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REVIEW

Gene therapy for liver regeneration: Experimental studies and prospects for clinical trials

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Abstract

The liver is an exceptional organ, not only because of its unique anatomical and physiological characteristics, but also because of its unlimited regenerative capacity. Unfolding of the molecular mechanisms that govern liver regeneration has allowed researchers to exploit them to augment liver regeneration. Dramatic progress in the field, however, was made by the introduction of the powerful tool of gene therapy. Transfer of genetic materials, such as hepatocyte growth factor, using both viral and non-viral vectors has proved to be successful in augmenting liver regeneration in various animal models. For future clinical studies, ongoing research aims at eliminating toxicity of viral vectors and increasing transduction efficiency of non-viral vectors, which are the main drawbacks of these systems. Another goal of current research is to develop gene therapy that targets specific liver cells using receptors that are unique to and highly expressed by different liver cell types. The outcome of such investigations will, undoubtedly, pave the way for future successful clinical trials.

Key words: Liver regeneration; Gene therapy; Genetic vectors; Growth factors

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INTRODUCTION

The need to enhance the capacity of liver regeneration has long been recognized but only avidly pursued recently. The clinically successful massive liver resection or small-for-size liver transplant carry the risk of liver failure due to impaired regeneration of the remnant/split liver[1]. Liver regeneration is also an integral component of the recovery processes of liver cirrhosis, fibrosis or liver failure[2]. Major advances in understanding the molecular mechanisms that govern liver regeneration have been made over the past few years[3,4]. Identification and molecular characterization of specific growth factors that promote liver regeneration has allowed the development of recombinant growth factors and their use to promote liver regeneration. The success of this strategy was hampered by the short half-life of these proteins in the circulation and the need for them to be administered continuously[5-9]. To overcome this problem, investigators successfully used gene transfer technology to transfer the genes that encode these growth factors. The intrinsic production of growth factor proteins following the transfer of their encoding genes enhances liver proliferation in various animal models with partial hepatectomy and/or chemical injury. Despite the success of proof of principle studies of gene therapy to enhance liver regeneration, and the potential of translation into clinical settings, no systematic review of published studies has appeared so far. Therefore, an evaluation of the current literature on gene therapy for liver regeneration is required and a look at future perspectives is

warranted. This article briefly summarizes the current key concepts in liver regeneration and gene therapy as they are related to each other, gives an overview of the published studies, and envisions future progress in the field.

LIVER REGENERATION: BASIC CONSIDERATIONS

Following two-thirds partial hepatectomy, the residual liver lobes enlarge within a week to make up for the mass of the removed lobes. Liver regeneration is carried out by proliferation of all adult liver cells including hepatocytes, sinusoidal endothelial cells, biliary epithelial cells, Kupffer cells and hepatic stellate cells (HSCs)[10]. It has been firmly established that mature hepatocytes are not terminally differentiated and that they have an almost unlimited capacity to proliferate, so that the liver can be entirely repopulated by intact hepatocytes that represent 1% of the hepatocyte population[10-15].

The molecular mechanisms of liver regeneration can be divided into two critical steps: the transition of the quiescent G_0 phase hepatocyte into the cell cycle (priming phase), and progression beyond the restriction point in the G_1 phase of the cycle (progression phase). These phases are under separate control; priming by the cytokines tumor necrosis factor (TNF) and interleukin-6 (IL-6), and cell cycle progression by the growth factors hepatocyte growth factor (HGF) and transforming growth factor (TGF)-α[11]. The priming phase does not lead to DNA replication unless the cells can progress through the cell cycle which is accomplished by growth factors. Once hepatocytes pass the G_1 restriction point they are irreversibly committed to replication (Figure 1)[16,17].

