partial hepatectomy, the overall graft size at its maximum is insufficient to support replication at a detectable level, if at all. After the initial period of regenerative signaling post-PH, graft function declines in a linear fashion to extinction at around 6 weeks. During this period of time, any virus taken up by human cells after the initial inoculum would be in a period of latency, as the virus attempts to replicate and thereby increase its copy number within host cells; at the same time host hepatocytes are declining in number in steady fashion.

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These two features are at cross-purposes, and ultimately render this system inadequate for viral support.

Despite the negative results produced in this experiment, the theoretical possibility of infecting established human hepatocyte graft remains intact. It appears, however, that for any such system to have practical utility a considerable expansion of the overall graft cell mass will be required. There are two major models of hepatocyte regeneration which might prove useful in this regard: the Alb-uPA mouse[28] and the FAH-deficient mouse[29]. In each case, the individual genetic manipulation results in an accumulation of toxic substances within individual hepatocytes which in turn causes a persistent stimulus to liver regeneration; cells free of the genetic defect are capable of a vigorous response to these persistent signals. In this fashion, transplanted cells can be expanded through multiple cycles of cell division to occupy a significant proportion of the total hepatocyte mass[30]. An alternate approach to graft expansion might be through the administration of pyrrolidizine alkaloids, substances commonly found in plant such as hogwort and ragwort, and known to cause liver disease in cattle and sheep. The substances are taken up and rapidly metabolized within host hepatocytes to a toxic derivative which effectively blocks the hepatocyte cell cycle at the late synthetic or early gap2 phase; this metabolic block prevent cells from undergoing normal mitosis for a period of 1-2 months. When administration of retrorsine, a pyrrolidizine alkaloid, was combined with transplantation of normal congeneic hepatocytes and concurrent partial hepatectomy to initiate a regenerative stimulus, extensive proliferation of donor hepatocytes was seen[31]. Although as yet tested only in a congeneic rat system, this approach might be adaptable for use in the human to SCID model used herein.

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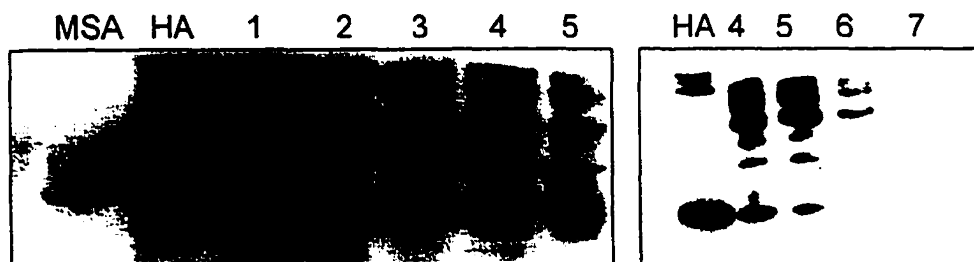

Figure 4-1. Representative Western blots showing detection of human albumin in the serum from an animal transplanted with human hepatocytes. Numbers represent weeks post-transplantation; MSA – mouse serum albumin (negative control); HA – human albumin (positive control).

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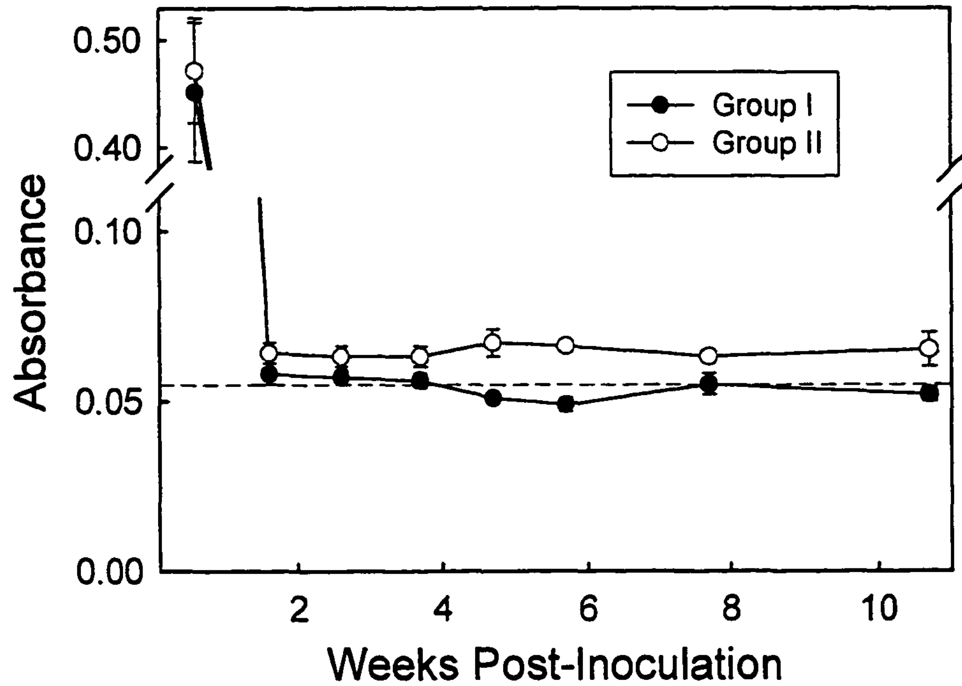

Figure 4-2. HBsAg in serum samples taken from transplanted mice after inoculation with HBV-positive human serum. Dashed line represents the mean background absorbance of blank samples; dotted lines represent the associated 95% confidence interval. * - groups different at $p<0.05$.

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CHAPTER 5

REPLICATION OF HEPATITIS C VIRUS IN MICE WITH CHIMERIC HUMAN LIVERS

INTRODUCTION

Human liver disease caused by the hepatitis C virus (HCV) has emerged over the past decade as one of the most difficult challenges facing the worldwide medical community. The elucidation of the viral sequence in 1989[1] established the beginning of an era of concerted study of HCV. Improved methods of viral detection have led to a fuller appreciation of the enormous magnitude of the problem: currently it is estimated that over 1% of the world population is infected with the virus[2]. At present there is neither an effective antiviral agent nor a vaccination against HCV. Progress in developing therapeutic strategies has been significantly hampered by difficulties in establishing *in vitro* and *in vivo* models of viral replication.

Currently the only accepted animal model of HCV infection is the chimpanzee, *pan troglodytes*[3-5]; replication of the virus in other higher primates is either nonexistent[6; 7], or at best erratic[8; 9]. The chimpanzee model is impractical for use by many investigators, due to inadequate animal facilities and high maintenance costs. Attempts to find a natural small animal host for HCV have met with very limited success; only the tree shrew *tupaia belangeri chinensis* has shown any promise in hosting the virus[10], and even this is dubious as a practical laboratory model. The development of a small animal model of HCV infection is critically important to the progress of investigators in establishing even the basic biology of the virus, notwithstanding attempts at developing antiviral therapies.

Theoretically, if human liver tissue could be transplanted into an appropriate murine host it might provide a suitable environment for supporting replication of hepatotrophic viruses such as HCV. We have shown that human hepatocytes can be isolated and purified from surgical liver biopsies (see Chapter 2), and can be successfully transplanted into immunodeficient murine hosts (see Chapter 3); without significant increases in the extent of engraftment, however, it is unlikely that human hepatocyte transplantation alone will provide a suitably large graft cell mass to sustain a meaningful

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viral infection (see Chapter 4). The inability of the SCID-beige mouse to support hepatrophic viral replication prompted an examination for an alternate model which might prove more suitable to our purposes.

The Alb-uPA transgenic mouse was developed in 1990 as a model for the study of neonatal bleeding disorders[11]. By placing a tandem array of murine urokinase genes under the control of an albumin promoter, high level urokinase production was targeted to the liver, producing a profoundly hypofibrinogenemic state. Subsequent experimentation with this model demonstrated that after spontaneous somatic deletion of the transgene, individual hepatocytes within the transgenic liver acquired a significant survival and replicative advantage over surrounding cells, and repopulated the liver with largely nontransgenic cells[12], an advantage which could be extended to transplanted hepatocytes derived from nontransgenic mouse and rat donors[13; 14]. Our intention in this series of experiments was to exploit this proliferative stimulus to generate a series of mice with chimeric human/mouse livers, and then utilize these chimeric organs to produce much-needed *in vivo* models of human hepatotrophic viral replication.

In this series of experiments, we have developed an immunodeficient SCID-beige mouse carrying the Alb-uPA transgene (SCID-uPA) which is capable of stimulating rapid expansion of human hepatocytes transplanted into its liver. These grafts can be reproducibly initiated in all animals carrying the transgene, and a subset of animals go on to develop sustained human chimerism. Animals developing sustained human chimerism in turn have been shown to be capable of supporting viral replication in hepatocytes taken from virally-infected human donors. Finally, we have demonstrated that mice carrying noninfected human grafts can be infected with hepatitis C virus taken from an unrelated donor, and can go on to develop chronic persistent infections with levels of viral replication identical to those of infected humans. This represents the first successful small animal model of hepatitis C developed, and should provide the much needed platform for development of antiviral strategies.

METHODS

Animal Care and Ethics

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All animals were kept in virus/antigen free conditions, and were stored and manipulated in accordance with guidelines established by the Canadian Council on Animal Care (1993). Ethical approval for use of human tissue was obtained from the University of Alberta Faculty of Medicine Research Ethics Board, and approval for animal experimentation was obtained from the University of Alberta Animal Welfare Committee.

