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Advancing the Alb-uPA/SCID/Bg Chimeric Mouse Model for Hepatitis C Virus Infection

by

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Abstract

The feasibility of the Alb-uPA/SCID/Bg chimeric mouse as a model for Hepatitis C Virus (HCV) infection was assessed experimentally by (1) the infection and treatment with another hepatotropic virus, Hepatitis B Virus (HBV) and (2) the infection of the model with HCV and the subsequent treatment of that infection with a pro-apoptotic factor (BID) targeted to infected hepatocytes. In the former, the infected mouse responded favorably, and in the manner of human patients, to a standard imunoglobulin therapy. In the latter, HCV-infected hepatocytes were successfully targeted for cell death, with repeated doses of Adenovirus-delivered BID being the most effective at inhibiting virus spread. Efficacy and toxic side-effects of BID treatment could be reconciled by modulating the timing between doses, the most effective tested being three doses of BID at 7-day intervals. Analyses of chimeric model production were undertaken to improve the quality of human hepatocyte engraftment (typically only 25-35% of mice receiving grafts are currently used experimentally). Minor variations in success rates were experienced with respect to donor age or health status, or the age of recipient mice within an operational window of 5 to 13 days from birth. The greatest obstacle to useful engraftment (aside from technical challenges) was deemed to be the genetic/cellular integrity of the recipient mouse. This conclusion was based on variable engraftment success with 'healthy' donor cell preparations and a consideration of variability in immune deficiency arising in mice within a SCID/Bg mouse colony.

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List of Abbreviations

AAT	alpha-1-anti-trypsin
Ad	adenovirus
Alb-uPA	albumin-urokinase plasminogen activator transgene
ALT	alanine aminotransferase
Apaf	apoptotic protease activating factor
BSA	bovine serum albumin
CBC	cord blood cells
AFP	alpha fetoprotein
cDNA	copy deoxynucleic acid
CTL	cytotoxic T lymphocytes
DAB	Diaminobenzidine
DMN	Dimethylnitrosamine
E	envelope protein
ECM	extracellular matrix
EGF	epidermal growth factor
eGFP	expressed green flourescent protein
ELISA	Enzyme Linked Immunosorbent Assay
FACS	Fluorescent Activated Cell Sorter
FADD	Fas-associated protein with death domain
FGF	fibroblast growth factor
H and E	hematoxylin and eosin
hAAT	human alpha-1-anti-trypsin
HbIg	hepatitis B immunoglobulin
HbsAb	hepatitis B surface antibody
HBV	hepatitis B virus
HBSS	Hank's Buffered salt solution
HCV	hepatitis C virus
HGF	hepatocyte growth factor
HIV	human immunodeficiency virus
HRP	horseradish peroxidase

HSPG	heparin sulfate proteoglycans
HVR	hypervariable region
IFN	interferon
ICG	indocyanine green
Ig	immunoglobulin
IGF	insulin-like growth factor
IGFBP	insulin-growth factor binding protein
Il	interleukin
IRES	internal ribosomal entry site
LPS	lipopolysaccharide
ISDR	interferon sensitivity determining region
IU	international unit
mBID	modified BCL-2 interacting domain
mHGF	murine hepatocyte growth factor
MHC	major histocompatibility complex
MUP	major urinary protein
NANBH	non-A, non-B hepatitis
NK	Natural killer cell
NS	nonstructural protein
ORF	open reading frame
OSM	oncostatin M
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PG	proteoglycan
RBC	red blood cell
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SCF	scatter cell factor
SCID	severe combined immune deficiency
siRNA	small interfering ribonucleic acid

STAT	Signal Transducers and Activators of Transcription protein
TBS-T	Tris-buffered saline – tween
TCR	T cell receptor
TdT	Terminal Deoxynucleotidyl Transferase
TGF	transforming growth factor
TNF	tumor necrosis factor
TUNEL	deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling
UTR	untranslated region

Chapter 1

Introduction

Part A: Hepatitis C Virus Infection, Impact and Treatment

History

Viral hepatitis is a major cause of chronic liver disease and cirrhosis. Upon the characterization of hepatitis B virus in 1970 and Hepatitis A virus in 1973, there remained a large population of patients with viral hepatitis that tested positive for neither virus. This group of patients was generically classified as having non-A non-B hepatitis (NANBH). In 1989, with the application of novel molecular biological techniques, a new hepatitis genome was sequenced (as a cDNA copy) from the sera of NANBH patients [1, 2]. The new virus, called Hepatitis C (HCV), was deemed responsible for the majority of the previously identified cases of non-A non-B hepatitis. Since its identification, HCV has emerged as the most common cause of chronic liver disease in the developed world, touted as the most serious complication of blood transfusions and is the number one indication for liver transplantation in North America.

Epidemiology

An estimated 170 million people worldwide are infected with the Hepatitis C virus. Seventy to eighty percent of infected people become chronic carriers. Chronic infection can result in a gradual progression toward significant morbidity and mortality. For example, twenty to thirty percent of these patients develop cirrhosis, end stage liver failure and hepatocellular carcinoma [3, 4]. Transmission of the virus is usually through the parenteral route. Most commonly, HCV-positive patients have previously received blood transfusions, or engaged in some kind of parenteral contact with blood products. Prior to the screening of blood products for HCV antibody, the rate of NANBH was reported as 10-13% (0.45% per

unit transfused) in recipients of blood components [5]. Eighty percent of these patients were later shown to be infected with HCV [6]. With the advent of surrogate marker testing for alanine transferase (ALT, indicative of hepatocyte damage) and for antibody to hepatitis B core antigen, this rate was reduced by greater than fifty percent [7, 8]. With the addition of anti-HCV antibody testing, the risk of seroconversion was reduced by 70-85% per unit transfused. The current reported risk of transfusion-associated HCV infection is now less than 0.03% per unit transfused [5, 9].

HCV transmission has also been demonstrated to occur via intramuscular injections, transplantation of tissue or organs from infected donors and, the use of improperly sterilized reusable medical materials. Known risk procedures through which patients have been inoculated with HCV include ear piercing, acupuncture, tattoos and cultural practices involving contact with blood [10, 11].

Studies have identified a number of high-risk lifestyles and demographic groups. In the United States, the majority of newly acquired HCV infections are due to intravenous drug abuse. Among drug-using populations, the prevalence of HCV ranges from fifty to almost one hundred percent. Although it has not been directly demonstrated to be sexually transmitted [12], those individuals with multiple sexual partners are at a significantly increased risk of being infected with HCV [10]. Vertical transmission from infected mothers to their infant is approximately 5% [13]. It is hypothesized that transmission occurs during labor or at delivery. There is a direct relationship between maternal viral titres and the rate of chronic HCV infection in the newborn.

Populations at higher risk of being infected with HCV include healthcare workers, dialysis patients, hemophiliacs and HIV infected individuals. Health care workers are at increased risk because of the potential for needlestick injuries [14]. There is a five to ten percent probability of becoming chronically infected after a hollow-point needlestick injury from an infected patient. The overall risk in hospital from a random needlestick is 0.1% [15]. Dialysis patients and hemophiliacs have a higher rate of HCV

seroconversion because of their recurrent exposure to blood products and through the use of shared dialysis machines [11]. Because HIV and HCV share similar modes of transmission there is a high incidence of co-infection. The literature reports rates of HCV co-infection in HIV positive patients of between eight and fifty-one percent [3]. Liver disease secondary to HCV co-infection is now the most common cause of death in the North American HIV population. Regardless of the numerous risks, there are up to 20% of infected individuals who deny exposure to any of the above identified risks. These individuals are said to have community acquired HCV.

Virology

HCV is a single-stranded positive sense RNA virus. It is a member of the *Flaviviridae* family (other members include yellow fever and dengue fever viruses) but also shares similarities to the *pestivirus* family [16, 17]. The positive-stranded RNA genome is ninety-four hundred base pairs in length [18, 19]. Through genome sequence homology, six major genotypes of the virus have been identified, along with at least forty major subtypes. HCV has been estimated to replicate at 10^{12} copies/day. Given the poor fidelity of the viral RNA dependent RNA polymerase, a very high rate of viral mutation occurs. Within infected individuals, quasispecies appear that are a result of cumulative mutations appearing in the primary genotype – a mutation rate estimated at 0.001 nucleotide substitutions per replicative cycle (or roughly nine substitutions per genome) [20-22]. Although the entire genome can show genetic variability, a hypervariable region in the E2 5' region is where the majority of mutations occur [23]. It is hypothesized that hypervariation of HCV promotes chronic infection; the mutations allow the virus to escape from neutralizing host antibodies. This is a challenging factor in the development of an effective HCV vaccine strategy.

A comparison of complete genomes and subgenomic regions has identified six major HCV genotypes. A sequence similarity of less than seventy-two percent of any known sequence is evidence for a new genotype. A new subtype is identified if the maximum sequence similarity to other genotypes is 75 to 86

percent [24]. Of the known genotypes, there are some that have broad worldwide distribution (types 1 and 2) while others are localized to certain geographical areas (refer to Fig. 1.1). In North America, the most frequently seen infections are with genotypes 1a, 1b, 2a, 2b, and 3a. Type 4 is predominantly seen in Central Africa, Egypt, and the Middle East. Patients in South Africa are mainly infected by type 5a [11, 24].

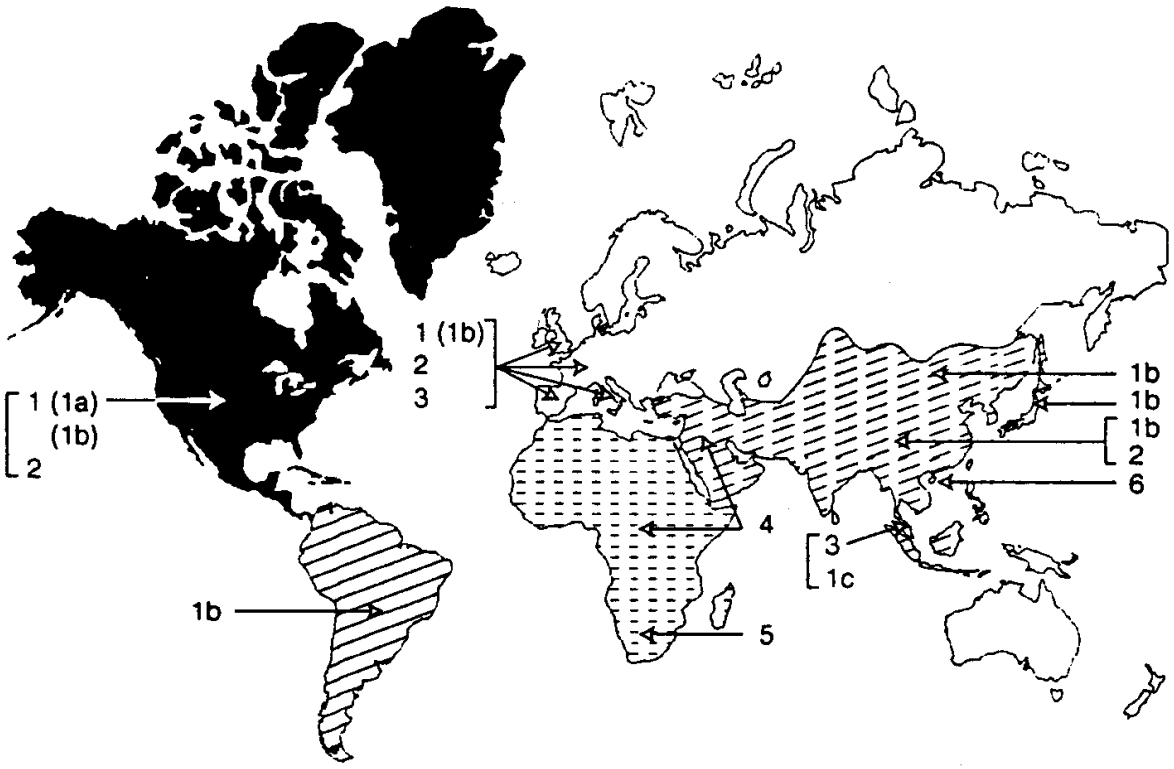

Figure 1.1. Geographical distribution of HCV genotypes [21].

Knowledge of the different genotypes is important in the development of universal vaccines and antiviral treatments. Genotyping is now accomplished through PCR hybridization and RFLP (Restriction

Fragment Length Polymorphism) analysis. Though not precisely defined clinically, evidence suggests that genotype bears profoundly on disease progression and the response to specific treatments. Type 1b has been associated with a lesser likelihood of responding to interferon therapy and to a higher risk for progression to cirrhosis. Genotypes 2 and 3 show much better success when treated with interferon and better prognosis overall [25].

Molecular Biology

The viral genome of HCV contains a single open reading frame, which encodes a polyprotein of approximately three thousand amino acids (3010-3033). Flanking the open reading frame, or coding region, are 5' and 3' untranslated regions (UTR) [16]. The 5' UTR (341-344 nucleotides) is responsible for internal ribosome binding. In the downstream portion of the 5'UTR, a large conserved stem-loop structure forms. Functional in initiating translation, this region is highly conserved amongst genotypes [26-28]. The 3' UTR is divided into three regions – a highly variable region, a stretch of polyU, and a conserved 98 base terminal region which forms a stable stem-loop structure [29].

The structure and function of the HCV proteins have been deduced through comparative analysis with other known genomic sequences. This was in large part due to the lack of an *in vitro* or *ex vivo* means of expressing the cloned HCV genome. Through homology comparisons, HCV shares similar characteristic to both *flaviviruses* and *pestiviruses* (although it is classified with the former). The relatively long 5' UTR contains several AUG codons preceding the actual initiation codon for translation. This characteristic closely resembles that of the *pestivirus* RNA. Because in *pestivirus* the 5' end forms a complex secondary structure that is the internal ribosome entry site, it is proposed that the same purpose is served in the HCV genome [30]. If point mutations are inserted into this region the secondary structure is disrupted, and translation is reduced significantly [26]. The organization of the remaining HCV genome has areas resembling regions of the *flavivirus* family.

The HCV polyprotein is proteolytically processed by both viral and host proteins to form at least ten protein products. The N terminal region contains the structural proteins, and the nonstructural proteins are contained in the C terminal region. The structural proteins include a nucleocapsid core protein, and two envelope proteins, E1 and E2. The nuclear core protein is designated p22 (it is approximately 22 kD in size). It is encoded by the 5' proximal portion of the viral genome [31, 32]. The appearance of anti-p22 antibody precedes antibodies to other HCV antigens, suggesting that the core protein is synthesized relatively efficiently in HCV-positive patients. Findings that the nuclear core protein is detected mainly in the cytoplasm of hepatocytes of patients chronically infected with HCV correlates with the fact that HCV RNA replication events occur predominantly in the cytoplasm [33].

The HCV core protein is divided into an N-terminal hydrophilic end and a C-terminal hydrophobic end. The antigenic epitopes of HCV core protein are found on the N-terminus. The hydrophobic C' end is composed of two domains [34]. When one or both of these domains are deleted, the core protein is no longer found in the cytoplasm but in the nucleus of the host cell. In a hepatoma cell line that supports HCV, 10-20% of the corresponding mutant virus is found in the nucleus when stained with a monoclonal antibody [35]. Virus found in the nucleus had a truncated 18 kD nuclear core protein (compared to 22kD). Hence, it has been postulated that the C-terminal domains of the nucleocapsid protein target HCV to the cytoplasm [36]. Post-translation processing by either a host or a viral proteinase during viral assembly results in the loss of the hydrophobic domain and translocation of the core protein into the nucleus. The significance of this processing and translocation is unknown. A comparison of HCV sequences with known *pestiviruses* indicates that the core protein has a putative RNA binding domain indicative of a role in genome encapsidation. Core protein also contains DNA-binding motifs and dimerization domains, consistent with the core protein's playing a role in the regulation of viral and host gene expression [35].

The envelope proteins E1 (gp31) and E2 (gp70) interact to form a heterodimeric complex. These proteins are membrane associated and are localized to the endoplasmic reticulum. Within the N terminus of the E2 protein there is a hypervariable region, HVR1, between amino acids 384-410. HVR1 is exposed on the virion surface and its variability is a factor in the generation of quasispecies within one host as well as contributing to the definition of the major genotypes [17, 31, 37]. The variability of HVR1 destabilizes this peptide, hampering antibody recognition in immune responding hosts. Host immune pressure drives quasispeciation by selecting for mutant viruses that, in part, bear variable E2 proteins and thus escape immune elimination.

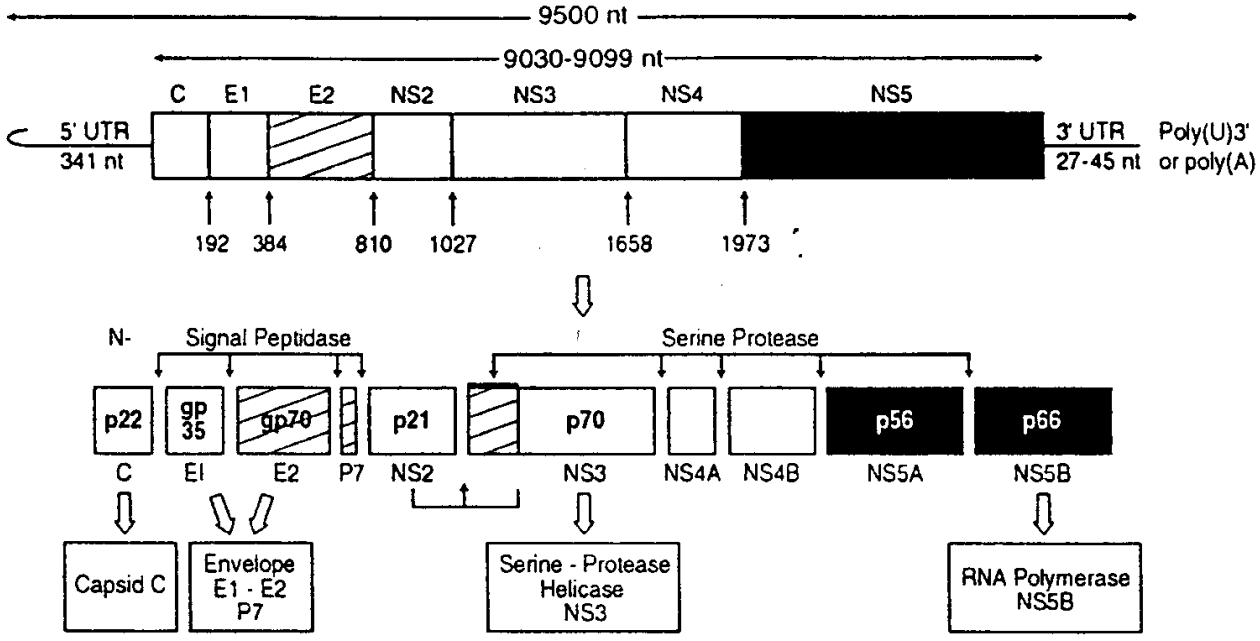

Figure 1.2. HCV genome organization [21].

The nonstructural proteins (see Fig. 1.2 above) are called NS2, NS3, NS4A, NS4B, NS5A and NS5B [16]. NS3 has three putative enzymatic activities including: 1) a serine protease 2) a helicase and 3) an ATP-dependent nucleotide triphosphatase [38]. NS2 and the N terminal end of NS3 encode a second protease [39,

40]. NS4A acts as a cofactor for the NS3 proteinase activity and together they form an active heterodimeric complex. The NS5B sequence is consistent with an RNA-dependent RNA polymerase with terminal transferase activity. NS5A is a serine phosphoprotein whose function has not been surmised.

The core and envelope proteins are cleaved from the HCV polyprotein by host signal peptidases (see Fig. 1.3). In contrast, the nonstructural proteins are processed by the viral proteinases. NS2-NS3 cleaves NS2 from NS3. NS3 acts in *cis* to cleave NS3-NS4A and in *trans* to sequentially cleave NS5B, NS5A and NS4B [19]. The individual proteins undergo additional post-translational processing. Phosphorylation of serine residues on the core protein and NS5A is thought to regulate these proteins functions [21, 41].

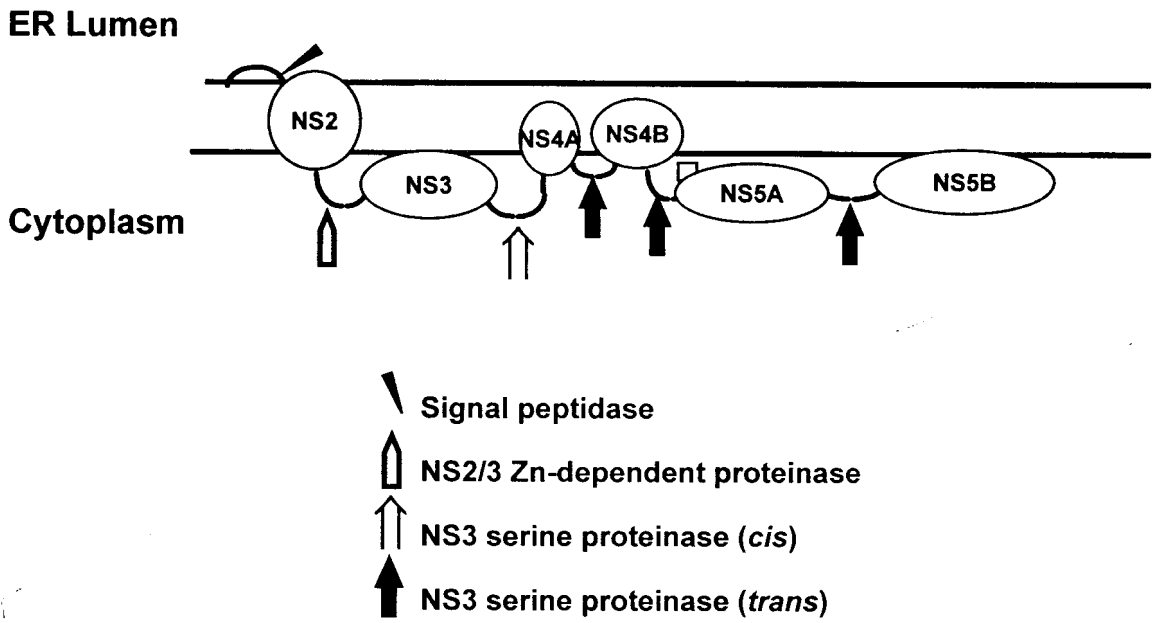

Figure 1.3. Proteolytic processing of HCV polyprotein [42].

Although its replication is not clearly defined, the HCV genome is copied to a negative RNA strand intermediate (common to the *flavivirus* and *pestivirus* families). This intermediate strand serves as a template for the formation of new positive-stranded RNA genomes. Detection of negative stranded HCV RNA is evidence for the existence of viral replication [21].

The development of the cell culture replicon systems has aided the progress in understanding the molecular biology of hepatitis C. The JFH-1 replicon has the capacity to replicate and release infectious particles, thus allowing studies on HCV life cycle. [43]

The 5' non-translated region of the HCV genome is one of the most conserved regions. It is capable of binding the 40S ribosome subunit of host cells as well as the eukaryotic initiation factor 3 (eIF3). The interaction of these three components, IRES of HCV, 40S subunit and eIF 3 modulates translation of the HCV genome. [44]

Once the HCV polyprotein precursor is translated, it undergoes further processing by host and viral proteases into the structural and nonstructural proteins. Host signal peptidases process the junctions between core/E1, E1/E2, E2/p7, and p7/NS2. The viral nonstructural proteins are further processed by viral proteases – NS2-3 protease processes the junction between NS2 and NS3, and the remaining 4 nonstructural protein junctions are processed by the NS3-NS4A complex.

Once the viral proteins have been processed, the HCV genome has the necessary components to replicate. Replication occurs in the cytoplasm of the virus-infected cells and involves the endoplasmic reticulum, golgi apparatus, endosomes and lysosomes. The nonstructural proteins NS3-5B form a replication complex in a vesicular membrane structure ("memebranous web"). [45] The HCV genome generates a full-length negative strand RNA. Positive strand RNA is then produced from the negative strand template, likely via double-stranded RNA intermediate. Although not all components of the viral replication complex are known, it is felt both host and viral factors are involved. NS4B's function is not completely elucidated, but it forms a membranous web complex which becomes the site where HCV

RNA replication occurs. NS5B has polymerase activity and is likely essentional in catalyzing HCV RNA synthesis. NS3, through its helicase/NTPase activies is thought to unwind any double stranded intermediates or secondary structures so that the NS5B polymerase can copy both strands of the viral RNA. Positive strand RNA is then used for further translation, replication and packaging into viral progeny. [43, 46]

HCV: Chronic Infectivity versus Viral Clearance

The majority of patients infected with HCV do not experience the characteristic symptoms of acute hepatitis – i.e. malaise, fatigue, and jaundice. Instead, it is estimated that only 10-25% of patients develop an acute hepatitis. The percentage of people that end up with chronic hepatitis is significantly higher. Compared to hepatitis B infection where there is a 5% chronic liver disease rate, the probability of HCV infection being chronic is between sixty and eighty percent [47]. HCV infection is best regarded as a chronic disease and therefore it is of considerable research interest how a small proportion of individuals can clear the infection.

Host defenses against viral pathogens can be divided into an innate, nonspecific reaction and an antigen-specific acquired immune response. The innate response involves the stimulation of NK (natural killer) cells and macrophages and the release of inflammatory cytokines, including type I and type II interferons. The antigen specific immune reaction, on the other hand, relies on the production of antigen-specific antibodies (a humoral response) and the activation of CD8 lymphocytes (the cytotoxic T cell response). Any viral infection, to some degree, overcomes these host mechanisms. In general, viral evasion of the host immune system can be accomplished in the following ways:

1) Compromise the immune system directly – infecting lymphocytes and antigen presenting cells (like HIV-1)
2) Avoid recognition by infecting immunologically privileged sites or remaining latent
3) Interfering with antigen presentation

4) Modulating the immune response by producing viral cytokine-like or cytokine receptor-like proteins (like poxviruses)
5) Immune exhaustion causing selective deletion of virus-specific CD8 cytotoxic T lymphocytes (CTLs)
6) Emergence of variants that escape antigen recognition
7) Modulate the anti-viral effects of type I interferon

A significant factor in the success of HCV to evade the immune system is the emergence of mutants that escape the adaptive CTL response. Viral mutants can escape immune recognition if critical viral epitopes (such as HVR) deviate significantly from the original infecting virus [48, 49]. In the early phase of infection, if mutant viruses arise that can avoid the dominant CTL response, chronic infection is greatly enhanced because this period is when the virus is most susceptible to elimination. Early host responses to HVR1 correlate with early control of the infection and an increased likelihood of an acute, self-limiting infection [50].

Beyond antigenic variation, certain viral genotypes have been shown to have an intrinsic insensitivity to the antiviral effects of inflammatory cytokines. Clinically, this is apparent in patients with type 1 genotypes who do not clear the HCV virus despite being treated with interferon-α (a type I interferon used to treat HCV infections, as discussed in a later section). Resistance correlates with NS5A protein which bears what is classified as an IFN sensitivity-determining region (ISDR), conferring IFN resistance. Recent *in vitro* studies have suggested that the ISDR can bind and suppress IFN-sensitive protein kinase, an enzyme that mediates the antiviral activity of type I IFN [51].

HCV persistence is not for lack of a demonstrable host immune response. When examining chronic HCV infections, there is evidence of histologic inflammation, increased major histocompatibility complex class I expression and the presence of virus-specific T cells. There have been proposals that HCV has low viral protein expression and immunogenicity. This would allow the virus to establish a chronic, slow-brewing

infection that would not elicit a strong, clearing, immune response. To this point, over-expression of HCV core expression *in vitro* sensitizes cells to tumor necrosis factor (TNF) leading to cell death by apoptosis [52]. Although the importance of this has not been established *in vivo*, a 'slow' infection may reduce the overall immunogenicity of the infecting virus, promoting the establishment of HCV infection. HCV titres, virus-specific CTL responses, and intra-hepatic viral expression are typically low in comparison to other viruses, such as influenza.

Current evidence highlights the significance of the CTL response in control of HCV. The CTL response may be insufficient to clear the virus, but is enough to establish a level of control as demonstrated by the inverse correlation between viral titres and CTL response. Certainly early in the course of the infection, a strong T cell response is required for clearance. When comparing self-limiting infections with chronic infections, patients who were able to clear the virus early as indicated by normal serum ALT levels and negative HCV RNA screens, did so with demonstrably higher T cell responses. In contrast, persistent liver inflammation correlated with high viremia and weak T cell responses [53].

CD4 responses are important in stimulating and maintaining antiviral immune responses (see Fig. 1.4). As helper T cells, they support antibody production by B cells and prime CD8 T cells for cytotoxic action. The cytokine milieu to which a T-helper cell is exposed determines the differentiation of a TH0 cell (an uncommitted T helper cell) into either a TH1 type cell (for priming and maintaining a cellular response) or a TH2 type cell (for stimulating a humoral response). The intra-hepatic mRNA in chronic HCV infections shows up-regulated levels of IL-2 and IFN-γ, critical cytokines for promoting TH1 development. Paradoxically, this TH1 response may contribute to tissue damage, and promote a chronic HCV infection [54]. In an HBV transgenic mouse model, the cytokines associated with TH1-skewed immunity were important factors in the development of progressive liver injury and hepatocyte destruction. Enhanced TH1 type cytokine expression in these cases may reflect a futile cellular immune response that ultimately leads to direct hepatic injury.

There is as yet no clearly identified humoral or cellular mechanism conferring protective immunity against HCV (general mechanisms of immunity are summarized in Fig. 1.4). Another difference in immune responses characteristic of acute infections and chronic infections is the predominant viral epitope recognized by responding T-cells. In a study involving fourteen patients with acute hepatitis C and sixty-five patients with chronic Hepatitis C (anti-HCV antibody positive and HCV RNA positive), peripheral blood mononuclear cells (PBMC) from each patient were stimulated with different viral proteins. Those acute patients who were able to clear the virus had developed a strong response to the NS3 protein.

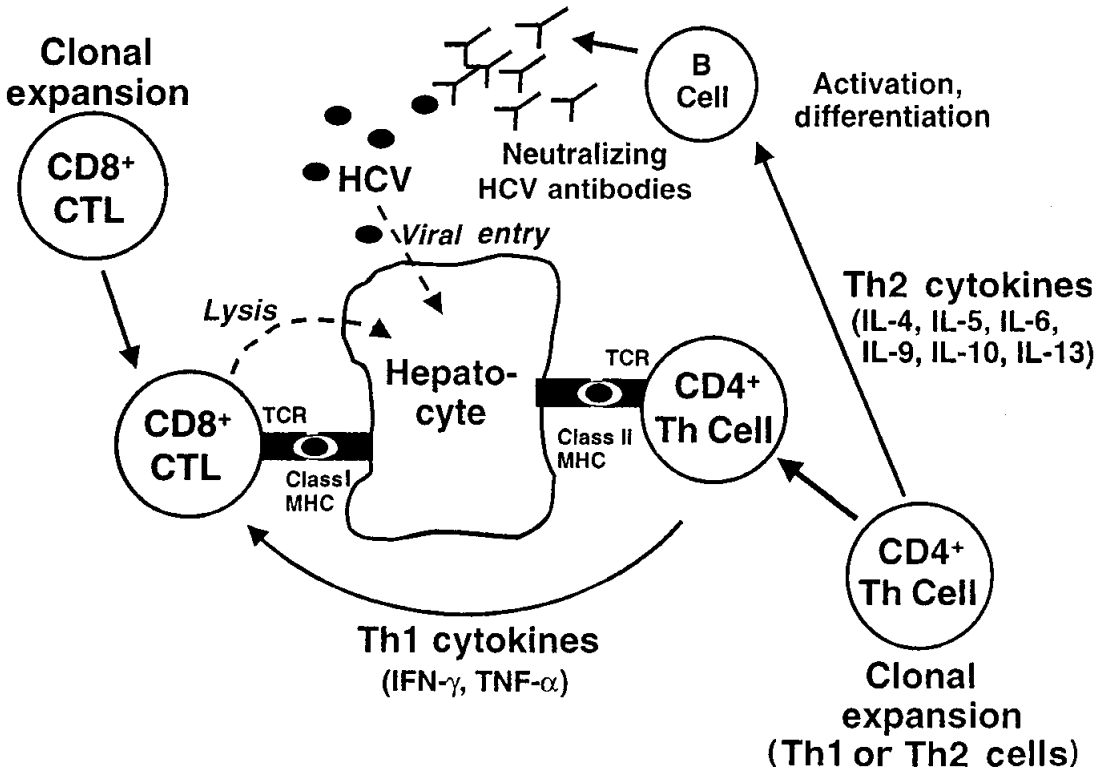

Figure 1.4. Components of anti-HCV immune response [55].

Those patients proceeding to chronic hepatitis had a higher trend of response to the HCV core proteins. When the sixty-five chronic patients were tested, their PBMCs had a negative response to NS3 proteins [56]. Although a larger study was required, this data suggested that a T-cell response to the NS3 protein is important in viral elimination. Because of this information, NS3 may well be an important target in the future development of vaccinations and therapeutic drugs.

Innate immunity in HCV infection may also be key in determining the outcome of the infection, but the HCV virus has adapted many mechanisms to evade this immune response.

Interferons are synthesized and secreted by mammalian cells in response to viral infections. Adjacent cells recognize the secreted interferons and subsequently express antiviral proteins, thus allowing the host to slow the rate of viral replication and the implication of an adapted immune response.