The mechanisms that initiate cytokine cascade liver regeneration have not yet been fully identified. It has been proposed that liver injury causes the release of reactive oxygen species and lipopolysaccharide (LPS), which trigger the activation of the complement system. After complement activation, cleavage of C3 or C5 leads to generation of the potent anaphylatoxins C3a and C5a. LPS, C3a and C5a in turn activate the non-parenchymal cells (NPCs) such as Kupffer cells, through the cell surface receptor TLR4 and C3aR and C5aR, which causes activation of the transcription factor nuclear factor (NF)-κB signaling pathway and the production of cytokines such as TNF-α and IL-6[18,19]. Also, the cytokine cascade can be triggered through the binding of TNF to its receptor TNFR1, which leads to activation of the NF-κB in NPCs, with the production of TNF and IL-6. Thus, the released TNF acts on the same NPCs in an autocrine fashion and on hepatocytes by a paracrine mechanism. Released IL-6 binds to its receptor on hepatocytes and leads to activation of the transcription factor STAT3 (signal transduction and activator of transcription), which translocates to the nucleus where it induces transcription of a number of target genes (Figure 2). The precise role played by each cytokines is, however, debatable[3,11]. TNF is not a direct mitogen for hepatocytes. It does, however, enhance the mitogenic effects of direct mitogens such as HGF. For example, it has been shown in stellate cells in culture that TNF and IL-6 activate the transcription factor C/EBPβ (CCAAT/enhancer-binding protein β), which induces HGF mRNA expression[20]. TNF is also involved in the activation of TGF-α[4]. IL-6 has both mitogenic and anti-apoptotic effects on hepatocytes and protects the regenerating liver against ischemic injury[11]. IL-6 has a crucial role in initiating acute phase response in hepatocytes, with the production of many proteins that assist in controlling acute or chronic inflammation[21].

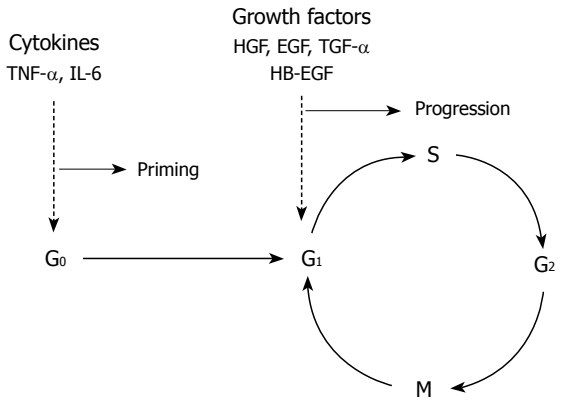

Figure 1 Effect of cytokines and growth factors on hepatocyte cell cycle. TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; HGF: Hepatocyte growth factor; EGF: Epidermal growth factor; TGF-α: Transforming growth factor-α; HB-EGF: Heparin-binding EGF-like growth factor.

While cytokines are responsible for the passage of quiescent hepatocytes into the cell cycle (G_0 to G_1), cell cycle progression is then driven by growth factors, which override a restriction point in the late G_1 phase[3]. HGF and ligands of epidermal growth factor receptor (EGFR) are important growth factors that drive cell cycle progression during liver regeneration. Studies have shown that despite the expression of many mitogenic receptors, including receptors for platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF), the only mitogens for hepatocytes are HGF and ligands of EGFR. The family of ligands that bind EGFR, in addition to EGF, includes TGF-α, heparin-binding EGF-like growth factor (HB-EGF), and amphiregulin (AR)[3,22]. Stimulation of the tyrosine kinase receptors for HGF and the EGF ligands activates numerous intracellular signaling pathways that regulate transcription factors involved in liver regeneration[3,4]. It is important to mention, with the possible exception of HGF, that complete elimination of a single growth factor does not entirely abrogate liver regeneration.

HGF is the most extensively investigated growth factor for liver regeneration. It stimulates regeneration in normal and injured liver. It is produced by NPCs and stimulates hepatocytes by a paracrine or endocrine mechanism. Following binding to its receptor, cMet, on hepatocytes, it stimulates DNA synthesis. HGF effects are multiple including mitogenic, motogenic, morphogenic and anti-apoptotic effects[4,11,17,23].