Development of the SCID/-uPA Strain

Mice heterozygous for the Alb-uPA transgene (strain TgN(*Alb1Plau*)144Bri; The Jackson Laboratory, Bar Harbor, ME) were crossed with immunodeficient mice homozygous for the *scid* and *beige* traits (strain C.B-17/GbmsTac-*scid-bg*N7; Taconic Farms, Germantown, NY). The progeny from this cross were screened for presence of the Alb-uPA transgene (henceforth, "the transgene") by PCR analysis of genomic DNA extracted from tail biopsies, using two 18-mer primers (5'-CAT CCC TGT GAC CCC TCC-3' and 5'-CTC CAA ACC ACC CCC CTC-3') which amplify a 151 bp product from the 3' UTR of the transgene construct (Jackson Laboratories technical support); the PCR reaction conditions were as follows: in a final volume of 25µl of 10X PCR buffer containing 1.5mM $MgCl_2$ and 0.5 U AmpliTaq polymerase (Perkin-Elmer-Cetus; Foster City, CA), 50ng of genomic DNA was amplified with 1mM of each primer; after denaturing at 94°C for 2 min, samples were subjected to 30 cycles of 30 s at 94°C, 60 s at 60°C, and 90 s at 75°C, followed by a final extension at 75°C for 5 min. Samples were stored at 4°C, and separated by electrophoresis through 2%, 1x TBE agarose gels. Animals not carrying the transgene were euthanized. Animals carrying the transgene were then backcrossed against the parental (C.b-17 SCID/bg) strain, and the progeny of this second cross were screened for homozygosity of the SCID trait. The homozygous SCID trait was identified phenotypically by quantification of total serum IgG using a sandwich ELISA technique. Sheep anti-mouse IgG (ICN Biomedicals, Aurora OH) was used as the primary capturing antibody, with a goat anti-mouse IgG conjugated to horseradish peroxidase (ICN Biomedicals) as the secondary antibody. Signal was developed using o-phenylenediamine dihydrochloride (OPD) chromagen solution (Sigma, St. Louis, MO) and absorbance was read using a Dynatech MRX microplate

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spectrophotometer (Dynex; Middlesex, England). Quantification of IgG was calculated from a standard curve prepared on each plate using a mouse IgG standard (ICN Biomedicals). "Leakiness" of the SCID-beige trait was defined as >1% of normal serum IgG[15]– animals with serum IgG levels above this cutoff were euthanized. All immunoglobulin-deficient offspring were then screened for presence of the transgene, as previously described. Animals selected as founders for the new colony were homozygous for the SCID trait, and heterozygous for the Alb-uPA transgene. All littermates from a cross of two founder parents were used in subsequent transplantation experiments, thus blinding the investigators to recipient genotype and providing wild-type controls within each litter.

Isolation of human hepatocytes

A detailed description of isolation methods is presented in Chapter 1. Essentially, segments of human liver tissue (15-20 cm^3) obtained at laparotomy were perfused with ice-cold Ca/Mg-free PBS (ICN Biomedicals, Aurora, OH) containing 0.5 mM Na_2EDTA, prominent perfusing vessels were cannulated and the tissue was perfused for 30 minutes with recirculating carrier solution (35 mM NaCl, 3.5 mM KCl, 2.5 mM $CaCl_2$, 50mM HEPES, pH 7.6) containing 0.38 mg/mL Liberase CI collagenase (Roche-Boehringer Mannheim, Indianapolis, IN)[16; 17]. Dispersed hepatocytes were filtered through 100 µm stainless steel mesh, purified by density-gradient centrifugation (Percoll, density 1.04 g/mL; Sigma) at 400 *g* for 5 minutes, and washed twice in ice-cold HBSS prior to suspension in Belzer-University of Wisconsin solution (DuPont Pharmaceuticals, Scarborough, Ontario) at 0°C for short-term storage prior to transplantation. Cell counts and viability were confirmed by trypan blue exclusion prior to transplantation; final viability was routinely >80%.

Transplantation of human hepatocytes

Unselected offspring (5-14 days old) were anesthetized with Halothane/O_2 (4% induction, 1-2% maintenance), and a small left flank incision was made. Under operating magnification, hepatocytes were injected into the inferior splenic pole with a 27g butterfly injection set (Abbott; Sligo, R. Ireland), and a single sterile titanium clip was

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placed across the injection site for hemostasis. Initially 3×10^5 viable human hepatocytes were transplanted per animal, but this number was increased to 1×10^6 viable cells after the first litter of transplants. After ensuring hemostasis, the spleen was returned to the abdomen, and the flank incision was closed in two layers. Animals were returned to their parents for further care, and were weaned in standard fashion at 21 days.

Inoculation with HCV-infected human serum

Serum was obtained from a patient with high-titer chronic active HCV infection unresponsive to interferon-α therapy, and was stored on ice prior to inoculation into mice, which occurred within 2 hours of procurement. Mice at six weeks post-transplantation were inoculated with 0.25 ml of undiluted serum intravenously via the internal jugular vein. Animals were then followed in standard fashion with serum sampling by jugular venous puncture at weekly intervals. The human serum donor was confirmed positive for HCV RNA, with viral titers of approximately 1×10^7 copies per ml serum. Each animal thus received approximately 2.5×10^6 viral copies per inoculum.

Monitoring of graft recipients

Beginning at four weeks after transplantation each animal had serum samples (200-250μl) taken by repeated jugular venous puncture at weekly intervals, all of which were stored at -70°C. Tail biopsies were taken for analysis of genomic DNA and stored at -70°C. In earlier experiments, randomly selected animals were euthanized, and livers were immediately harvested. Multiple biopsies were taken from each lobe, placed in OCT compound and snap-frozen in liquid nitrogen. The remaining liver tissue was placed in formalin. In later experiments, harvesting and biopsy of livers occurred only at termination of experiments, or in the event of unexpected death, shortly after the death of the animal.

Detection of human albumin in serum samples

Aliquots of mouse serum (20 μl) were incubated with an anti-human albumin monoclonal antibody (Clone HSA-9; Sigma), and antigen-antibody complexes were

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precipitated with protein G-agarose (Boehringer-Mannheim). Immunoprecipitates were then heated for 5 minutes at 98°C in SDS buffer containing 0.2 M dithiothreitol, separated on a 7.5%SDS-polyacrylamide gel by electrophoresis and transferred to nitrocellulose. Western blots were prepared in standard fashion[18] using a second anti-human albumin monoclonal antibody (Clone HSA-11; Sigma) conjugated to biotin as the primary. A streptavidin-horseradish peroxidase conjugate (Pierce, Rockford, IL) was employed as the secondary, and photodetection of immune complexes was accomplished by addition of enhanced chemiluminescence reagents (Pierce).

Autoradiographs were scanned with a Umax Astra 1200S scanner and VistaScan DA v. 1.2.2 imaging software (UMAX Corp.; Fremont, CA). Quantification of HA peaks was performed using NIH Image 1.60/fat software (National Institute of Health; Bethesda, MD)

Immunohistochemical detection of human MHC Class I

Cryosections of recipient livers were fixed in acetone and embedded in paraffin for use in immunohistochemical studies. Immunohistochemistry was performed in standard fashion, as previously described[19]. A murine monoclonal antibody specific for human MHC Class I was expressed and purified from the BB7.7 cell line (ATCC, Manassas, VA), conjugated to biotin using a standard protocol and used as the primary antibody. An avidin/peroxidase complex (Vector; Burlingame, CA) was used as a secondary, with peroxide/diaminobenzidine employed as the chromagen. This monoclonal antibody was tested against sections of human and non-transplanted mouse liver to determine the specificity for human MHC Class I, and degree of cross-reactivity, if any, with murine MHC Class I.

Detection of hepatitis B infection

Aliquots of serum (20 μl) taken at selected timepoints were screened for presence of HBsAg using a sandwich ELISA kit (Heprofile HBsAg; ADI Diagnostics) with plate analysis performed using a Dynatech MRX microplate spectrophotometer (Dynex; Middlesex, England). Essentially this is a sandwich ELISA system using a monoclonal anti-HBsAg antibody for capture and a polyclonal chimpanzee anti-HBsAg:peroxidase

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conjugate as a reading antibody. Both positive and negative human serum controls, as well as negative murine serum controls were included in all assays. Detection of HBV DNA in serum was performed by polymerase chain reactions (PCR) on DNA isolated from 12.5 µl of mouse serum, using primers specific to highly conserved regions of the HBV genome (5'-AGACCACCAAATGCCCCTAT-3' and 5'-GGCGTTCACGGTGGTCTCC-3'), with DNA isolation and PCR amplification conditions as previously described[20]. All analyses were performed in blinded fashion, and included negative and positive controls.

Detection of hepatitis C infection

Qualitative analysis for HCV RNA in serum was performed on samples of mouse serum (generally 80-140µl) diluted to 220µl total volume by human serum confirmed negative for HCV RNA. Analysis was performed by reverse transcriptase PCR (RT-PCR) using the Cobas Amplicor system (Roche Diagnostics, Indianapolis, IN), according to manufacturers instructions. Selected serum samples were analyzed for quantitation of HCV RNA levels within serum, using the Cobas Amplicor HCV Monitor system (Roche Diagnostics), again according to manufacturers instructions. The qualitative RT PCR analysis has a detection limit of approximately 10^1 viral copies per ml, while the quantitative analysis has a detection limit around 10^3 copies per ml.

RESULTS

Hepatocyte Isolations

A total of 5 human hepatocyte isolations (2 female, 3 male) were performed for use in transplantations. Mean donor age was 49.6 ± 6.4 years (mean ± SEM), and average warm ischemic and total isolation times were 6.6 ± 1.2 minutes and 141 ± 5 minutes, respectively. Final hepatocyte preparations were 82.0 ± 2.5% viable, with a mean of $1.52 \pm 0.58 \times 10^8$ viable cells per isolation. All transplantations were completed within 4 hours of hepatocyte isolation.

Establishment of Human Hepatocyte Engraftment

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To establish the capacity of human hepatocytes to migrate to the liver via the portal venous system and engraft within the transgenic liver parenchyma, 2 sets of offspring were transplanted with human cells. The first group of 8 animals, pooled from two separate litters, received 3×10^5 viable hepatocytes at 5 days of age and all survived the perioperative period without incident. The second group of 7 animals taken from a single litter received 1×10^6 viable hepatocytes at 11 days of age: although the transplantation procedure was successful, 4 of the recipients were attacked by the mother and cannibalized in the 12 hour period post-operatively; the remaining 3 animals survived the perioperative period without incident. One further animal from each group was found missing from each litter at subsequent points after transplantation, and each was presumed cannibalized by the parents; this left a total of 9 animals available for follow-up.