The detection of double stranded RNA molecules initiates a signaling cascade to initiate type I interferon gene expression. Both interferon alpha and beta bind to a common receptor (type I interferon receptor) which is present on almost all host cells. Once binding occurs, the interferon signaling pathway is activated resulting in the transcription of antiviral proteins and cellular protective mechanisms. [57]

Interferon regulatory factors (IRF) are key in the activation of type I interferons. The engagement of Toll-like receptors can stimulate the activation of IRF, especially IRF-3. [58] Activation of IRF 3 results in suppression of HCV replication. [59] Toll-like receptors (TLR) are employed by immune cells to recognize specific pathogenic patterns. In the case of HCV, a number of different toll-like receptors are activated in the presence of virus. TLR3 recognizes double stranded RNA, and TLR7/8 recognizes single-stranded RNA, both which are both present during the HCV life cycle. TLR2 is activated by the HCV core protein. [60] Upon activation, TLRs induce pathways resulting in the production of proinflammatory cytokines, including TNF-α. Interestingly, part of the pathogenesis of HCV is the

ability of the NS3/NS4A protease to disrupt TLR3 signalling and thus inhibiting the expression of anti-viral type I interferons. [61]

HCV Treatment

Current treatments of HCV are limited. Successful treatments are slow in developing because the virus has only recently been characterized, *in vitro* cell culture systems propagate the virus poorly, and reliable animal models of HCV infection are only now in development. There are currently two approved therapeutic agents available in the treatment of HCV, interferon-α and ribavirin. As single agents, they have suboptimal effects on HCV, but have synergistic effect when used together. However, the response rate to current regiments is not ideal, and there is a need for continued development of more efficacious anti-HCV agents.

Interferon-alpha. Interferons are well-studied cytokines known for their anti-viral, anti-proliferative and immunomodulatory effects. In 1957, Issacs and Lindenman first reported the anti-viral effects of interferons. Since then, there have been three interferons discovered which are differentiated by their structural, biochemical and antigenic properties. The type I interferons include interferon-alpha (IFNα), produced by monocytes and transformed B cells when stimulated by the presence of viruses and other antigens, and beta-interferon (IFNβ), produced by fibroblasts in response to double-stranded RNA, polyribonucleotides and viruses. TH1 cells (primarily) secrete the type II, or gamma, interferon (IFNγ) in response to a number of different mitogens and antigens. In contrast to the other IFNs, IFNγ has more immunoregulatory than antiviral properties [62].

IFNα has been widely used in the treatment of viral hepatitis, especially hepatitis B. Thus it was a logical choice in the treatment of chronic hepatitis C. The first randomized, multi-centre study on the effects of IFNα in HCV was undertaken by Davis *et al.* [64]. The design of the study involved three arms. One

group was given 3 million units of IFNα subcutaneously three times a week for twenty-four weeks. A second group was given 1 million units, and a third group was untreated. There was a significant difference between the patients treated with IFNα and those receiving no treatment. There was also a dose-response, as a dosage of 3 million units was more effective in normalizing serum ALT and maintaining a sustained remission. Histologically, there was an improvement in the lobular and periportal inflammation in the patients treated with 3 million units. A number of other investigators have substantiated the effect of IFNα and have demonstrated a dose response up to 6 million units per administration of recombinant IFNα [63-65]. However, with increasing doses of IFNα, deleterious side effects were pronounced.

There are two proposed mechanisms for the action of IFNα on HCV. The first is the classical inhibition of viral replication through cellular processes that contribute to the induction of the non-specific antiviral state [68]. The second process involves a multifunctional immunomodulatory role, in which the expression of class I and class II major histocompatibility complex (MHC) molecules are induced, cytotoxic T cells are activated, and macrophages are stimulated to release inflammatory cytokines.

Although IFNα as a single agent has efficacy in treating chronic hepatitis C, the overall rate of patient responsiveness is dismally low at only 22%. When a patient responds to initial treatment with IFNα, success rates are high (80%) in terms of a sustained response (by biochemical parameters) and reduced viral load. Independent predictors of a successful treatment include age, presence of cirrhosis on liver biopsy, titre of anti-HCV core IgM, and virologic parameters such as viral load, HCV genotype and the genetic heterogeneity of HVR1 [66, 67]. In retrospective comparisons of viral hepatitis, moderate to severe chronic active hepatitis responded better to IFNα compared to mild disease, but follow-up cases favor milder disease as a favorable prognosis. It was also found that a shorter duration of disease (acute hepatitis) correlates with increased responsiveness to interferon. This is likely due to the correlation

between shorter disease duration and less histological damage and cirrhosis. Cirrhotics as a group generally have a poor response to interferon compared to any other histological group mainly due to complications from the side effects of treatment [63, 66]. Again, the development of multiple quasispecies is problematic. The virus species least susceptible to IFNα action has the greater chance of persisting. Genotypes of HCV that are more resistant to IFNα treatment include types 1a and 1b. When HCV type1b was studied, the IFN sensitivity-determining region in NS5A was characterized [25]. This 40 amino acid stretch in the NS5A gene is highly variable among quasispecies, but for three conserved serine residues [51]. NS5A is an integral part of the HCV polymerase complex and phosphorylation of these serine residues is important for the core function of the protein. Synonymous mutations in this region increase the diversity of the mutants but do not alter the viral replication kinetics.

Recently, there have been changes in the modes of administration as well as the interferon compound. The current standard therapy uses a combination of pegylated IFNα and ribavirin [71]. Pegylated interferon is an IFNα molecule with a polyethylene glycol moiety attached. This improves the pharmocokinetics of interferon by endowing a longer half-life. In two large randomized control clinical trials comparing interferon plus ribavirin versus pegylated interferon plus ribavirin [72, 73], there is a higher sustained virological response with the latter. Genotype 1 HCV infected patients have a 40% response rate and genotype 2 and 3 have 80% response rates. There are two forms of pegylated interferon, α2a and α2b. Although there have been no direct trials comparing the two, both forms have similar virologic response rates. Current recommendations use 1-1.5 μg/kg weekly injections of pegylated interferon in combination with ribavirin as first line therapy.

Ribavirin. Ribavirin (1-β-ribofuranosyl-1, 2, 4-triazole-3-carboxamide) is an antiviral agent used widely since 1986, for the treatment of respiratory syncytial virus (RSV) in children. In addition, it has been used in the treatment of other viral illnesses including influenza, Lassa fever virus, Hanta virus and

human immunodeficiency virus (HIV). In the treatment of HCV, ribavirin is used as a synergistic agent with interferon-α.

The mechanism of action of ribavirin is unclear, with different modes proposed. One hypothesis is that this agent suppresses the synthesis of viral nucleic acid by decreasing the intracellular availability of guanosine triphosphate. An alternative hypothesis is that ribavirin treatment results in the synthesis of viral RNA with an abnormal or absent 5'cap structure. These abnormal RNA products result in inefficient translation of viral transcripts (there is no 5' cap structure in HCV, however). A third proposed mechanism of action is the suppression of the viral polymerase. There is no consensus on the mechanisms of ribavirin and the above hypotheses may not be mutually exclusive [68]. In the early 1990's, ribavirin was used in the treatment of chronic HCV with modest result on viral load and replication. Pilot studies looked at the effectiveness in combination with IFNα and found a synergistic response when both agents were used combined. The Swedish Study Group in 1998 designed a randomized, double-blind, placebo-controlled trial of interferon-α2b with and without ribavirin in chronic hepatitis C infection. There were one hundred patients enrolled who were assigned to receive subcutaneous interferon-α2b three million units three times a week with either ribavirin or a placebo for twenty-four weeks. The results showed that in follow-up there was a significant difference between the control and experimental groups. At week forty-eight, 21/46 patients receiving IFNα and ribavirin had negative HCV RNA levels compared to 10/48 in the placebo group ($p=0.02$). Similar to treatments with interferon alone, the genotype of the HCV virus seemed predictive of long-term outcomes. Even though ribavirin improved the overall response rate of IFNα in this study, there was a significant number of patients unresponsive to treatment [69]. When patients with IFN-resistant hepatitis C are studied alone, there was no significant improvement with ribavirin [70]. Thus, one can conclude that ribavirin is synergistic in cases where IFNα is effective, but does not enhance interferon effects in non-responders. The need for alternative antivirals continues to be addressed.

***Prospective treatments for HCV infection*.** The proportion of patients non-responsive to IFNα and ribavirin is high, so alternative antivirals are required. In recent years, there has been an explosion of information and *in vitro* developments on potential anti-HCV agents. Many of these agents are in pre-clinical trials and clinical trials. Much research is focused on directly inhibiting proteins of HCV.

NS3 is an important serine protease in the processing of the viral polyprotein. NS3 is likely to be essential for the production of active HCV proteins. Potential mechanisms for inhibiting NS3 are by (i) blocking the enzyme cleavage site with competitive inhibitors, (ii) allosteric interference or (iii) inhibition of interactions with essential co-factors (NS2B for NS3 of yellow fever virus and NS4A for HCV). Inhibition of NS2B/NS3 protease activity by mutagenesis crippled yellow fever virus replication [74]. Kakiuchi *et al.* have published a study of over two thousand nonpeptide, low-molecular weight compounds randomly selected from a chemical library [75]. These agents were thought to have inhibitory action against HCV NS3. Because they are small and non-peptide, they have the advantage of being less susceptible to hydrolysis and degradation. One compound was determined to be a competitive inhibitor and reduced the activity of NS3 by fifty percent. Two other compounds that were specific non-competitive inhibitors for HCV NS3 (did not bind to the substrate cleavage site) reduced activity but not as efficiently. Other groups have investigated antibody fragments targeted to NS3 as well as anti-sense RNA [71], demonstrating some efficacy for these agents as antivirals. Confirmation of this efficacy in *in vivo* HCV infection models is required.

Indicative of the excitement and challenges in developing prospective new treatments for HCV infection was the recent experience with HCV NS3/4A protease inhibitor BILN 2061. Initially described by Lamarre *et al*. in 2003, BILN 2061 is a competitive inhibitor that was developed using the Huh7 hepatoma line with a subgenomic HCV type 1b NS2-NS5b replicon. It is most potent against the 1a and 1b genotype forms of NS3 [72]. In *in vitro* studies with the HCV replicon system, BILN 2061 blocked NS3 mediated processing of the viral polyprotein. Subsequently, in a phase I, randomized control, proof

of concept study, patients treated with BILN 2061 showed reduced viral titres of 2-3 logs in 24-28 hours after administration (25-200mg twice a day for two days). When used in conjunction with IFNα, BILN 2061 acted synergistically in replicon cells leading to greater than a three log decrease in viral loads [73]. Studies were also performed in genotype 2 and 3 patients. A similar protocol of 500mg of BILN 2061 was administered twice a day for two days. The effect of BILN 2061 was a 1-2 log decrease in 5 of 8 patients. The remaining patients were unresponsive. The affinity of BILN2061 to the non-genotype 1 NS3 protease is markedly decreased, accounting for the variable response in the genotype 2 and 3 patients. This experience revealed the difficulty in developing a specific anti- HCV agent due to the significant differences amongst viral genotypes [74]. Collectively, these studies showed significant promise in developing a cocktail of pharmacologic treatments for HCV, however others reported that at high doses of BILN2061, over a four week course treatment, cardiac toxicity was evident in rhesus monkeys [80]. Consequently, development of BILN 2061 was teminated.

Another NS3/4A protease inhibitor, VX-950 (telaprevir) was developed by VERTEX. First described in 2004, VX-950 is a small molecule selective protease inhibitor of NS3/4A and functions by covalently binding to the catalytic site of the protease. It has been shown to be effective in both the replicon systems as well as fetal hepatocytes infected with HCV positive sera. In the replicon system, VX-950 has been shown to be effective against BILN-2061 resistant mutations and vice versa. As well, in combination with IFN-α, VX-950 has eliminated the HCV virus in the replicon system [73, 75]. Recent phase Ib clinical trials have shown that treatment with oral VX-950 in genotype 1 patients can reduce viral loads by 3 logs in 2 days and after 14 days of treatment, an average decrease of viral titres by 4.4 logs [76]. Two current trials (Prove 1 in the USA and Prove II in Europe) are underway. Thus far, this oral drug has been well tolerated and has been used in combination with pegylated IFNα and ribavirin with no serious side effects [77].

The second protein targeted in anti-HCV therapy that seems promising is the viral RNA polymerase, NS5B. A number of different polymerase inhibitors have been developed and undergone further testing. During clinical trials, further use has been halted for significant side effects. These included a 2'-C-methylcytidine derivative, NM283 (Idenix), which was discontinued for gastrointestinal toxicity [86], and HCV-796 (Viropharma/Wyeth) which was discontinued for hepatoxicity. A 2'-C-methyladenosine derivative is still in development by Merck [87]. All had initially shown promise in reducing virus titres in animal models or replicon systems as reported at the 46th Interscience Conference on Antimicrobial Agents Chemotherapy in San Francisco (September 2006) (personal communication, N. Kneteman).

Another target is the RNA genome itself. In the 5'UTR lies an internal ribosome entry site (IRES) directly at or just upstream from the AUG start codon. A number of investigators are studying the interaction between ribosomes and the 5'UTR IRES because interruption of this process could block viral replication [71, 78], with little to no effect on host machinery. Ribozymes could also attack the viral genome with high specificity. These are compounds developed as a result of the discovery that RNA can have enzyme-like capabilities, like RNA splicing and cleavage. As therapeutic agents, ribozymes can be targeted to transcripts of viral proteins. Their specificity is defined by Watson-Crick basepairing. Once bound to their target, ribozymes destabilize the phosphate backbone, subsequently cleave the RNA, rendering the mRNA untranslatable and susceptible to degradation by ribonucleases [79]. Heptazyme is a ribozyme directed against the 5'UTR of the HCV genome. Developed in cultured cells, heptazyme was shown to inhibit HCV replication by 50 –90%. The target site is a conserved region amongst the HCV genotypes with potential effectiveness for most patients [79, 80]. Despite their specificity, their poor efficacy in recent clinical trials and adverse reactions in toxicity trials in chimpanzees, has hampered their development [81, 82].

Similarly, siRNA is a new treatment approach that exploits RNA interference. SiRNAs are 22-mer double stranded RNA fragments that were designed to target cleavage of the HCV RNAs at various

regions of the genome. Randall *et al*. first described the ability of siRNAS targeted to the core protein to decrease HCV RNA in replicon cells eight fold by 4 days and more than 98% of the cells had cleared the virus [83]. Targets of the HCV RNA that result in silencing replication of the virus are 5'- UTR, core, NS3, NS4B, NS5A and NS5B [84]. Strategies investigated in using combinations of siRNAs targeting different HCV RNA sequences as well as siRNAs directed at both positive and negative strands of viral RNA result in higher rates of clearance from the replicon system [85].

With the enormous diversity of new anti-HCV anti-virals being developed, it is becoming increasingly important to have a viable animal model available for testing these agents. The development to the clinical applicability of these antivirals can potentially be expedited with an effective model for trialing novel drugs.

Experimental HCV Infection Models

The development and testing of new anti-HCV therapies has been hindered by the lack of an HCV-susceptible cell culture system or a reliable, low-cost animal model. Unlike HBV, no natural liver cell lines support hepatitis C virus replication. No small animals are susceptible to infection. Chimpanzees can support HCV infection, but there are severe limitations to their use in therapy and vaccine development. Limitations are related to availability, high cost, housing and ethical issues.

Cell culture models. Prior to the development of the replicon system there has not been a robust, *in vitro* culture system to study the replicative cycle of HCV. Current cell culture systems include (i) primary hepatocytes or hepatocyte cell lines that can be infected, (ii) cultured cell lines that are transfected with cloned viral sequences and derivatives of these, (iii) replicon systems.

Attempts have been made to propagate HCV in cultured human hepatocytes and human cell lines through infection. While there was success in infecting human cells, viral titres and rates of viral replication were

low, and reproducibility was poor. Different immortalized cell lines (hepatocytes, and others) could propagate HCV, but again there was difficulty maintaining a persistent infection and reproducing results. While HCV RNA-transfected human Huh7 hepatoma cells could propagate virus, greater success was achieved in a cell assay system reported by Moradpour *et al*. in which U-2 OS human osteosarcoma cells were made transgenic for HCV (UHCV cell line) [97]. This cell line supported high levels of the entire HCV open reading frame and reliably produced high viral titres. The viral transgene was the HCV ORF that was under the control of a tetracycline promoter, such that adjusting levels of tetracycline could regulate the steady state level of expression over a broad range.

A breakthrough in HCV *in vitro* systems was the replicon system first described by Lohmann *et al*. These systems were developed in Huh7 hepatocyte cell lines upon transfection with a cloned viral genome. Initially, the RNA replicon consisted of the 5' untranslated region (5' UTR) of HCV 1b, a neomycin resistance gene (NEO) for selection, an internal ribosomal entry site (IRES) from encephalomyocarditis virus, the nonstructural genes of HCV, and then the 3' UTR of HCV. Thus, the selective marker NEO was under the control of the HCV IRES and the HCV nonstructural proteins, including the viral replicase, was under the control of the ECMV IRES. The HCV replicon Huh7 cells were positively selected in the presence of neomycin sulfate (G418). Cells with high amplification of the replicon survived, having selected for robust replication of the HCV RNA genome by virtue of the co-transcribed NEO gene [86, 87]. Since its development, mutations adaptively introduced into the HCV RNA have improved the efficiency of replication [87].

In 2003, Kato *et al*. described the JFH-1 *in vitro* system for modeling HCV replication [100]. Using the same principles as the replicon system, Huh-7 cells were transfected with a library of NS3 to NS5b fragments isolated from the total RNA isolated of HCV infected livers. The JFH-1 clone was isolated from a patient with fulminant hepatitis C with genotype 2. The replicon from this clone had unprecedented replicative capacity and did not require any adaptive mutations. The inherent replicative

ability of this clone is unclear and could be due to the source coming from a fulminant hepatitis and having natural occurring adaptive mutations within the genome [101]. The replicon system and in particular the JFH-1 clone have been important *in vivo* HCV models for developing and testing anti-virals as well as studying the replicative molecular biology of HCV.

Animal models: the chimpanzee. In the late 1970s and early 1980s, four groups independently transmitted non-A non-B hepatitis from human into chimpanzees. Furthermore, they were able to demonstrate serial transmission from one infected primate to another. Chimpanzees develop an acute andchronic course similar to human infections. The primary infection closely parallels that in humans with an acute phase, development of the host response and long-term sequelae of the HCV infection [1, 16]. Chimpanzees often develop an early viremia (usually less than seven days) and an acute hepatitis phase that is milder to that in humans. Similar to humans, the majority of chimpanzees become chronically infected, with persistent viremia and a mild hepatitis but not necessarily end-stage liver disease as in humans [88]. The chimpanzee, though an excellent model for HCV infection, for reasons already discussed, is not widely used. However, experimentation in chimpanzees did permit a biological characterization of the virus and the entire sequence of the HCV genome was cloned from animal sera. During this early period in HCV research, limited studies in chimpanzees was an indispensable tool to our understanding of HCV biology and disease.

Transgenic and in vivo gene transfer HCV rodent models. To develop a small animal model, various methods were used to introduce the HCV genome into mice and rats. Takahara *et al*. used retrograde biliary injection of liposomes containing HCV cDNA to establish a viral infection in rat livers [103]. Expression of HCV RNA in the rat livers was confirmed with a reverse transcriptase-PCR analysis two days post-liposome injection. However, the existence of HCV transcripts and proteins was temporary and by a week post-transfection, HCV was no longer detectable.

Making mice transgenic for one or more HCV genes was a successful means to establish, or model, chronic viral gene expression *in vivo*. Transgenic mice were not infectious nor did they normally mount an antiviral response, but they did model important aspects of polypeptide processing and they revealed the inherent pathogenicity of viral proteins. Mice transgenic for the structural proteins of HCV failed to develop a histologically significant change (i.e. evidence of liver disease), indicating a non-cytopathic model [89]. However, when Moriya *et al*. developed transgenic mice containing the HCV core gene, histologic changes in the livers were observed [105]. Hepatic steatosis is observed in chronic HCV infections and was observed in these transgenic mice, replicating an pathological process seen in human disease. Similarly, mice transgenic for the entire 1b genotype of HCV developed liver disease coincident with lymphocytic infiltration [106]. Although not an infectious model (because murine cells can't be infected and the 5' UTR of HCV was replaced by a heterologous promoter), HCV transgenic mice represented a significant step towards understanding the pathogenesis and function of HCV genes/proteins in an *in vivo* context. In an attempt to create an infectious mouse, a putative human receptor, CD81, was expressed in multiple tissues of transgenic mice [107]. These mice could not be infected using HCV-positive chimpanzee serum, despite evidence of HCV glycoprotein binding to transgenic murine cells.

***Infectious human/mouse chimeric models*.** Making a small animal model of HCV infection that would not only sustain infection but replicate immunity and immune-related disease dictated humanizing rodents with susceptible human liver tissue and a human immune system. Homozygous SCID (severe combined immune deficient) mice lack functioning B and T cell lymphocytes (due to a mutation in the recombinase enzyme necessary for maturation of B and T cell receptors) lack an effective immune system and thus are capable of supporting xenografts. Early attempts at humanizing SCID models involved engrafting portions of human liver under the skin or intraperitoneally [108,109]. The engrafted liver was either healthy or HCV-infected. HCV-infected adult human liver fragments tolerated in SCID mice showed

proliferation of bile ductules and ductular epithelium. Ten weeks post-engraftment, the transplanted tissue remained HCV positive by PCR and was also positive for negative-stranded RNA for HCV by in situ hybridization – an indication of active viral replication. Maeda *et al.* demonstrated that transplanted uninfected human livers could be infected with HCV by injecting the mice with human HCV-positive serum [109]. HCV RNA, indicative of virus propagation, was detected in these liver grafts.

A trimera model, reported by Ilan *et al*., was developed as an infection model for HBV and HCV [110,111]. In this model, CB6F1 mice were lethally irradiated, radio-protected by reconstitution with SCID mouse bone marrow, and then engrafted with HCV-infected human liver. In 85% of the animals, there was histologic evidence of engraftment and evidence of negative strand RNA (via RT-PCR) in the implanted tissue. However, the grafts were only sustained over the short term and virus titres were low, limiting the models usefulness [90].

Dandri *et al.* described an immunodeficient model based on the RAG-2 mouse [91, 92]. RAG-2 mice have non-functional T and B cells. Similar to the SCID mice, the defect in these animals causes a disruption of enzymes required in the functional rearrangement of IgG and TCR and subsequently the maturation of B and T cells. In SCID mice the defect is a point mutation in the DNA dependent protein kinase that is responsible for rejoining the DNA in immunoglobulin (Ig) and TCR rearrangement. The RAG-2 mouse on the other hand is a null mutation of the enzyme recombinase-activating gene-2 leading once again to a defect in the TCR and Ig rearrangement. The RAG-2 mouse in their model system was a hemizygous transgenic for urokinase-type plasminogen activator, whose expression was directed to the liver (linked to the albumin promoter) to poison endogenous murine hepatocytes [113]. These animals were capable of accepting human hepatocytes that would repopulate the murine liver, and these human hepatocytes were capable of sustaining an HBV infection [92]. A successful propagation of HCV in this system has not been reported.

Mercer *et al.* was the first group to report an HCV mouse model based on an immunodeficient mouse bearing the uPA transgene [114]. The transgene in this model, different than the RAG-2 mouse, was homozygous. This Alb-uPA/SCID/Bg mouse developed a form of subacute liver failure. This resulted in a profound stimulus for hepatocyte proliferation that endogenous resident mouse cells are unable to effect. These animals were "rescued" by transplanting human hepatocytes, isolated from patients undergoing liver resections, through intrasplenic injections. Transplanted human cells replicated prolifically in the mouse liver and were able to support an HCV infection. Mice were infected by intraperitoneal or intravenous injection of human HCV-positive serum. Virus titres rose over time and negative-strand HCV RNA was detected in liver tissue, indicative of an active infectious process. Since this report, HCV infections have been established in mice by two other groups using similar model systems [115, 116].

Part B: Liver Regeneration and Hepatocyte Growth Factor

A damaged liver naturally repairs itself through regenerative processes. Relevant to the body of work presented here, these processes support the reconstitution of Alb-uPA/SCID mice [114] with human liver grafts. By understanding and exploiting these processes, it may be possible experimentally to enhance the repopulation and long-term survival of human grafts in order to improve this model of HCV infection.

In mammals, the liver has a unique capability among organs to regenerate and restore function by the generation of new parenchymal cells. The cellular players in liver regeneration are the adult hepatocyte and the resident stem cell. If no liver injury were to occur in the life-cycle of the hepatocyte, this cell would undergo one or two mitoses in its lifespan. In the face of tissue loss (partial hepatectomy, transplantation, or pathological change), the hepatocyte has the ability to regain the ability to proliferate to replace the lost hepatic parenchyma. The hepatocyte responds to a complex network of stimulatory factors, with negative and positive feedback mechanisms, to maintain tissue homeostasis.

Cellular Mechanisms of Regeneration

In hepatocyte regeneration, the liver cell makes a transition from a resting stage (with its inherent blocks to cell cycling) to replication and mitosis. This process follows a general pattern in which the resting hepatocyte enters a pre-replicative phase that may be subdivided into priming and progression components. At the time of insult, priming is initiated and lasts for four to six hours. At this time, the cell progresses from the G_0 (resting) to the G_1 stage [93, 94]. The inciting signal involves a change in membrane potential of the hepatocyte that induces metabolic changes within the cell [95]. Together with signals from extracellular mitogenic factors, the expressions of proto-oncogenes within the hepatocyte are induced. The proto-oncogenes involved in the priming stage include *c-fos*, *c-jun* and *c-myc* (Figure 1.5, immediate-early genes).

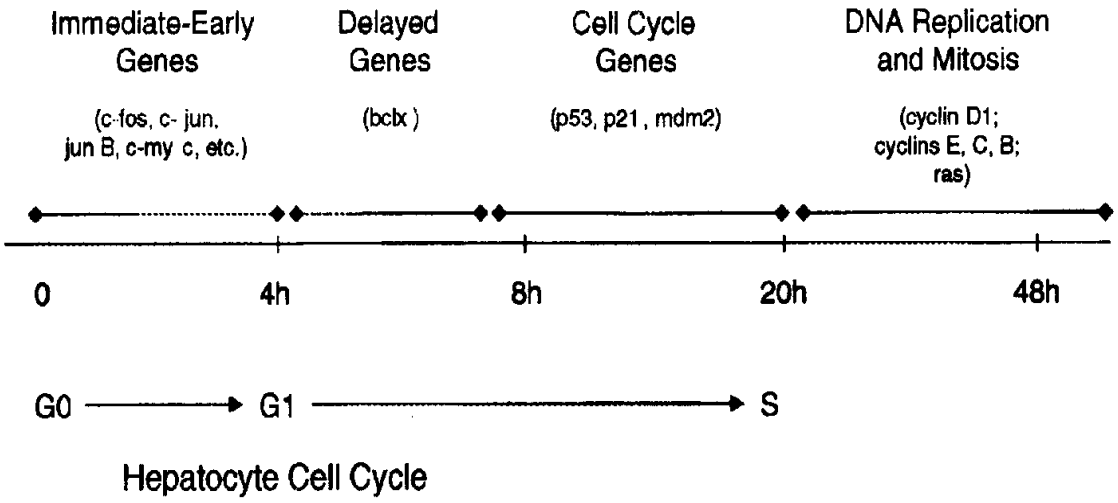

Figure 1.5. Sequence of gene activation in liver regeneration [96].

The chronologic integration of injury, mitogenic stimuli, and proto-oncogene expression are important in the regenerative process [97]. *C-fos* and *c-jun* expression are detectable within 10 minutes of partial hepatectomy in rats and return to basal levels within two hours. *C-myc* expression peaks at two hours and falls off by four hours. Up to seventy other immediate-early proto-oncogenes have been identified that play a regulatory role in hepatic regeneration following partial hepatectomy [97]. One, the *c-met* proto-oncogene, encodes the cell receptor for hepatocyte growth factor (HGF), a central player in liver regeneration. Delayed-early gene expression marks the progression stage (G_1 to M) of pre-replication. In this stage, growth genes (*ras*, *mdm2*, *bcl-2*) are expressed whose products block negative cell cycle regulators (p53, p21). This signals progression beyond G1 into DNA synthesis, or S phase. Promoted by the effect of local growth factors, the hepatocyte proceeds through the complete cell cycle [98].

For the liver to restore its normal size and return to full function, multiple cycles of replication may need to occur. As more hepatocytes are produced, they are organized into a histologically and anatomically

correct architecture. Initially, as the liver regenerates, there is one-sixth the normal hepatocyte-to-hepatocyte contact. This is a stimulus for growth. With reduced cellular contact, gap junction proteins and cell adhesion molecules decrease, favoring proliferation. Hepatocytes become more responsive to growth stimuli through an increased expression of HGF receptors. When greater cell densities are achieved and cellular interactions normalize, a plasma protein known as cell surface modulator (CSM) senses this and hepatocyte responsiveness to mitogenic stimuli is reduced. Tissue organization is then favored.

Hepatic Growth Factors

Hepatic growth factors can be complete mitogens, acting independently to stimulate hepatic regeneration, or co-mitogens in that they require serum co-factors to stimulate cell growth. Some known factors are briefly discussed below.

Insulin and Glucagon. Neither insulin nor glucagon has the ability to stimulate hepatocyte proliferation *in vitro*. However, insulin has been established as an effective co-mitogen in liver regeneration [94, 99]. Insulin and glucagon have synergistic actions in promoting mitotic amplification, DNA synthesis and increased liver weight. This synergy in not well understood, but it is postulated that glucagon increases the number of insulin receptors, which enhances the effect of insulin [124, 125]. These hormones may induce particular proto-oncogenes and/or specific growth factors in a fashion resembling that typically observed in hepatocyte regeneration [100].

Insulin-like growth factors. Insulin-like growth factor I (IGF-I or somatomedin C) and IGF-II (somatomedin A) are structurally related to insulin and share functions similar to the hormone. These cytokines promote cell division and differentiation in a number of different cell types including liver [101]. Kupffer cells and sinusoidal epithelial cells synthesize both IGFs and their receptors in the liver. IGF-II is normally expressed in the liver during fetal development whereas levels are virtually

undetectable in the adult liver. It is thought that the IGFs and their regulatory factors, IGF binding proteins (IGFBP), modulate hepatic regeneration. In partially hepatectomized rats, IGF, IGFBPs and IGF receptors are involved in a coordinated pattern of expression. IGF levels seem to decrease or stay constant, whereas the receptors and the binding proteins increase significantly. IGFBP increases rapidly within the first hour but declines thereafter within twenty-four hours. The receptors gradually increase over the first twenty-four hours and remain elevated for several days [102].

Other hormones. Adrenal cortical hormones induce hepatic enlargement, perhaps as a consequence of their influence on hypertrophy. Levels of cortical hormones increase post-hepatectomy, most likely due to stress. Cathecholamines and prolactin are two other hormones with positive effects on liver regeneration, but any role in the regulation of hepatic growth is unclear. Both parathyroid and thyroid hormone have also been found to enhance the metabolic activity related to liver regeneration. Their role is likely due to regulatory effects on vitamin D and calcium metabolism [103].

Metabolic and nutritional demands. Regenerating liver has high metabolic requirements and adequate energy supplies are required by the hepatocytes for DNA synthesis and mitotic activity. It has been demonstrated that protein depletion in rats results in decreases in DNA synthesis and mitotic activity [104]. Important elements required for DNA synthesis include Vitamin D and calcium. Both hypocalcemia and vitamin D deficiency independently reduce DNA synthesis [103].

Prostaglandins. Prostaglandins E2 and F2α are produced by nonparenchymal liver cells and provide paracrine stimulation to regenerating hepatocytes. Undetectable in normal conditions, the prostaglandins are measurable in the serum post-partial hepatectomy. Exogenous administration of PGE2 results in increased DNA synthesis, as demonstrated by tritiated-thymidine uptake in regenerating liver. Upregulated levels of PGE_2 are detectable in Kupffer cells post-partial hepatectomy [105].

Hepatocyte Growth Factor. Hepatocyte growth factor (HGF) was discovered in serum of partially-hepatectomized rabbits and in platelets of rats with regenerating livers. When its sequence and structure was determined, HGF was found to have significant homology to heparin binding growth factors [106]. HGF is a stimulant of many cell types and is two to three times more potent than either epidermal growth factor (EGF) or transforming growth factor-alpha (TGFα). HGF has actions not limited to hepatocyte regeneration (its mitogenic activity) such as being a liver motogen and morphogen. Further actions and the molecular biology will be discussed in a later section.

Epidermal Growth Factor (EGF). Although not as potent as HGF, EGF is still an effective mitogen. Like HGF, it has actions on a number of cell types. It is not derived from liver but produced in the Brunner's glands and salivary glands [107]. Experiments have shown that the removal of these glands lead to delayed liver regeneration. EGF's effects are enhanced and modulated by other hormones/factors. There is a definite sex-specific effect of EGF. Female hormones are less effective than male hormones in increasing expression of hepatic EGF receptors. *In vitro*, estrogen antagonizes binding of EGF, thus inhibiting hepatic regeneration. *In vivo*, however, estrogen has a definite hepatotrophic effect [108]. There is currently no explanation for this discrepancy. EGF is affected by norepinephrine, a co-mitogen. Norepinephrine appears to act by modulating the EGF receptor [107].

Transforming Growth Factor-Alpha. TGFα is a 50 amino acid peptide that has significant homology to the EGF superfamily. It shares the same receptor as EGF but with a four-fold lower affinity. TGF-α transcripts are up-regulated post-hepatectomy. Levels rise within 4 hours and peak at 22 hours, simultaneous with the peak of DNA synthesis. There is evidence that HGF is an early signal in the cell cycle and TGFα acts during progression through the cell cycle [109, 110]. Since TGFα is produced by

both hepatic stems cells and perisinusoidal cells, it is both an autocrine and a paracrine mediator of hepatocyte proliferation.