EGFR ligands are direct mitogens for hepatocytes. EGF is continually available to the liver through the portal

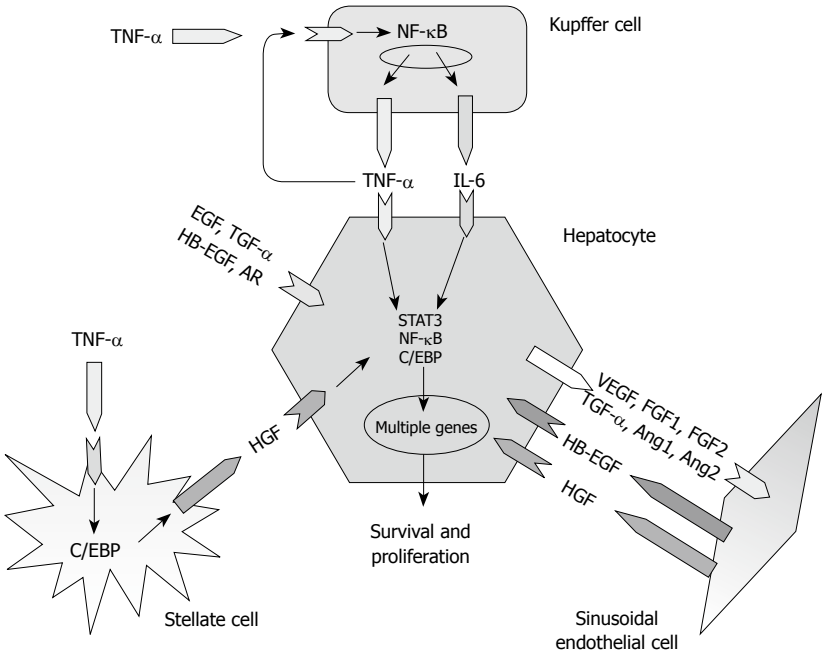

Figure 2 Major cytokine and growth factor signals during liver regeneration. NF-κB: Nuclear factor-κB; TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; EGF: Epidermal growth factor; TGF-α: Transforming growth factor α; VEGF: Vascular endothelial growth factor; FGF: Fibroblast growth factor; HB-EGF: Heparin-binding EGF-like growth factor; AR: Amphiregulin; C/EBP: CCAAT/enhancer-binding protein; HGF: Hepatocyte growth factor.

vein, and is produced from Brunner's glands of the duodenum. EGF given to intact animals causes hepatocyte proliferation. TGF-α is an autocrine growth factor that is produced by and active on hepatocytes. Transgenic mice that overexpress TGF-α display hepatocyte proliferation and develop tumors[24]. On the other hand, TGF-α knockout mice have no defects in liver regeneration; probably because of the overlap between various ligands of the EGF family. TGF-α is also a mitogen for endothelial cells and bile duct epithelial cells. HB-EGF is produced by endothelial and Kupffer cells and is a key factor for hepatocyte progression through G_1/S transition during liver regeneration[25]. AR also contributes to liver regeneration, because mice deficient in AR have deficient liver regeneration[26]. It is likely that the different growth factors have independent but partially overlapping functions in liver regeneration[3,4].

Cytokine and growth factor pathways interact during different phases of liver regeneration[3,27,28]. For example, TNF activates TGF converting enzyme (TACE) that results in release of TGF-α, activation of EGFR and hepatocyte proliferation[3]. It should be noted at this point that there is significant redundancy between the components of each pathway, such that the lack of a single component generally causes a delay and/or reduction of regeneration. In other words, loss of an individual component gene rarely leads to complete inhibition of liver regeneration[3,23]. In contrast to the large number of hepatocyte growth promoters, very few inhibitors of liver regeneration have been identified. The most potent of these inhibitors is TGF-β[22]. For a more detailed review on molecular mechanisms of liver regeneration, readers should refer to references[1,3,4,23,29-33].

The molecular events involved in liver regeneration are significantly influenced by the extent of resection, as massive (85%-90%) liver resection leads to suppression and delay of liver regeneration, compared to 70% partial hepatectomy (PH), because of suppressed and delayed induction of the regenerative genes TNF-α and IL-6 after 90% PH. Moreover, apoptosis rates are also elevated in 90% PH compared to 70% PH[34]. Several studies have shown that growth factors that promote liver regeneration (HGF and TGF-α) are upregulated in 70% PH, whereas no or only reduced induction occurs after 90% resection. These findings suggest that expression of the factors relevant to the regeneration of liver tissue is influenced by the extent of resection[17,34-36].