At two weeks post-transplantation, two animals were euthanized for detailed examination. On gross inspection both livers were noted to be uniformly pale white, with no evidence of nodularity, similar in appearance to that shown in Figure 1. Cryosections taken from these livers were immunostained with a specific anti-human MHC Class I antibody to detect the presence of human cells within murine liver parenchyma (Figure 5-2A and B). Sections at two weeks revealed clusters of cells staining positive for human MHC scattered uniformly throughout the host liver, comprising an estimated 1-2% of all hepatocytes (Figure 5-2C). Individual clusters appeared to be comprised of 8-10 cells each. Both of these animals would later be shown to be carriers of the Alb-uPA transgene, and were positive for production of human albumin with moderate signal intensity (Table 5-1).

At four week post-transplantation, two additional animals were sacrificed. The first animal sacrificed was larger than the other, and on necropsy its liver appeared normal in colour, size, and structure. This animal was later shown to be wild-type with respect to the Alb-uPA transgene (Table 5-1). The second animal sacrificed was smaller, and its liver appeared distinctly abnormal: the normal lobular appearance of the liver was distinctly disturbed, with the major left and right lobes appearing fused into one structure. The smaller central "butterfly" lobes of the liver were primarily composed of white tissue, similar in appearance to that seen at 2 weeks, with evidence of multiple red

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nodules ranging in size from 2-5mm; the remainder of the liver was normal in colour. Immunohistochemical analysis of cryosections from the latter animal showed diffuse clusters of human MHC Class I expressing cells throughout the sections, comprising 3-5% of the total surface area. Again, this animal would later be confirmed to carry the Alb-uPA transgene, and showed a strong human albumin signal (Table 5-1).

The remaining five animals were sacrificed at 6 or 8 weeks. Four of the five animals had livers grossly normal in size, colour, and appearance. The remaining animal has a liver roughly normal in colour and lobular structure, but the entire liver surface had a nodular appearance, suggestive of confluence of multiple smaller nodules; no evidence of the white parenchyma typical of transgenic liver was seen. Sections taken from this animal revealed a significant percentage of the liver parenchyma replaced with hepatocytes expressing human MHC Class I, in some cases with up to 40-50% of a section being composed of human cells (Figure 5-2D). The interface between human and mouse cells was distinct, with cords of human cells extending into the surrounding murine parenchyma (Figure 5-2E). Individual human cells maintained a normal appearance and developed sinusoidal architecture, although portal triad structures were notably absent from the regenerating nodules. Of the five animals, only this animal carried the transgene and expressed strong human albumin signals (Table 5-1).

The detection of human albumin (HA) in mouse serum samples was assayed using immunoprecipitation and Western blotting, as a marker of differentiated human hepatocyte graft function. Recipient mice were sampled by jugular venous puncture at four weeks post-transplant (or earlier, if sacrificed earlier) and at weekly intervals thereafter. A clear HA signal was demonstrated in the serum of 5/9 of the initial group of transplants. Subsequent genotype analysis revealed that all HA-positive animals carried the Alb-uPA transgene, whereas all the animals negative for HA, except one who was weakly HA positive at a single timepoint, were also negative for the transgene; this information is summarized in Table 5-1. Clear HA bands were detected as early as two weeks post-transplant, with an increase in intensity at the 4 and 6 week timepoints (Figure 5-3A). In one case, an incremental increase in signal intensity was seen over the period from 4-6 weeks (Figure 5-3B). All animals were screened for leakiness of the SCID trait by measuring total serum immunoglobulin levels using a sandwich ELISA

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technique: in all cases, serum immunoglobulin levels were well below the defined cutoff for "leakiness" (Table 5-1).

Transplantation of HBV-Infected Hepatocytes

Hepatocytes isolated from a 46-year old male known to be a chronic carrier of hepatitis B virus (positive serum HBsAg levels and negative serum HBV DNA, indicating a chronic carrier state without active viral replication[21]) were isolated and transplanted into the spleens of a single litter of 8 pups at 11 days of age. Two randomly selected animals were sacrificed at 4 weeks for histologic analysis, and the remaining 6 animals were followed at weekly intervals indefinitely. Of the 8 animals in the litter, 6 carried the Alb-uPA transgene, all of which showed extremely strong HA signal intensity at 4 weeks post-transplantation; the two remaining littermates were wild-type controls, and showed no evidence of albumin production at any timepoint. Sections of liver taken from the two animals sacrificed at four weeks showed diffuse clusters of human hepatocytes, similar to those described above.

The remaining 6 animals were followed at weekly intervals for a period of 17 weeks. In animals carrying the transgene, HA signals remained near maximal to 8 weeks at which point two distinct patterns of graft function emerged (Figure 5-4A). In three animals graft function began to slowly decline, with extinction of the HA signal at 10, 15 or 16 weeks (graft failure was defined as two consecutive weeks of no HA signal; graft duration was measured at point of last positive signal). In contrast, the fourth transgenic animal showed maximal HA signals at all measured timepoints (Figure 5-4B) and appeared to have stable engraftment of human hepatocytes up to 17 weeks post-transplant, with no signs of diminution of signal intensity. This animal died during week 17 post-transplant from hemorrhage complicating the jugular venous sampling procedure.

Serum samples taken from all 6 mice at each timepoint were analyzed by ELISA for the presence of HBsAg. The results of this analysis are summarized in Table 5-2. Signals remained at background levels for all animals except that with sustained HA production. In this animal, although HBsAg levels were initially below those of infected human controls, they were clearly considerably above those of the remainder of the group. After 8 weeks post-transplantation, there was an abrupt increase in HBsAg levels, to well above the range of human controls (0.30-0.40 absorbance units). Samples of

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serum taken from this animal at 8, 10, 12 and 16 weeks were then analyzed by PCR for the presence of HBV DNA. At each timepoint, serum samples were strongly positive for HBV DNA (Figure 5-5).

Infection of Transplanted Mice with HCV-Positive Human Serum.

Hepatocytes isolated from a 61-year old male serologically-negative for both HBV and HCV were transplanted into a single litter of 8 mice at 7 days of age; one animal died at four weeks post-transplant as a result of blood sampling. The remaining mice appeared normal throughout the period of experimentation, and were followed weekly until graft failure at which point they were sacrificed and tissues were harvested. After confirming graft function in some animals at 4 and 5 weeks after transplantation, each animal was inoculated with HCV-infected human serum. The results of this experiment are displayed in Table 5-3.

Three of the 7 littermates did not carry the Alb-uPA transgene, and all were negative for human albumin production at each timepoint tested. The remaining four animals had strong HA signals initially, similar to the previous group of transplanted mice. Over time, three of the four animals showed a transient pattern of graft function, with loss of HA signal at around 15 weeks. The fourth animal maintained a pattern of persistent high levels of HA production. Serum samples taken at 11, 12 and 13 weeks post-transplantation (5, 6 and 7 weeks after infection with HCV) were analyzed qualitatively for the presence of HCV RNA using RT-PCR: the animal with persistently strong graft function showed strong evidence of viral RNA at all tested timepoints (Table 5-3). Subsequent periodic testing demonstrated persistent presence of HCV RNA in serum samples up to 24 weeks post-transplant; this animal is currently alive, and further analysis is ongoing. Selected stored serum samples taken from this animal were analyzed for quantitation of viral RNA at 9, 16, 20 and 24 weeks post-transplant (3, 10, 14 and 18 weeks post-inoculation). Viral levels were 1.81×10^5 copies/mL and 1.22×10^5 copies/mL at 3 and 24 weeks post-transplant, respectively; the remaining two samples were below the limit of detection of the assay.

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A second litter of 7 pups was transplanted with non-virally-infected human hepatocytes at 4 days of age. Initial graft function was confirmed at 5 and 6 weeks in 5/7 littermates prior to inoculation with HCV-infected human serum (taken from a different donor than previous group) at 7 weeks post-transplant. One animal with strong graft function died of unknown causes during week 6 post-transplantation, prior to receiving infectious serum. All animals with graft function were tested for HCV RNA by qualitative RT-PCR at 10, 11, 12 and 15 weeks post-transplant (3, 4, 5 and 8 weeks post-inoculation); all samples were negative for HCV RNA.

In one animal, graft function began to decline slowly after 8 weeks, and HA signal was extinguished by 12 week. In the remaining three animals with good initial graft function, HA signal remained at maximum intensity over all timepoints measured. Based on this continued graft function, animals were re-inoculated at 17 weeks post-transplant with HCV-infected serum taken from the same donor used in the first HCV-infected group. Despite ongoing strong graft function (currently measured up to 22 weeks post-transplantation), samples taken at 3 and 5 weeks post-inoculation were negative for HCV RNA.

DISCUSSION

Hepatitis C has emerged over the past decade as a dominant cause of liver-related morbidity and mortality worldwide. Despite active research by various groups, the scientific community has been unable to develop models of HCV replication and infection, either *in vitro* or *in vivo*, with which to explore viral pathophysiology and novel antiviral strategies. This has proven to be one of the most significant, if not the most significant impediment to development of therapies against HCV. While *in vitro* assays have been reported which are useful for screening candidate compounds for activity against key viral enzymes such as the NS3 protease[22; 23], there is no system to evaluate the efficacy of these compounds in selectively inhibiting replication either in culture or in the more complicated animal host.

Although an accepted cell culture-based HCV viral replication system does not exist, systems employing nontransformed hepatocytes in culture seem to have shown at

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least some potential for supporting viral replication[24; 25]. These models are currently impractical for use due to low levels of viral replication and difficulties in maintaining cultures long-term. Transplanting nontransformed human hepatocytes into an appropriately receptive murine host might significantly enhance their longevity. Theoretically they could be infected with HCV, and might develop a productive infection which would then be detectable in the serum. This would represent an *in vivo* model of viral replication, facilitating progress toward the identification of candidate antiviral agents.

Based on our earlier work, it appeared that a significant mass of cells within a murine host was required to produce viral particles at levels suitable for use in laboratory investigation. To effect this increase in cell mass, we chose to employ the hepatocyte-proliferative environment which has been shown to accompany expression of the Alb-uPA transgene[12; 13]. Recent work has suggested that this proliferative environment may be the result of enhanced levels of numerous growth factors within the liver[26], especially hepatocyte growth factor (HGF), the most potent stimulus to hepatocyte proliferation[27]. To render mice carrying this transgene receptive to a xenogeneic hepatocyte graft, they were bred onto a SCID-beige background which produces severe deficiencies in T-cell, B-cell, and NK-cell function.