Heparin binding or acidic fibroblast growth factor. After partial hepatectomy, Heparin Binding Growth Factor (HBGF-1) expression is elevated, prior to the detection of TGFα mRNA. HBGF-1 levels peak at twenty-four hours and remain elevated for 4 days, normalizing after one week. HBGF-1 is produced from both hepatocytes and non-parenchymal cells. It is an important regulator of liver development and regeneration because of its resistance to the negative effects of TGFβ [111].

Growth Inhibitory Substances

Homeostatic control of liver regeneration involves proliferation inhibitory signals from factors such as TGFβ1. TGFβ1 is expressed in acute and chronic liver injury and apart from inhibitory effects on hepatocyte proliferation, it induces fibroblast proliferation and the accumulation of extracellular matrix [112]. Following partial hepatectomy, TGFβ1 expression in non-parenchymal cells increases, peaking around 72 hours [139]. This establishes a paracrine regulatory loop with TGFα that peaked two days earlier. TGFβ is also a potent inducer of apoptosis in primary hepatocytes and in regressing livers [140].

Other cytokines with inhibitory effects are IFNγ and IL-2. These up-regulate MHC class II expression on Kupffer cells (resident macrophages of the liver) and promote the presentation of hepatic antigens to infiltrating T cells, activating them to effect the immune elimination of hepatocytes. This proposed mechanism of action contrasts with the effects of the drugs FK-506 and cyclosporine which inhibit MHC class II antigen expression on Kupffer cells and promote hepatocyte proliferation [113]. IL-1β, IL-6 and hepatocyte proliferation inhibitor also suppress hepatocyte proliferation. IL-1β acts like TGFβ, depressing DNA synthesis but to a lesser extent.

IL-6 is a major inducer of acute phase reactant proteins, and via a mechanism that remains unclear, can inhibit the proliferation of mouse hepatocytes in primary culture [114, 115]. However, an early role for IL-6 in the regenerative process may be positive. Cressman *et al*., studying IL-6 and TNFα knockout mice, defined the following signaling cascade following partial hepatectomy: TNF →TNF-Rc → NFκ B → IL-6 → STAT 3. STAT3 is a transcription factor (signal tranduction and activators of transcription) in a family of nuclear factors that regulate cellular activation [143]. Figure 1.6 summarizes the players in the initiation of liver regeneration. The Kupffer cell is activated by inflammatory signals (TNFα, Toll-like receptor or TLR ligands, and complement components), releasing TNFα and IL-6. IL-6 acts on hepatocytes to induce growth-related genes, to replace cells sensed as damaged (and triggering the inflammatory response). This response is accentuated by the coincidental activation with SCF, or scatter cell factor, later called HGF. In contrast to this scenario, a persistent (or high) IL-6 presence may signal stress due to a chronic infection to which the organ responds by inhibiting hepatocyte growth to counteract the infection of hepatocytes.

Hepatic regeneration post injury is a complex, multifaceted process. However, an integral and potent factor appears to be hepatocyte growth factor (HGF). I expected that in the Alb-uPA/SCID/Bg model of hepatitis C infection, HGF would play an important role in the development of the human hepatocyte transplant. There is little data in the literature regarding the species specificity of HGF. Because the Alb-uPA model incorporates the transplantation of human hepatocytes into a murine background, there was the possibility that human HGF would be more mitogenic for the transplanted human cells than the endogenous murine HGF. One of the aims of this study was to incorporate human HGF into the Alb-uPA/SCID/Bg model to enhance transplantation success. The following sections elaborate on the actions of this factor.

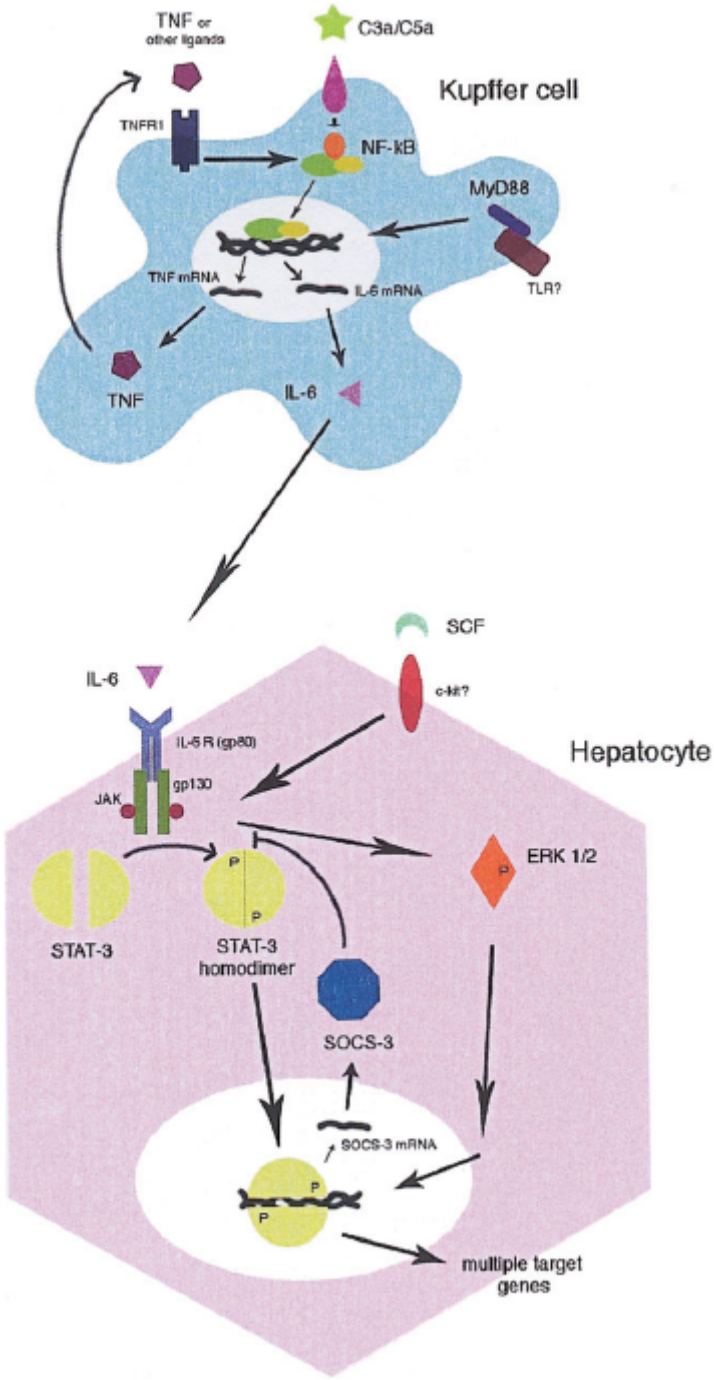

Figure 1.6. Initiation of liver regeneration [96]. SOCS-3, or suppressor of cytokine signaling, negatively regulates IL-6 effects. SCF, scatter cell factor, or HGF.

Hepatocyte Growth Factor (HGF)

The unique ability of liver to regenerate after injury fuels a search for the underlying mechanisms. Early in the 1960's it became evident that there were hepatotrophic factors that were largely responsible for potentiating/initiating these events. By the early 1980s, many research groups had discovered that the serum from hepatectomized rats could stimulate growth of adult rat hepatocytes in primary culture. Hepatocyte growth factor (HGF) was characterized by three independent investigators to have multiple points of action. Michalopoulos *et al*. identified HGF as a potent mitogen that markedly enhanced regeneration of the liver in rats and stimulated growth of primary hepatocytes in culture [116, 117]. At the time, they called it hematopoeitin A. Two other groups identified a potent hepatocyte mitogen from platelets of rats [118, 119] which, on further characterization, showed remarkable homology and similar properties to hematopoeitin A. They called their factor hepatocyte growth factor. A potent hepatocyte mitogen was isolated in patients with acute, fulminant liver failure [120, 121]. It was also called hepatocyte growth factor. As the above factors were purified and characterized, their identity was verified.

HGF: Molecular Structure

After the purification of HGF, the molecular structure and characteristics of the protein were rapidly identified. HGF is a 82kD protein that forms a single band on gel electrophoresis under non-reducing conditions. The glycoprotein precursor protein undergoes maturation, whereby the N-terminal pro-sequences are cleaved and a single intra-chain site is cleaved by a trypsin-like protease to form a heavy chain (α chain) and a light chain (β chain). A heterodimer of the α and β chain forms, covalently linked by a disulphide bridge [149]. The α-chain is approximately 69 kDa and corresponds to amino acids 55-494 of the pro-sequence [122-124]. The 54 amino acids preceding the α chain are divided into two domains. The first 29 amino acids are mainly hydrophobic and resemble a typical signal sequence. The following 25 amino acids constitute the pro-sequence. Its tertiary structure is composed of four

homologous triple-looped, 3-disulfide-bridge kringle structures (Fig. 1.7). The β chain encompasses amino acids 495 to 728.

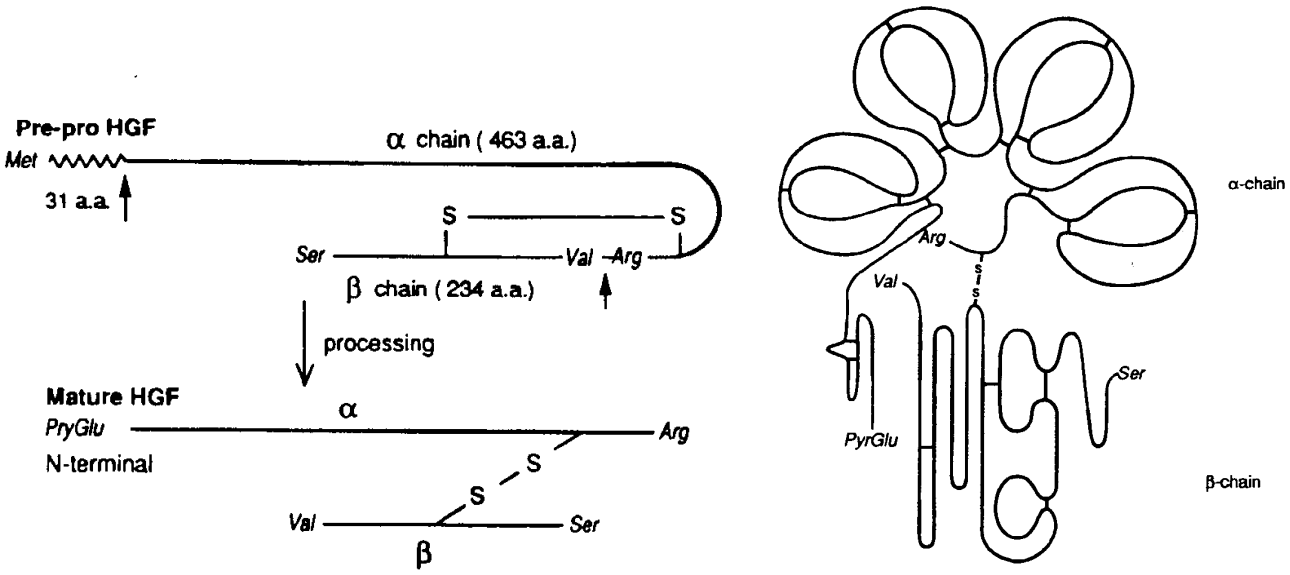

Figure 1.7. Processing of pre-pro HGF and tertiary structure of mature HGF [125].

HGF has no homology with other known growth factors. However, it does show considerable similarities with several proteins involved in fibrinolysis and blood coagulation factors (plasmin, prothrombin, tissue plasminogen activator and coagulation factor XII). In particular, the common structural feature is the kringle factors. Plasminogen and its activators contain five kringle structures which correlate with the protease function of the protein. HGF has not retained this activity because of altered key amino acids at the potential active site (replacement of His and Ser in plasminogen to Gln and Tyr in the β chain of HGF) [126]. In addition to its similarities to plasminogen, HGF also shares a heparin-binding affinity site with the fibroblast growth factor superfamily [118]. As yet, there is no direct evidence that HGF requires heparin binding for its basic biological activity as seen for basic FGF. There is also no evidence showing regulatory effects of proteoglycan interactions on HGF. Because of the presence of this binding site,

HGF or an HGF-like substance is sequestered in the extracellular matrix. This could potentially serve as a source of accessible growth factor, such that when HGF is required, it can be accessed from the extracellular milieu [127].

HGF: Genomic Organization

Circa 1990, several groups isolated the cDNA for hepatocyte growth factor from rat liver, human liver, human placenta, and human leukocytes [150, 152, 155, 156]. With the cDNA, human genomic libraries were screened and the gene found on chromosome seven. It consisted of eighteen exons. The 3'-untranslated region was about 3.6kb and contained a polyadenylation signal sequence, AATAAA. When comparing the hHGF cDNAs derived from placenta, leukocytes and liver, the liver-derived forms displayed differences in the 3' end [126]. This may indicate gene polymorphism or a form of transcriptional regulation (i.e. RNA editing) that exists in liver tissue.

When comparing the sequence of HGF and the Serps (serine proteases) involved in blood coagulation, there is significant homology in sequence/exon organization and functional domains of proteins. When examining Exon I, it contains the 5'-untranslated region as well as the signal peptide required for secretion. Exons II and II correspond to an area homologous for the pre-activation peptide of plasminogen. The kringle-containing peptides are encoded by the next eight exons. Typically, each kringle region is encoded by two exons. Exon XII is the region between the α- and β- chains. The remaining five exons encode a Serp-like domain analogous to the catalytic domain of plasminogen and tPA [126].

HGF: Evolutionary Conservation

When Liu *et al*. published the structural organization of the mouse HGF gene in 1994, significant similarities between mouse and human were identified. The mouse gene HGF is approximately 65 kb compared to the human gene of approximately 70 kb. Both genes had eighteen exons. The homology amongst the exons ranged from 69.5 to 99.3 percent homology [128, 129]. Between mouse, rat and

human HGF there is about ninety percent homology in the amino acid sequence. For example, in the α-chain, the first kringle amino acid sequence between mouse and rat, mouse and human, and rat and human are 100%, 97.5% and 97.5% identical. In addition, the promoter sequence for HGF amongst the different species is identical for 110 bp upstream of the TATA box [158]. Hence, it is assumed that rodent and human cytokines are functionally similar.

Regulation of HGF Activity

HGF is produced by Kupffer cells and peri-sinusoidal endothelial cells in the liver (Ito cells, or stellate cells) [130], endothelial cells in the kidney [131], and alveolar macrophages and endothelial cells in the lung [132]. Human embryonic fibroblasts in the lung also are capable of producing HGF. No HGF mRNA was found in parenchymal hepatocytes, renal tubular epithelial cells or epithelial cells of the lung [133]. Thus, it can be concluded that HGF is produced by cells of mesenchymal origin but not by epithelial cells.

Transcriptional regulation. There have been several potential transcriptional regulatory regions identified upstream of the basal HGF promoter. *In vivo* analysis in mice showed 0.7 to 0.1 kb upstream of the promoter region to be essential for transcriptional activity. Through deletion analysis of the HGF promoter in transgenic mice, Bell *et al.* were able to demonstrate that elements located between 0.1 and 2.7 kb upstream of the HGF transcriptional start site were responsible for tissue tropism and robust expression [134, 135].

A 30 base pair sequence identified at –260 to –230 of the promoter region has specific interactions with members of nuclear factor 1 (NF1) and upstream transcription factor 1 (USF1). NF1 suppresses HGF promoter activity whereas USF1 binding upregulated promoter activity. Following a partial hepatectomy, NF1 expression is down-regulated and binding to the HGF promoter region is decreased. In contrast, USF 1 expression is up-regulated and binding activity is increased [136].

Other *cis*-acting transcription factors that may impact HGF gene expression *in vivo* have been identified. An estrogen response element (5'-ggtcagaaagacc-3') is present at the –872 to –860 bp region. Binding of this site by a member of the steroid/thyroid hormone receptor superfamily virtually eliminated the basal expression level of the HGF gene. Estrogen, by contrast, induced the HGF promoter. In murine models, estradiol stimulated HGF promoter activity in tissues such as mammary glands and ovaries. In fibroblasts of the cell line NIH3T3, IL-6 and tumor necrosis factor-α were also capable of up-regulating the HGF promoter [135].

Post-translational processing. As noted, precursor HGF must be processing for activity. Pro-HGF can bind to the HGF receptor (*c-met* gene product) with high affinity, but is unable to activate it. Processing of the precursor may follow one of two pathways. Tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) can convert pro-HGF into α and β chain HGF in *in vitro* models. uPA and its receptor form a stable complex on the cell membrane of hepatocytes. Together, they are capable of cleaving the single chain HGF into the active two-chain HGF. Following partial hepatectomy, active uPA is presented almost immediately to cleave HGF. A regulatory feed back loop controls uPA activity. Activation of the HGF receptor in certain cells results in the down-regulation of uPA transcription. In addition, TGFβ also decreased uPA transcription. Certain growth factors activate uPA expression, such as HGF, EGF and TGFα [137]. The second pathway involves an activator which has yet to be fully characterized. When tissue damage occurs, epithelial cells produce a factor activated by thrombin that is able to process pro-HGF [138]. Recent findings indicate that epithelial cells secrete two potent inhibitors of this HGF activator: HAI-I and HAI-II. Under normal physiological conditions, the regulation of HGF through proteolytic processing may be important at stages in development and response to injury [139].

Heparan sulfate-mediated sequestration. To ensure that HGF acts locally, mechanisms are in place to sequester the cytokine to neighboring cells. Heparan sulfate proteoglycans (HSPGs) are present within the extracellular matrix and they have a high binding affinity for HGF, limiting HGF to the milieu proximal to the producing cells. HGF does not require binding by HSPGs for activation or for adequate

signaling between HGF and its receptor. Besides full length HGF, two naturally occurring truncated versions are also present. These truncated forms are designated NK1 and NK2, named by their site of truncation, i.e. after kringle 1 and kringle 2 respectively. NK1 and NK2 have been shown to be HGF antagonists in hepatocytes, but in the presence of heparin binding proteins, are partial agonists. This unique role that HSPGs have on truncated forms of HGF may be important in certain cell types [140].

HGF Receptor

HGF cellular effects are mediated by a receptor present on the surface of responding cells. Higuchi and Nakamura, in 1991, identified HGF binding sites on rat hepatocytes and on isolated rat liver plasma membranes [168]. On rat hepatocytes, there were over 500 binding sites per cell, with a K_d of 20 to 30 pM. The receptor was specific for HGF given the absence of competition between HGF and other growth factors. The receptor was not restricted to the liver, and was present in the lung, kidney, adrenal glands and pituitary. It was also expressed on a number of different epithelial cells: renal tubular epithelial cells, normal human keratinocytes and melanocytes [141].

The HGF receptor, a 220 kDa protein, was later discovered to be the product of the *c-met* proto-oncogene [142]. It has structural and functional similarities to the tyrosine kinase family of growth factor receptors. It is a transmembrane protein that is synthesized as a single polypeptide chain of 1436 amino acids. Like its ligand, it undergoes proteolytic cleavage, resulting in a two-chain heterodimer. The N-terminal α-chain is outside the membrane and the C-terminal β chain consists of an extramembrane domain, transmembrane domain and cytoplasmic protein kinase domain. The intracellular kinase domain shares 44 percent homology with the human insulin receptor. The extracellular, ligand-binding domain shows no apparent sequence homology with other receptors [143]. Upon binding HGF, a signaling cascade is triggered downstream of the receptor. The details of the pathways are not completely understood, but important players identified thus far include the Ras-mitogen-activated protein (indicative of a growth

response pathway), Ras-Rac/Rho and PI 3-kinase (motility pathway); and Gab1 and STAT3 (signal transducers and activator of transcription 3) (morphogenesis pathway) [144].

Activities of Hepatocyte Growth Factor

Hepatocyte growth factor has demonstrated itself to be a ubiquitous cytokine with multiple modes of action. This cytokine was actually identified as three other separate factors: lung fibroblast-derived growth factor, scatter factor, and tumor cytotoxic factor. As these other factors were characterized and their amino acid and genomic sequences determined, they were all determined to be hepatocyte growth factor. Thus, known functions of HGF now include: mitogenic/growth promotion, cellular migration, cell stimulation, growth inhibition, angiogenesis, and cell survival [131, 141, 145-150]. Clearly, HGF's effects on cell function are not limited to hepatocytes. HGF has growth stimulating effects on normal rabbit renal tubular epithelial cells, normal human epidermal melanocytes and keratinocytes and nontransformed epithelial cell lines. The potency of HGF is similar to both EGF in renal tubular epithelial cells and basic fibroblast growth factor in human keratinocytes [131, 141]. HGF also enhances colonies of both fetal and adult hematopoietic lineages including erythrocytes, macrophates, megakaryocytes and granulocytes [151]. As HGF's multiple functions were delineated, it was also found to be involved in hematopoeisis and angiogenesis. When administered in vivo, it had a profound effect on new blood vessel formation [152].

HGF has also been shown to significantly increase the motility of certain epithelial cells. 'Scatter factor', a factor secreted by MRC-5 fibroblasts, (line of human embryonic lung fibroblasts) was first purified by Stoker *et al*. [153]. It was later found to be produced by other fibroblasts from both human and murine origin. Scatter factor is not produced by epithelial cells, but enhances epithelial cell motility. When further purified, scatter factor in the mouse consisted of two subunits 57 kDa and 30 kDa. The 30kDa peptide was found to be very similar to the β-subunit of HGF. In addition, when scatter factor was added

to cultured rat hepatocytes, the former stimulated DNA synthesis in the rat cells. When antibodies were added to a milieu of HGF and hepatocytes, the stimulatory effects of HGF on DNA synthesis were blocked. Since immunologically and biologically, the two factors acted similarly, HGF and scatter factor were considered the same entity [153].

The growth of certain cell types are inhibited HGF. Several tumor cell lines including B6/F1 melanoma and HepG2 hepatoma cells are inhibited in the presence of HGF. Hepatocellular carcinoma cells with transfected HGF vectors produced tumors only 10% the size of controls when these cells were transplanted into nude mice [182]. This anti-tumor effect, especially in hepatoma cells, could have a substantial impact in future treatments and therapies for liver carcinomas.

The functions of HGF can be summarized in two broad categories. The first is epithelial-mesenchymal interactions and development, and the second is tumor-stromal interactions. As a paracrine mediator, HGF serves as an important mediator in normal tissue induction (mitogenic activity), organogenesis and morphogenesis of various tissues. The coupling of HGF from mesenchymal cells and the *c-met* receptor on epithelial cells promotes proliferation of developing tissue because of effects on stimulation of DNA synthesis, proliferation of developing cell populations, and migration of cells to their final destination (i.e. in developing limbs, branchial arches, lung bud formation). In tumor-stromal interactions, HGF's effects on cell growth, motility and angiogenesis have implicated it as a significant player in growth, invasion and metastasis of tumor cells.

HGF in Liver Disease

A number of groups have reported that HGF protects liver tissue from injury. Fulminant hepatitis can be induced in mice by treatment with α-naphtylisothiocyanate. HGF protected animals from this injury [154]. Mice treated with D-GaIN and bacterial lipopolysaccharide (LPS), display significant hepatocyte apoptosis and animals so treated die of hepatic failure. When animals were given HGF pre- and post-administration of the toxin, hepatocyte apoptosis was reduced and 75% of the mice survived [184]. Two

mechanisms were proposed to explain this protection. HGF can stimulate the production of prostaglandin E2 (PGE2) which is cytoprotective by regulating the liver cells' membrane microviscosity. As well, HGF induces BcL-xL (an inhibitor of apoptosis), blocking the induction of apoptosis. HGF has also been shown to be protective against damage mediated by interferon gamma and carbon tetrachloride [146].

In a model of sepsis and endotoxemia causing fulminant hepatic failure, HGF was once again found to reduce and/or prevent liver injury. Lipopolysaccharide (LPS), the endotoxin from gram-negative bacteria, causes the release of inflammatory mediators. These factors are responsible for the development of the systemic inflammatory response syndrome and multiple organ failure, including fulminant hepatic failure. Mice injected with LPS and D-GalN release TNFα from Kupffer cells, leading to massive hepatocyte apoptosis. Otherwise untreated mice died between 6 to 7 hours post injection of LPS but mice treated with HGF were normal at 5 hours and by 48 hours had only mild injury to the liver parenchyma. This protection existed despite the tissue infiltration of activated macrophages and neutrophils (elicited by TNFα from Kupffer cells). This demonstrates that HGF does not interfere with the induction of inflammation but rather diminishes its consequences on cells in the inflamed tissues. HGF suppresses the activation of the apoptosis by inhibiting the expression a principal apoptosis transducer capase-3 (CPP32) [155]. As stated, HGF also up-regulates the expression of Bcl-2 and Bcl-xl, inhibitors of apoptosis [156]. HGF may condition cells for survival under toxic conditions such as might occur during inflammatory processes.

Compared to normal livers, cirrhotic livers have significantly impaired regenerative capacity and hepatic function. In partially hepatectomized rats with cirrhosis, liver failure is high for this reason. An animal model of cirrhosis was developed in rats treated with dimethylnitrosamine (DMN). Pathological changes in the rat liver resembled changes found in humans with liver cirrhosis: collapse of parenchymal cells, and the formation of regenerative nodules separated by fibrous septa (micronodular pattern in parenchyma). When these animals were injected with a human HGF expression vector liposome, both endogenous and human HGF were induced [176]. The vector also elevated the level of *c-met* protein.

With repeated injections of the HGF vector, there was a decrease in the fibrosis of the periportal and centrilobular liver (greater than 70% fibrosis reduction in image analysis techniques). With increases of HGF, apoptosis induced by DMN was prevented. Compared to control rats, those transfected with the HGF DNA were rescued from fatal liver cirrhosis. Independently, similar benefits were attributed to HGF in cirrhotic rats with partial hepatectomies [175]. In these rats, livers had less fibrous tissue in the periportal region and no pseudolobular formation, unlike the control rats. Following the operation, there was no evidence of centrilobular damage, liver regeneration was accelerated and a higher survival rate was achieved compared to the control group. Although the mechanism is not clearly known, there are three proposed hypotheses for the beneficial effects of HGF in this model. The first two involve HGF's effect on collagenase and the extracellular matrix. HGF increases hepatic collagenase activity resulting in increasing degradation of the ECM. Secondly, HGF acts by downregulating the levels of procollagens and TGFβ. Both are important factors in the promotion of tissue fibrosis. And third, cells are more capable of resisting apoptosis. Thus HGF therapy either through direct administration or gene therapy has the potential to protect against the development of liver cirrhosis.

HGF in Partial Hepatectomies

In the clinical setting, liver surgery is important in the management of both primary and metastatic liver tumors. Because of the liver's ability to regenerate after removal of part of its parenchyma, partial hepatectomies can be performed safely without permanent loss of liver function. HGF was initially discovered in the serum of rats post partial hepatectomy [117]. Following liver surgery, HGF expression is upregulated from a low constitutive level. In rats, the amount of liver resected influences the timing and peak level of HGF mRNA expression. In the standard two-thirds resection, (or greater volume resections) HGF mRNA up-regulation was seen by two hours and peaked between six and twelve hours post surgery [157]. However, if a lesser resection was done (30%), there was a more gradual increase by six hours and a peak mRNA level at twenty-four hours.

HGF in Liver Transplantation

The effects of HGF in liver transplantation have been studied at the cellular and organ levels. In hepatocyte transplantation, there are two means to repopulate the liver. Progenitor cells (or stem cells) have the potential when transplanted into suitable environments to differentiate into the hepatocyte lineage. Should the liver be chronically depleted or devoid of healthy hepatocytes (for example, in the Alb-uPA mice), differentiated hepatocytes can repopulate the host liver without the participation of progenitor cells. There has been conflicting evidence on the effect of HGF in these cases - whether the effect is increased survival of transplanted cells or proliferation of the cells, a question complicated by the different model systems implemented. For example, Gupta *et al*. studied the effect of HGF on engraftment following injury to the liver (partial hepatectomy and CCl_4 toxicity) [188]. Selective injury to host hepatocytes stimulated engraftment but the additional treatment of HGF had little or no effect on cell number or mass. The authors questioned whether a sustained treatment of HGF or simultaneous treatment with additional growth factors would have beneficial results. Kato *et al*., in contrast, reports that IV administration of recombinant human HGF produced a three–fold improvement in transplanted cell mass in engrafted rats [189]. However, the recipient rats in this case were not subjected to injurious pre-treatment and doses between the two groups differed: 2.4 mg/kg /day for seven days as a continuous infusion with the former group and 360 μg/kg every 24 hours for five days in the latter group. Another uncertain variable is the source of HGF (human vs. rat), despite the significant homology between rat and human HGF (90%).

A splice variant of HGF (dHGF; d, for deleted) had a clear beneficial effect in partial and whole liver transplants (in rats). dHGF was isolated from human embryonic lung diploid fibroblasts and lacks 5 amino acids of HGF in the first kringle domain. dHGF alters the immunological, solubility and biological activities of HGF and is more potent in stimulating DNA synthesis in rat hepatocytes. It also has an anti-fibrotic effect for rat livers [158]. When dHGF was administered to orthotopic liver transplant recipient

rats, the overall graft survival was increased (11.8 +/- 0.4 days compared to 21.4 +/- 1.3 days) and there were larger numbers of proliferating cells (as demonstrated by immunohistochemical staining of proliferating cell nuclear antigen) compared to non-treated rats. When rejection took place in the dHGF treated mice, there was no change in the cellular infiltration and mechanisms of rejection, but dHGF was able to decrease the amount of apoptosis (2.5% +/- 0.4% vs. 1.3 +/- 1.3% in treated mice). When these experiments were repeated with reduced-size transplants (40% reduction in liver mass by removing the left lateral lobe and two caudate lobes prior to transplant), dHGF had a positive effect on promoting hepatic regeneration to restore a normal hepatic graft/body weight ratio in syngenic and allogenic transplants, coincident with a higher rate of DNA synthesis and cell replication [158, 159]. Thus, through promotion of DNA synthesis, replication and possible prevention of hepatocyte apoptosis, HGF may play a significant role in successful transplantation.

Liver Regeneration and the Alb-uPA/SCID/Bg Model

The objectives of the research described in the following chapters were to improve the success of human liver transplants in the Alb-uPA/SCID/Bg mice, and to validate the use of this model to study HCV infection. One of the problems of engraftment was to overcome the outgrowth of murine cells (in which the toxic transgene was inactivated) that out-competed the human grafts [192]. I chose to explore the use of human HGF, believing that it may favor the human graft. Also, I wished to explore the use of alternative graft tissue (hepatocytes stem cells) as the availability of human liver tissue was limiting. To explore the utility of the model, I tested the anti-HCV activity of a prospective antiviral (BID) in engrafted mice infected with HCV. Finally, I undertook a statistical analysis of our transplants with the hope of identifying unappreciated factors that may impact the successful transplantation of human liver cells in the Alb-uPA/SCID/Bg mice.

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Chapter 2

Modeling HCV Immunotherapy in Chimeric Mice: Surrogate Evidence from Immunoglobulin Treatment for HBV Infection

Introduction

Hepatitis C virus (HCV) infection is endemic, with an estimated 170 million carriers worldwide. Recent developments in both *in vitro* and *in vivo* systems have helped to define the challenges in developing effective treatment and preventing transmission. HCV replicon model systems have greatly expanded the capacity to screen potential approaches active against the machinery of viral replication and to study the basic biology of the virus [1,2]. We have reported on a chimeric mouse model of HCV infection and moved towards its validation for study of cytokine and small molecule based therapies for HCV infection [3,4]. Here we supply surrogate evidence for the use of this model in developing immunotherapies for HCV infection.

To date, no therapeutic or prophylactic immune based therapies for HCV have demonstrated efficacy in clinical application, nor have effective regimens been established in the laboratory setting. Hence we chose a passive immunotherapeutic approach for hepatitis B virus (HBV) infection as a surrogate study for immune intervention effectiveness in the chimeric mouse model. A standard protocol utilized to prevent re-infection of the liver in HBV infected patients after transplantation was applied to study the prevention of *de novo* HBV infection in SCID/Bg mice carrying the albumin linked, urokinase-type plasminogen activator gene and transplanted with human hepatocytes (chimeric mice). HBIg, or hepatitis B immune globulin, is a polyclonal hepatitis B surface antibody (HBsAb) purified from pooled patient serum. HBIg is used to prevent HBV infection in instances of high-risk needlestick injuries and community contacts, and

to prevent the reinfection of transplanted livers in HBV positive recipients [5-7]. Our goal was to confirm that chimeric mice carrying an effective plasma level of HBV surface antibody (HBsAb) would be protected from inoculation with high titre HBV human serum.

As a single agent in a post-liver transplant multicenter trial, HBIg was shown to decrease the rate of HBV recurrence from 75% in untreated controls to 33% [8]. The conversion to HBV infection was a consequence of overproduction of HBV or insufficient titres of HBsAb. The level of HBsAb required to maintain prophylaxis has been clinically shown to be >100-150 IU/L. At this level, recurrences are prevented in up to 75% of patients [6,9]. If a level of >500 IU/L is maintained, a recurrence in HBV transplant patients may be prevented in 90% of patients [10,11]. No such product is currently approved for HCV prophylaxis in similar situations. Trials of pooled anti-hepatitis C antibody preparations have been carried out in Canada (Cangene) and USA (NABI) but have failed to demonstrate effectiveness in preventing HCV re-infection after liver transplantation. Their development is still evolving [12,13].

Materials and Methods

Animals. Alb-uPA/SCID/Bg mice were homozygous for the uPA transgene and housed in the University of Alberta Health Sciences Laboratory Animal Housing under approved conditions. All experimental protocols complied with Canadian ACUC guidelines. The mice were transplanted with fresh human hepatocytes according to a protocol previously published [3]. Pups between five and fourteen days of age were engrafted with one million fresh human hepatocytes via intrasplenic injection. Recipient mice were anesthetized with halothane/O_2, and, through a small left-flank incision, 1×10^6 viable liver cells (in saline solution; ~ 100 μl) were injected into the inferior splenic pole. A single titanium clip was placed across the injection site for hemostasis, and the incision was closed [3].