A simplified summary of the interactions between cytokines and growth factors and between different cell types during liver regeneration is shown in Figure 2. Hepatocytes are the first to undergo proliferation, based on external stimuli from a variety of sources. HGF is rapidly becoming available to hepatocytes very rapidly through local matrix release and activation induced by urokinase-type plasminogen activator. Stellate and endothelial cells are sources of new HGF, which is synthesized after 3 h following PH. Hepatocytes produce growth factors that are mitogenic for stellate cells (PDGF)[37] and for endothelial cells (VEGF, FGF1, FGF2, stem cell factor, angiopoietins 1 and 2, and TGF-α). Proliferation of endothelial cells aims to restore the network of sinusoids that occurs over a long period of time, from days 3 to 6 after PH. Kupffer cells have not been clearly proven to proliferate during regeneration; however, they do produce TNF and IL-6, which appear to have a contributory role in STAT3 and NF-κB activation during the early stages of liver regeneration. Of note, the original hepatocyte mass is not restored through proliferation of stem cells, but through replication of residual mature hepatocytes. Hepatic stem cells (oval cells) are mobilized and differentiate into hepatocytes, only when proliferation of hepatocytes is totally blocked or when hepatocytes are chronically destroyed[10,23,33,38,39].

Identification and molecular characterization of spe-

Table 1 General characteristics of most commonly used vectors

System	Size of insert (kb)	Infect non-dividing cell	Genomic integration	Duration of expression	Immune response
Adenovirus					
1st generation	5	Yes	No	3-4 wk	High
2nd generation	8	Yes	No	Longer with	High
Gutless	35	Yes	No	Immuno-supression	Less
Adeno-associated virus	< 4.8	Yes	Yes and episomal	Long-term	Low
Herpes simplex virus 1	35	Yes	No	Long	High
Retrovirus	≤ 8	No	Yes, random	Long-term	Low
Lentivirus	≤ 8	Yes	Yes, into active genes	Long-term	Low
Baculovirus	> 20	Yes	No	Transient	Low
Plasmid-naked	Large	Yes	No	Short	Low
Plasmid-polymer	Large	Yes	No	Short	Low
Plasmid-lipid (liposomes)	Large	Yes	No	Short	Low

cific growth factors that promote liver regeneration allow the development of recombinant growth factors and their use to promote liver regeneration[6,40-43]. The success of this strategy is hampered by the short half-life of these proteins in the circulation and the need for them to be administered continuously. To overcome this problem, investigators have successfully used gene transfer technology to transfer the genes that encode these growth factors into liver cells.

GENE THERAPY FOR LIVER REGENERATION: KEY CONCEPTS

The strategy of introducing genetic material into liver cells to enhance proliferation or to inhibit apoptosis has been employed in experimental liver research for more than a decade. The transferred genetic material can be a natural gene[44-46], gene segment[47], chimeric gene[48], oligodeoxynucleotides (ODN)[49,50], or siRNAs. To facilitate transfer (transduction) into cells, the foreign gene (transgene) is packaged into construct named vectors. Gene transfer vectors are classified as either viral or non-viral. Viral vectors provide a powerful means for delivering therapeutic genes to targeted cells due to their high transduction efficiency. They are made replication-defective by deletion of viral genes involved in the replication and pathogenesis of the virus. This allows for the inclusion of non-viral genetic material in the viral genome. The general characteristics of most commonly used vectors are shown in Table 1. The most commonly used viral vectors are retrovirus, adenovirus, adeno-associated virus (AAV), herpes simplex virus, lentivirus and baculovirus. For a gene to be expressed inside a cell, its coding DNA sequence should be linked to an appropriate promoter. These regulatory DNA sequences can be categorized as viral (universal) promoters, which allow transgene expression in most transduced cells, housekeeping promoters, or tissue-specific promoters, which drive gene transcription only in selected cell types[51]. Because of their universal activity, viral promoters were components of many first-generation vectors. However, many of the viral promoters, such as the cytomegalovirus (CMV) promoter, are attenuated or completely shut-off in organs such as the liver. In comparison to viral or housekeeping promoters, tissue- or liver-specific promoters direct higher levels of expression *in vivo*. Successful application of gene therapy depends on the choice of relevant therapeutic genes, appropriate promoters, and effective vectors that allow an adequate level and duration of transgene expression[52-54].