The first series of experiments established that human hepatocytes can successfully engraft and proliferate within the SCID-beige/Alb-uPA hepatic environment, forming a "chimeric" human/mouse liver. By two weeks after transplantation, human hepatocytes were seen throughout the liver parenchyma, in clusters of 8-10 cells. Assuming that each cluster is the result of clonal proliferation of a single cell, this would imply that 3-4 cycles of replication had occurred, or about one division every 4-5 days. Sections taken at later timepoints showed significant expansion of transplanted hepatocyte populations, with some sections containing 40-50% human cells at 6 weeks. If one estimates conservatively that 20% of the entire hepatocyte mass is composed of donor cells, and that the entire hepatocyte mass of a murine liver is about 6×10^8 hepatocytes[28], the final human hepatocyte mass would represent approximately 1.5×10^8 cells. As 1×10^6 cells were transplanted, and roughly 20% of transplanted cells engraft into the surrounding parenchyma[28], this would suggest that the initial cell number has

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increased in size by ~750 times, roughly equivalent to 9-10 cycles of cell division. Interestingly, this yields a time per cell division of approximately 4.5 days, consistent with the estimations made above. These numbers are very similar to those calculated by Rhim *et al.* in their original papers on transplantation in the Alb-uPA model[13; 14]. This data support the conclusion that human hepatocytes are capable of responding to prolonged proliferative stimuli in the form of murine-derived cytokines and growth factors. The presence of high levels of human albumin with murine serum supports the further conclusion that the cells are differentiated hepatocytes, with intact protein synthetic function.

Transplanting human hepatocytes taken from a donor already infected with HBV allowed assessment of the capacity of transplanted hepatocytes to support viral replication. From the onset of this experiment, one animal was shown to consistently produce HBsAg at levels in the serum significantly higher than its littermates. Although HBsAg is one of the earliest markers of a *de novo* hepatitis B infection[29], its presence in the serum does not necessarily imply active viral replication[21]. The abrupt increase in HBsAg levels at 8 weeks post-transplantation was suggestive of restoration of active viral replication, however; active replication of the virus was confirmed by detection of HBV DNA within the serum. This proved that the transplanted human hepatocytes retained all of the functional capacity necessary for replication of a hepatotrophic virus. This result was of special interest in that despite not actively replicating within its human donor, the virus was reactivated within the immunodeficient murine host. This reactivation may have been the result of inadequate antiviral immunity, similar to what is observed in chronic HBV carriers given pharmacologic immunosuppression after organ transplantation[30].

Also of interest was the occurrence of such an abrupt increase in HBsAg production at 8 weeks after transplantation, rather than a gradual increase which might be expected to accompany expansion of the donor hepatocyte population. In most models of liver regeneration, the period of active hepatocyte replication is confined to the first 8 weeks after transplantation[14; 31; 32]. It may be that during this period the hepatocyte intracellular machinery is occupied with active cellular replication in response to the microenvironment of high levels of growth stimulants. When this period ends, the virus

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may then able to begin active replication: this might have implications for the timing of infection of chimeric mice with human viruses, as sufficient cellular resources may not be available to support viral replication until after the period of proliferation is completed.

To confirm the chimeric human/mouse liver as a true model of hepatitis C infection, it was necessary to establish an infection in human cells which were not previously exposed to hepatitis B or C. This was done by first producing a series of mice with functional human grafts, and then inoculating them with human serum containing high titers of the hepatitis C virus. In this fashion, a chronic productive HCV infection was established in a chimeric mouse which has continued for almost six months at the present time. Quantitative analysis of HCV RNA revealed levels of 1-2 x 10^5 viral copies per mL of serum, well within the range of infected humans[33]; further analysis is ongoing to determine the pattern of viral production, either stable or fluctuating over time. This experiment proves that all of the components necessary for viral entry and replication are contained either within the mouse itself, or as a product of the human hepatocyte grafts. Thus cell surface markers which appear critical for viral attachment and/or entry such as CD81[34] must be intact, and other substances which might be required for hepatocyte targeting and entry either share sufficient structural homology between mice and humans to be cross-reactive between species or are produced by human hepatocytes in sufficient quantities to facilitate infection.

Although a prolonged HCV infection has been definitively established in this mouse model, a second group of transplant recipients did not develop an infection after the initial inoculation with HCV-infected human serum. Preliminary results suggest that active infection did not occur after a second inoculation with serum taken from the same donor used to establish the first infection. Although HCV RNA typically is present within weeks after primary infection[2], because the latency within the model is unknown further testing is required before this can be firmly concluded. The reasons for the failure to establish infection in these animals are not yet clear. It may be that a critical level of human hepatocyte engraftment is required before an HCV infection becomes measurable. At present the nonlethal method of graft monitoring, assaying for human albumin production by immunoprecipitation and Western blotting is inadequate for accurately determining the overall extent of human engraftment. Studies are underway to accurately

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quantitate the amount of human albumin within mouse serum and correlate it with histological evidence of graft extent, as a nonlethal determinant of graft size. One will also have to entertain the hypothesis that not all human livers may be susceptible to infection with the hepatitis C virus, perhaps due to variations in cell surface markers or to circulating cofactors required for viral entry. As more infections are established in chimeric mice, the answers to these issues will likely become more clear.

An important issue arising from these studies is the reproducibility of developing stable long-term engraftment of human cells within the recipient mouse liver. One of the factors which likely would impact on the overall extent of engraftment is the zygosity of the Alb-uPA transgene. The original work by Rhim *et al.*[14] suggested that recipients homozygous for the Alb-uPA transgene had consistently high levels of engraftment with concordant xenogeneic (rat) hepatocytes compared to heterozygous recipient, which had more variable levels of engraftment, ranging from <1 to 92%. Despite relative ease in determining presence or absence of the transgene, our attempts at determining its zygosity in hepatocyte recipients have proven less successful, and we are presently unable to conclusively distinguish any animals as being homozygous for the transgene. It has been noted on numerous occasions, however, that although litters are seen to contain 6-8 or more pups shortly after birth, at the time of transplantation the litter sizes are reduced to 4-5 animals, with no evidence of the missing pups. Furthermore, it is known that animals homozygous for the transgene have a very high perinatal mortality rate (~80%) secondary to bleeding complications[11]. It may be that the homozygous pups in each litter are dying of bleeding complications, and are subsequently being cannibalized by the parents prior to discovery by investigators. As such, all of the transplant recipients in this study may actually be heterozygous for the transgene, explaining the difficulties in determining zygosity.

Of all the animals reported on in the present study a total of 20/30 animals taken from 5 separate litters showed evidence of initial graft function, all of which corresponded to animals carrying the Alb-uPA transgene (counting the single animal who was nontransgenic with a single weak HA signal as a negative, and excluding all animals for whom genotyping was not performed e.g. secondary to early post-operative death). This number in itself is only slightly less than the 75% carriers of the transgene we would

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expect from a typical monoallelic Mendelian cross. However, the total number of offspring from these 5 litters is considerably less than what one would expect to see assuming the normal average of ~8 pups per litter (i.e. 40 pups total). If one makes the further assumptions that all the deaths occurred in pups homozygous for the transgene and the remaining mice carrying the transgene are heterozygotes, the hypothetical group of 40 pups would contain 10 homozygotes, 20 heterozygotes, and 10 wild type animals – numbers which correspond exactly to those of a Mendelian cross. Future investigations will include sacrificing a series of litters shortly after the time of birth and performing genotype analysis to determine if the homozygous animals are indeed being lost after birth.

It has also become apparent over the course of experimentation that two distinct patterns of graft function emerge over time. Although all animals carrying the transgene appear to have initial graft function, a significant proportion (7/12 animals followed long-term) develop a progressive decline in graft function which appears to begin between 8-10 weeks post-transplantation, and leads to graft failure at 12-16 weeks. The remaining animals appear to maintain persistent strong HA signals at all timepoints, with no evidence of decrement at up to and beyond 6 months – this subset appears to have stable permanent engraftment. Although the reason for these two patterns of graft function is not yet known, they may relate to rejection of human xenografts by natural killer cells present in some recipients. While the SCID trait was selected for in breeding the initial transgenic recipients by screening serum immunoglobulin levels, the beige trait was inadvertently unselected during breeding. As such, the mice employed as recipients will carry the beige trait in heterozygous, homozygous, or wild-type form. Animals heterozygous for the beige trait have an immune profile more similar to wild-type animals than to homozygous beige animals[35]. Furthermore, animals which are SCID only, and do not carry the beige trait are known to have higher than normal circulating pools of NK cells[36]. Natural killer cells have been implicated as a major cause of rejection of human tissues and concordant xenografts transplanted into SCID mice lacking the beige mutation[36-39]. Thus, the decline in graft function seen in some mice may actually represent NK-mediated rejection of human grafts in animals either

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heterozygous or wild-type for the beige transgene. Investigation is underway to determine if the zygosity of the beige trait correlates with ultimate graft fate.

This series of experiments establishes the capacity of the SCID-beige/Alb-uPA transgenic mouse to generate and sustain a chimeric human liver for prolonged and perhaps indefinite periods of time after transplantation of human hepatocytes. These chimeric organ can be infected *de novo* with HCV-positive human serum, and can support long-term replication of human-specific hepatotrophic viruses at clinically relevant levels. The model is both cost-effective and relatively easy to produce, and with optimization will allow investigators to directly explore strategies for inhibiting viral replication *in vivo*. With the potential for reconstituting the SCID-beige immune system with immune cells isolated from hepatocyte donors[40; 41], the model could also be applied to the design of vaccinations and immune therapies. Transplanting human hepatocytes obtained by percutaneous needle biopsy might permit individual tailoring of therapies most effective against a individual's specific viral complement. Finally, the utility of this chimeric liver model does not appear limited to the study of hepatotrophic viruses, and may be equally applicable to the study of a wide variety of human liver diseases.