***Primary human liver cells*.** Resected human livers were dispersed by collagenase treatment and cryopreserved according to an established protocol [3]. Thawed cells were centrifuged and washed free of preservative prior to resuspension in a small volume of saline. Cell viability was determined by trypan blue exclusion before being injected into recipient mice.

Testing for the presence of human hepatocytes via ELISA for hAAT. At four and eight weeks of age, animals were tested for the presence of human alpha-1-anti-trypsin (hAAT) production. Immulon2 96 well plates (*Corning, Inc*) were coated overnight at 4° with a 1:1000 dilution of goat anti-human AAT primary antibody (*Diasorin*) in 0.1M $NaHCO_3$. Following 3 washes with 0.025% Tween in Tris-buffered saline (TBS-T), the plates were blocked for one hour at room temperature with TBS-T/5% skim milk powder. 1:100 dilutions of mouse serum were serially diluted 1:4 and 50 μL aliquoted to wells of the assay plate. Controls included non-transplanted Alb-uPA/SCID/Bg mouse serum and serum from an Alb-uPA/SCID/Bg mouse with known good transplant. Standard curves were generated from serial dilutions of human reference serum, Calibrator 4 (*Diasorin*). Samples and standards were incubated for 2 hours at room temperature then washed with TBS-T. Goat anti-human AAT antibody linked to horseradish peroxidase (HRP) (using EZ-Link Plus Activated Peroxidase Kit - *Pierce, Rockford IL*) was added to each well (secondary antibody) with 2 hour incubation at room temperature. Following washes with TBS-T, HRP substrate solution (1mg of 3,3', 5,5'-tetramethyl benzidine dihydrochloride (*Sigma*) in 0.05M phosphate-citrate buffer, pH5 with 0.02% H_2O_2) was added for 5 minutes. Reactions were terminated by the addition of 2N H_2SO_4. HRP activity was measured spectrophotometrically at 450nm and hAAT levels computed with *Softmax* software. Animals with levels of hAAT greater than 25 μg/ml at four weeks, and 100 μg/ml at eight weeks, were used for experiments.

Hepatitis B infection and immunoglobulin administration. At eight weeks of age, eight experimental animals were administered 1 ml/kg of HBIg (Nabi-HB^{TM}; 312 IU/ml) by intraperitoneal injection (day 0 of our experiment). Twenty-four hours later (day 1), the mice were inoculated intraperitoneally with 100 μL high titre human HBV positive serum collected from a lamivudine naïve patient (obtained with informed consent as approved by the University of Alberta Faculty of Medicine and Dentistry Human Ethics Committee). Single maintenance doses of HBIg injections (0.12 ml/kg) were given daily for the next 14 days. Six control mice were inoculated with the same human serum but received normal saline sham injections of HBIg at the same volume. The mice were bled (via the jugular vein) to test for levels of antibody to hepatitis B surface antigen (HBsAb) and the presence of HBV DNA at two week intervals post-inoculation.

HBV DNA PCR. Viral DNA from 20 μL of serum from HBV inoculated mice was extracted using phenol chloroform. The DNA was then precipitated using isopropanol and washed with 70% ethanol. PCR was done on a thermocycler with 1.5 millimolar magnesium chloride with a 58°C annealing temperature for 30 cycles (forward primer 5' ggtcctaggaatcctgatgt; reverse primer 5' gtggagccctcagtgctcagg). PCR samples were run out on 0.8% agarose gel. Positive and negative samples were determined by ethidium bromide staining and visualization by ultraviolet light.

Results

To ascertain the serum level of HBIg achieved, blood samples of infected mice were analyzed for HBV surface antibody by the Alberta Provincial Laboratory of Public Health. HBsAb reached a mean level of 5102 IU/L by week 1 (range of 2536 to 8325, Figure 2.1). At four weeks post HBV administration (2 weeks after antibody administration had ceased), none of the treated animals tested HBV DNA positive by PCR analysis of blood samples. In contrast, there were

positive indications of HBV DNA in 5 of 6 unprotected mice. By week six, the levels of antibody had diminished but remained above 100 IU/L in 6 of the eight animals, and all six of these mice tested HBV DNA negative (Figure 2.2). The two animals that were below 100 IU/L HBIg were HBV positive by this time, and were consistently so for the 12 week observation period. Interestingly, three other treated mice tested positive sporadically after 6 weeks but were negative at week 12 (Figure 2.2). By week 12 all the controls tested positive for HBV DNA. Both treatment group mice that had seroconverted and all control mice were HBV positive by immunohistochemical analysis for Hepatitis B surface and core antigen (Figure 2.3). Treated mice that were HBV DNA negative were also negative for core Ag by immunohistochemistry, including mice that had sporadically tested positive prior to 12 weeks.

When comparing the level of HBIg in HBV negative and positive animals, the difference was significant with a p-value of 0.046 (Mann Whitney-U test). The levels of HBV surface antibody in those mice that never developed a positive HBV infection were higher than in those that seroconverted (Figure 2.4). There appeared to be a threshold level of antibody required to suppress or prevent a detectable infection. Animals that fell below 10 IU/L within the first 4 weeks post-HBV inoculation were confirmed to be HBV positive by PCR analysis and immunostaining. The three treated animals that sporadically tested positive for HBV DNA (once or twice in the 12 week study) did so at antibody levels below 10 IU/L, at 60 IU/L or at 310 IU/L. The three treated mice that were consistently negative for HBV DNA by PCR analysis maintained serum HBIg levels above 160 IU/L through week 6.

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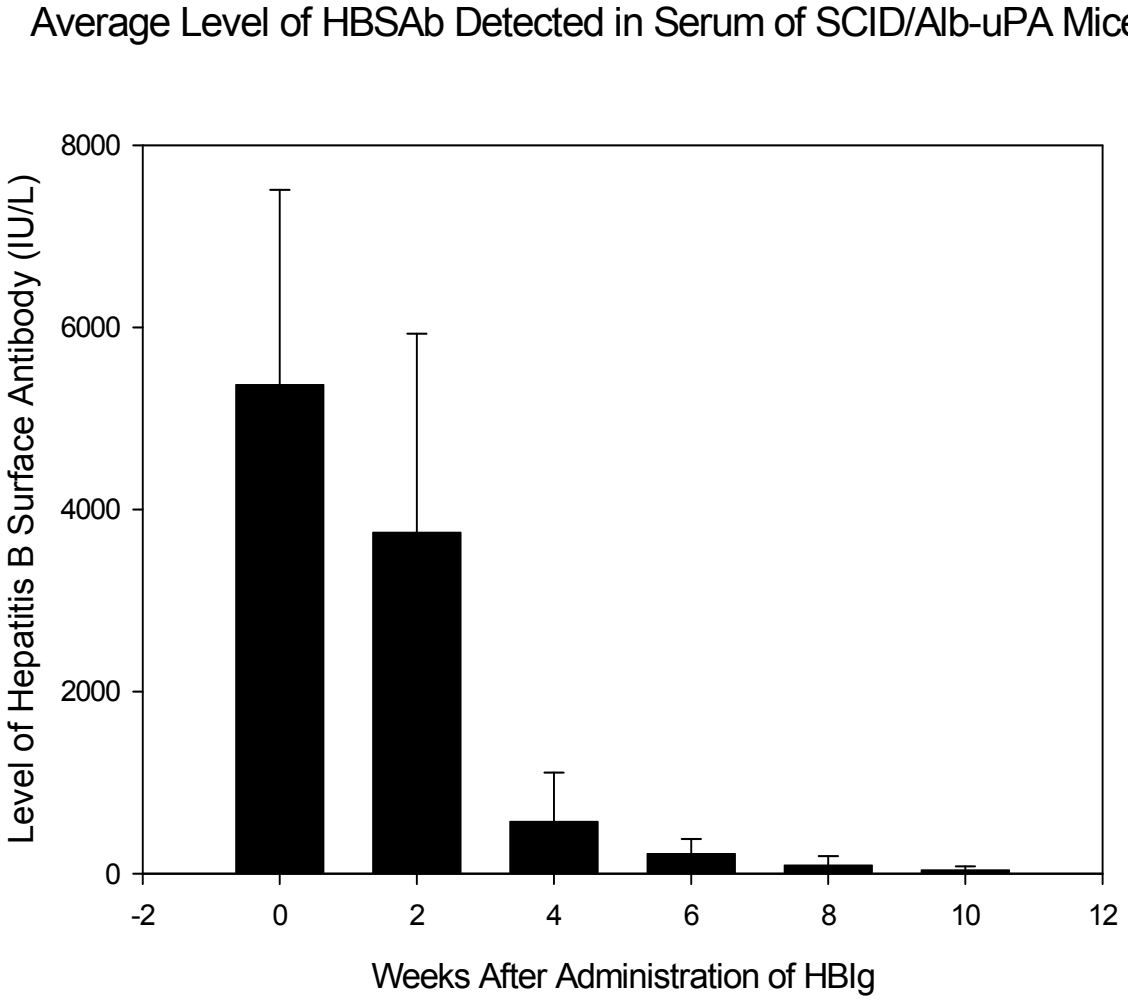

Figure 2.1. Serum levels of HBV surface antibody in chimeric mice. Mean values are plotted after intraperitoneal injections of HBIg. Peak levels were attained after an initial high dose (1ml/kg) injection. Target levels were maintained for up to four weeks with daily injections of 0.12 ml/kg for 2 weeks.

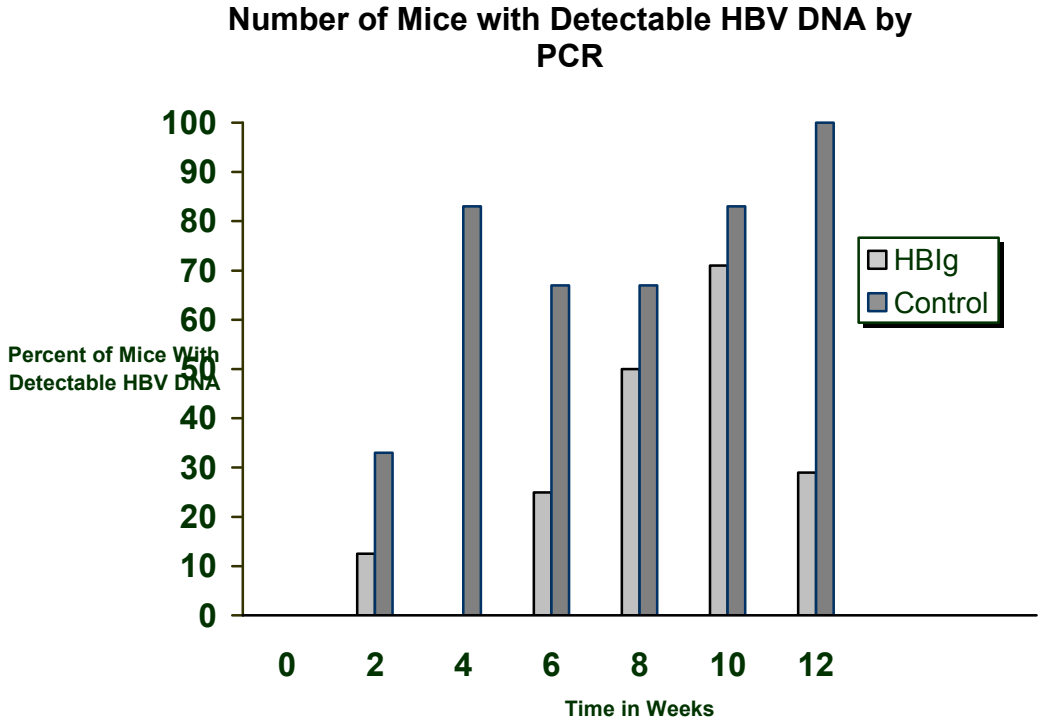

Figure 2.2. Mice with detectable HBV DNA by PCR. The presence of HBV DNA in treated and placebo mice was measured via non-quantitative PCR. By twelve weeks, all control mice had HBV DNA present in the serum, however, only thirty percent of HBIg treated mice had breakthrough HBV present in the serum.

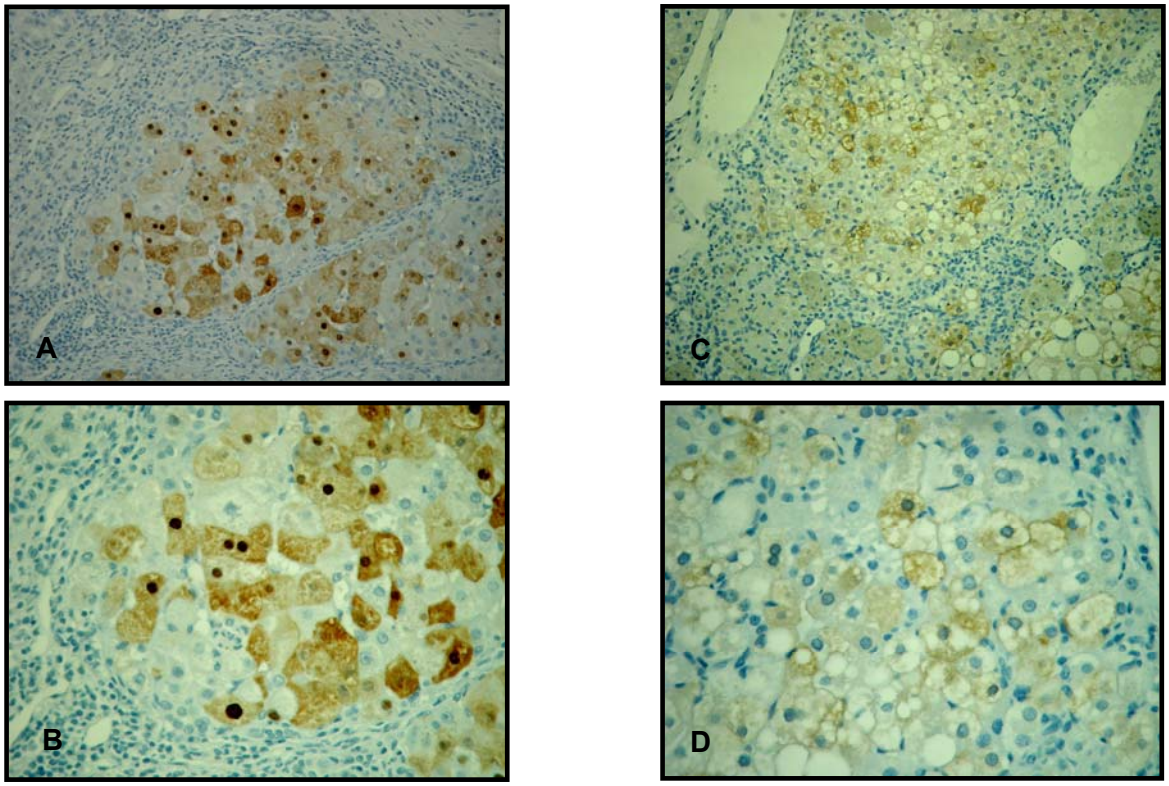

Figure 2.3. Hepatitis B core antigen immunostaining. Figures A and B low and high power of a human positive control - a hepatitis B positive patient. Figures C and D are from an HBV-positive Alb-uPA/SCID/Bg animal after breakthrough in association with low HBsAb levels.

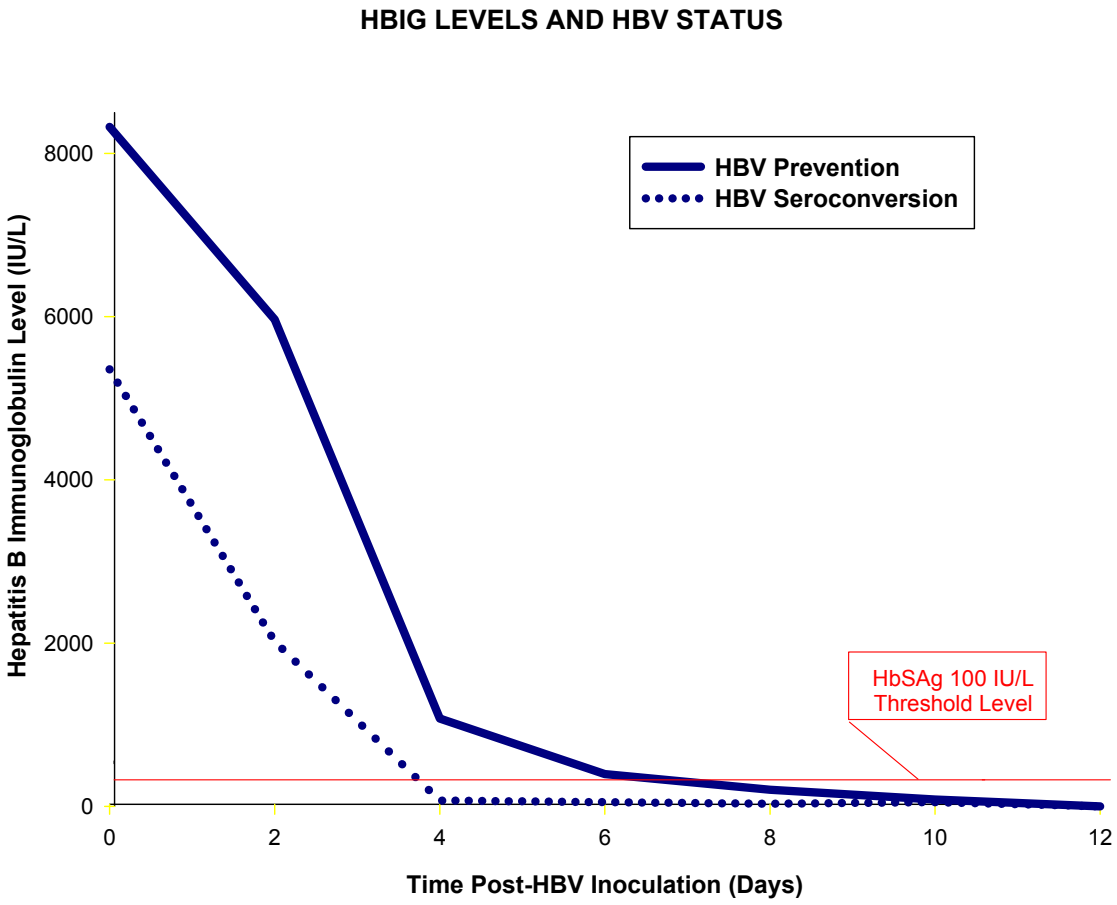

Figure 2.4. HBIg levels and HBV status. The presence of detectable HBV in the serum is related to initial levels of serum HBIg. The animals that becme to be positive for HBV had lower mean starting levels of HBIg as well as a faster drop off to HBIg levels below the 100 IU/L threshold.

Discussion

The lack of a small animal model for HCV has been a stumbling block to understanding HCV pathogenesis in the context of the associated liver damage and the development of effective anti-virals. Chimeric Alb-uPA/SCID/Bg mice are able to sustain an HCV infection in the engrafted human liver cells. The validity of the model is supported by evidence of a spreading infection that can be transmitted experimentally from animal to animal (based on the presence of negative strand viral RNA as confirmation of virus replication) [3]. The tropism of HCV was restricted to the human liver cells as non-transplanted mice could not be infected. Inoculated virus in these non-chimeric mice was undetectable twenty-four hours post-HCV positive serum administration (unpublished data), therefore the persistent presence of virus in chimeric mice indicated an ongoing infection.

To further examine the utility of the Alb-uPA chimeric mouse *vis-a-vis* the development of passive immunotherapy, we performed a proof of principle experiment with hepatitis B immune globulin – an established passive immunotherapy agent for prevention of HBV infection in high risk clinical situations. HBIg is widely used and accepted in the clinical setting to prevent transmission of HBV post-needlestick exposure and prevent recurrence after liver transplant in HBV positive patients. For effective prevention of a recurrent hepatitis B infection post liver transplant, a threshold level of hepatitis B surface antibody above 100 IU/L needs to be maintained in the serum of patients [9,14]. This antibody level was effective in reducing the recurrence rate of HBV in post-transplanted HBV positive patients from 70% to 25% [8,9].

Our results showed that HBIg suppressed a primary HBV infection in Alb-uPA chimeric mice with an efficacy similar to that experienced in humans. Of 6 mice treated with HBIg that maintained HBsAb titres over 100 IU/L, only one transiently tested positive for HBV DNA. In the same time course, all 6 control mice were positive for HBV infection. Two treated mice

whose HBsAb titres fell below 10 IU/L consistently tested positive for HBV DNA. In the context of the experiment described here, it can not be resolved that infection was prevented by HBIg treatment. The occasional positive HBV DNA result, interestingly at times of apparent low serum levels of HBIg, suggests that a low-grade chronic infection existed that was below the level of detection. Nevertheless, virus replication was clearly suppressed in chimeric mice treated with HBIg. It is reasonable to hypothesize that HBV presence in treated mice was due to an overwhelming of the HBsAb present by HBV replication, either through enhanced virus production or altered HBIg pharmacokinetics in positively tested mice compared to negatively tested mice. Maintenance of HBIg at above 100 IU/L was generally suppressive, whereas dips in measured HBIg levels to 10 IU/L generally corresponded with positive HBV DNA in results. These results are consistent with HBIG sequestering viral spread in the human hepatocytes of infected chimeric mice.

The positive outcome in the surrogate model system presented here corroborates the assertion that this model will be useful to study passive immunotherapy for HCV infection. Antibody preparations could then be evaluated in protocols similar to that reported here. Recently, the chimeric alb-uPA/SCID/Bg mouse model was used to test the passive immunity of two monoclonal antibodies (mAb) that were developed from the serum of HCV patients. The two antibodies tested were developed against two antigenic regions, AR3A and AR3B in the E1-E2 proteins of genotype 1a HCV. After administration of the monoclonal antibodies AR3A and AR3B, the mice were challenged with an inoculation of genotype 1a HCV infected serum. Protective immunity was seen in two of the five mice treated with mAb AR3A and three of four mice treated with mAb AR3B at six weeks from inoculation. [15] The ability to achieve neutralization of HCV in the chimeric mouse with antibodies to specific epitopes gives insight into developing targets for effective immunization in an HCV vaccine.

Similar to the development of HBIG, the generation and recovery of antibodies to HCV could be accomplished by vaccinating immunocompetent animals with HCV antigens or recombinant proteins. This would necessitate an enhancement to the model, one with the potential benefit of replicating additional aspects of HCV immunopathology. The mice would have to be chimeric in terms of a reconstituted human immune system as well as hepatocytes. Such a model would be useful in the development of a range of immunotherapies that counteract pathogenic sequelae as well as restrict virus replication or spread.

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Chapter 3

Targeted Apoptosis of HCV-infected Hepatocytes in Alb-uPA/SCID/Bg Mice

(Contents of this chapter, those containing initial outcomes with single dose mod-BID therapy, have been published elsewhere [8].)

Introduction

The targeting of viral proteins is a leading area of research in the development of new and improved therapies for viral infections. The HCV genome is translated into a single polyprotein that is cleaved into three structural and six nonstructural proteins. The structural proteins include the nuclear core and envelope proteins (p7, E1, E2). The nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B) have been the main targets for anti-viral therapeutics because the serine protease (NS3/NS4A), helicase (NS3) and polymerase (NS5B) are essential for viral replication [1-3]. Apart from targeting viral components, an alternative strategy would be to target the infected cell which, for HCV, is chronically and productively infected. Generally, if immune mechanisms (innate and adaptive) fail to protect an infected cell, that cell undergoes apoptosis as a last resort to spare the host. A cell chronically infected with HCV does not apoptose, but if apoptosis could be induced in a therapeutic fashion then the productive infection could be abrogated. In this chapter, the efficacy of targeted BID activation in infected cells as an anti-viral approach is explored.

Apoptosis or programmed cell death is a normal process that is highly regulated during development and later in life to maintain cellular homeostasis. Although there are different

mechanisms to induce apoptosis, hepatocytes are primarily triggered via exposure to ligands of the death receptor family. In particular, fas ligand (FasL)/Fas and tumor necrosis family (TNF)/TNF receptor interactions [4]. Fas is a cell surface glycoprotein expressed in the liver. TNFRs are widely expressed in both normal and neoplastic cells. The two receptors share a common C-terminal intracellular domain known as the death domain. When ligand interaction occurs, recruitment of the adapter protein FADD occurs. This results in the binding and activation of procaspase-8 at the cytoplasmic domain complex and the stimulation of the signaling cascade towards cellular death. The general process of apoptosis is a pathway of sequentially activated proteases or caspases [5]. Inducing apoptosis through death domain associated cell surface receptors in HCV infected cells may be a mechanism to therapeutically eliminate cells harboring virus while sparing healthy, uninfected cells.

In collaboration with Eric Hsu and Chris Richardson from the Ontario Cancer Institute in Toronto, we tested a gene therapy approach targeting the HCV virus using a modified BID molecule. BID is a member of the Bcl-2 family of proteins. Bcl-2 proteins are important because they are regulatory players in the mitochondrial pathway of apoptosis. Receptor-mediated death signals are potentiated by involvement of the mitochondria pathway to apoptosis. Certain death signals induce mitochondria to release cytochrome c into the cytoplasm. BID, along with other Bcl-2 family members, potentiate the release of cytochrome c [6]. Death receptor signaling is linked to the mitochondrial pathway through the activation of BID by caspase-8. Cytochrome c release signals the apoptosis cascade by binding to Apaf 1 (apoptosis protease activating factor 1). Apaf-1 then recruits pro-caspase 9 to form a complex that activates caspase 9 which, like caspase 8 in the alternative pathway, activates the downstream cell death effector caspase 3 [7].

The development of a modified BID molecule (mBID) that could be selectively activated in infected cells was achieved by engineering the molecule to contain a cleavage site recognized by

the NS3/NS4A serine protease of HCV. Cleaving this pro-molecule activates the modified BID leading to activation of the mitochondrial apoptotic pathway downstream cell surface ligand/death receptor interactions. *In vitro*, Hsu *et al.* demonstrated the effectiveness and the mechanism of action of modified BID in three model systems [8]. In one, rat muscle fibroblast cells (H962C) were infected with a recombinant retrovirus that expressed the NS3/NS4A fusion protein. These cells were infected 24 hours later with a recombinant adenovirus encoding mBID. Co-infected cells showed evidence of apoptosis as demonstrated by annexin V staining (annexin V binds phosphatidylserine exposed on the surface of apoptotic cells) and microscopy (membrane blebbing and nuclear condensation). Control cells lacking the protease did not display enhanced apoptosis.

In a similar fashion, apoptosis was induced in a human hepatoma cell line (Huh7). More importantly however, apoptosis was demonstrable in human cells with virally produced NS3/NS4A [8]. Hsu *et al*. employed the HCV replicon cell system described by Lohmann *et al.* [9, 10] to accomplish this. Huh7 hepatocytes were transfected with a cloned viral consensus genome sequence, an RNA replicon. The RNA replicon consists of the 5' and 3' untranslated regions (UTR) that are required for replication of HCV, a neomycin resistance gene within the 5' UTR for selection, and the nonstructural genes of HCV under control of an internal ribosomal entry site (IRES) from encephalomyocarditis virus. The HCV replicon in Huh7 cells was positively selected through culture in the presence of neomycin sulfate (G418). This system selects for efficient replication of the HCV replicon as it is dependent upon replication of the 5' UTR element bearing the resistance gene. After infecting the replicon cells with recombinant adenovirus containing the modified BID molecule, there was significant cell death within 6-9 hours relative to cells without mBID. Over 80% of the cells were dead by apoptosis (annexin V positive).

Inhibition of a primary infection with mBID was reported by Filicamo *et al.* who developed a recombinant Sindbis virus encoding the NS3 protease of HCV. Sindbis, like HCV, is a single-stranded positive RNA genome, however its genome is arranged in two open reading frames. These authors generated a family of chimeric viruses that would only replicate in the presence of an active HCV NS3 protease. This was accomplished by cloning the NS3 gene into the Sindbis virus upstream of the 3'ORF such that downstream processing of the polyprotein was dependent on NS3 proteolysis. These chimeric viruses infect a wide range of cell lines including Huh7 cells. Naïve Huh7 cells were infected with adenovirus-mBID or adenovirus without mBID (controls). Once expression of mBID was confirmed, cells were challenged with Sindbis virus. Modified BID prevented a primary chimeric Sindbis virus infection [11, 12].

Consideration of this data led us to hypothesize that gene therapy exploiting mBID might be applied to treat HCV infection and testable in the Alb-uPA/SCID/Bg chimeric mouse model. Gene therapy has been controversial due to adverse effects of the vectors used to administer the potentially therapeutic gene and, at times, the unforeseen consequences of therapeutic gene expression. It is widely felt that the significant impediment of gene therapy as a viable therapeutic option is the lack of an efficient, non-toxic means of delivering the genes [13]. Currently, two streams of vector delivery systems exist: viral vector systems and synthetic, liposome-mediated vectors [14]. Most clinical applications have utilized viral vectors as the synthetic vectors are still early in development. Viruses that are in use include adenovirus, adeno-associated virus, herpes simplex, retrovirus and lentivirus. The adenovirus was one of the initial viruses used and continues to be widely used because of its ability to infect efficiently and deliver its genes to a range of host cells [15]. Adenoviruses are advantageous in liver directed gene therapy because they have significant tropism for human hepatocytes and have a low efficiency rate of integration into the host genome [13]. [13, 16]Thus, for delivery of the mBID gene to the human hepatocytes in the Alb-uPA/SCID/Bg mice, non-replicating adenovirus was

chosen. This report describes the impact of multi-course treatment with mBID in the mouse model, and describes efforts to overcome some of the challenges inherent in multi-course treatment with viral vectors.

Materials and Methods

Animals. Alb-uPA/SCID/Bg mice were maintained in level 2 VAF animal housing and fed sterilized, high-fat (9%) chow. Homozygosity for the Alb-uPA transgene was maintained. Between 5 and 10 days, animals were rescued by injection of 1 x 10^6 human liver cells into the spleen according to a published protocol [16]. Eight weeks following the transplant, surviving mice were tested for serum levels of human α-1-anti-trypsin (hAAT) as an indicator of transplant success. Mice with serum levels of hAAT over 100 were used for the study. Animal studies were in compliance with regulations set forth by the Canadian Council on Animal Care as approved by the Health Sciences Animal Welfare Committee at the University of Alberta.

Infection of chimeric Alb-uPA/SCID/Bg mice. Alb-uPA/SCID/Bg/ mice were infected intraperitoneally with 100 uL of HCV positive human serum, genotype 1a or 1b. HCV viral titres in the mice were determined by RT-PCR analysis of serum using the Roche Amplicor kit. Serum levels of alanine aminotransferase (ALT) were measured as indication of hepatocyte death following infections. ALT levels and HCV titres were routinely determined by the Alberta Provincial Laboratory of Public Health.

Adenovirus infections. Three forms of non-replicating recombinant adenovirus, supplied by C. Richardson [1], were used to infect chimeric mice. Adenovirus vectors carried the gene for enhanced green fluorescent protein (eGFP), or for modified BID (mBID), or were empty (control). The gene for mBID was genetically modified with an epitope tag attached to the carboxy-terminus of the recombinant protein (FLAG) [1]. Various doses of adenovirus

containing the coding sequence for eGFP were administered by injection via the internal-jugular vein, portal vein/intrasplenic, or intraperitoneal routes. Mice injected with recombinant eGFP adenovirus were sacrificed after 36 hours for analysis. Infection of the liver was estimated by immunoflouresence on cryostat sections or by flow cytometry of hepatocytes. Dual infections of mice with HCV and adenovirus (jugular vein injections) were sequential. Adenovirus (Ad) infections followed HCV infections by two weeks. The timing of subsequent Ad virus infections is outlined in the Results.

Liver hepatocyte suspensions. Whole livers or partial hepatectomies (left lobe) were flushed with phosphate buffered saline until the effluent was cleared of blood. The liver was grossly diced and then underwent collagenase digestion at 37°C for 10 minutes. The digested tissue was then passed through a 0.25 mm wire mesh filter. After washing with HBSS, cell viability was assessed by trypan blue dye exclusion.

Flow cytometry. Isolated liver cells (10^6 cells) were resuspended in 20 μl of PBS/0.1%NaN_3 on ice. Five microlitres of PE-labelled anti-CD46 was added and the mixture was mixed gently and and left on ice for 20 minutes. After washing the cells by centrifugation samples were fractionated in a Becton Dickinson fluorescence cytometer and analyzed using CellQuest software. CD46 is expressed ubiquitously on human cells and was used to distinguish human from murine cells.

Liver immunohistology. For the detection of eGFP-positivity in chimeric livers, liver samples were embedded in OCT preservative, frozen and sectioned. Flourescence was observed directly by fluorescence microscopy. Tunel staining of liver sections, for indications of apoptosis, was performed using the In Situ Cell Death Detection, POD Kit (Roche Molecular Biochemicals). In this application, paraffin embedded tissues were deparaffinized and hydrated with xylene and

ethanol washes. Sections were subsequently digested with ProteinaseK (20µg/mL in 10mM Tris, pH8.0) for 20 minutes at 37°C and permeabilized with 0.1% Triton-X in 0.1% sodium citrate for 2 minutes on ice (4°C). Sections were labeled following the manufacturer's protocol. Slides were incubated with terminal deoxynucleotidyl transferase (TdT) for one hour at 37°C in a humidified chamber. The signal was subsequently amplified with an anti-fluorescein antibody conjugated to horse-radish peroxidase and treated with diaminobenzidine tetrachloride (DAB) (Vectastain ABC Kit) for analysis.