Although retroviral vector transfection results in long-term survival of the gene in the transduced cell, its major disadvantage is the risk of insertional mutagenesis as a result of random integration of the virus into the host chromosome. Moreover, the transduction rate after retroviral gene transfer into hepatocytes *in vivo* is disappointingly low. Efficient retrovirus integration into the host-cell genome requires the active proliferation of target cells with DNA replication and nuclear membrane breakdown during mitosis. Under normal physiological conditions at any given time, only 0.005% of hepatocytes divide. For retrovirus liver transduction, hepatocyte proliferation induced by PH must occur on or about the time of retroviral delivery. To increase gene transfer without hepatectomy, mouse hepatocytes have been transduced *in vivo* with a recombinant adenovirus that transiently expressed urokinase[55], or with recombinant HGF[56]. The induced liver regeneration allowed persistent and efficient retroviral-mediated gene transfer in hepatocytes[55,56].

Adenoviral vectors are the most investigated vectors in animal and human gene therapy studies. Adenoviral vectors exhibit several merits that make them suitable for liver regeneration gene therapy. Adenoviruses are highly hepatotropic and it is relatively easy to produce high titers of recombinant adenoviral particles[57]. Unlike retroviruses, adenoviruses transduce dividing and non-dividing cells and do not integrate into the host chromosomes, thereby eliminating the risk of insertional mutagenesis. These merits make adenoviral vectors suitable for proof of principle experimental studies to verify the effect of overexpression of a specific growth factor gene on liver regeneration. The major limitation of adenoviral vectors is their serious and potentially fatal toxicity as exemplified by the death of an 18-year-old man who received 6×10^{11} viral particles/kg of E1/E4-deleted human adenovirus type 5 vector that contained human ornithine transcarbamylase cDNA[58,59]. Moreover, the severe immune response of the host contributes to the limited survival of the adenovirus

DNA in targeted cells and results in transient expression of the therapeutic gene. Until resolved, adenoviral-vector-induced toxicity will limit its application in clinical gene therapy studies. The transient nature of gene expression with adenoviral vectors may be advantageous because the process of liver regeneration is usually completed in approximately 1 wk. However, liver regeneration is seldom the only goal of therapy. Treating associated liver fibrosis or cirrhosis requires a longer period of gene expression. Furthermore, transduction efficiency of diseased liver is much lower than that of healthy liver. Garcia-Bañuelos *et al*[60] have demonstrated that adenovirus-mediated gene transfer *via* the iliac vein at 3×10^{11} viral particles per rat resulted in approximate 40% transduction in livers made cirrhotic by chronic intoxication with carbon tetrachloride, compared with approximate 80% in control non-cirrhotic livers. In rats made cirrhotic by bile-duct obstruction only, 10% efficiency of transduction was observed. Yu *et al*[61] have shown that NPCs are transduced with greater frequency than hepatocytes at all adenoviral titers tested, both *in vitro* and *in vivo*. After liver injury, adenoviral transduction is reduced for all liver cell types compared with that for cells from normal livers (at all virus titers). Again, transduction efficiency remains greater in NPCs than in hepatocytes after liver injury.

Non-viral vectors can be divided into two categories: physical and chemical. Physical methods involve the introduction of plasmid DNA into cells using electroporation, ultrasound, or hydrodynamic delivery. Chemical methods use lipid or polymer carriers that complex with DNA to deliver the transgene into cells[62,63]. Several non-viral vectors have been used for *in vivo* liver gene therapy including various liposome preparations, protein-DNA conjugates, nanoparticles, and naked or complexed DNA[57,64,65]. Expression is usually both transient and at low level because the DNA is not stable in cells. Despite these limitations, non-viral vectors offer many advantages including being simple to use, ease of production of large quantities, and absence of host immune response.