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Figure 5-1. Photograph of livers harvested at 2 weeks of age from a single nontransplanted litter. The rightmost two livers represent the wild-type, the remaining 5 carry the transgene; regenerative (red) nodules are seen in transgenic livers.

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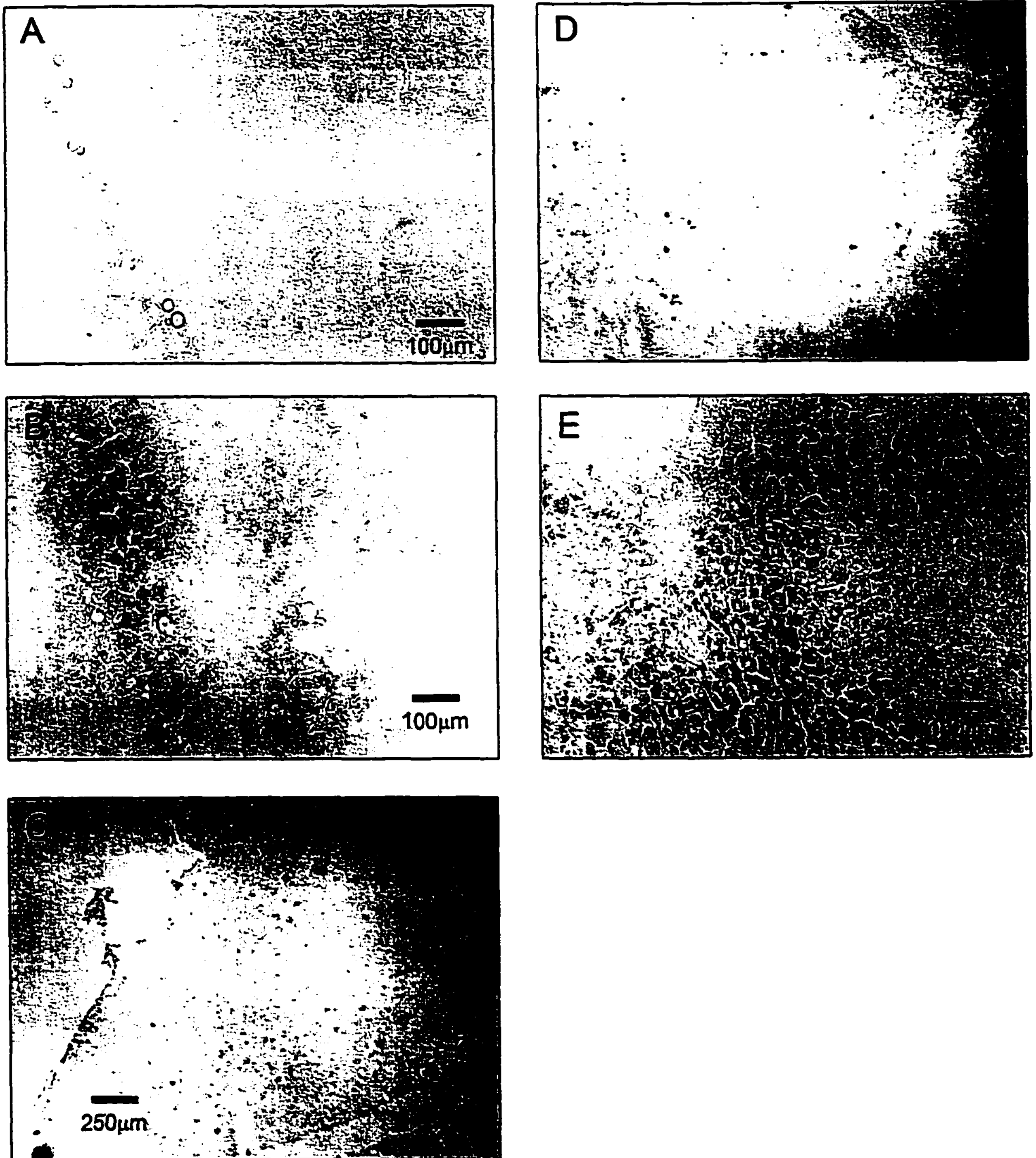

Figure 5-2. Immunohistochemistry of sections of recipient liver after transplantation of human hepatocytes. (A) mouse liver (negative control) and (B) human liver sections showing specificity of monoclonal for human Class I MHC; (C) – low power magnification of liver at two weeks post-transplant; (D) low power magnification of recipient mouse liver at four weeks post-transplant; (E) Same section at higher magnification, showing border between human and murine tissue.

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A

Weeks Post Transplant

HA 2 2 4 4 4 4 C HA 4 5 6 4 5 6

+ + -* + + + - - - + + +

Alb-uPA

B

Weeks Post Transplant

HA 4 5 6 4 5 6 C

Alb-uPa (-) Alb-uPa (+)

Figure 5-3. Immunoprecipitation and Western blotting of human albumin (HA) from recipient mouse serum after transplantation. (A) Signal intensity correlates with presence of Alb-uPA transgene; * positive at a single timepoint and not transgenic by PCR. (B) – Increasing HA signal intensity detected over weeks 4-6 post transplant; (-) and (+) indicate absence or presence of the Alb-uPA transgene; C – control non-transplanted mouse

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Mouse Number	Week Sacrificed	HA Production	Alb-uPA Genotype	Serum Ig (mg/dL)
1	2	+	+	0.007
2	2	+	+	0.003
3	4	+	-	0.007
4	4	++	+	0.05
5	6	+++	+	<0.001
6	6	-	-	0.002
7	6	-	-	<0.001
8	8	-	-	0.04
9	8	-	-	0.007

Table 5-1. Summary of first group of transplants of human hepatocytes into *scid-bg*/Alb-uPA mice. HA – human albumin (Western blot); Ig – immunoglobulin. Leakiness of the *scid* trait is defined as total serum Ig>5 mg/dL.

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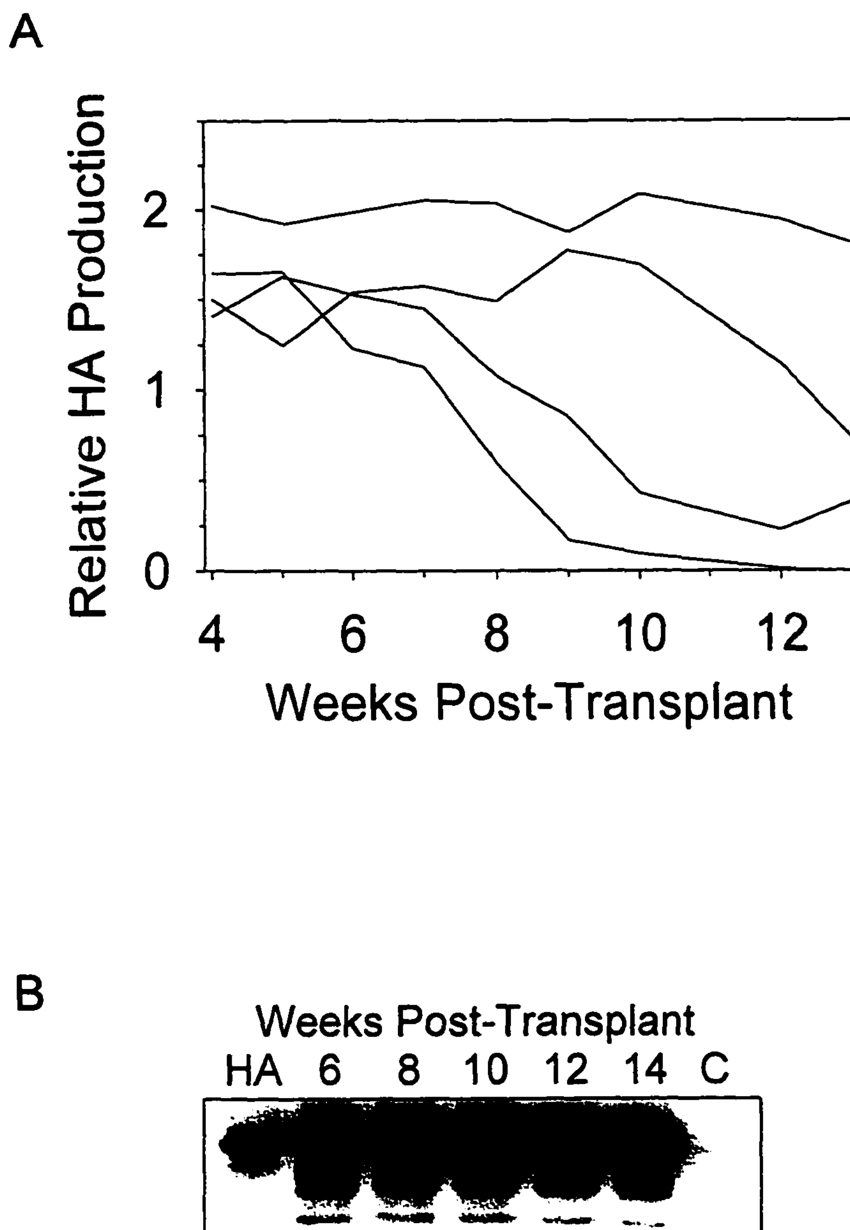

Figure 5-4. Long-term graft function in litter transplanted with HBV-infected human hepatocytes. (A) HA bands from Western blots were quantified using image analysis software and band densitometry, and normalized to a 50ng HA standard present on each blot; (B) Western blot from HBV (+) mouse (•) showing consistent strong signal intensity over time. Minor bands below HA represent degradation of albumin secondary to prolonged storage and freeze-thaw cycles.

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Mouse	Alb-uPA Genotype	HA Expression Pattern	HBsAg Level (weeks post-transplant)*				
			6	8	10	12	16
1	-	Absent	ND	0.04	0.04	0.04	ND
2	-	Absent	0.04	0.03	ND	0.02	ND
3	+	Transient	004	0.03	0.08	0.05	ND
4	+	Transient	0.12	0.04	0.07	0.04	ND
5	+	Transient	0.04	0.03	ND	0.04	ND
6	+	Permanent	0.13	.013†	3.18†	3.78†	3.44†

Table 5-2. Analysis of serum markers of hepatitis B infection after transplanting 6 littermates with 1 x 10^6 hepatocytes obtained from a known carrier of the hepatitis B virus. HA - human albumin; ND - not done; * HBsAg (hepatitis B surface antigen) levels measured by ELISA, data expressed as absorbance units; † Samples positive for HBV DNA by PCR analysis.