Immunohistochemistry for anti-FLAG (SIGMA) and anti-BID (Cell Signalling Technology) Paraffin embedded tissues were deparaffinized and hydrated with xylene and ethanol washes. Antigen unmasking was performed by microwaving sections in 10mM sodium citrate buffer for 1 minute at full power followed by 9 minutes at medium power and then cooled for 20 minutes. Endogenous peroxidase was blocked with 3% H2O2 in methanol for one hour at room temperature. Primary antibodies were added (incubated overnight at 4°C for anti-BID and one hour at room temperature for anti-FLAG). Slides were analyzed using DAB.

Results

Adenovirus infects the livers of chimeric mice following jugular or portal vein injection. Prior to the infection of chimeric mice with adenovirus encoding mBID, conditions for infecting experimental mice were optimized by using adenovirus encoding the gene for eGFP. Infection efficiency was evaluated on the basis of fluorescence associated with the liver or isolated human hepatocytes. Fluorescence due to eGFP could be observed in liver sections following injection of 5.5×10^9 pfu recombinant adenovirus into either the jugular vein or portal vein, but not after injection into the peritoneal cavity. Suboptimal fluorescence was observed at the two lower dosages tested. Human hepatocytes were differentiated from mouse hepatocytes by staining with

anti-human CD46 antibody and sorting by flow cytometry. Following one injection of adenovirus (5.5 x 10^9 pfu), 61.9% of all cells in the transplanted liver (mouse and human) expressed eGFP. Almost all of the CD46-positive human cells in the liver (91.6%) expressed eGFP.

HCV monotherapy with adenovirus-delivered modified BID. A pilot experiment was designed to test the efficacy of a single dose of recombinant adenovirus encoding mBID (Ad-mBID) to suppress HCV replication in chimeric mice. Ad-mBID was delivered intravenously (jugular vein) at a concentration of 5 x 10^9 pfu/chimeric mouse. The mice had been infected two weeks prior with HCV patient's serum. Damage to the human hepatocytes in mBID treated mice was apparent from grossly elevated serum levels of the human liver enzyme alanine amino transferase (ALT) measured 2 and 5 days after mBID delivery. The leakage of this cytoplasmic enzyme signaled hepatocyte destruction. Enzyme levels eventually returned to baseline after one week, consistent with the persistence of human hepatocytes in the Alb-uPA/SCID/Bg mice following treatment with mBID. Histopathologic examination of mBID treated mouse livers revealed focal areas of damage to the liver tissue surrounded by healthy hepatocytes (discussed in more detail later in the context of a multi-dose therapy). With respect to the effect of mBID on HCV titres, there was considerable variation in individual responses. Three of six mice displayed a greater than 10-fold drop in viral titre (Table 1) whereas three mice appeared uniformly unprotected. Statistically, there was no significant protection offered by mBID in this protocol.

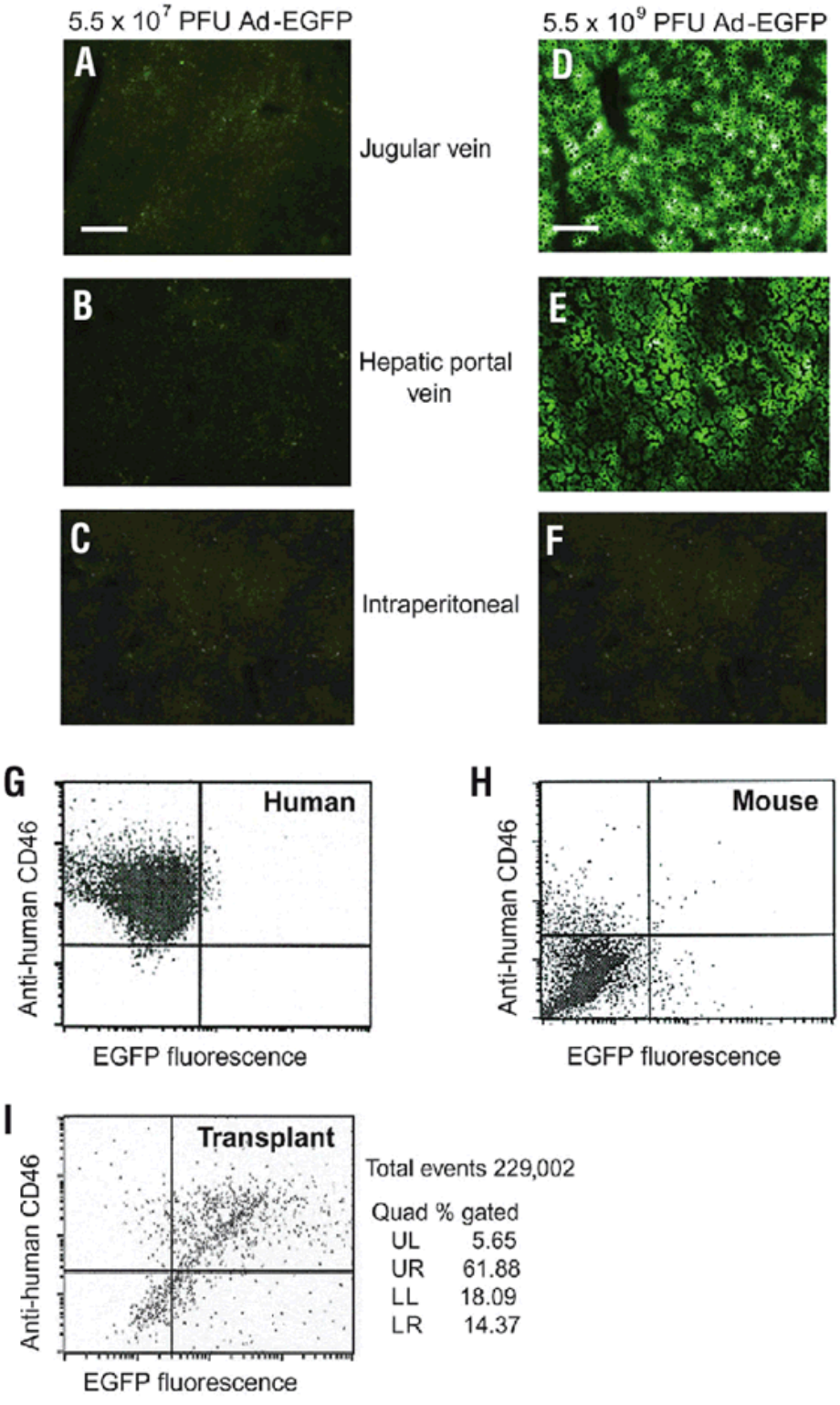
5.5 x 10^7 PFU Ad-EGFP
5.5 x 10^9 PFU Ad-EGFP
A
D
Jugular vein
B
E
Hepatic portal vein
C
F
Intraperitoneal
G
Human
Anti-human CD46
EGFP fluorescence
H
Mouse
Anti-human CD46
EGFP fluorescence
I
Transplant
Anti-human CD46
EGFP fluorescence
Total events 229,002
Quad % gated
UL 5.65
UR 61.88
LL 18.09
LR 14.37

Figure 3.1. Efficacy of adenovirus infection of murine livers. Mice containing human liver cells were injected with either 5.5 ×10^7 or 5.5 ×10^9 PFUs of adenovirus eGFP reporter virus into the jugular vein (A,D), into the hepatic portal vein (B,E), or into the peritoneal cavity (C,F). Bar, 250 μm. Mice were killed after 36 h, and liver cryosections were viewed by fluorescence microscopy. eGFP fluorescence was observed throughout the livers of chimeric mice injected by jugular and hepatic portal vein routes (D,E). The percentage of human liver cells that expressed eGFP after a single intrajugular injection of nonreplicating adenovirus encoding eGFP was calculated by FACS (I). Human (G) and mouse hepatocytes (H) were differentiated by CD46 expression on their cell surface. Engrafted human liver cells were the predominant cell type expressing eGFP in the liver sections taken from adenovirus infected chimeric mice (I).

Treatment	**Initial HCV Titre (Log)**	**Final HCV Titre (Log)**	**HCV Titre Log Difference**
Control	5.63	5.32	0.31
	6.18	5.63	0.55
	2.78	2.83	-0.05
	3.08	2.78	0.3
			Mean Dif. 0.28
mBID	5	3.77	1.23
	5.9	4.35	1.55
	5.89	5.63	0.26
	6.26	5.71	0.55
	6.93	5.87	1.06
	6.14	5.89	0.25
			Mean Dif. 0.82

Table 3.1. HCV suppression with mBID monotherapy. The mBID treated mice received one injection of Ad-mBID (5 mice) and the controls (4 mice) received a sham injection of empty adenovirus vector. All mice had been previously infected with HCV-positive patient's serum. 'Final' titres were measured two weeks after mBID delivery. On statistical analysis with the Mann-Whitney U test, the p-value for the data set is not significant with ($p=0.24$).

Multi-dose recombinant adenovirus with modified BID induces HCV-dependent apoptosis.

The poor protection from HCV infection by adenovirus-delivered mBID was thought to be due to inefficient levels of adenovirus infection attained in the engrafted human hepatocytes. Thus, to maximize the number of HCV infected cells exposed to mBID, experiments were undertaken with multiple doses of recombinant adenovirus. Mice containing human chimeric livers and previously infected with HCV, were injected 3 times via the spleen with the non-replicating adenovirus (10^9 pfu) that expressed modified BID (Figure 3.2A). The animals were inoculated intraperitoneally with HCV-positive human serum eight weeks after human hepatocyte grafting. HCV titers were determined 2 weeks later, and then the mice were inoculated three times with 10^9 pfu of recombinant adenovirus encoding mBID over three day intervals. In addition to experimental animals (group 1), HCV-infected mice were injected with adenovirus that did not contain the gene for mBID (group 2), and HCV-uninfected mice were injected with adenovirus that expressed modified BID (group 3). Two of eight experimental mice were found dead on days 7 and 10. A third was euthanized on day 10. A fourth animal was euthanized on day 14 due to signs of morbidity. Euthanized animals had uniformly pale livers with no signs of healthy tissue.

Liver biopsies were collected at 0, 2, 5, and 10 days following the initial adenovirus injections. Haematoxylin and eosin (H & E) stained sections revealed extensive cell death and hepatocyte dropout in liver samples of mice that were inoculated with both HCV and the adenovirus that expressed mBID (Figure 3.2B). No pathology was evident in the livers of mice infected with Ad-mBID alone, or HCV-infected mice super-infected with adenovirus lacking mBID. Additional liver sections were stained for FLAG-tagged mBID expression using an anti-FLAG antibody to verify mBID expression, or stained for signs of apoptosis using the TUNEL assay (to detect DNA fragmentation).

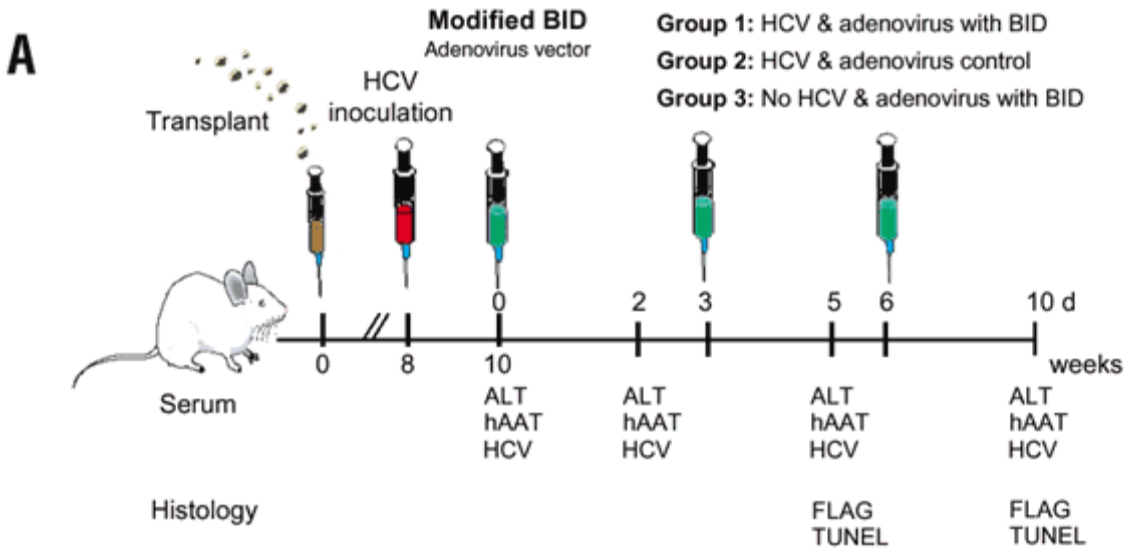

Figure 3.2. BID expression in HCV-infected mice. (A) Regimen for treatment of infected mice with modified BID.

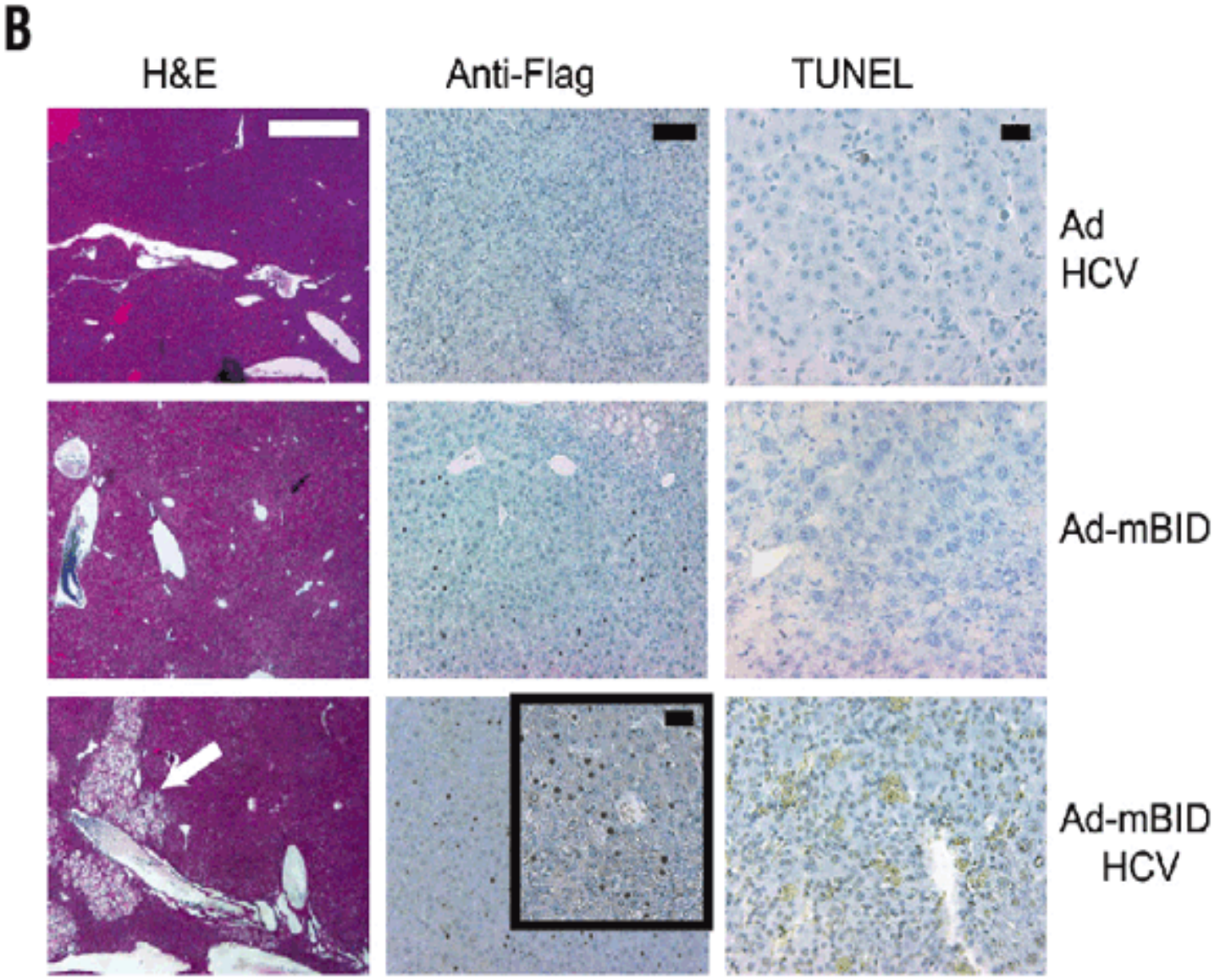

Figure 3.2. BID expression in HCV-infected mice. (A) Regimen for treatment of infected mice with modified BID. (B) Liver biopsies were stained for H&E. Bar, 1,000 μm. Cell death was evident as 'dropped out' hepatocytes, shown with an arrow (H&E, Ad-mBID HCV). Liver sections were stained with anti-FLAG antibodies to detect the expression of modified BID fused to FLAG tag. Bar, 250 μm; inset square, 50 μm. Liver sections of the human xenograft from mice were analyzed for apoptosis with TUNEL stain. Bar, 50 μm.

Anti-FLAG staining confirmed the expression of modified BID in the livers of mice injected with adenovirus encoding modified BID. Furthermore, TUNEL staining indicated that apoptosis occurred in mBID-positive tissue but only in animals co-infected with HCV (Figure 3.2B, lower right panel). Control mice treated with adenovirus without mBID and mice that were injected with Ad-mBID alone were negative for TUNEL staining.

Toxicity of modified BID correlates with HCV co-infection and the suppression of HCV replication. Serum samples from infected chimeric mice were assayed for liver transaminase (ALT) and serum HCV RNA levels at specified days after the initial injection with recombinant adenovirus. In mice co-infected with Ad-mBID and HCV, there was a spike of serum ALT between 2 and 4 days following the first injection of recombinant adenovirus (Figure 3.3A). ALT levels were mildly elevated between 6-15 days after the third injection with modified BID and thereafter receded. Injection of control adenovirus produced no change in ALT levels, while delivery of mBID in the absence of HCV produced the mild elevation in ALT over the 6 to 15 day window. Hence the apparent induction of cell death occurred predominantly after the first Ad-mBID dose in HCV-infected animals.

Of the 8 mice infected with both HCV and Ad-mBID, 4 animals survived all the manipulations, and exhibited significant decreases in serum HCV RNA levels (Figure 3.3B, and Table 2). Initial levels of HCV in these four mice ranged from 10^4 to 10^8 RNA genome equivalents/ml. Mice with the lower initial titers of HCV had undetectable levels of virus at the completion of the experiment. One of the surviving mice displayed no protection. Early analysis of the mice that succumbed during mBID treatment revealed a consistent reduction in HCV titres of two logs after the second mBID treatment. Figure 3.3A includes a one such animal that died ten days after the first treatment. Morbidity was not observed in HCV infected or Ad-infected chimeric mice.

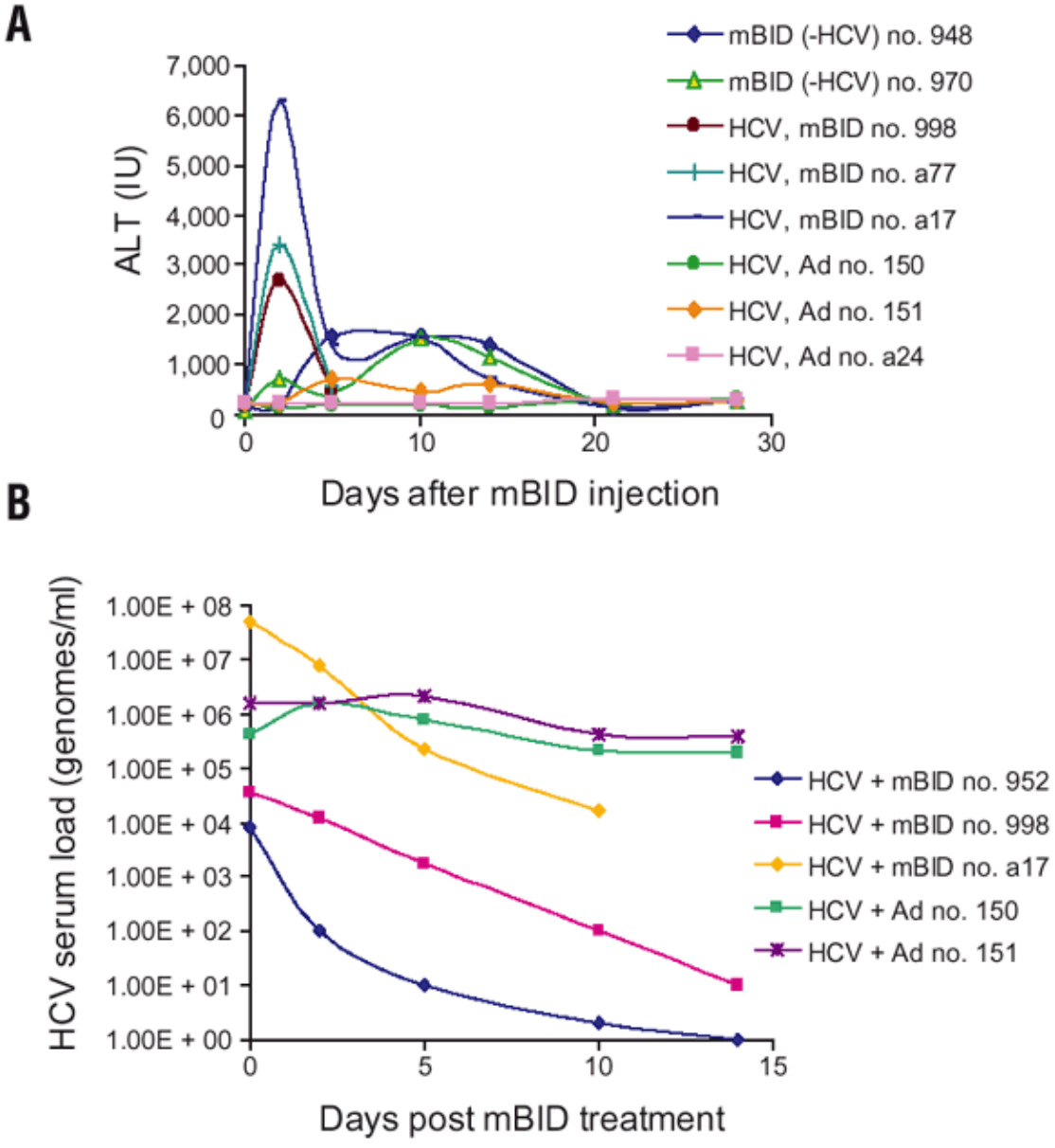
A
ALT (IU)
7,000
6,000
5,000
4,000
3,000
2,000
1,000
0
0
10
20
30
Days after mBID injection
mBID (-HCV) no. 948
mBID (-HCV) no. 970
HCV, mBID no. 998
HCV, mBID no. a77
HCV, mBID no. a17
HCV, Ad no. 150
HCV, Ad no. 151
HCV, Ad no. a24
B
HCV serum load (genomes/ml)
1.00E + 08
1.00E + 07
1.00E + 06
1.00E + 05
1.00E + 04
1.00E + 03
1.00E + 02
1.00E + 01
1.00E + 00
0
5
10
15
Days post mBID treatment
HCV + mBID no. 952
HCV + mBID no. 998
HCV + mBID no. a17
HCV + Ad no. 150
HCV + Ad no. 151

Figure 3.3. Cell death and HCV load in chimeric mice. A: Hepatocyte death after mBID delivery. Mice containing human liver xenografts were sequentially infected with HCV then recombinant adenovirus encoding modified BID (Ad-mBID). Serum samples were taken at 0, 2, 5, 10, 14, 21, and 28 d after the first inoculation with Ad-mBID or empty adenoviruses. Injections of Ad-mBID (mBID) by itself, or HCV and Ad-mBID, or HCV and control adenovirus (Ad no.) were given to the mice. ALT was measured in international units per ml. B: Titers of HCV after delivery of mBID. Serum samples taken at 0, 2, 5, 10, and 14 d after the initial injections of Ad-mBID or control adenovirus were assayed for HCV genomic RNA. For both A and B, representative responses are shown.

Seven-day interval Ad-mBID therapy. The high degree of morbidity associated with the every-third-day regimen prompted an experiment in which mBID was delivered every seventh day. Consideration was given to the recovery of serum ALT measurements which had normalized about 7 days after the first delivery of mBID. Five animals received 7 day interval injections at 10^9 pfu/ml and all survived. Four of the 5 mice had their viral titres drop below detectable levels (<100 RNA viral particles/ml) (Figure 3.4). The fifth animal responded well early (following two doses of mBID) but serum HCV levels declined only 1.5 logs over the 28 day experimental period. This animal also had the highest initial HCV titre of the five mice. While the overall protection was comparable to or better than the 3-day regimen (Table 2), the serum profiles of HCV titres were distinctly different. Ten days after the initial Ad infection in the 7-day regimen, HCV titres rose, attaining in some cases the levels of virus present prior to the onset of mBID treatments. The third, or day-14, injection of Ad-mBID then successfully pushed titres down to undetectable levels, resulting in a bi-phasic viral response with the 7-day regimen. The change in viral titres of all treated animals (Table 2) was a mean log drop of 1.89 with a standard deviation of 0.90 compared to the control group which had a mean change of 0.53 with a standard deviation of 0.45 (p-value = 0.019).

Discussion

Modified BID was shown *in vitro* to inhibit HCV replication. As a proof-of-principle, the experiments reported here demonstrated the ability of mBID to restrict HCV replication in an *in vivo* model setting. That cell death and specifically apoptosis were observed when mBID was delivered to HCV-positive liver cells supports the critical assumption that this lethal gene product was predominantly targeted to HCV-infected cells through its activation by NS3/NS4A protease.

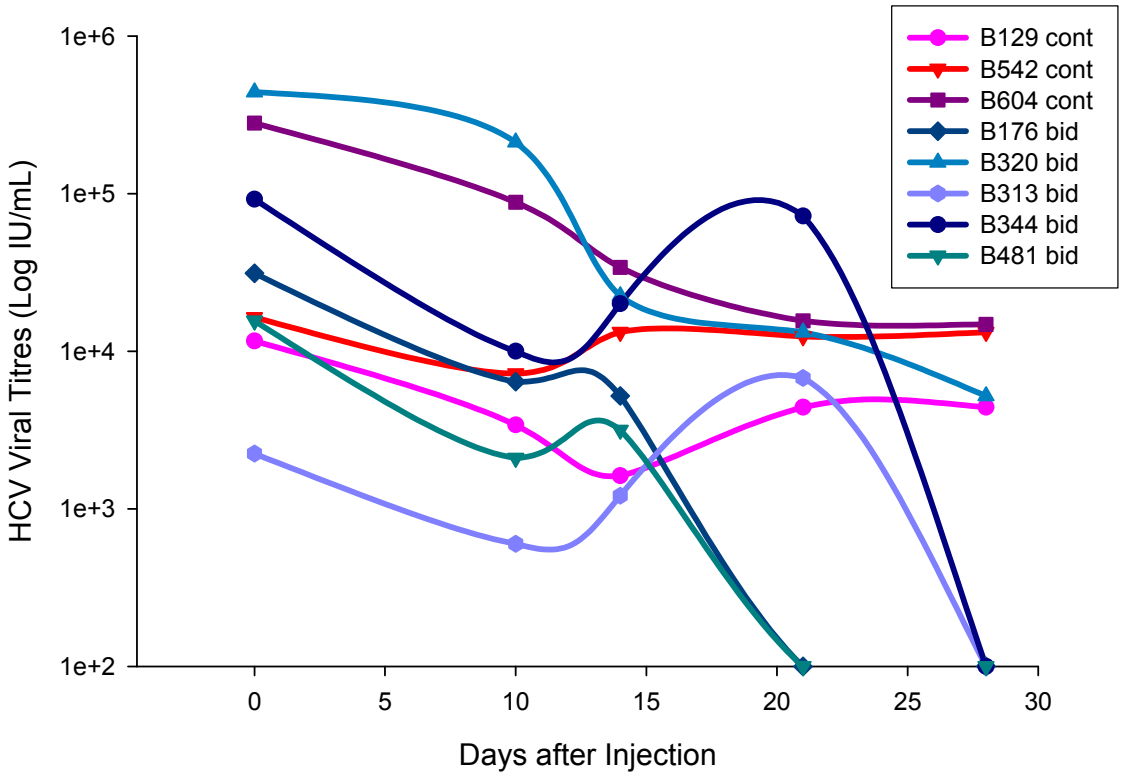

Figure 3.4. HCV load following multiple mBID treatments. HCV titers were determined after initiation of three doses of mBID given seven days apart. Chimeric mice were infected with Ad-mBID on days 0, 7 and 14 and serum samples were analyzed for HCV RNA on days 10, 14, 21 and 28 after the initial injection of Ad-mBID or control adenovirus. Samples were assayed for HCV genomic RNA.

Mouse	Initial HCV Titre (Log)	Final HCV Titre (Log)	Difference in Titres (Log)
CONTROL	4.06	3.64	0.42
	4.21	4.12	0.09
	5.45	4.17	1.28
	6.18	5.64	0.54
	5.63	5.32	0.31
			Mean drop 0.53 logs
mBID	4.49	<2	>2.49
	5.64	3.72	1.92
	3.35	<2	>1.35
	4.96	<2	>2.96
	4.19	<2	>2.19
**	3.91	<2	>1.91
**	4.54	<2	>2.54
**	6.90	5.08	1.82
**	5.56	5.72	-0.16
			Mean drop >1.89 logs

Table 3.2. HCV titres in mice receiving multiple injections of mBID. The first five mBID animals received three injections at 7 day intervals. The remaining four (marked by**) received three injections at 3 day intervals. Compared to the control animals receiving empty adenovirus vectors, the mean drop of HCV titres was >1.89 logs compared to 0.53 log in control mice ($p=0.019$).

There were indications that mBID alone was cytotoxic. In Figures 3.3, serum ALT levels rose in some control animals (+ mBID, - HCV) after multiple dose applications. No morbidity was associated with this treatment nor was there direct evidence of apoptosis in the liver after 10 days of treatment (Figure 3.2).

With each of three dosage regimens, there was evidence of the efficacy of mBID to reduce HCV titres. Despite the failure of single dose therapy to garner statistically significant results, a consideration of the responses of individual mice groups them into responders (3/6) and non-responders (3/6). Multi-dose approaches produced non-responder as well (or weak responders; 1/4 and 1/5). Why this variation occurs is important to the understanding of the mouse model as it is to the efficacy of mBID. Results from the single course of therapy suggest technical limitations such as in the delivery of mBID, or perhaps the state of the recipient mice such as the state of the infection or success of the engraftment. That repeated doses mBID reduced the frequency of non-responders is consistent with the delivery (or infection) of Ad-mBID being a significant variable. Consistency in the preparation and delivery of Ad-mBID was not tested and no conclusions are drawn regarding the cause of this variation. However, if overcome, it could lead to therapeutic efficacy with single doses of mBID with much reduced toxicity.

Comparing 3-day and 7-day regimens highlighted another variable that could impact on the success of treatments such as mBID. One dose of mBID led to cell death (rapidly elevated serum ALT) and, in some cases, a 2 log drop in viral titres. Hence, mBID acted quickly when it did act. Repeated doses, in the 3-day regimen, presumably maintained the effect to the termination of observations. As no direct examination was made of the impact of these subsequent doses, it remains open to discussion if one or both were important to levels of protection achieved. The 7-day regimen offers an intriguing insight. First, reductions in titres after 10 days were only one log. This could be misleading if, as seen in the 3-day regimen, one dose can induce a 2 log drop.

As the first titre wasn't determined until day 10, it is possible that titres dropped then rebounded in this time frame. In fact, that is what is seen at later time points: a rebound in virus titres around the time of the third Ad-mBID injection. The timing of rebounds may be critical because the 3rd dose of mBID reduced HCV titres to undetectable levels. A key unknown is the importance of timing subsequent doses relative to hepatocyte regeneration, as rebounding virus most likely involves the recent infection of cells not exposed to Ad-mBID. A better understanding of the appearance of regenerative cells and their impact on the experimental HCV infection would benefit the design of mBID protocols that would be more effective and less toxic.

Modified BID was observed to be toxic on two levels. In the context of an HCV-infected cell, it was by design toxic. If most liver cells were infected, then the liver could be destroyed by Ad-mBID. Single dose was not toxic in this fashion, perhaps because enough cells escaped infection by mBID or HCV. And, importantly, regenerating cells were not exposed to mBID. Repeated exposure to mBID in quick succession, in an environment with HCV, could conceivably target every surviving and/or regenerative hepatocyte. Waiting between doses may allow enough hepatocyte survival to sustain liver function while the anti-viral effects of Ad-mBID are imposed. The second level of toxicity is unrelated to HCV activation of mBID. Some cell death was attributed to mBID in the absence of HCV (Figure 3.3A). Interestingly, this could be during the period of liver regeneration. Additional work would have to address the possibility that mBID is more toxic to regenerating hepatocytes than it is for non-replicating hepatocytes.

No conclusions can be drawn regarding the long-term effects of Ad-mBID treatments. It could be that one or two doses could effectively reduce titres to a point where immune elimination of infection might be achievable. However, it is reasonable to assume that treatments would be undertaken in the presence of protease or polymerase inhibitors that would reduce the likelihood of productive re-infections of healthy regenerating liver cells. The chimeric mouse model is an

ideal tool with which to address all possible permutations in protocols to improve mBID and other cytotoxic anti-virals. In humans, a targeted therapeutic approach using cytotoxic agents such as modified BID may be most useful in the early stages of hepatitis, during limited infection of the liver. It is conceivable that modified BID administration will reduce virus loads in chronically infected patients, and in conjunction with interferon/ribivarin therapy eradicate HCV from the infected host. It could also be used as a prophylactic to protect individuals following accidental exposure to the virus or to prevent re-infection of the transplanted liver in transplant patients who are HCV positive. The rate of recurrence of an active HCV infection in the transplanted livers of HCV positive patients is 100%. By treating the liver *ex vivo* with modified BID prior to transplantation, a recurrent HCV infection could potentially be prevented.