A major advance in the intravascular delivery of vectors followed the development of the hydrodynamic injection technique. The technique involves rapid tail vein injection of a large volume of the vector (around 10% of the body weight of a mouse or rat) in a short time period (5-7 s in mice and 15-20 s in rats). The hydrodynamic method results in dramatically higher hepatic transfection efficiency compared to conventional injection. Typically, 10%-15% of hepatocytes are transfected in mouse liver following injection of 10 μg plasmid, but levels up to 40% have been reported[66]. Liver enzymes are transiently elevated and liver histology shows minimal damage that resolves within a week, which is similar to the results obtained from intravascular delivery into liver vessels[66,67]. It has been postulated that increased pressure in the inferior vena cava causes retrovenous blood flow from the central to the portal vein, and the resultant increased intrahepatic vascular pressure promotes massive endocytosis that generates intracellular water movement that facilitates gene entry[68,69]. There are multiple lines of evidence that the species differences in the diameter of sinusoidal fenestrae are a critical determinant of transgene expression after adenoviral transfer. The small diameter of fenestrae in humans should be considered in any rational design of gene transfer technology for hepatocyte-directed transfer. Hydrodynamic gene transfer is highly successful in rodents. The significantly lower efficacy in higher species may also partially be due to species differences in liver architecture[70]. Intrinsic factors, in particular compliance (elasticity) of the liver are likely to be crucial in determining the degree of swelling for a given level of intrahepatic vascular pressure. Liver compliance is likely to be the major reason for the low level of hydrodynamic gene delivery in the pig model, and will influence the effectiveness of the approach in humans, both in general and in different disease states[71].

This procedure has great limitations for application to clinical practice, therefore, a clinically relevant method for regional hydrodynamic delivery of vectors has been developed. The method entails the use of an occlusion balloon catheter into the inferior vena cava and retro dynamically injecting towards the liver and through the hepatic vein, 100 mL of the plasmid in saline solution (20 mg/mL), at a rate of 7.5 mL/s. This retrodynamic hepatic vein gene delivery method has been performed in pigs, and was as well tolerated as in mice and led to liver transgene expression, however, the plasma levels of the transgene protein were four orders of magnitude lower than those reached in the murine model[68,72]. A variety of different modifications have been reported recently[73,74].

Recently, retrograde administration of adenoviruses into the common bile duct has been shown to induce efficient transgene expression in the liver without causing severe adverse effects, thus supporting the feasibility of adenovirus-mediated gene transfer into the liver in clinical settings by means of endoscopic retrograde cholangiography[75-77]. Repeat administration of adenoviruses into the common bile duct is successful in re-expressing the transgene in the liver[78]. This contrasts with the failure of re-expression of transgene following intravenous readministration of an adenoviral vector long after the initial administration[79].

OVERVIEW OF PUBLISHED STUDIES

The general features of the reviewed gene therapy studies for enhancing liver regeneration are summarized in Table 2. Gene therapy investigations that fulfilled the following criteria were selected for review: (1) demonstrated, objectively, enhanced liver cell proliferation and or increased survival as compared with controls; (2) animals and/or livers receiving gene therapy were not genetically modified as they do not directly represent human liver diseases (e.g. liver cirrhosis, fibrosis or failure) in which liver regeneration has a critical role in recovery; and (3) gene therapy was administered *in vivo*. The selection of homogeneous cohort studies based on these criteria allows us to delineate the main characteristics of these studies, and more importantly, envision what needs to be done in fu-

Table 2 Main features of reported gene therapy experiments[39,44-50,80-96]