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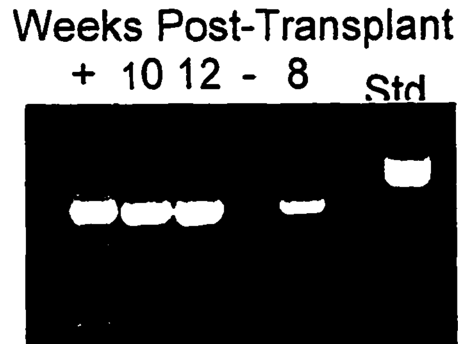

Figure 5-5. PCR analysis of mouse serum for HBV DNA; (+) infected human control, (-) noninfected mouse serum. Sixteen week timepoint not shown.

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No.	Alb-uPA Geno-type	Graft Duration (weeks)	HCV RNA (weeks post-Tx)							
			9	11	12	13	14	15	21	24
1	-	0	ND	-	-	-	ND	ND	ND	ND
2	-	0	ND	-	-	-	ND	ND	ND	ND
3	-	0	ND	-	-	-	ND	ND	ND	ND
4	+	14	ND	-	-	-	ND	ND	ND	ND
5	+	15	ND	-	-	-	ND	ND	ND	ND
6	+	15	ND	-	-	-	ND	ND	ND	ND
7	+	28*	+†	+	+	+	+	+	+	+‡

Table 5-3. RT-PCR analysis for HCV RNA in recipient mouse serum after transplantation of 1 x 10^6 non-infected human hepatocytes into 7 littermates followed by inoculation with HCV-infected human serum at 6 weeks post transplant; times expressed as weeks post-transplant. HA - human albumin; *currently ongoing; † 1.81 x 10^5 viral copies/mL serum; ‡ 1.22 x 10^5 viral copies/mL; ND - not done

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CHAPTER 6

GENERAL DISCUSSION

SUMMARY OF THE PROCESS OF DEVELOPING THE HCV MODEL

The long-term morbidity and mortality associated with chronic hepatitis C infection carries a significant cost for society through mandatory testing of the blood supply, treatment with interferon, and providing supportive settlements to patients infected with contaminated blood products[1; 2]. As such, developing an understanding of the viral lifecycle and developing strategies to prevent or cure its manifestations are concerns not only of the scientific and medical communities, but also of society at large. Despite the advances made in the past ten years since discovery of the virus[3] there are no good therapies yet available, nor do any such therapies appear imminent on the horizon. The inability to develop models of viral replication, either *in vitro* or *in vivo*, has led to a bottleneck in the forward progression of scientific advancement in the field of hepatitis C – there is no bridge yet available between the worlds of molecular biology and clinical application. To this end, we began a program to develop a small animal model of HCV infection, with the intention of alleviating this obstruction to progress in the development of a successful antiviral therapy for hepatitis C.

The hepatitis C virus has a very narrow host range, which has made development of animal models difficult. It appeared from cell culture systems that nontransformed human hepatocytes might hold promise for supporting an infection with HCV[4], but that the maintenance of such cells in culture would be difficult. This lead to the notion that through hepatocyte transplantation, a mouse liver might provide superior long-term support for human hepatocytes, in effect functioning as a "living culture dish"; this became our approach to model development. We proceeded forward on the general hypothesis that human hepatocyte grafts could be established within an appropriate immunodeficient murine host and then infected with human HCV, thus providing a small animal model of viral replication for use in laboratory study.

Initially, it was important to develop methods for consistently isolating hepatocytes from human tissue. A unique advantage to experimentation in human liver diseases at the University of Alberta is the close collaboration between basic science and

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hepatobiliary surgery, which provides a unique access to significant volumes of normal human liver tissue. To maximize the utility of each surgical liver specimen in a cost-effective fashion we developed a small volume perfusion system which, when combined with high-purity enzyme preparations, provides consistent yields of high-viability human hepatocytes which are comparable or superior to previously published methods. The use of the purified enzyme blends improved consistency of yield and viability as compared to crude enzyme preparations, allowing for isolations of hepatocytes in numbers well in excess of what is required for hepatocyte transplantations. This in turn allows for the development of banks of cryopreserved hepatocytes for use in later transplantation experiments, as will be discussed further below.

Despite improvements related to enzyme type used, it is clear from the experiments in Chapter 2 that other factors are equally important in overall outcome of isolations. Patient factors are likely to influence the yield of cells from liver specimens, with conditions such as cirrhosis producing poorer yields in general. When comparing our experimental data with that of other groups, it also appears that efforts at minimizing the total warm and cold ischemic time from occlusion of blood flow to suspension of cells in cold preservative solution have a major impact on overall yield. This has practical importance in that it suggests that centers where liver tissue is available on a regular basis should also perform hepatocyte isolations, in as timely a fashion as possible, to maximize the utility of this valuable and limited resource. We have preliminary data which suggests that human hepatocytes, once isolated, are stable in a cold-preservative such as UW solution for a period of 24-48 hours with only minimal loss of viability (Figure 6-1). If hepatocytes were to be shipped to distant centers for experimentation, this would appear to be the optimal path to follow; when others have attempted to initially flush and store liver tissue in preservative solution, with hepatocyte isolation performed in delayed fashion, overall viable cell yields per gram of tissue were at least an order of magnitude poorer than our results[5]. Finally, it is clear from our data that the quality of perfusion is extremely important in determining the final outcome of a perfusion. In some cases this factor is dominated by characteristics of the tissue biopsy itself, and cannot be modified to improve outcome. In other cases, however, techniques

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such as multiple-cannula perfusions might be used to improve the overall tissue distension with enzyme solution.

Our initial attempts at using hepatocyte transplantation to develop a model of HCV infection focused on transplantation of human cells into immunodeficient C.B-17-SCID-bg mice, which lack the functional capacity to reject xenografts. In chapter 3, initial experiments with the fluorescent cell label CFSE showed that hepatocytes transplanted intrasplenically could successfully translocate to the recipient liver and engraft within the host parenchyma. It became evident from our own work and the work of others, however, that methods to stimulate proliferation of engrafted cells would be necessary to increase the total mass of human cells and improve upon functional graft longevity. Although methods to stimulate hepatocyte grafts such as partial hepatectomy (PH) had been shown effective in stimulating graft function in congeneic and allogeneic transplant systems[6; 7], it was unclear whether the regenerative signaling after PH would effectively stimulate cells from a discordant xenogeneic species. The data from the experiments in Chapter 3 shows that human cells can respond to murine signals. The performance of a 50% partial hepatectomy concurrent with transplantation of human hepatocytes lead to a doubling of the median graft survival, from 3 weeks to 6 weeks. This finding would have additional importance in later experiments, in that it suggested that human cells would be capable of responding appropriately to the sustained high-proliferative stimulus of the Alb-uPA transgenic mouse.

Despite improvement in overall graft survival, the lifespan of transplanted human hepatocytes is considerably shortened relative to their lifespan within their native environment. Although human cells can respond to murine growth signals, there are structural differences between murine and human growth factors[8] which might lead to inadequate or partial agonism on human receptors[9]. As human hepatocytes transplanted into a murine host are bathed in a microenvironment of murine cytokines and growth factors, this in turn might lead to a foreshortened lifespan of the transplanted human cells. The experiments in Chapter 3 showed that the provision of a specific human growth factor (hepatocyte growth factor, HGF) at the time of transplantation significantly impacted on the overall longevity of human grafts. This indicates that in future experimental systems incorporation of a source of human growth factors, either through

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co-transplantation of specific cell lines producing such factors or through development of animal strains transgenic for human growth factor genes, might have an impact on the duration or natural function of human grafts.

Although significant advances were made in the development of techniques of hepatocyte isolation and in our knowledge of the behaviour of human hepatocytes in a xenogeneic environment, it was not clear whether the extent of engraftment achieved in the SCID-bg transplant model would be adequate to support a viral infection at clinically-meaningful levels. In Chapter 4, we used the human hepatitis B virus to evaluate the possible efficacy of this system as a model for later studies in hepatitis C. Hepatitis B virus was a reasonable surrogate for HCV at this point because of higher infectivity, generally higher serum levels in clinical infection, and relative safety of use because of active immunization of investigators against HBV infection. The SCID-bg system was shown in this manner to be inadequate for use in further experimentation as a model for hepatitis C infection. The total amount of cell engraftment likely to be achieved, probably less than 1% at best, was inadequate for support of viral replication in any clinically meaningful sense. This suggested that any system dependent on the simple abrogation of an immune response against human liver tissue to produce an animal that supports HCV infection is very likely to fail, in this case most notably the "trimeric" mouse model proposed by Galun *et al*[10].

At this point, it became clear that success in developing an animal model of HCV would require a significant shift in paradigm in our approach to sustaining human hepatocyte grafts long-term. Building upon the concept that a significant increase in graft cell mass would be required to support a viral infection at reasonable levels , we turned to animal models of hepatocyte regeneration. The two major models in this field were the Alb-uPA transgenic mouse[11; 12] and the FAH-deficient mouse[13], and of the two, the former had been more extensively studied with application to xenogeneic transplantation[14]. Based upon our knowledge that human cells would likely respond to murine proliferative signals, we felt that if this transgene could be expressed on an immunodeficient murine background such as SCID-bg, it might provide the significant increase in graft cell mass believed to be necessary for supporting viral replication.