Adenovirus has innate qualities that make it advantageous for gene therapy. With regards to safety, the wild-type adenovirus causes relatively mild disease in the human host. In a study examining the effects of wild-type virus on tumor cells, patients had minimal side effects from the adenovirus administration – i.e. flu-like symptoms that lasted anywhere from 2-7 days. [17] Although there has been one tragedy of an 18 year old being treated for ornithine transcarbamylase deficiency with a gene carried within an adenovirus vector, this was likely due to a toxic dose of adenoviral proteins injected intravenously resulting in acute hepatic failure secondary to a massive immune response to the foreign protein load [15]. Adenovirus utility in liver-directed gene therapy was realized [18, 19] but innate immunity and the inflammatory response unfortunately limited the use of first generation adenovirus vectors in humans [20]. Over-efficient neutralization of the virus by the host immune system, broad target cell specificity, and a need to optimize the route of delivery has further complicated the use of adenovirus vectors.

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Chapter 4

Evaluation of Animal Model Production

Reported in this chapter are four approaches explored to further the understanding and utility of the Alb-uPA/SCID/Bg mouse model. In previous chapters, the validity of this system to model HCV infections and to respond predictably to anti-viral therapies was documented. Value can be added to the model with measures designed to attain consistently high levels of human hepatocyte engraftment. One approach was to better define the parameters that determine successful grafts through an historical evaluation of transplants. A characterization of the cell types involved in the repopulation of the liver was a second avenue explored. A third approach was to supplement the mouse model with hepatocyte growth factor (HGF) by exogenous supply or through transgenesis. And last, the limited supply of human graft tissue spurred an exploration of stem cells as an alternative source of hepatocytes.

PART A. Umbilical stem cells as a source of hepatocytes.

There are ongoing developments in the search for an alternative source of hepatocytes for transplantation in end stage liver disease. Recent advancements in the field of stem cell research have led many to believe that there is great potential in pluripotent cells as a building block for hepatocyte transplant. There is interest in using both adult and embryonic stem cells, but ethical issues surrounding the use of embryonic tissue prompted our choice to investigate the use of adult stem cells as transplant material to create chimeric mice. There is evidence for the derivation of hepatocytes from blood borne stem cells isolated from bone marrow. One report characterized the differentiation of bone marrow cells into hepatocytes following bone marrow

transplants in rodents [1]. Human studies, with mismatched bone marrow transplants, demonstrated the same by detecting the presence of Y chromosome hepatocytes in XX recipients [2]. We postulated that Alb-uPA/SCID/Bg livers could be repopulated with hematopoetic stem cells. The strong stimulus for regeneration in our mice due to the presence of the uPA transgene should be conducive to differentiation, maturation and proliferation of the transferred stem cells.

Liver stem cells. In the liver there are three replicative cells thought to contribute to the regenerative process. These are adult hepatocytes, intrahepatic stem cells, or oval cells (tissue determined stem cell derived from ductular progenitor cells), and extrahepatic stem cells that are derived from circulating bone marrow stem cells. By definition, the stem cell is capable of self-renewal. In addition, it has the potential of producing at least one, but often, many different specialized cell types; they are pluripotent. In the adult, stem cells are important for maintaining tissue homeostasis and repair.

Stem cells can be broadly classified into totipotent, embryonic and determined. Totipotent cells can produce adult cell types of all germ layers (ectoderm, mesoderm and endoderm). Although these cells are mainly found in embryonic tissues, there has been some recent controversial evidence that a few of these cells persist in the adult bone marrow [3,4]. Embryonic stem cells, which are also totipotent, are unique because they have the ability to enter the germ line and are derived from pre-implantation or peri-implantation embryos. In the adult animal, the majority of the stem cells are determined and/or committed progenitors. Determined stem cells are pluripotent but cannot give rise to all possible cell types. Committed progenitors, however, are precursors for only a single cell type.

Mature hepatocytes have the ability to replicate up to two cycles when the appropriate signals are present but have no multipotentiality. That is, they can only produce more hepatocytes [5,6].

These cells are important because they are numerous and respond rapidly to liver injury. It has been proposed that the level of injury determines the cell response in the liver. Acute loss of normal liver tissue results in proliferation of the mature hepatocyte. However, if there is massive insult, extensive chronic injury or any inhibition to hepatocyte proliferation by viral infections, the amount of replication from the remaining adult hepatocytes is not always sufficient. Small-hepatocytes generated from oval cells (periductular liver stem cells), are recruited to the liver parenchyma. These cells have the ability to differentiate into hepatocytes or biliary epithelial cells and their replication accounts for a significant amount of the resulting regeneration [6].

The mechanisms of stem cell recruitment and activation are not well understood, however, there are significant similarities in the stem cell model of tissue renewal and stem cells in embryonic development. Embryonic and adult stem cells share many of the same receptors (c-kit, Thy-1, CD34) and respond to many of the same cytokines and growth factors. From this one can hypothesize that in the environment of the Alb-uPA/SCID/Bg mouse liver, stem cells can be directed from different sources to hone to the liver, proliferate and repopulate the injured liver.

Oval cells. Following significant parenchymal cell loss (toxins, surgery), hepatocytes re-enter the cell cycle at the G_0 phase and, in a standard partial hepatectomy where up to two-thirds of the liver is removed, the few cycles of replication adult hepatocytes can undergo is sufficient to regenerate to the pre-operative cell mass [7,8]. However, in developing the techniques of hepatocyte transplants as an alternative to whole organ transplants, significant re-expansion of the grafted cells led to the hypothesis that these adult cells have the capacity of clonal expansion. Interestingly, the contribution of hepatic stem cells to this re-population has not been well characterized. Within the liver, there are resident hepatic stem cells, known as oval cells, which respond to massive hepatic injury. This population of cells resides in the canals of Herring, the point where the terminal bile ducts are adjacent to periportal hepatocytes. Upon injury, oval cells

are recruited to the hepatic lobules where they differentiate into either liver parenchymal cells or biliary epithelial cells [9]. There are two theories on the origin of resident hepatic stem cells. Initially it was believed that oval cells were the product of biliary epithelial cells de-differentiating to become pluripotent. More recent findings have demonstrated the presence of hepatic cells of donor origin in bone marrow transplant recipients, leading to the proposal that a certain number of liver stem cells are derived from hematopoetic stem cells [1,2,10,11]. Following hepatic insult, these latter stem cells are recruited to the liver and undergo differentiation into hepatic cells.

Cell differentiation during liver embryogenesis. The liver develops from the ventral foregut endoderm [12]. The developing liver has temporal and spatial relations to both the pancreatic bud as well as cardiac mesoderm. Both pancreas and liver develops from a common endoderm that is diverted to one of two fates. The default pathway is to pancreatic tissue. The adjacent cardiac mesoderm provides the necessary signal to promote hepatogenesis. Fibroblast growth factor (FGF) produced by the cardiac cells is sufficient to divert the non-committed endoderm cell into a hepatoblast. In tissue explant studies, the proximity of cardiac mesoderm to developing ventral endoderm is required for the activation of liver specific genes and further liver development. After this initial differentiation, a concert of cytokines is involved sequentially to continue maturation and proliferation of the hepatocyte [12,13].

Early in embryogenesis, the major site of hematopoeisis is the liver. In late fetal and early neonatal stages, the liver appears to lose this hematopoetic function and takes on metabolic functions as a result of a cascade of events involving cellular differentiation and signaling [13]. Oncostatin M (OSM) is produced by hematopoetic cells and OSM receptors are mainly expressed on hepatocytes. It is a member of the interleukin-6 (IL-6) cytokine family. It is expressed in mid-fetal to perinatal liver but decreases significantly after birth. OSM stimulates progression of

hepatocytic development through the induction of morphological changes in the cells, at the same time inducing liver-specific marker genes (glucose-6-phosphatase and tyrosine amino transferase. OSM also stimulates functional maturation of the liver cell: glycogenesis, lipogenesis and ammonia clearance. In development, as the mass of differentiated hematopoeitic cells increases in the fetal liver, there is consequently an increased amount of OSM produced [13,14]. This later increase results in the maturation of the liver parenchymal cells and termination of the embyronic liver as a hematopoeitic organ. As OSM expression decreases after birth, the presence of other cytokines becomes more influential in the continuation of liver maturation and proliferation. There are a number of growth factors that are essential in hepatic maturation and proliferation including hepatocyte growth factor (HGF), tumor necrosis factor and IL- 6. In development, HGF expression is mainly seen at the perinatal stage [14,15]. HGF provides the initial stimulus for differentiation and IL-6 is required for continued replication.

To manipulate the differentiation of stem cells into hepatocytes, understanding of the underlying mechanisms for both liver development and regeneration are integral. Liver regeneration is very likely to involve endogenous and exogenous stem cells. Applying the principles of stem cell differentiation clinically could be a major benefit to hepatocyte transplantation. Because the Alb-uPA transgene is toxic to the murine liver, essentially resulting in acute liver failure, there is a tremendous endogenous stimulus for hepatic regeneration. The environment should provide the necessary signals for differentiation, maturation and subsequent proliferation of stem cells into hepatocytes, providing an experimental model to define these differentiation principles.

The following experiments were designed to test two hypotheses regarding the repopulation of the Alb-uPA/SCID/Bg mouse liver with stem cells.

1) The efficiency and success rate of long-standing human hepatocyte grafts will be increased by enriching the progenitor cell population in the transplanted material.

2) Human hematopoietic stem cells from umbilical cord blood have the potential to differentiate into the hepatic lineage and establish a significant hepatocyte graft.

Grafts composed or containing exogenous stem cells will be evaluated for hepatitis C (HCV) infectability. The goal is to substantiate the use of stem cells as a constant source of hepatocytes for transplantation, in the chimeric mouse model and ultimately in the treatment of chronic metabolic liver disease in humans.

Materials and Methods

Cord blood cells. Fresh cord blood cells (CBC) were obtained from research samples collected by the Alberta Cord Blood Bank. Prior to receiving the samples, the cells had undergone a Ficoll purification by the blood bank. Maternal consent for research was obtained prior to donation of the blood sample.

Characterization of CBCs by flow cytometry. Fresh CBCs were treated with RBC lysis buffer (Sigma) and were suspended in PBS/0.1% sodium azide at a concentration of 10^6 cells/0.02 ml. Antibodies to stem cell markers (in 5 μl) were mixed with the cells on ice for 20 minutes (FITC-conjugated anti-CD34, CyChrome-conjugated anti-Thy-1, and PE-conjugated anti-c-kit). PE-conjugated anti-C-met was used to identify cells potentially responsive to hepatocyte growth factor. Samples were analyzed in a Bectin Dickinson fluorescence cytometer using CellQuest software.

Alb-uPA/SCID/Bg Mice. Animals were reared and housed in a level 2 biocontainment facility, and fed sterilized, high fat (9%) chow. All procedures were approved by the institutional Animal Welfare and Ethics committee and conformed to Canadian ACUC guidelines. The animals were rescued with transplants of primary hepatocytes, or cultured cord blood cells, between 5-7 days of age. Cells were injected intra-splenically while animals were anesthetized with 5% enflurane.

In vitro culture of cord blood cells. Cord blood cells were transferred from the blood collection bag, diluted to $2x10^6$ cells/ml and plated on collagen coated plates. Media of 1:1 Dulbecco's Minimal Essential Media and Hams F-12 containing 1x insulin/transferrin/selenium, 10 ng/ml epidermal growth factor, 1%BSA, and 100 mg/mL dexamethasone. The cells were cultured at 37°, 5% CO_2 overnight (14 hours). Supplemented media with fibroblast growth factor (20 ng/ml), oncostatin M (20 ng/ml) or hepatocyte growth factor (10 ng/ml) were added sequentially with media changes every two days. The cells were treated for 7 days with FGF, then 5 days each with OSM and HGF. Supernatants were harvested at various time points.

Western blots. Supernatant fluids or mouse sera were collected and fractionated by SDS-PAGE run under reducing conditions. A positive control of human serum diluted 1:500 was ran simultaneously as a positive control. Protein was electroblotted unto nitrocellulose membranes overnight at 4°C at 10 V. Membranes were then probed with goat anti-human albumin at a 1:5000 dilution and washed. Protein was visualized following reaction with HRP-labeled donkey anti-goat IgG antibody (1:5000 dilution) and peroxidase staining (Sigma).

Immunohistochemistry. To prepare slides, cells were either grown directly on Biocoat Collagen coated plates or cell suspensions were dropped onto Histobond slides and dried. Slides were fixed in a 9.25% formalin/10% glacial acetic acid solution and then stored in 70% ethanol until ready to stain. For both albumin and α-fetoprotein antibodies, antigen retrieval was done on the sections prior to staining. Albumin antibody was used at a 1:5000 dilution and alpha-feto-protein was used at a 1:10000 dilution. Visualization was with an HRP-linked secondary antibody and subsequent DAB reaction (Sigma).

Results

Characterization of isolated hepatocytes from human liver biopsies. Standard protocol for engraftment of human hepatocytes into the Alb-uPA/SCID/Bg mouse model entailed the isolation of hepatocytes from liver resections via collagenase perfusion and percoll density gradient purification. A number of cell preparations were screened for the presence of stem cells and, secondarily, to see if there was a correlation between stem cell number and the success of related transplants. The number of stem cells (oval cells/hepatic stem cells) may have varied due to the presence of metastatic tumors in the liver resections. It has been reported that oval cells increase in reaction to viral hepatitis, toxic injury, hepatocellular and metastatic carcinoma [16].

After isolating the hepatocytes, two markers were used to identify potential oval cells and hepatic stem cells. C-kit and Thy-1 are two hematopoetic stem cell markers that are also found on isolated oval cells. Cell preparations were distinguished on the basis of donor age and the presence of tumors. The first sample was from a 5 year old donor patient. These cells were revived from cryopreservation and engrafted into mice resulting in transplants of human alpha-1-anti-trypsin levels over 100 μg/L at 4 weeks post-transplant. The second and third samples were taken from a 46 year old and a 69 year old patient both of whom had a liver resection for metastatic colon cancer to the liver. These cells were not frozen prior to transplantation but were frozen prior to FACS analysis. A fourth sample was a resection from a 64 year-old with metastatic disease. Fresh cells were used for both murine transplants and FACS analysis. Successful murine transplants were achieved at rates of 55%, 16%, 14%, and 11% for the four samples, respectively (human alpha-1-anti-trypsin levels greater than 100 μg/L). Cell preparations were stained individually, or in combination, for c-kit, thy-1, CD34, and c-met and analyzed by FACS and the percentage of presumptive stem cells determined (Table 4.1).

Though limited in scope, this analysis confirmed the presence of stem cell forms in the transplanted human cell preparations. Concern over the loss of cell surface markers following cryopreservation seemed unwarranted, though direct comparisons were not undertaken. What the data failed to show was a correlation between stem cell content and the rate of successful murine transplants. There was an apparent correlation with age of the donor, and this topic is taken up in a later section.

Differentiation of umbilical cord blood cells in culture. Cord blood cells were obtained from the Alberta Cord Blood Bank and cultured for twenty-four hours in media without any added growth factors. These cells were sorted and CD34+ stem cells were determined to make up about 2% of the cell population (Figure 4.1). A larger percentage of cells (24%) were shown to express the HGF receptor (c-met). Growth factors were added sequentially in a fashion that mimics what is expressed locally in the developing embryonic liver bud. The protocol followed was the sequential addition of fibroblast growth factor, oncostatin M and finally hepatocyte growth factor as reported by others [17].

Age of Donor (yr)	Transplant Success	c-kit	Thy-1	c-kit/thy-1
5[1]	55%	3.4%	2.5%	1.3%
46[2,4]	16%	22%	31%	24%
69[2,4]	14%	9.0%	12%	8.0%
64[3,4]	11%	1.8%	3.0%	1.7%

Table 4.1. Stem cell composition of hepatocyte preparations used in murine transplantation. Percentages of stem cells (c-kit+/thy-1+) identified by flow cytometry in different liver cell preparations are shown in relation to mouse transplant success rates and the age of each liver donor. [1]Transplanted and sorted as cryopreserved hepatocytes. [2]Transplanted as fresh hepatocytes, sorted after freezing. [3]Transplanted and sorted as fresh cells. [4]Donor with metastatic carcinoma.

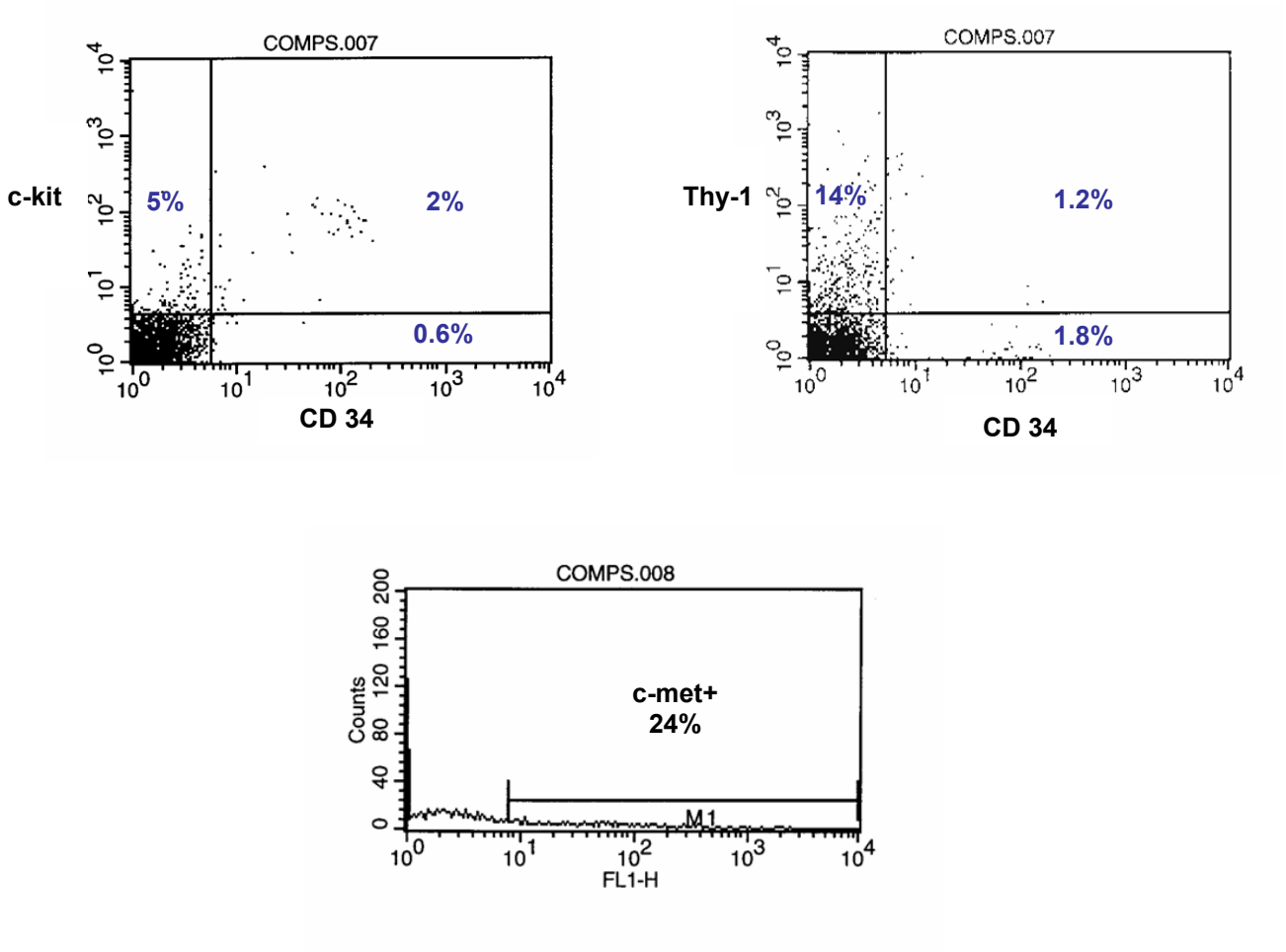

Figure 4.1. Flow cytometry of fresh cord blood cells. Cell preparations were stained for CD34 to identify stem cells. C-kit and Thy-1 are other surface antigens that appear on stem cells. C-met (receptor for HGF) was detected to estimate the number of CBCs that might be responsive to HGF during liver regeneration.

However, the timing of growth factor additions was based on measured amounts of albumin released into the medium (Figure 4.2). Media was assayed from day three post-addition of fibroblast growth factor (FGF). By day five, there was production of albumin in the supernatants which was the signal to add oncostatin M. Subsequently, following seven days of oncostatin M, there was an increase in confluency on the cell culture plates as well as a stronger albumin band on western blot. Cells were then treated with human hepatocyte growth factor for seven days at which point, colonies of cells developed. At this time, albumin production was increased over that from treatment following OSM.

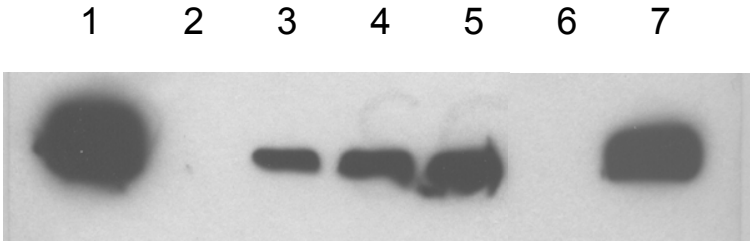

Figure 4.2. Albumin production by differentiated cord blood cells. Culture medium was analyzed by Western blot following growth factor treatment. Lane 1: human serum; Lane 2: culture media; Lanes 3, 4, and 5: CBCs plus fibroblast growth factor (day 5 post-treament), FGF plus oncostatin M (day 12), and FGF, OSM and hepatocyte growth factor (day 15); Lane 6: untreated cells; Lane 7: FGF, OSM, plus HGF (day 19 - day of transplanting into Alb-uPA/SCID/Bg mice).

Hepatocyte differentiation by indocyanine green uptake. Indocyanine green (ICG) is a nontoxic organic anion used clinically to assess liver function. It is specifically taken up and then secreted by hepatocytes. When added to culture supernatants, differentiated hepatocytes take up the ICG and appear green under a stereomicroscope. After the treatment of CBCs with FGF, OSM and HGF, ICG was added at 1 mg/ml to the cultures. Following a fifteen minute incubation time, clumps of cells under the stereomicroscope had taken up the dye (Figure 4.3, panels A and B). When examined one hour later, the cells had excreted the ICG and were no longer green in color. This demonstrated that our cells in culture had acquired a liver specific function and differentiated to hepatocyte-like phenotype. Undifferentiated cells (untreated CBCs) did not take up the ICG dye.

Hepatocyte differentiation by immunohistology. Hepatocytes were also identified by immunostaining differentiated CBCs for α-fetoprotein and albumin. Cells were grown and differentiated on collagen coated slides. Both α-fetoprotein (AFP) and albumin staining were seen following treatment with fibroblast growth factor alone. At this stage, there were more AFP positive cells than albumin positive cells. After treatment with all three factors, FGF, OSM and HGF, there were significantly more albumin positive cells, though absolute numbers of AFP positive cells did increase (Figure 4.4). Both of these markers are liver specific and their expression demonstrates an evolution in the development of the hepatocyte. The presence of AFP in cells denotes early differentiation along the hepatocyte lineage but albumin staining is consistent with mature hepatocytes.

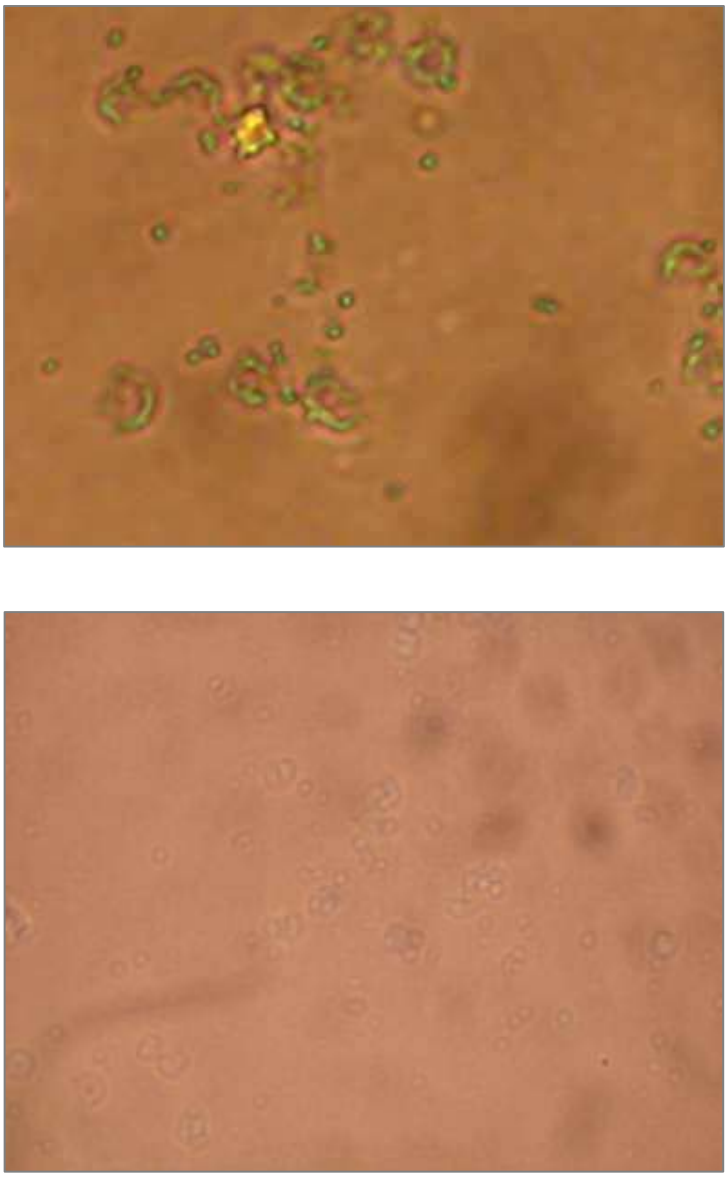

Figure 4.3. Hepatocyte features of growth factor-treated cord blood cells. CBCs treated with FGF, OSM and HGF took up indocyanine green as functional hepatocytes do (top panel) whereas cells cultured without growth factors did not (bottom panel). Cells were treated with ICG and photographed within 15 minutes.

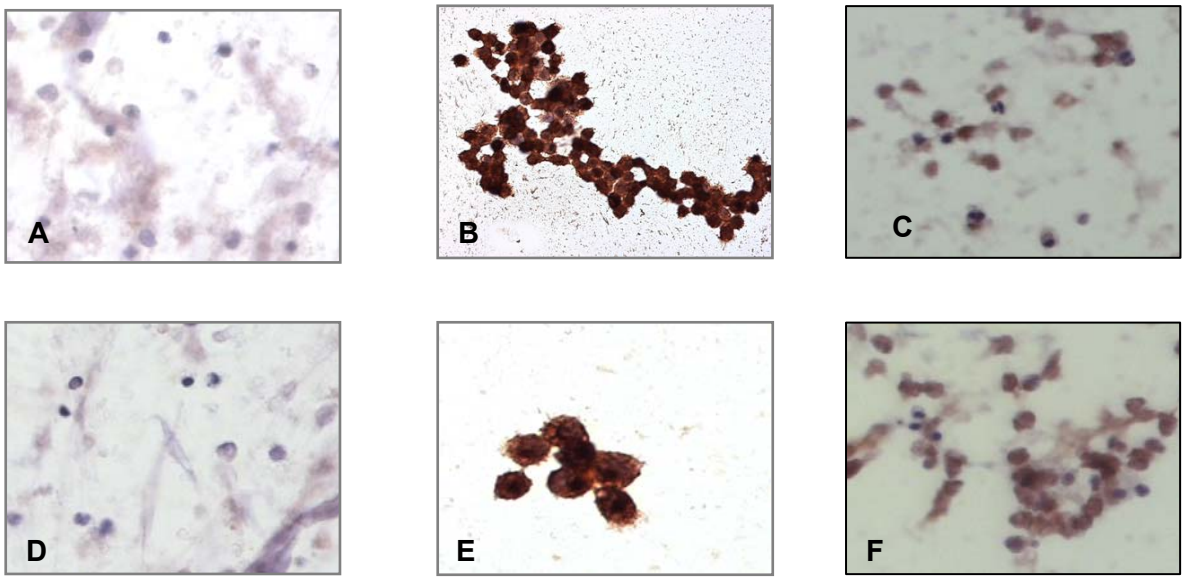

Figure 4.4. Differentiated stem cells produce hepatocyte-specific proteins. Differentiated (C,F), or undifferentiated cord blood cells (A,D), were immunostained for α-fetoprotein (A,B,C) or albumin (D,E,F) as markers of hepatocyte maturation. Panels B and E are Huh7 cells as a positive hepatocyte control.

***Transplantation of differentiated umbilical cord blood cells into Alb-uPA/SCID/Bg mice*.** Two million CBCs treated with growth factors, were transplanted into three 5 day-old Alb-uPA/SCID/Bg mouse pups via inferior splenic pole injection. After transplantation, we followed the mice for four weeks at which point they were sampled for the presence of human albumin in the serum. We were able to detect human albumin starting at four weeks post-transplant via western blot of diluted serum and again after two months (Figure 4.5). No human α-1-anti-trypsin was detectable in the serum by ELISA using standard assay procedures.

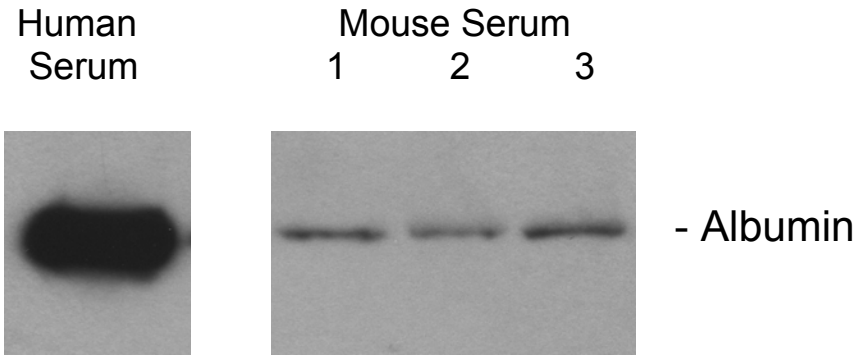

Figure 4.5. Human albumin production in engrafted mice. By western blot, human albumin was detected in chimeric mice transplanted with differentiated cord blood cells. Diluted human serum was compared with serum from three Alb-uPA/SCID/Bg mice transplanted with differentiated CBCs eight weeks post-transplantation.

Discussion

The number of available liver donors is not sufficient to satisfy the number of patients requiring liver transplants. There is two potential modes of liver replacement therapy – whole organ transplantation and cellular (hepatocyte) transplantation. Since primary hepatocytes are from the same pool of resources as whole organs, alternative cell sources have become increasingly important. Stem cell transplantation may be a feasible alternative since they can be induced to differentiate into functional hepatocytes. In addition, they are thought to be less immunogenic.

The chimeric mouse model can be used to test alternative means of liver replacement. The potential for using stem cells was explored, first by measuring the percentage of stem cell forms in liver cell suspensions currently used in creating the chimeric mouse model. Stem cells, or oval cells, that normally reside in the liver formed a small proportion of the transplanted liver cells in typical experiments (1-10%). This would imply a minor contribution in the re-population of Alb-uPA mouse livers given the abundance of adult hepatocytes transferred. Our limited data is consistent with this interpretation as stem cell numbers did not correlate with successful engraftment of Alb-uPA mice. Still, there could be an important role played by endogenous stem cells and their selective enrichment in transplanted material may be beneficial, in terms of supplying progenitors or supporting hepatocyte regeneration indirectly.

Were abundant stem cells available, it may be possible to use differentiated or undifferentiated stem cells to establish, or promote, a functional liver graft. Previous attempts to transplant undifferentiated mononuclear cells from umbilical cord blood, or isolated CD34+ population of cells from cord blood, into the Alb-uPA/SCID/Bg mice failed to establish life-sustaining grafts. Consequently, *in vitro* attempts to pre-differentiate stem cells along the hepatocyte lineage were made as a possible means of supplying graft material for making chimeric mice. Following an established protocol, FGF, oncostatin M, and HGF were sequentially added to cord blood cells

containing about 2% stem cells (CD34+ cells). Treated cells manifested a number of liver-specific attributes, such as the production of albumin and α-fetoprotein and the uptake of indocyanine green. After transplantation of CBCs containing a proportion of pre-differentiated cells into the Alb-uPA/SCID/Bg mice, human albumin was detectable in the mice eight weeks post-transplant. These transplants were successful in rescuing the Alb-uPA mice but the chimeric mice were rejected as models of HCV infection because serum α-anti-trypsin was undetectable.

Since these experiments were conducted, others have successfully differentiated human bone marrow stem cells into mature hepatocytes [18]. Murine stem cells lines have also been successfully transplanted into Alb-uPA/SCID mice [19] or MUP-uPA/SCID (major urinary protein-uPA) mice [20]. Human stem cell lines would be quite valuable but ethical considerations will slow their development. Rogier's group in Belgium demonstrated with sequential treatment of adult bone marrow stem cells and cryopreserved liver epithelial cells, a hepatocyte-like cell could be produced. These differentiated cells were able to rescue CCL_4 treated toxic mice [21]. The supply of fresh umbilical cord blood cells limited the studies I report here. When cryopreserved umbilical cord blood cells were used, they did not survive long term in culture. Bone marrow precursors may be a better alternative, short of the establishment of human stem cell lines.