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As outlined in Chapter 5, our experiments in the Alb-uPA/SCID model showed that human cells could respond to the proliferative signals characteristic of the model, and ultimately that animals could be produced that carried a significant degree of human chimerism within their livers. Through serial analysis of serum samples for the presence of human albumin, an exclusive product of human hepatocytes, we were able to demonstrate that transplanted human cells responded to the stimulatory environment of the Alb-uPA liver with rapid cycles of proliferation. Parallel analysis of liver sections by immunohistochemical staining for a human cell surface marker (MHC II) confirmed the presence of significant human chimerism within the murine liver, up to 40% of some sections. Ongoing long-term follow-up of transplanted mice has shown that grafts can be maintained with high levels of albumin production for well beyond 300 days, a period which comprises a significant portion of the host's lifespan. This demonstration of sustained human chimerism supported the first premise of our hypothesis, that human hepatocyte grafts could be supported within an appropriate animal host.

When transplanted with hepatocytes taken from a human donor chronically infected with the hepatitis B virus, it was next demonstrated that not only could the hepatocytes proliferate within the Alb-uPA liver, but the virus itself was stimulated into active replication from a state of latency present within the original human host. This proved that the transplanted cells retained all of the cellular machinery needed to support a viral infection, and that this infection could be detected easily by routinely performed tests, at levels which were clinically relevant to the same infection in a human host. These finding added further support to the notion that chimeric human/mouse livers would be useful in the study of human viral liver disease. Although viral replication was possible, this did not yet prove the system as a model for hepatitis C infection, nor did it prove that transplanted cells were capable of binding and uptake of human viruses, a quality which would be highly desirable from a model of HCV.

The establishment of the Alb-uPA/SCID mouse as the first small animal model of HCV infection was satisfied in a group of mice transplanted with cell taken from a human donor not infected with the hepatitis C virus, as described further in Chapter 5. A series of mice were produced with significant initial human chimerism within their livers.

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After confirming that initial graft proliferation had occurred, all animals were challenged with an inoculum of HCV-infected human serum. Of this initial recipients, one animal developed a chronic persistent infection with human HCV. This animal was extensively studied, and continued to produce virus at levels consistent with infected human patients (10^3 to 10^4 viral particles per mL serum) for up to 18 weeks after infection. These findings were well in excess of any findings published by other groups in potential models of HCV replication.

Subsequent to the data presented within these experiments, we have now replicated our results in a series of chimeric mice, and conclude with confidence that the SCID/Alb-uPA model is a robust, reproducible model of hepatitis C replication. Furthermore, we have had success in establishing a method for distinguishing the zygosity of the Alb-uPA transgene using a Southern blotting technique (Figure 6-2). This has provided critically important information confirming an earlier hypothesis that successful high-level human chimerism, and ultimately the establishment of HCV infection would be dependent on homozygosity of the Alb-uPA transgene. This ancillary study has demonstrated that in fact, homozygosity of the transgene appears to be crucial to establishing HCV infection (Table 6-1). This data not only lends further support to the validity of the model, but also points to the direction in which the recipient colony should be steered in order to begin mass production of infected murine hosts for further experimentation, as outlined further below.

ADVANTAGES OF THE SCID-bg/Alb-uPA MODEL OF HCV INFECTION

There are a number of important advantages to our model over any other proposed models of HCV that will increase its utility for investigation. The foremost advantage is that the model appears capable of sustaining viral replication for extended periods of time, at levels of replication which are directly relevant to clinical situations. The major flaw in all other systems of viral replication, excepting the chimpanzee, has been inconsistency of replication, or replication at levels approaching the limits of discrimination of available assays. Of natural small animal hosts, only the tree shrew *tupaia belangeri chinensis*, has been suggested as a potentially susceptible small animal host of human HCV[15]. Of all the animals tested in the only published study, a significant

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number had no evidence of infection at any timepoint, and the majority of the remainder (9/44 animals followed for 47 weeks) had a positive qualitative HCV RNA test at one or two timepoints. In the only other transplantation system touting itself as a model for HCV infection[10], the "trimeric" mouse, of 31 animals transplanted with HCV-infected human tissue, 19 showed evidence of HCV RNA in the serum at a single timepoint only in the first two months, and no animal was capable of infection by HCV-positive human serum.

In contrast, we have shown that our model is capable of sustaining a viral infection which is persistently detectable over prolonged periods of time at levels equivalent to those commonly seen in human patients. Furthermore, we take no special precautions in obtaining serum samples, and perform analysis for HCV RNA using a commercially-available automated system which is used extensively for human patients. In fact, in order to provide an adequate volume for use in the automated system, we have diluted samples 1:1 or 1:2 in normal noninfected human or mouse serum, and have still demonstrated qualitative signals equivalent to human patients. These factors argue to the robustness of the model, as the conditions of monitoring and analysis are those which would be employed in everyday laboratory situations and require no special precautions for detection of HCV RNA. Indeed, with the development of systems tailored to working with the smaller volumes of serum obtained from mice, the sensitivity of the assays should increase.

The second advantage to this model is the relative ease of use after a limited period of learning. A model which requires unique equipment, highly specialized techniques or rare talents would be less applicable to use in a variety of laboratory settings. The equipment utilized herein, however, is commonly available, and could be assembled in almost any laboratory or animal facility. Although initially requiring some practice, after a short period of learning an adequately skilled investigator should be able to reproducibly isolate human hepatocytes and perform transplantations.

Finally, because the Alb-uPA transgene has been bred onto a SCID-bg background, the possibility exists for reconstitution of the immune system of the recipient mouse with leukocytes taken from the donor of the liver tissue, thereby avoiding an allogeneic immune response[16; 17]. At present, little is known about the effects of the

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immune system on HCV infection[18]. The ability to study in *in vivo* cellular and humoral responses to HCV would be of tremendous benefit. Characterization of the immune response to acute and chronic HCV infection would have potential implications for developing immunomodulatory therapies, and vaccination strategies.

POTENTIAL DIFFICULTIES

While the SCID-bg/Alb-uPA transgenic mouse has proven successful as a model for HCV infection, there are still some areas of difficulty currently being addressed. A number of these issues have been explored in extensive detail in the discussion accompanying Chapter 5. As such, they will be briefly re-iterated here, along with potential solutions, but the reader is referred to Chapter 5 for a more detailed discussion.

The first difficulty is that as the model is based upon the transplantation of human hepatocytes into a permissive environment of replication, there is an absolute requirement for a source of human tissue. While the steady supply of tissue available for use in experimentation has been an invaluable resource in our laboratory, not all investigators will have access to such a supply. This problem may in part be alleviated by the transport of cold-stored human hepatocytes, as suggested above. Alternately, certain centers may need to develop programs whereby animals are transplanted and monitored for initial graft function prior to transport for investigations in other laboratories. While technically feasible, there may be additional legal and ethical issues associated with the procurement and potential "sale" of donated human tissues to other institutions.

The second difficulty is that it appears from our data that transplantation at an earlier time after birth, ideally from 4-7 days of age, might be associated with one or two extra cycles of cell division, which over a prolonged period of potential doubling, might lead to large increases in final graft cell mass. To optimize this aspect of transplantation, the supply of pups available at the appropriate age must be coordinated with the supply of fresh human liver tissue, which is not always consistent. The former factor can be corrected by increasing the number of breeding pairs at any one time, and staggering the production of new generations, such that mice of an appropriate age are available at any given time. The latter factor might by corrected if a bank of human hepatocytes could be established, such that at any time an aliquot of viable cells could be made available for

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use in transplantation. We have preliminary data which strongly suggests that not only can human cells be cryopreserved for a period of months, but that they proliferate normally when transplanted into transgenic recipients after this period of storage. Furthermore, we have been successful in establishing HCV infection in an animal which was the recipient of cryopreserved human hepatocytes. The development of a tissue bank would obviate the need for high numbers of breeding pairs, as cells could be made available at the optimal time for each litter of recipient pups that is produced.

A final difficulty has been variation in the extent and duration of engraftment in recipient mice. The variation in overall percent engraftment is very likely to be related to the zygosity of the Alb-uPA transgene[14], with homozygous recipients producing more consistent high percentage chimerism. We have determined by Southern blot analysis that homozygosity of the transgene is critical to establishing HCV infection. Methods for determining extent of engraftment suggested by other groups are rather arbitrary in nature, dependent largely on selection of biopsy sites and qualitative evaluation of species-specific DNA content in tissue samples[19]. It is our belief that the most reliable method for quantification currently is direct immunohistochemical analysis of the percent human chimerism within randomly selected liver biopsies. This analysis is time consuming, requires sacrifice of the transplant recipient, and is still subject somewhat to the vagaries of sampling bias, however. We are developing methods currently to quantitate human albumin production within the mouse liver, using protein dot-blot analysis of serum samples with image analysis and computerized densitometry. This information will then be correlated to immunohistochemical analysis, to provide a simple, reproducible and non-lethal method of quantitative graft size analysis which is not at all dependent on biopsy sampling. This information will then be used to analyze the conditions which lead most reproducibly to high-level engraftment.

FUTURE DIRECTIONS

Optimization of the Model

The extent of engraftment of human cells within the transgenic Alb-uPA liver is dependent on the duration of the stimulus to proliferation[14; 20]. This stimulus in turn is dependent on the amount of diseased liver present within an individual mouse[21].

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Transplanted normal cells which are stimulated to divide do so in competition with native hepatocytes which have spontaneously deleted the Alb-uPA transgene[12]. In homozygous animals, the chance of deleting both copies of the transgene in a given cells are considerably smaller than in hemizygotes, which need only delete a single copy. As such, homozygous animals have a prolonged period of hepatocyte stimulation, which results in more consistent high levels of chimerism within the liver[14]. However, it is also known that homozygous animals are less robust that hemizygous animals, due to an enhanced propensity for hemorrhagic complications[14]. It appears from our data that a significant proportion of the homozygotes produced from a (hemizygous) X (hemizygous) cross may be dying shortly after birth, perhaps as a result of bleeding complications. There are a number of potential solutions to this problem:

1. As homozygotes would be the most desirable recipients, transgenic pups carrying the homozygous transgene could be transplanted with hepatocytes, ideally from normal mouse donors, to "rescue" them from the lethal effects of the homozygous transgene which occur at 2-3 weeks after birth. Although it may require a number of litters of transplants for this to occur (in order to get sufficient numbers of homozygotes which survive the first 48 hours post-partum), in this fashion, a series of homozygous transgenic breeders could be produced, which would then yield only homozygous offspring. While a reasonable number of these offspring may continue to die in the early post-operative period secondary to bleeding complications, one would expect any "early survivors" to develop high levels of chimerism within their livers after transplantation, making them useful for viral infection studies. By expanding the number of breeding pairs is a colony, sufficient numbers of homozygous recipients could easily be produced to support viral studies.