An interesting question has arisen over the involvement of transdifferentiation (cell fusion) of exogenous transplanted adult stem cells in liver tissue. Cell fusion is a mechanism to explain the plasticity of stem cells and their ability to differentiate into multiple lineages upon transplantation. Essentially, transplanted stem cells hybridize with recipient cells to form multiploidy cells. These fused cells subsequently take on the phenotype on the recipient cells [22,23]. Cell fusion was documented when monkey embryoid bodies were transplanted into Alb-

uPA/SCID mice [24]. However, cell fusion was not demonstrated in the experiments with bipotential liver stem cells transplanted into Alb-uPA/SCID mice or with differentiated hepatocyte-like cells transplanted into MUP-uPA/SCID mice [19,20]. Because embryoid bodies are at a different state of maturation compared to pre-differentiated cells, they may be prone to hybridization through cell fusion. More differentiated cells may preferably undergo integration although low levels of fusion cannot be completely disregarded. Using adult hepatoctyes for transplantation in the Alb-uPA/SCID/Bg mice, repopulation occurs without fusion. In our experiments with pre-differentiated hepatocyte-like cells transplanted, repopulation was also likely to occur without fusion, however, to demonstrate this would require additional studies.

Our results using undifferentiated cord blood cells as a source for hepatocyte transplantation was encouraging in as much as hepatocyte differentiation was achieved and recipient mice were rescued. The limited success of the transplants was likely due to the small number of differentiated hepatocytes transferred compared to comparable transplants using murine stem cells [19,20]. Perhaps with the enrichment of stem cells in CBC populations or a similar application with more abundant human bone marrow stem cells, an alternative supply of hepatocytes could be realized. Our results support the further exploration of differentiated stem cells as both a possible source for hepatocyte transplantation in experimental models and clinical therapies.

PART B: Human HGF and Liver Reconstitution in Chimeric Mice

The overall efficacy of hepatocyte transplantation and HCV infection in the SCID/Bg/Alb-uPA model has been about 25%. For every four mice transplanted with fresh human hepatocytes, one mouse was engrafted with human hepatocytes that could sustain an infection with hepatitis C virus. Dissatisfied with this rate of success, different avenues were explored to enhance the rate of successful transplants as well as to increase the longevity of the transplanted cells. The literature reveals that liver regeneration and growth is dependent on a number of regulatory cytokines and growth factors. In particular, hepatocyte growth factor (HGF) is the most potent mitogenic growth factor identified. Initial uses of exogenous HGF administration *in vivo* demonstrated the factors ability to promote hepatocyte transplants of greater cell mass [25-27]. In preliminary experiments with engrafted Alb-uPA/SCID/Bg mice, human HGF showed promise in enhancing transplantation efficiency (unpublished data, D. Mercer). In these experiments, human HGF was delivered to the mice via an osmotic mini-pump, resulting in elevated levels of human albumin in the serum (indicative of stronger human grafts). Trophic differences between human and murine HGF, reflecting some level of species specificity, may be the basis of the augmenting effect of human HGF.

Reported in this section are attempts to substantiate the efficacy of exogenous HGF in the chimeric mouse model. In addition, to have human HGF present at birth, and to avoid another invasive surgical procedure on recipient mice, a double transgenic model was made in which a gene for human HGF was introduced into the Alb-uPA background. It was anticipated that transplantation success in recipient mice would be improved by both these experimental approaches.

Methods and Materials

Animal studies. Similar to previous chapters, all animal studies were done in compliance with regulations set forth by the Canadian Council of Animal Care. All protocols were approved by the Health Sciences Animal Welfare Committee at the University of Alberta. The mice were transplanted with human hepatocytes according to a protocol previously published [28]. Briefly, pups homozygous for the Alb-uPA transgene were transplanted with one million fresh human hepatocytes via intrasplenic injection between the ages of five and fourteen days. The serum of these animals was tested at four weeks and eight weeks post transplant for the level of human α-1-anti-trypsin to determine the success of the transplant.

Hepatocyte isolation from human liver biopsies. As previously described [28], liver wedge biopsies were obtained from the operating room after surgical resections of the liver. Specimens were flushed with cold phosphate buffered saline in the operating room once removed from the patient. They were placed on ice, in PBS, for transport back to the laboratory. Subsequently, the main feeding vessels to the liver were cannulated and the liver biopsy perfused with collagenase digestion solution via a peristaltic pump. The hepatocytes were disaggregated, filtered, and washed, before being purified on a Percoll gradient.

Human hepatocyte culture and stimulation. Hepatocytes were cultured on collagen (rat tail collagen I) coated dishes and the media of 1:1 DMEM and F12 was supplemented with epidermal growth factor, dexamethasone, insulin, transferin, selenic acid, and fetal bovine serum. Both human hepatocyte growth factor and murine HGF were produced in COS cells that had been transfected with human or murine HGF. Transfection success was confirmed by the detection of HGF mRNA via RT-PCR (forward primer: 5' attggatcaggaccatgtga, reverse primer: 5' aaaggccttgcaagtgaatg). Human HGF in the culture medium was assayed by an ELISA sandwich detection kit. Human hepatocytes were treated with the supernatants of transfected cells from

either the human or murine HGF transfected cells. Flow cytometry was subsequently performed on the cells labeled with BrdU to determine any difference in proliferation of the human hepatocytes.

Hepatocyte isolation from mouse livers. Liver was harvested from Alb-uPA/SCID/Bg mice at the time of sacrifice or from a partial hepatectomy. Using a 25 gauge needle, the tissue was flushed with PBS via the portal vein (in whole livers) or the left hepatic artery (in partial hepatectomies). Once the effluent was clear, the tissue was sliced into 5 millimeter pieces and digested with collagenase (5 mg of Liberase H1 [Roche Diagnostics] per gram of tissue) for ten minutes at 37° C with intermittent agitation. The digested material was pipetted through a 10mL pipet to mechanically disrupt the tissue. After 5 minutes incubation at 37° C, the digests were placed on ice for 5 minutes with continued intermittent agitation. The liver tissue was passed through coarse and fine filters (45 micron pore size) to separate the digest into a single cell suspension. The cells were pelleted by centrifugation at 500 rpm for 3 minutes, resuspended in Hanks Buffered Salt solution (HBSS), and RBCs were lysed (Sigma lysing buffer - 0.83% ammonium chloride in 0.01M Tris buffer). The cells were collected by centrifugation at 500 rpm for 3 minutes and resuspended in HBSS, cell culture media or FACS staining buffer (0.1% sodium azide, 1.0% bovine serum albumin in PBS). Cell viability was determined by trypan blue staining.

Flow cytometry. Isolated cells that were cultured *in vitro* and hepatocytes isolated from Alb-uPA/SCID/Bg mice were used as samples for cytometry. Cells were resuspended in PBS/0.1%NaN_3 at a concentration of one million cells per twenty microlitres. Five μL of PE-labelled anti-CD46 was added to one million cells, mixed gently and maintained on ice for 20

minutes. Samples were analyzed with a Becton Dickinson fluorescence cytometer using CellQuest software.

HGF Transgenesis. Two forms of the human HGF gene were injected into mouse embryos to create transgenic mice. The first was a genomic clone contained in a BAC vector supplied by Dr. J.F. Elliot. Microinjection of the entire genomic DNA segment into single-cell Alb-uPA/SCID/Bg embryos was performed by the University of Alberta Transgenic Facility. DNA was isolated from tail biopsies of the resulting pups using standard procedures [29] and PCR for HGF was performed. We screened for the presence of both the 5' and 3' prime ends of the genomic transgene. Alternatively, a cDNA clone of human HGF, kindly provided by the laboratory of Dr. G. Shiota, was microinjected. Similar to the Shiota transgenic mice made in the FVB mouse strain [30], the HGF gene was linked to the albumin promoter to direct expression to the mouse hepatocytes. Transgenic animals were identified by PCR amplification of HGF sequences in DNA obtained from tail biopsies (forward primer: 5' attggatcaggaccatgtga; reverse primer: 5' aaaggccttgcaagtgaatg). Expression of the transgene was confirmed by screening mouse serum for human HGF using a commercially available ELISA kit. Transgenic mice were back-crossed to non-transgenic sibling mice and the hemizygous offspring were used experimentally. To monitor relative levels of cell proliferation in the chimeric livers, one milligram of BrdU was injected into recipient mice intrapertioneally and livers collected from the animals 4 hours later by non-survival surgery. The numbers of BrdU cells (mouse and human) were compared using flow cytometry with an anti-BrdU antibody labeled with phytochrome (PE).

Implantation of Alzet mini-pumps. Alzet osmotic minipumps (Pao Alto, CA) were loaded with human HGF according to the manufacturer's directions (830µg of HGF plus 5 units of heparin). The alzet pump inserted into the proximal part of the left gastric vein of Alb-uPA/SCID/Bg mice with the assistance of Dr Lin Fu Zhu. Following recovery, 4.5 million fresh human hepatoctyes

were injected into the mouse spleen. The mice had weekly blood draws to determine if there was any human albumin via western blot or human α-1-anti-trypsin production via ELISA

Alpha-1-anti-trypsin ELISA assay. Immulon2 96 well plates (Corning, Inc) were coated overnight at 4°C with a 1:1000 dilution of goat anti-human AAT primary antibody (Diasorin) in 0.1M $NaHCO_3$. Following 3 washes with 0.025% Tween in Tris-buffered saline (TBS-T), the plates were blocked for a minimum of one hour at room temperature with TBS-T/5% skim milk powder. 1:100 dilutions of mouse serum were serially diluted 1:4 and 50 μL are aliquoted to the assay plate. Controls included on each plate were as follows: negative control: non-transplanted Alb-uPA/SCID/Bg mouse serum; positive control: Alb-uPA/SCID/Bg mouse with known good transplant. Standard curves were generated from serial dilutions of human reference serum, Calibrator 4 (Diasorin). Samples and standards were incubated for 2 hours at room temperature then washed with TBS-T. Goat anti-human AAT antibody linked to horseradish peroxidase (HRP) (using EZ-Link Plus Activated Peroxidase Kit - Pierce, Rockford IL) was added to each well and the plate incubated at room temperature for 2 hours. Following washes with TBS-T, HRP substrate solution (1mg of 3,3', 5,5'-tetramethyl benzidine dihydrochloride (Sigma) in 0.05M phosphate-citrate buffer, pH5 with 0.02%H_2O_2 was added for 5 minutes. Reactions were terminated with the addition of 2N H_2SO_4. HRP activity was measured spectrophotometrically at 450nm and hAAT levels calculated with Softmax software.

Results

Marginal human grafts fail irrespective of exogenous HGF. To evaluate the efficacy of exogenous HGF, liver transplants were prepared from resected liver removed from a 51 year old male undergoing a resection for a tumor secondary to MEN. The viability of the cells post-isolation was marginal at 83%. Twelve transplants were performed and the animals divided into

three experimental groups. One group underwent partial hepatectomy to increase endogenous murine HGF. A second group was implanted with a mini-osmotic pump supplying human HGF and the third group underwent a laparotomy without pump insertion (control group). There was one post-operative death in the partial hepatectomy group, and this animal was replaced by a thirteenth experimental animal with a partial hepatectomy that was transplanted with cryopreserved cells from the same donor.

Human albumin was not detectable in the sera of any of the twelve mice by Western blot, nor was human α-1-anti-trypsin detected by ELISA assay of sera from week one out to week eight in any of the twelve mice. The failure of engraftment in all experimental mice precluded an assessment of the potential benefit of hHGF, other than to say it did not rescue a 'poor' graft with what was deemed acceptable donor material. Limited by the availability of recipient mice, these experiments were abandoned in favor of the alternative approaches discussed later. It remains unexplained why engraftment with this donor material failed.

HGF responsiveness of cultured human cells. The effects of human and murine hepatocyte growth factor on human hepatocytes were characterized in cell culture. Cultured hepatocytes were treated with medium containing either murine or human HGF after which proliferation was measured by the incorporation of BUdR. Less than 5% of the untreated cells incorporated BUdR by FACS analysis. Cells treated with HGF proliferated at levels between 10 and 12%, but there was no significant difference between human or murine HGF supplemented cells (Table 4.2).

Sample	Percent of proliferating cells
mHGF 1	10.55
mHGF 2	11.40
hHGF 1	11.64
hHGF 2	10.61

Table 4.2. Effect of HGF on hepatocyte proliferation. Shown is the percent of proliferating human hepatocytes following treatment of cultured human hepatocytes with supernatants containing murine HGF or human HGF. Each data set represents triplicate experiments.

Engraftment of hHGF transgenic animals. Transgenic animals were created from the microinjection of human HGF genomic DNA. To verify the insertion of the complete transgene, both the 5' and the 3' ends of the gene were amplified by PCR. After multiple rounds of injections, we identified one positive male founder. This male was bred repeatedly to Alb-uPA/SCID/Bg females to generate F1 progeny. F1 pups were transplanted with human hepatocytes prior to screening for the HGF transgene. Therefore, HGF transgenic and non-HGF transgenic mice were treated similarly without bias. From seven F1 litters, 20 non-transgenic and 9 HGF transgenic siblings were recipients of hepatocytes. Measured levels of hAAT at four weeks post-transplantation were 5.4 ± and 3.6 ± μg/L for controls and HGF transgenic mice, respectively.

Two transgenic males were identified that carried the cDNA form of HGF as a transgene. Others have produced HGF transgenic animals using this vector containing the albumin enhancer/promoter and human HGF cDNA. HGF was produced in the liver and resulted in greater regeneration rates post-partial hepatectomy [30]. In contrast to the naturally regulated genomic HGF transgene, the Alb-linked hHGF gene would be constitutively expressed. The two male founders were rescued with hepatocyte transfers and crossed to Alb-uPA/SCID/Bg females. Their F1 progeny were rescued with transplants and tested for the incorporation of the human grafts. Human AAT levels in the male founders were comparable to controls (19.5 and 25.9 μg/L versus 83.8 and 18.5 μg/L for non-transgenic sibling mice). Twelve offspring of the two founders were analyzed following receipt of human hepatocytes. Human AAT levels (in μg/L, 8 weeks post-transplant) for the non-transgenic mice averaged 71 ± 67, and 102 ± 63 for the three transgenic mice. The fraction of human hepatocytes reconstituting the mouse livers was determined by human MHC Class I cell sorting. In the three transgenic mice, human cells made up 15%, 6% and 12% of the liver population. The corresponding percentages for three non-

transgenic siblings were 18, 24 and 13. These differences were not significant in accordance with Mann-Whitney U tests.

Despite the apparent absence of a positive effect on engraftment, we looked at the effect of human HGF on proliferation amongst engrafted human hepatocytes. Animals were injected with 1mL of BrdU 4 hours prior to sacrifice and isolation of the hepatocytes. The number of positive human cells staining positive for BrdU would demonstrate proliferating cells. For two transgenic animals, hepatocytes positive for human MHC I and BrdU totaled 1.2% and 4%. The liver of one non-transgenic control littermate contained 3.5% proliferating human hepatocytes. The absence of any significant difference between transgenic and non-transgenic recipients resulted in the termination of this experimental approach.

Discussion

These experiments were designed to evaluate the capacity of human HGF to support human hepatocyte engraftment in the SCID/Bg/Alb-uPA mouse. We hypothesized that if the mice had intrinsically high levels of human HGF, the transplanted human hepatocytes would have a greater survival advantage compared to endogenous murine hepatocytes. Experiments in which HGF was supplied exogenously from implanted osmotic pumps were inconclusive because human grafts could not be established in any of the twelve experimental mice. The failure of grafts was ascribed to the source of the human hepatocytes (a single donor liver), or the cohort of recipients (discussed further in chapter 5), in the absence of vagaries related to the technical procedures involved. Heparin was included in the HGF solutions and while heparin may have an inhibitory effect on HGF action [31], it was not expected to have any effect on engraftment. The viability of the hepatocyte preparation was not optimal but was within the range of viability typical of reconstitution procedures. Furthermore, the additional surgical procedure of pump implantation

was not expected to be a negative factor as engraftments have been successful in previous experiments conducted in the laboratory.

Two forms of human HGF transgenic mice were created and evaluated for their ability to sustain hepatocytes grafts. One transgenic line carried the genomic sequences for hHGF. Our data showed that the transgene did not enhance engraftment levels based on the expression of the human liver enzyme AAT. Two transgenic founders were created that bore a recombinant cDNA transgene in which hHGF was under the transcriptional control of the albumin promoter. This was a functional transgene in the hands of other researchers [30]. The founder mice were rescued with transplants but graft performance was not improved over that in non-transgenic chimeric mice. Transgenic offspring rescued with human transplants also failed to display any benefits related to the gene for human HGF.

Critical to the interpretation of the transgenic experiments was a demonstration that human HGF was produced in the livers of transgenic mice. How much human HGF could be expressed in the severely compromised livers of Alb-uPA mice? An ELISA for human HGF was used in an attempt to determine levels of human HGF in the transgenic mice but the ELISA could not differentiate between murine and human protein. Measurement of HGF transcript in the liver (by RT-PCR, for example) was not performed because a limited number of transgenic mice were available and would have to be sacrificed or subjected to partial hepatectomies. A partial hepatectomy would have impacted on graft performance in a way that would complicate experimental outcomes. HGF expression could have been attempted post-experimentation but was not due to the negative results of the experiment and the need to use surviving animals for breeding purposes. Because we were looking for specific expression in the liver, and HGF has works locally, we did not look into expression in other tissues in the mice.

There are reasons for believing that hHGF would not have a beneficial effect in the chimeric mouse model. Recombinant human HGF has been shown to be effective in promoting the proliferation of mouse, rat and human hepatocytes. The similarity in amino acid sequences between mouse, rat and human HGF is about ninety percent. In comparing the human and mouse sequences directly, there is 97.5 percent homology [32]. When examining the regulatory sequences of the human and mouse gene, there is significant homology. The promoter sequence for HGF amongst the different species, shows 100% homology up to 110 bp upstream from the TATA box [33]. These similarities in the two species may result in the human and mouse HGF protein working interchangeably on both human and mouse hepatocytes. Furthermore, it's possible that human HGF expressing cells are transferred over during engraftment and any contributing stimulation is optimally provided by these cells. And last, HGF is secreted as a pro-protein that is processed to an active form via urokinase and tissue-type plasminogen activators. In uPA transgenic mice, continued production of urokinase-type plasminogen activator together with an impetus for regeneration could result in the wildtype (non-hHGF transgenic) mice having maximally up-regulated levels of HGF. In conclusion, the supplemental supply of human HGF, if in fact it existed in transgenic mice, may have been irrelevant in the context of the reconstituting livers of the experimental mice. Because of the low probability for a positive result from the addition of endogenous human HGF, this set of experiments was terminated.

Part C. Cellular Attributes of Engrafted Livers.

The donor livers used to rescue Alb-uPA mice represent a highly variable source of human hepatocytes. Differences in the cellular composition of prepared hepatocytes are expected to influence the quality of the engraftment and the subsequent success of HCV infections. In addition to estimating the numbers of stem cells that might be transferred during reconstitution of mice (Ch. 4A), experiments were undertaken to determine the numbers of NKT cells that might be transferred coincidentally. The presence of NKT cells in graft material may influence HCV susceptibility by initiating immune reactions against infected cells. Another factor that may influence HCV susceptibility is the concentration of virus receptor on the engrafted hepatocytes. As a presumptive receptor of HCV, CD81 presence in graft material and in reconstituted livers was determined.

The interaction between HCV envelope protein E2 and human CD81 and has been proposed as a necessary part of viral entry [34-36]. CD81 is a member of the tetraspanin membrane superfamily. It has a role in organizing cell surface signaling by associating with other tetraspanins, integrins and signaling proteins. It is ubiquitously expressed on human cells *in vivo*. There is a number of candidate receptors for HCV viral entry, and CD81 is the best characterized. HCV E2 glycoprotein binds specifically and with high affinity (K_d~ 10^{-8}M) to CD81. Studies with HCV E2 pseudotyped retrovirus have shown that CD81 facilitates the interaction between HCV and hepatocyte cell surface. This interaction when blocked with monoclonal antibodies to CD81 prevented infection by the pseudovirus. HCV tropism for human hepatocytes may depend on co-receptors [37].

Natural killer T cells, as their name implies, are lymphocytes that share features of both T cells and NK cells. Surface receptors on NKT cells are variable and can include $\alpha\beta$ or $\gamma\delta$ T cell

receptors (TCRs), as well as NK cell markers (CD56, CD69, CD94, CD161) [37]. A subset of these cells, called invariant NKT cells ($CD56^{+/-}$, $CD161^{+}$), have a highly restricted TCR repertoire which recognize glycolipids associated with CD1d (in the human, these are Vα24JαQ and predominantly Vβ11). The majority of the CD1d reactive invariant NKTs demonstrate a Th1 type phenotype. A recently identified, $CD161^{+}$ non-invariant CD1d-reactive T cell in human bone marrow, was shown to display a Th2 like phenotype.

In rodent studies, invariant NKTs were shown to accumulate in the liver and thymus. Of all intrahepatic lymphocytes in rodents, invariant NKTs comprised about 50% of the lymphocyte population and in some models of hepatitis these cells were responsible for the liver damage [38]. Hepatocytes and other liver cells express CD1d, thus NKTs could potentially play a major pathologic role, despite their protective role in resisting infections and tumor cell propagation [39-41]. In the human liver, the number of NKT cells is enriched compared to peripheral blood, but the absolute number of invariant NKT cells is very low [42,43]. Exley *et al*. examined the intrahepatic lymphocytes (IHL) in livers of chronic hepatitis C patients. In hepatitis C patients, a CD1d reactive, $CD161^{+}$, Th1 biased IHL cell was identified. It was proposed that these cells could contribute to the pathogenesis of hepatitis C [43]. Because hepatocytes normally express CD1d, the IHL may be involved in the apoptosis of healthy hepatocytes as well as infected HCV cells. Hence the presence of IHL in reconstituted chimeric mouse livers could dramatically affect the performance and reliability of the murine model of HCV infection.

Materials and Methods

Hepatocyte isolation. Similar to previous descriptions, human hepatocytes were isolated from resected liver sections and samples of five to ten million cells were set aside in DMEM media for experiments. Some of the human samples were cryopreserved using the step-wise addition of

DMSO (SIGMA) and a technique involving nucleation and cooling to -40°C in a BIOCOOL freezer. Frozen samples were stored in liquid nitrogen dewars and, when needed, thawed rapidly in a 37°C water bath, collected by centrifugation and the overlaying media was discarded. The pellet was resuspended in sucrose-enriched media and thereafter treated in the same manner as fresh hepatocytes. Hepatocytes were isolated from reconstituted Alb-uPA/SCID/Bg mice using an *ex vivo* collagenase digestion as previously described. Control hepatocytes were similarly collected from unreconstituted Alb-uPA/SCID/Bg mice and wild type SCID mice.

***Flow cytometry*.** Isolated hepatocytes were resuspended in PBS/0.1% sodium azide at a concentration of one million cells per twenty microlitres. Five microlitres of labeled anti-body (PE-labelled anti CD81 or anti-CD161; FITC-labeled anti-CD3 or anti-MHC class I) was added to one million cells, mixed gently and then incubated at 4°C for 20 minutes. Samples were subsequently analyzed with a Becton Dickson fluorescence cytometer using CellQuest software.

Results

***Expression of CD81 on engrafted liver cells*.** The presence of CD81+ cells was confirmed in samples of cryopreserved human hepatocytes that had been used for transplantation in SCID/bg/Alb-uPA mice. The percentage of CD81+ cells was compared to the percentage isolated from the actual transplanted mice CD81. Three samples of human cells were tested, two from HCV naïve patients and one from a patient undergoing a liver resection who was HCV positive. The two naïve samples had CD81+ levels of 20.88% and 51.14%. The one sample from the HCV positive patients had 48.68% of the liver cells positive for CD81. The correlation between CD81 expression and susceptibility of chimeric mice to HCV infection was investigated by exposing mice to HCV-positive serum, re-isolated liver cells and staining for HCV using antibodies to intracellular NS5a or HCV core proteins.

Most human cells are positive for CD81 in the transplanted animals, exceeding the percentage of prepared human liver cells that bear CD81. Clearly this would suggest that CD81 cells are preferentially engrafted in the murine liver. However, there is no clear correlation between CD81 expression and the ability to infect reconstituted mice with HCV. It is clear from Table 4.3 that there is no correlation between infection and either serum sample or liver donor. The lone variable, barring any technical reason for the inconsistent infections, is the status of the recipient mouse. CD81 is expressed in a variety of human cell types and thus does not determine tropism for the virus. Other relevant co-receptors may play a tropism-defining role and bear more on infection than would the presence of CD81. These studies do demonstrate that the CD81 cell surface receptor is preserved in the process of isolating and transplanting the human hepatocytes in the Alb-uPA/SCID/Bg mice.

Transfer of human NK T cells with hepatocyte engraftment. From the same cryopreserved stocks of primary hepatocytes, FACS was performed to identify any possible $CD161^+$ cells in our hepatocyte isolations from surgical liver resections. On these three samples, the number of $CD161^+$ cells was 2.57% (5 year old), 24.6% (46 year old) and 9.05% (69 year old). These cells were doubly stained for CD3 to determine if they were NKT cells. Double stained CD161+CD3+ cells were 2.31, 20.82%, and 5.70% respectively. Thus, we determined that our transplanted cell population was not only a heterogeneous population but they were composed of widely variable fractions of immune cells. CD161 positive cells were most likely NKT cells that may have a role in the pathogenesis of hepatitis. An experiment was performed to determine if these immune cells persisted following transplantation into recipient mice. Hepatocytes were isolated from Alb-uPA/SCID/Bg mice that had received liver transplants. Complete hepatectomies were performed and *ex vivo* perfusion of the liver with collagenase solution was used to isolate the hepatocytes. The cells were stained to determine the number of human hepatocytes based on MHC class I staining in conjunction with the number of NKT cells present. In the three mice

sampled (A1, A2, and A3 from Table 4.3), the numbers of human NKT cells was consistently 5% of the human cells present (by FACS, data not shown).

MOUSE	MHC I	CD81	MHC I/CD81	HCV status
993	10.31	10.14	7.82	6.3×10^2 (BW)
992	12.87	13.14	10.83	1.4×10^3 (BW)
996	22.14	20.19	19.12	Negative (BW)
A1	2.61	3.41	1.93	Negative (GT)
A2	2.51	3.52	2.31	Negative (BW)
A3	2.32	2.98	2.28	Negative (BW)
A7	19.69	17.66	17.33	3.7×10^4 (BW)
A18	18.54	15.81	15.62	2.9×10^3 (GT)
A50	11.58	13.65	8.59	Negative (BW)
957	14.34	11.43	10.30	Negative (BW)

Table 4.3. Presence of CD81 (putative HCV receptor) in engrafted mouse livers. Liver cells from reconstituted Alb-uPA/SCID mice were analyzed by FACS. The percentage of human (MHC I+) cells in mouse livers that bear CD81 are shown relative to the susceptibility of these mice to HCV infection with two different patients sera: BW or GT). The first eight mice were reconstituted with the same donor liver cells; A50 and 957 had unique donors.

Discussion

In this section, two potential determinants of HCV pathogenesis in experimental chimeric mice were explored. Under consideration was the presence and maintenance of the presumptive viral receptor CD81 in reconstituted mice. CD81 is present on the majority of human cells including hepatocytes and immune cells. The data in table 4.3 are consistent with a direct relationship between the percent of CD81-positive cells and HCV infectivity, if one considers only those mice in which HCV infection was successful (mice 992, 993, A7 and A18). Viral titres rose with increasing percentages of CD81-positive cells. However, CD81 in this case merely reflected the number of human cells present in the chimeric liver, and no conclusion could be drawn regarding a possible role of receptor (CD81) density on HCV infection. It remains unclear in the cited experiment why infection failed in mice with comparable levels of engrafted human cells. Clearly this failure was unrelated to the presence of CD81, vagaries of the donor liver or the HCV serum.

The possible role of human NKT cells mediating HCV pathogenesis was also considered. NKT cells are a group of immunoregulatory and effector cells that are enriched in the liver. Classical NKT cells express both CD161 (NK cell marker) as well as CD3 (T cell marker). When stimulated they produce both pro- and anti-inflammatory cytokines. In hepatotropic viral infections, NKT cells are important in immunity as well as inflammation. The role of NKT cells has not been clearly defined in HCV pathogenesis. We were able to demonstrate the presence of human NKT cells in fractionated donor livers as well as in reconstituted Alb-uPA/SCID/Bg mice. It is significant that different donor samples may contain very different numbers of immune cells like NKT cells, and the presence of these immunologically reactive cells may impact on liver reconstitution as well as contribute to HCV pathogenesis. The chimeric mouse model could be a valuable aid in determining the role of NKT cells (and T cells generally) in liver reconstitution, HCV pathogenesis, and immune protection.

Part D. Retrospective Analysis of Engraftment in Alb-uPA/SCID/Bg Mice

The Alb-uPA mouse was developed by pioneers in the field of transgenesis in 1990. It was used to study neonatal bleeding and the pathophysiology of plasminogen hyperactivation [44,45]. A surprising outcome was the survival of transgenic offspring with profound morphological changes in the mouse liver. The uPA transgene caused significant hepatoxicity, but occasionally hepatocytes reverted to an apparent non-transgenic state. In fact, the transgene had been inactivated through an intra-chromosomal recombination event. Transgene-inactivated hepatocytes, in a regenerative fashion, proliferated and formed functional liver nodules [45]. This model became a powerful tool with which to study hepatocyte regeneration and has been a useful resource for studying hepatocyte transplantation.

Transplantation of hepatocytes into the Alb-uPA mouse was first described in 1994. Labeled donor hepatocytes from a different mouse were infused via splenic injection into the recipient mouse liver. The transplanted hepatocytes formed regenerative nodules and could replace 5-80% of the recipient liver. It was calculated by the authors that the donor cells could undergo up to twelve replicative cycles in the recipient liver [46]. The next set of studies with the Alb-uPA model was to perform xenogenic transplants. Rat hepatocytes were transplanted into immunodeficient athymic nude mice carrying the Alb-uPA transgene [47]. In homozygous Alb-uPA nude mice, rat hepatocytes could reconstitute up to 100% of the mouse liver. The biliary epithelial cells and other accessory cells were of murine descent, thus creating a chimeric liver. This model has subsequently been adapted by our laboratory in the development of the Alb-uPA/SCID/Bg HCV mouse model [28]. Transplantation of the Alb-uPA mouse model is technically challenging. Up to one half of the progeny normally succumb to neonatal hemorrhage and, for those animals that survive hemorrhaging, transplants must be completed at an early age to rescue them from acute liver failure. Transplants must also be done at a young age to give the

exogenous hepatocytes a growth advantage over any regenerating endogenous hepatocytes that may have inactivated the Alb-uPA transgene [48]. And last, the Alb-uPA/SCID/Bg mouse is a peculiarly vulnerable model due to its multiple immune deficiencies.

There have been less than 100 reported cases of hepatocyte transplants for metabolic liver disease. The number is low because of the success of whole organ transplants and the limited source of hepatocytes. The same issues of optimizing the transplants are similar to those encountered in the Alb-uPA/SCID/Bg mouse model, such as increasing the efficiency of transplantation and engraftment of donor hepatocytes [49]. Another major obstacle that we have encountered is maintaining long term survival of the graft. Obstacles exist at a number of different steps that influence the target outcome of sustaining an HCV infection in an experimental mouse. We looked at factors such as the status of the hepatocyte donors, the quality of isolated hepatocytes, and attributes of recipient mice. Our initial studies supported the exclusive use of homozygous Alb-uPA mice for transplantation and infection [28]. The success rate for high level engraftment with fresh hepatocytes was approximately 32%, as determined by target serum levels of human α-1 anti-trypsin. Our goal was to improve the model such that 50% or more of the mice could sustain an HCV infection. In this section, data collected on laboratory experiments performed from March 2001 to July 2004 are reviewed with intent to identify those technical limitations with the most negative influence on the model and yet pose the best opportunity for improvement. The majority of hepatocytes isolations and transplants into recipient mice were performed by the author. The remainder (about 25%) was undertaken by Lin Fu Zhu of the Surgical Medical Research Institute of the University of Alberta.

Data and Interpretation

Hepatocyte donor information included age of the patient, reason for the resection (i.e. colorectal liver mets, primary liver tumor – benign or malignant), lobe of liver resected, and quality of the liver tissue (i.e. normal, fatty or cirrhotic). The hepatocyte isolation data weighed warm and cold ischemic exposure time, collagenase perfusion time, and mass of the liver tissue digested. Success of transplantation was measured by the percentage of mice per group transplanted that displayed hepatocyte engraftment sufficient to yield levels of human α-1-anti-trypsin of at least 80μg/mL (in the mouse serum). Recipient mouse performance was considered in terms of the age at which engraftment was attempted. Statistics were done using SSPS version 10. Univariate as well as multivariate analysis was performed to determine if donor and/or recipient factors had an effect on overall transplant success (as determined by detectable human α-1- anti-trypsin in the transplanted mice).

From March 2001 to July 2004, one hundred and thirty-four human hepatocyte isolations performed in our laboratory. Most of the donors were undergoing resections for metastatic colon carcinomas to the liver. For this reason, the majority of the patients were over the age of fifty (the mean age of donors was fifty-two years) however, donors as young as one year old were used. Most pediatric liver donors were the result of receiving the residual unused segments of liver after cutdown resection for reduced-size liver transplantation in smaller children. A few were the resected liver from children undergoing transplantation for metabolic liver diseases where the liver was anatomically and structurally normal. The surgical resections were right or left lobectomies or segmental/multisegmental resections. Liver segments that were used for hepatocyte isolations were wedged off the original surgical resections (which were subsequently sent of to the University of Alberta Hospital Pathology department). These ranged from fifteen to ninety grams. The liver sections that were greater than a fifty to sixty gram weight were digested

in two portions because of the limitations of our apparatus. The overall viability of the isolated hepatocytes ranged from 33% to 97%. The mean of the viabilities was 80% with a standard deviation of 9.3%.

Quality of liver tissue. The quality of our liver sections was graded on a scale of 1 to 3 depending on gross appearance. From the one hundred and thirty-four specimens, seventy-six percent of the specimens were of normal quality (102 of 134). The remaining livers were almost equally distributed between fatty livers (grade 2, 11%) and cirrhotic livers (grade 3, 9%) (figure 4.6 below).

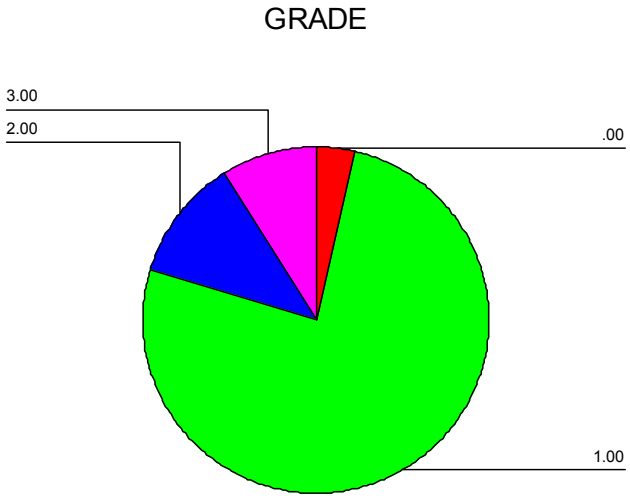

Figure 4.6. Graded health of donor livers. Donor livers were categorized by health status: grades 1, 2, and 3 represent normal, fatty and cirrhotic livers respectively (grade 0: ungraded).

Cell yields were better with grade 1 livers. Cirrhotic livers were digested poorly due to poor perfusion of the tissue and/or perhaps intrinsic resistance to collagenase. Not surprisingly, the

better grades produced better quality cells (greater viability), with a Pearson correlation value of –0.223 and a p-value of 0.021 (Fig. 4.7). However, the overall transplant success rate was not significantly different between the three grades of liver specimens (grade 1: 29.36% success; grade 2: 36.78%; and grade 3: 21.42%; ANOVA p-value of 0.06). As expected, more viable cell preparations were more likely to result in higher engraftment and transplant success. The p-value for this correlation was significant at 0.002. Thus, grade and viability can influence the success of a transplant. Although there was no significant difference in transplant success with respect to grade, these data were biased because only isolations with reasonable hepatocyte viabilities were used as donor cells (preparations were pre-selected on the basis of viability).

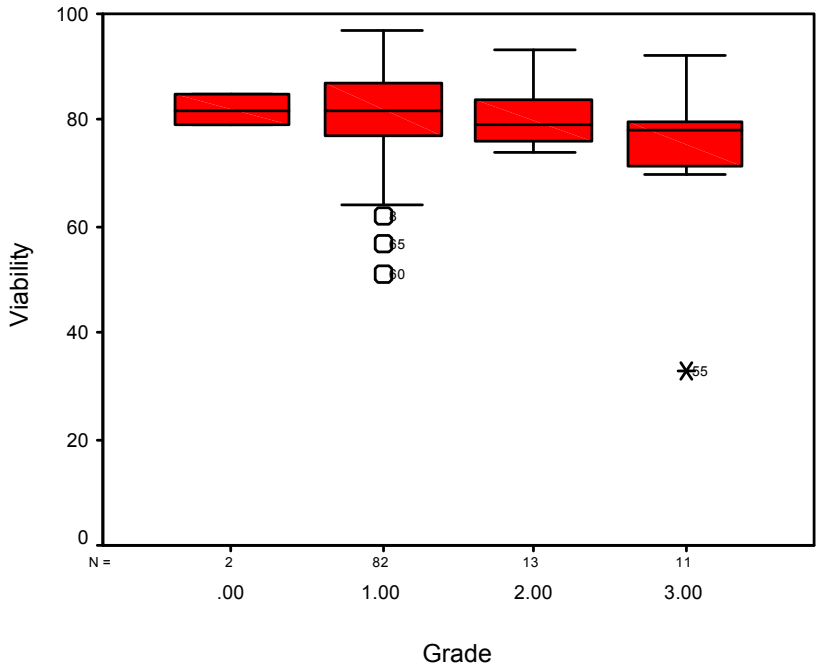

Figure 4.7. Viability of hepatocyte preparations as a function of liver health. Donor livers are assigned grades 1 to 3 (see text), with ungraded livers represented as .00. Sample sizes are noted (N).

***Donor age*.** An overview of the viability of freshly prepared hepatocytes relative to the age of the donor can be seen by a scatter plot of the two variables. While there is a tendency to reduced viability with age of donor tissue, the differences are not significant (Fig. 4.8 below).

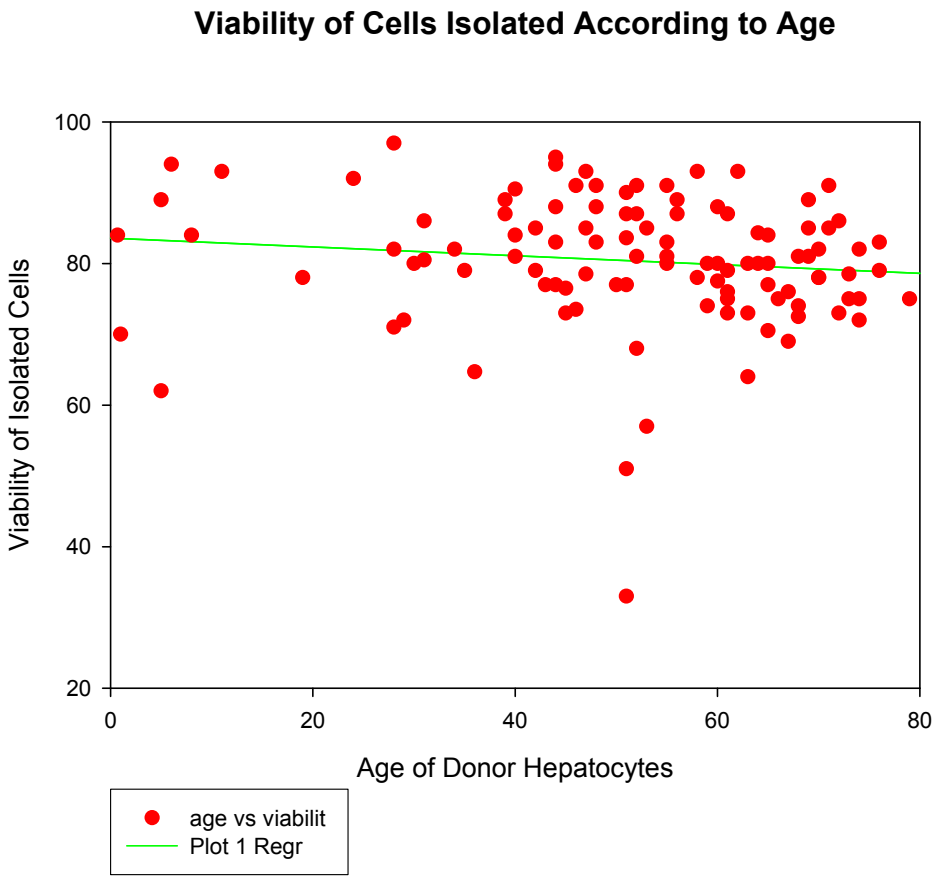

***Figure 4.8*.** Viability of hepatocyte preparations as a function of donor age. Individual cell preparations are shown.

Collective data comparing success of transplants with donor age also fails to support a broad correlation (using freshly prepared hepatocytes). However, the plot in figure 4.9 does show a significant reduction in success when tissue of donors exceeding 60 years of age is used.

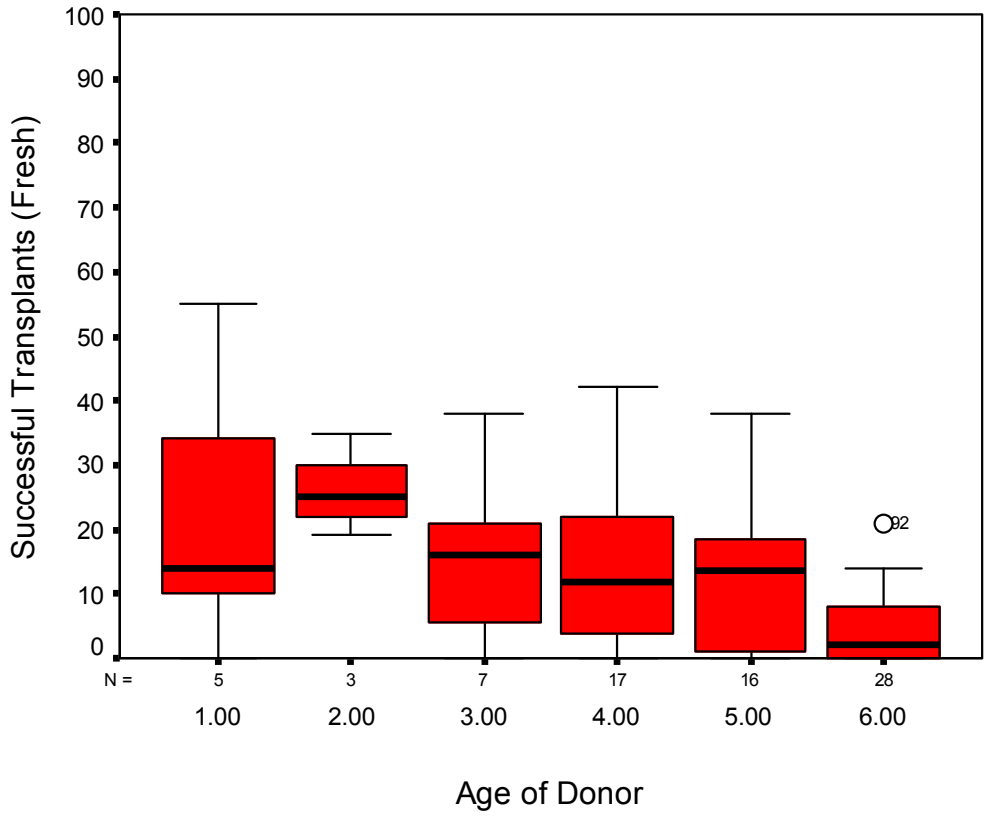

Figure 4.9. Transplant success as a function of donor age. Donors are grouped according to decades of age and hepatocytes were transplanted without being frozen. Sample sizes (N) are indicated.

The success of using of cryopreserved hepatocytes mirrored that of freshly prepared hepatocytes. Importantly, the use of frozen cells was significantly less successful than was the use of freshly prepared cells (compare figures 4.10 and 4.9).

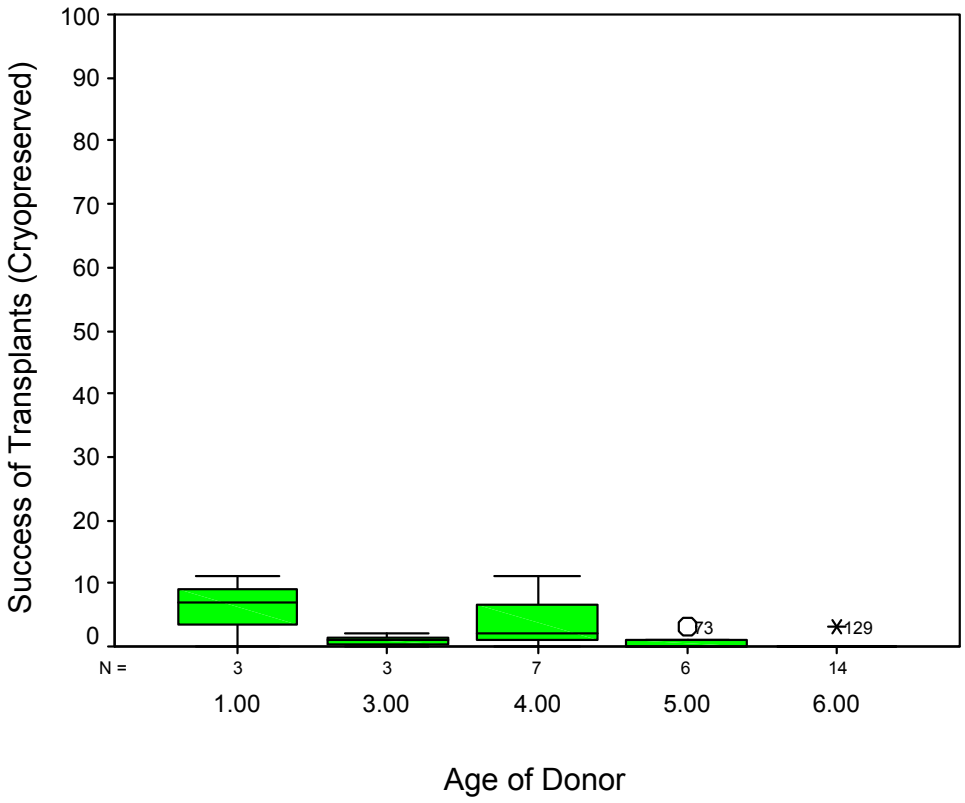

Figure 4.10. Effect or age and cryopreservation on transplant success. Transplant success is plotted according to donor age having used cryopreserved hepatocytes. Age groups as in figure 4.9. Sample size (N) is indicated.

The Pearson correlation coefficient value for the success of transplants with regards to age is -0.340 for fresh cells and -0.346 for cryopreserved cells. The respective p-values are 0.003 and 0.049, statistically supporting a correlation between higher success rates with younger donors compared to older donors. When we analyze the percent success of each of the groups, the overall mean was thirty-three percent from seventy-six of the cases that had all the data available. Using the ANOVA statistic, the p-value is significant at 0.001 and through post-hoc analysis, the differences are between donors aged sixty and older and all other age groups except for the donors that are under twenty years of age. There is no significant difference between any of the other groups. We see from this data set that there is a greater chance of achieving successful transplants using tissue of younger donors. There appears to be a significant drop in success rates (to about 17%) when older donors (>60 years) are compared to younger donors (>35% success).

Age of recipient mouse. Human liver becomes available sporadically, necessitating the transplantation of mice at different ages. There are limits to the age of mice transplantable because older untransplanted mice had a higher morbidity and mortality than age matched transplanted mice, due likely to an increased sensitivity to anesthesia with progressive liver failure. The youngest mice in which transplant was attempted were 4 days of age due to progressively increasing technical difficulty and the parallel increase in morbidity and mortality with these very early neonates.

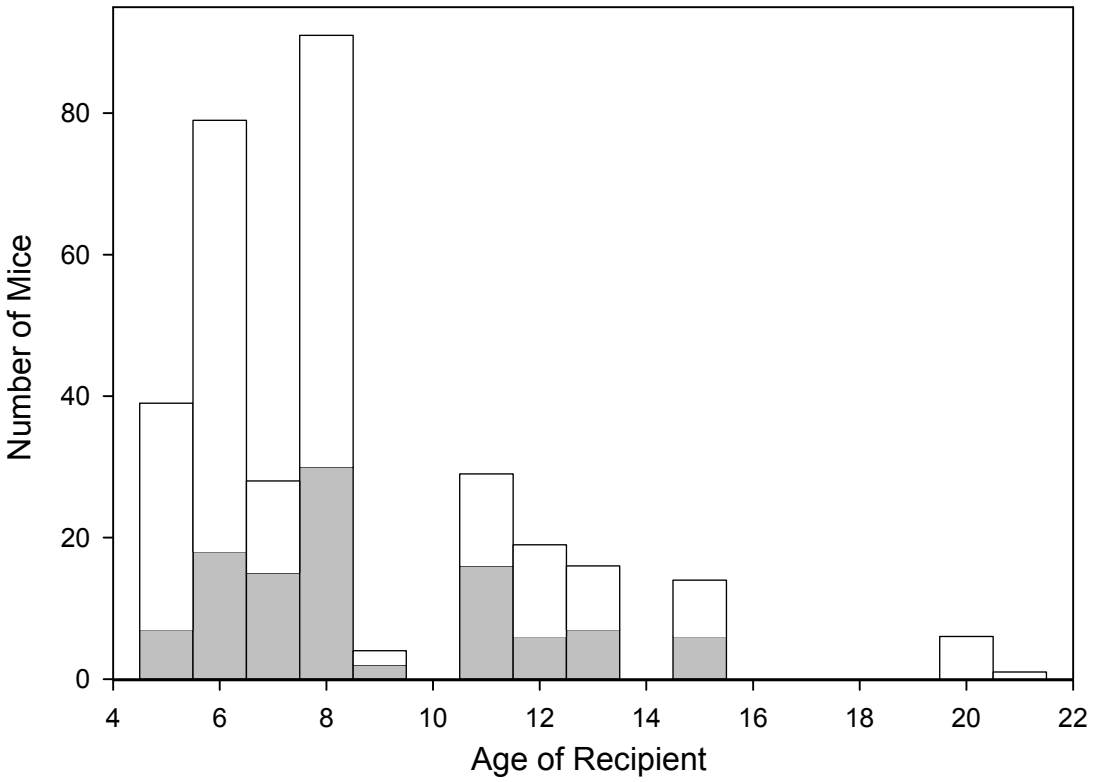

Figure 4.11. Transplant success as a function of recipient mouse age. The number of successful (shaded bars) and unsuccessful (open bars) transplants into mice are plotted relative to recipient mouse age (in days).

Initially, we arbitrarily divided the mice into two groups – mice that were transplanted at ten days of age or greater (10-14 days), or mice that were less than ten days of age (4-9 days). In the different cohorts of animals transplanted with fresh hepatocytes, we found that at the younger age 36.4% of the mice had "strong" grafts. The older mice had only a 25% success rate. Although technically it is more difficult to transplant younger age mice, there is a higher success rate in producing mice with high level chimeric human livers.

Many animals were successfully transplanted between the ages of 10-13 days. A marked decline in success was observed in recipient mice over 13 days old related, we believe, to the morbidity associated with endogenous liver failure. Evaluating success for each day after birth, based on the hAAT level as the determining factor, the mean age of successful transplants was 7.8 +/- 2.5 days and for unsuccessful transplants was 8.5 +/- 2.8 days. The difference between these two groups was significant with a t-test p-value of 0.019. We also analyzed the data according to recipient mouse age using the hAAT level as the dependent variable at the eight week time point. Using the Kruskal-Wallis test statistic, there was a significant difference between the groups with a p-value of <0.001. Stated differently, from the values of hAAT at each transplant age, there was greater success achieving high hAAT levels with the younger mice used.

Discussion

Our lab has exploited the Alb-uPA transgenic mouse to develop a humanized model for hepatitis C virus infection. There are different aspects of the model (including characteristics of the human hepatocyte transplants) we hoped to optimize to improve its reliability and efficiency. Overall, only one of three transplants ended in a successful graft and a mouse that could be used experimentally. To assess factors that may determine engraftment success, we considered human donor and recipient mouse contributions.

Hepatocyte preparations of higher viability tended to come from younger donors, and donors with more normal hepatic parenchyma. As expected, younger donors, healthier livers and cell preparations with higher viability resulted in the more successful transplantations. One can speculate that younger donor livers are healthier and contain cells with a greater number of potential replication cycles. The higher regeneration capacity could be related to the number of stem cells present in the isolated hepatocyte populations. Stem cell numbers may also be greater in younger donors and decreased in hepatocyte preparations from cirrhotic liver, where regeneration has been an ongoing process for some time, with potential depletion or diminished replicative power of remaining stem cell populations. It may also be related to the liver content of immune cells such as macrophages that function in the regeneration of liver tissue.

Hepatocytes that are isolated from fatty and cirrhotic livers may be more prone to cellular damage through the isolation and transplant process (resulting in lower viabilities in the isolated cells). Fatty livers are technically more difficult to transplant because they are more prone to clumping. Often instead of a homogenous cell suspension, hepatocytes isolated from fatty livers stuck together making injections into the murine liver more difficult and increased the risk of bursting the recipient spleens. Because the cells were likely also not single cell suspensions anymore, there was concern whether these clumps of cells may have blocked off the sinusoids or alternatively were not able to disperse as well in the recipient liver. Cirrhotic livers on the other hand were more difficult to process and often the *ex vivo* digestion with collagenase was not as successful. Although we try to compensate for viability discrepancies by transplanting one million live cells into each mouse, transplanting the increased number of nonviable cells may be counter productive. The lower viability may indicate the cells themselves are not healthy and may not have the capacity to regenerate once embolized to the recipient liver. In turn, greater numbers of dead cells transplanted may increase the possibility of thrombosing of liver sinusoids and vessels, preventing an optimal environment for engraftment of the healthy cells. This is

consistent with other hepatocyte transplant models that ninety percent viability is required for optimal transplant success.

Successful human hepatocyte transplants capable of sustaining an HCV infection must be delivered to animals homozygous for the Alb-uPA transgene [28]. Another important factor is the age at which we transplant the mice. The youngest mice that are transplantable without increasing the mortality rate is four to five days. If we transplant as close to this age as possible, we have a higher success rate of transplant. We can hypothesize that if human cells are transplanted and start to regenerate in the murine liver before its endogenous hepatocytes can delete the transgene and begins its own repopulation, the human hepatocytes may have a survival and subsequent repopulation advantage.

Even though our system is tailored for the HCV mouse model, the information obtained may be applicable to hepatocyte transplantation in general. It is an effective model that demonstrates that cellular transplantation can effectively reverse severe liver disease. In order for cellular transplantation to be successful, two conditions must be met: a signal to promote proliferation of the donor hepatocytes and second, a growth advantage for the transplanted hepatocytes over endogenous hepatocytes [50]. This is demonstrated in the Alb-uPA/SCID/Bg model. Previous experiences with hepatocyte transplants do suggest hepatocytes or liver fragments from fetal sources perform better than do adult liver segments. In addition, it is likely important that the donor cells be transplanted early enough to bridge complete hepatic failure and engraftment of the transplanted hepatocytes [51]. This also ensures that the donor cells are present when the signal for regeneration is at its height. In the context of our model, recipient mice had a higher chance of success if the animals were transplanted earlier, prior to regeneration of endogenous cells with deleted transgenes, and with younger donor cells. The difficulty with murine transplants is twofold: the efficiency of engraftment and graft longevity. To enhance both of these, the optimal

mass of hepatocytes to be transplanted as well as the number of transplants are variables that should be evaluated in the Alb-uPA mouse model.

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Chapter 5

Summary Discussion and Conclusions

The hepatitis C mouse model based on the Alb-uPA/SCID/Bg mouse was developed in the laboratory as a tool to study the pathogenesis and molecular biology of HCV, and for the rapid development of novel anti-HCV agents. Over the last few years we have undertaken studies to further validate the model for this purpose. At the same time, we endeavored to characterize features of the transplanted livers to better understand the physiologic process of liver reconstitution and, importantly, to improve the production of the chimeric mice through an appreciation of the parameters that may contribute to the variable success of model creation. This thesis considers features of these objectives.

Chapters 2 and 3 highlight two experimental applications that attest to the capacity of the chimeric mouse model to sustain an HBV/HCV infection of human liver and then respond in a reproducible fashion to treatments for that infection. It was necessary through this demonstration to equate responses of the transplanted hepatocytes to the infection and anti-viral treatment of livers in human patients. In chapter 2, chimeric mice infected with HBV responded in a predictable fashion to an accepted therapeutic treatment with anti-HBV antibodies. The salient result can be duly emphasized: anti-viral protection was achieved in susceptible experimental (chimeric) mice pre-selected on the basis of having strong human hepatocyte grafts. Hence, the animal model demonstrated the responsiveness typically associated with HBV-infected human patients.

In chapter 3, an experimental approach to treating HCV infection was evaluated in human hepatocyte-engrafted mice. The basis of this therapy was an apoptotic factor (BID) that, when introduced into engrafted mice, would be specifically activated in cells infected with HCV. The anticipated result was the elimination of infected cells through BID-mediated apoptosis. Chimeric mice were infected with HCV, and subsequently co-infected with adenovirus bearing the inactive BID (mBID). Therapeutic results were mixed, and complicating factors were identified. A single treatment with Ad-mBID showed some efficacy in limiting HCV infection. Multiple Ad-mBID infections showed greater potential in limiting HCV spread but, in one multi-dose regimen, resulted in significant mortality due presumably to liver failure.

Cytotoxicity associated with the Ad-mBID vector was observed in two forms. In the absence of HCV co-infection, cell death was evident from increases in plasma hALT, indicative of hepatocyte rupture. Controls vectors (empty Ad vectors) produced no comparable toxic effect, ruling out the viral vector as a cause of HCV-independent cell death. It is reasonable to surmise then that modified-BID had partial activity or was activated to some degree through the action of cellular proteases. This cellular toxicity was not observed by histopathologic examination and did not jeopardize the gross health of the experimental mice. It's possible that toxicity of this form could be eliminated with lower doses of mBID that remain effective in inhibiting HCV growth and/or spread. Of greater concern, is the degree of hepatocyte death associated with BID in HCV-infected cells as this resulted in 50% of experimental mice dying in one multi-dose experiment. The experiment in question involved Ad-mBID infections administered 3 days apart following HCV infection. When the doses were repeated at 7 day intervals, morbidity was not observed. Longer intervals may have been protective because they allowed for a more favorable balance between hepatocyte cell death and hepatocyte regeneration. The benefit of subsequent doses is then apparent in limiting HCV spread in the regenerating population of hepatocytes.

This pattern was, in fact, observed in figure 3.4, where rebounding HCV was observed consistently around day 12 following mBID treatment.

Statistically, Ad-mBID was effective in reducing HCV infection in the animal model with a single administration. A limiting factor in its effectiveness could be the degree of exposure of hepatocytes to Bid as delivered by adenovirus. Multiple infections with Ad-Bid more effectively limited HCV replication, presumably because more hepatocytes were being exposed to the vector. Experimental controls (Ad-GFP) demonstrated that while the adenovirus vector could reach the vast majority of hepatocytes, a small percentage of cells could have escaped Ad-infection. HCV spread in experimental mice may have been sustained in these cells that were unexposed to, or uninfected by, Ad-mBID. This may explain why a percentage of mice were not significantly protected by Ad-mBID (3/9; table 3.2). Protection may be variable because the activation of mBID may depend on the infectious state of the cell (chronic vs. active) thus allowing HCV replication irrespective of its presence in the cell.

BID is, by design, hepatotoxic in this experimental scheme. A high degree of HCV infection implies that BID will mediate the destruction of the host liver. In the mouse model, it's conceivable that reverted murine hepatocytes may have sustained liver function during episodes of BID-mediated apoptosis. It is also possible, as suggested earlier, that regenerating human cells were sufficient to protect treated mice from liver failure. Hence, regenerating hepatocytes are a critical factor both in terms of preserving liver function and supporting HCV spread on the other. The success of treatments that eliminate infected hepatocytes will have to account for, and most probably promote, the regenerative capacity of hepatocytes. Timing of treatments to exploit natural regenerative mechanisms will be important. In addition, cytokine therapy (promoting either host immunity or regeneration of hepatocytes) or transplantation of stem cells that conceivably are genetically resistant to HCV infection may be beneficial.

In our studies and those that preceded it, the most restrictive feature of the chimeric mouse was not its capacity to model human viral infection but our ability to reliably and routinely produce chimeric mice. At most, about one-third of the surviving transplanted animals were useful experimentally based on our measure of transplant success. We reviewed data collected from one hundred and thirty-four hepatocyte isolations. This translated into 2,386 hepatocyte transplants. Overall success rate in the production of mice with a successful engraftment was thirty-seven percent. Not surprisingly, our data revealed that the quality of the donor hepatocytes was a factor in determining the success of the transplant. The quality of the hepatocytes (reflected by the viability of the cells) is influenced by attributes of the donor liver. Two important variables are the age of the donor and the grade, or health, of the liver. The younger and healthier the source of human hepatocytes, the more likely healthy cells will be isolated and the greater the likelihood of a successful transplant. The variable content of stem cells in the transplanted material seemed not to have an impact on the success of transplants in the chimeric mice. This latter observation was a surprise and its validity remains unclear. The participation of stem cell populations in liver regeneration and HCV infection duly requires more study, particularly in light of evidence indicating the presence of stem-like cells in populations of 'adult' hepatocytes [1].

It was disappointing that while minor variables were demonstrated to influence graft success, no 'breakthrough' information arose to markedly enhance engraftment above the ~35% level already achieved. A recent report (published after the completion of this study) compared similar approaches to making an HCV infection model and concluded that the chimeric Alb-uPA/SCID/Bg was the most successful [2]. There is no indication in this report of success rates in creating 'experimentally useful' chimeric mice. It is noteworthy, to say the least, to observe the 100% success achieved by another group [3] that engrafted SCID mice with hepatocyte cells (8/8 mice). In this 2004 paper studying the differentiation capacity of hepatocyte lines,

engraftment success was contingent upon the selective poisoning of endogenous murine macrophages. This approach deserves attention in the SCID/Bg model because the Bg mutation cripples the cytotoxicity of granular cells like NK cells but not the spectrum of cellular functions associated with activated macrophages. The Strick-Marchand [3] report implies that macrophages and not NK cells are critically important to graft rejection in their SCID-based mouse model. It is certain that activated macrophages would be recruited to repair a uPA-damaged murine liver and these could impair colonization by human cells. While this thesis work focused largely on variables in donor hepatocytes preparation, it concludes that the genetic and cellular integrity of the recipient mouse are factors demanding serious consideration. If the Bg mutation is less important than the action of murine macrophages as suggested, the genetic constitution of the model mouse could be simplified (avoiding possible genetic drift) at that same time that engraftment is improved. This possibility warrants examination in a Alb-uPA/SCID model background.

Our understanding of the literature led us to consider supplementing engrafted mice with hepatocyte growth factor to promote the growth of transplanted human hepatocytes. This approach, however, was not productive, possibly because the Alb-uPA mouse is in a regenerative state already with an optimal milieu of cytokines. A possible alternative would be to give the transplanted human hepatocytes an advantage over the endogenous murine cells by priming them with growth-promoting cytokines prior to transplantation. Treatments could include hepatocyte growth factor and/or IL-6 (an important cytokine that primes the hepatocytes to respond to HGF). The Alb-uPA/SCID/Bg mouse is in a state to promote liver regeneration, and advantages to optimize transplanted human cells could be intrinsic to the cells themselves.

The Alb-uPA/SCID/Bg mouse is ideal for developing techniques for cell transplants with differentiated adult cells as well as multipotential or stem cells. Within this mouse, there are

intrinsic signals for hepatic regeneration that promote the expansion of transplanted cells. Our model is based on the transplantation of adult human hepatocytes: a heterogenous population composed mainly of parenchymal cells but also containing epithelial and immune cells. Oval cells are bipotential cells in the adult liver that are capable of differentiating into both hepatocytes and biliary epithelial cells. Repopulation in the Alb-uPA/SCID/Bg mouse by human liver cells is most likely a combination of expansion amongst adult hepatocytes and oval cells. Other stem cells (exogenous to the liver) could be tapped to supplement liver grafts. Umbilical cord blood cells are a rich source of stem cells that have been used in murine models of liver disease for regeneration. We attempted to differentiate cord blood cells along the hepatocyte lineage and then use these to transplant into the Alb-uPA mouse. We achieved low level repopulation in the liver so the mice could survive, but grafts were not sufficient to sustain an HCV infection. Alternative approaches with stem cells that are still worthy of consideration should include, (1) optimizing the differentiation of the cord blood cells with cytokines other than three tested in this study (FGF, OSM and HGF), such as IL-6, and TNF-α, (2) engrafting with larger numbers of differentiated stem cells or, (3) transplanting a mixture of adult hepatocytes (sych as we routinely used to establish grafts) together with differentiated cord blood cells. This may provide a more conducive environment for the immature hepatocytes to further proliferate and engraft. It would also be interesting to identify the cells that differentiate into hepatocytes since cord blood contains a variety of stem cells. This could be tested in a fundamental way by comparing transplant success with cord blood cells plus or minus CD34 positive cells, or alternatively with populations enriched in c-met-expressing cells. Generally speaking, identification of cell characteristics involved in the regenerative process could be exploited to improve the engraftment process.

This study was extended to identify factors that may influence HCV infection of transplanted animals since viral replication and spread were elements of the model impinging on pathogenesis

and the treatment of disease. CD81 is a reputed receptor for HCV and could play a role in mediating viral entry in conjunction with one or more co-receptors. We were able to detect CD81 on the human cell preparations used for transplants and on the human cells successfully engrafted into murine livers. In animals transplanted and subsequently inoculated with hepatitis C, the cells that were infected also expressed human CD81. While there was a trend towards higher numbers of CD81 cells associated with positive infections, differences were not significant, given the low numbers of mice we were able to test.

NKT cells may influence both HCV infection and pathogenesis. We detected human NKT cells in both hepatocyte preparations and in engrafted mice. Although we were not able to correlate the presence of NKT cells with a positive HCV infection, the presence of these human immune cells in liver cell preparations does offer the opportunity to assess their role in greater detail. By manipulating hepatocyte preparations, depleting them of CD3/CD161 cells for example, the effect of NK cells on HCV infectivity could be evaluated further. Just knowing that immune cells can be transplanted and maintained after splenic injections, it may be possible to repopulate the host immune system in a fashion that endows the model with additional clinical relevance.

In conclusion, this thesis outlines the performance status of the Alb-uPA/SCID/Bg mouse model of HCV infection currently used in our laboratory. The usefulness of this model as a tool for studying the pathogenesis of HCV has been established in a practical way and in a theoretical way by confirming the presence of factors likely to play a role in HCV pathogenesis (CD81 and NKT cells, for example). The humanized mouse model mimics the clinical scenario since HCV-infected human serum can establish an active infection in the reconstituted mouse liver. This is important advance for testing and developing new anti-viral therapies *in vivo*. We have demonstrated the success of this model through our proof and principle experiments as well as the testing of novel gene therapy. As our understanding of it grows, the Alb-uPA/SCID/Bg mouse

model will become increasingly powerful in the study of the mechanisms of liver regeneration, aiding the development of treatments for a variety of human liver diseases.

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