2. As the early lethality of the homozygous Alb-uPA transgene is associated with bleeding complications secondary to elevated levels of urokinase, pharmacologic intervention to reduce this bleeding diathesis may significantly improve early survival rates. To this end, we have begun preliminary experiments utilizing the

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drug tranexamic acid, employed clinically in human patients to reverse the state of fibrinolysis. This drug is known to cross the placenta at high levels, and if administered to pregnant mothers in the week prior to parturition, may serve to prevent early bleeding complications in offspring secondary to birth trauma.

3. It is competition between donor hepatocytes and recipient hepatocytes which delete the transgene that ultimately control the duration of graft proliferation, disadvantaging the host cell proliferation in a heterozygous animal might have the same effect as rendering the animal homozygous. This disadvantage might be accomplished through the administration of pyrrolizidine alkaloids, as performed by Laconi *et al* in a rat model[22]. These compounds are taken up and rapidly metabolized within host hepatocytes to a toxic derivative which effectively blocks the hepatocyte cell cycle at the late synthetic or early gap2 phase, preventing cells from undergoing normal mitosis for a period of 1-2 months. A certain type of pyrrolizidine alkaloid, lasiocarpine, has been shown to cross the placenta when administered in a single dose to pregnant rats at one week pre-parturition, and to effectively block mitosis in the livers of the rat pups for a period of one month[23; 24]. In the Alb-uPA model, this might provide a significant growth advantage to the transplanted cells over the host cells which have deleted the transgene, leading to high levels of engraftment with human cells. It is unclear whether the administration of such substances would be lethally toxic to offspring.

Development of a Hepatocyte Bank

As described above, we have good evidence that human hepatocytes can be reliably cryopreserved for extended periods of time, and upon thawing respond completely appropriately to the growth signals in the Alb-uPA liver. Furthermore, active HCV infection can be established in animals which have received grafts of cryopreserved cells. In collaboration with Dr. Jonathan Lakey, we are working to establish the factors which are most critical to successful freezing and storage of human hepatocytes, with the intention of establishing a bank of human hepatocytes which would be available for transplantation at any time. As more information is gained about the susceptibility of

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individuals to infection by HCV, factors which influence this outcome can be analyzed for individual donors, and their cells marked as such, for use in later studies. The development of a tissue bank will also allow for reduction in variability between transplants which might be in some manner relate to donor characteristics.

Development of an HCV Serum Bank

In addition to banked hepatocytes, a collection of infectious sera frozen and available for thawing and use in infection would be highly desirable. Factors which might influence the viability and infectivity of viral particles after storage would be determined through a series of infectivity experiments, so that each frozen sample would be maximally infective when thawed. Samples would ideally be genotyped and quantitated prior to storage, as these factors may prove important in the ability to establish infections in chimeric mice.

Reconstitution of Mice with Donor Specific Immunity

As little is known about the effects of the immune system on HCV infection[18], the ability to study *in vivo* cellular and humoral responses to HCV would be of tremendous benefit. As alluded to above, mice could be reconstituted with donor-specific lymphocytes[16; 17], providing recipient mice with both a chimeric human liver and a human immune system. Although typically SCID mice are reconstituted with human PBL at 6-8 weeks of life, this might be performed earlier with equal success. This would require experimental optimization. Having achieved some degree of reconstitution, the nature of the new immune system would need to be characterized to determine which cells lines have been restored, as well as whether their immune responsiveness is qualitatively similar to the normal human immunity. After optimizing the conditions of reconstitution, a number of critical studies could be performed, including the analysis of acute-phase serologic markers of HCV infection, the critical viral epitopes involved in provoking an immune response, the development of viral quasispecies over time in the face of immune pressure, and ultimately methods to provoke protective humoral- and cell-mediated responses to acute infection by vaccination

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Development of Patient-Specific Chimeric Human/Mouse Livers

The SCID-bg/Alb-uPA model would be applicable to use in patient-specific circumstances. In this case, a percutaneous liver biopsy, routinely taken to assess degree of liver damage in infected patients, would be used as the source of hepatocytes. Techniques for dispersion and purification of hepatocytes from such samples could be adapted from current protocols. Cells from an infected patient would be processed, and transplanted into a series of animals, which could then be assayed for various parameters of viral infection. This might then allow pharmacologic studies with antiviral drugs given alone or in combinations, to determine which strategies will work best for that patient's specific viral genotypes, without exposing the patient to prolonged drug trials, or potentially harmful side effects without benefit. The same transplanted mice might also be reconstituted with PBL from the patient in question, to further explore the interplay of the patients immune system with different therapeutic strategies.

FINAL CONCLUSION

Based upon the data compiled from the series of experiments comprising this thesis, we are confident in concluding that the SCID-bg/Alb-uPA system represents the first robust animal model of hepatitis C infection ever developed. Our hypothesis that human hepatocyte grafts could be established within an appropriate immunodeficient murine host and then infected with HCV, thus providing a small animal model of viral replication for use in laboratory study has been supported by the experimental data. Rather than being a conclusion, however, this finding presents a fantastic new vista to investigators pursuing a cure for hepatitis C. We anticipate that this model will provide the platform upon which further progress in the study of hepatitis C will proceed, and steps have been taken to establish programs of antiviral drug and vaccination development at the University of Alberta. Now at the forefront of HCV study worldwide, it is hoped that this animal model will provide a critical bridge between drug design and clinical application. This may lead ultimately to the development of immunization strategies and antiviral compounds which can be employed in the eradication of this major human pathogen.

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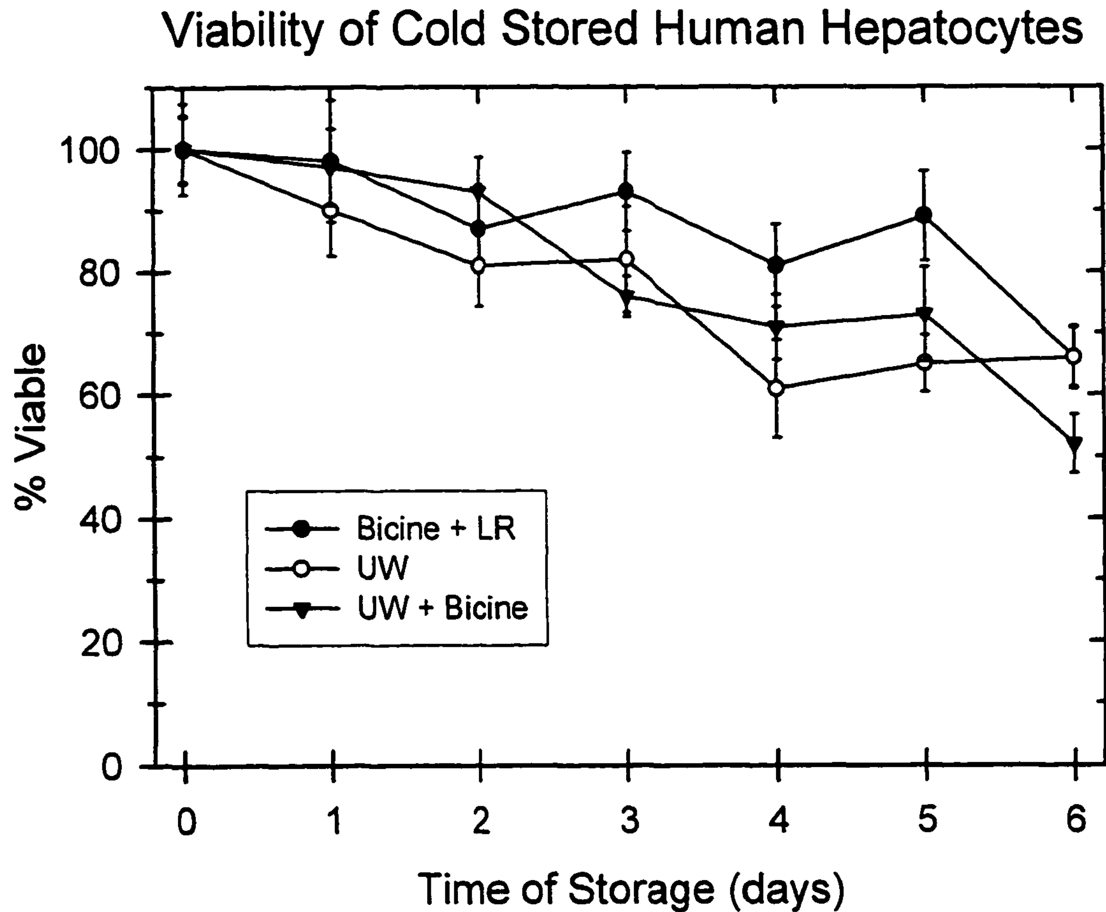

Figure 6-1. Short-term viability of human hepatocytes stored in various organ preservation solutions, as assessed by trypan blue dye exclusion. UW – University of Wisconsin solution; LR – Lactobionate-raffinose storage solution.

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Figure 6-2. Determination of Alb-uPA transgene zygosity by Southern blot analysis. T – transgenic urokinase; E – endogenous urokinase; T/E – ratio of signal intensities

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HA Signal (4-5 wks)	HA Pattern	n	Alb-uPA Copies	Geno -type	HCV RNA*
None (n=8)	Absent†	8	0	-/-	0/8
Strong (n=17)	Transient	10	4	+/-	0/10
	Persistent	7	4	+/-	0/3
			8	+/+	4/4

Table 6-1. Summary of 25 mice transplanted from 4-13 days with $1x10^6$ normal human hepatocytes and inoculated with 250μl HCV-infected human serum at 6 weeks post-transplant. HA – human albumin; * Results of qualitative RT-PCR analysis(27) performed at 3 and 5 weeks post inoculation; † 3/8 samples were weakly positive at a single timepoint (5 weeks) only

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