Vector, Ref.	Dose	Transgene (promoter)	Liver model, animals, route	Measured parameters
Adenovirus vector				
Hogaboam *et al*[80], 1999	1×10^8 pfu	r-MIP-2	Acetaminophine injury, mice, IV	↑DNA synthesis, ↑survival
Phaneuf *et al*[46], 2000	1-4 × 10^{11} vp	h-HGF (CMV)	Healthy, mice, IV	↑DNA synthesis, ↓apoptosis and ALT
Shiota *et al*[39], 2000	1×10^9 pfu	r-HGF (CAG)	AAF/70% PH, rats, IV	↑Oval cell proliferation
Nomi *et al*[95], 2000	1×10^9 pfu	r-HGF (CAG)	D-Gal/LPS liver failure, rats, IP	↓Apoptosis, ↑survival
Hecht *et al*[48], 2001	1×10^8 TU	h-HIL-6 (CMV)	D-Gal liver failure, mice, IP	↑Survival, ↑proliferation
Hwang *et al*[81], 2003	1×10^{11} vp	h-HGF (CMV)	TAA liver failure, mice, IV	↑Survival, ↑DNA synthesis, no hepatic necrosis
Iwaki *et al*[49], 2003	2×10^9 pfu	m-MIF antisense	BCG-LPS liver failure, mice, IV	↑Survival
Oe *et al*[45], 2004	7×10^8 pfu	h-VEGF + or r-HGF (CAG)	DMN cirrhosis 70% PH, rats, IV	↑SECs and hepatocytes proliferation
Oe *et al*[82], 2005	7×10^8 pfu	r-HGF, or h-VEGF (CAG)	AAF/70% PH, rats, IV	↑Oval cell proliferation, ↑regeneration
Wullaert *et al*[84], 2005	2.5×10^9 pfu	m-ABIN-1 (CMV)	TNF + Gal-liver injury, mice, IV	↑Survival, ↓apoptosis,
Ichiba *et al*[94], 2005	1×10^9 pfu	r-TPO (CAG)	AAF/70% PH, rats, IV	↑Oval cell proliferation
Khai *et al*[44], 2006	1×10^{11} vp	h-HB-EGF or h-HGF (RSV)	Fas-induced injury, mice, IV	↓Apoptosis and ↑proliferation by both
Ozawa *et al*[47], 2006	5×10^8 pfu each	r-HGF, +/or h-TGFβ2R (CAG)	DMN cirrhosis 10% PH, rats, PV	↑Proliferation, ↑survival, ↓cirrhosis
Tan *et al*[96], 2006	1×10^{11} vp	m-HNF6 (CMV)	70% PH, mice, IV	↑Proliferation
Yuasa *et al*[85], 2007	1×10^9 pfu	r-HGF, (CBA)	85% PH, rats, IV	↓Apoptosis, ↑proliferation, ↑survival
Ueno *et al*[83], 2007	5×10^8 pfu	r-HGF (CAG)	DMN cirrhosis 70% PH, rats, sPV	↑Proliferation, ↑survival, ↓cirrhosis
Atta *et al*[93], 2009	7×10^9 pfu	h-HGF, h-VEGF (CMV)	Healthy, dogs, IV	↑SEC and hepatocytes proliferation
Naked plasmid DNA				
Yang *et al*[90], 2001	10-40 μg/wk × 8	h-HGF (CMV)	Healthy, mice, IV	↑Proliferation
Xue *et al*[89], 2003	50 μg × 3	r-HGF	CCl_4 cirrhosis 70% PH, mice, IM + EP	↑Proliferation
Zhang *et al*[91], 2005	200 μg/kg per 12 h × 4	r-ALR	CCl_4 liver injury, rats, IV, IP	↓ALT and AST, ↑proliferation, ↑survival
Horiguchi *et al*[86], 2009	-	h-HGF	DMN cirrhosis, dogs, IA	↓ALT and AST, ↓fibrosis, ↑survival
HVJ Liposomes				
Ueki *et al*[88], 1999	20 or 40 mg weekly × 4	h-HGF (SRa)	DMN cirrhosis, rats, IM	↓Apoptosis, ↑survival, ↑r-HGF, ↓fibrosis
Ogushi *et al*[92], 2003	50 nmol	NF-κB decoy ODN	*P. acnes*-LPS liver injury, mice, PV	↑Survival, ↑proliferation, ↓apoptosis
Nishino *et al*[87], 2008	20 μg	h-HGF (SRα)	DMN cirrhosis 70% PH, rats, PV	↑Proliferation, ↑survival, ↓apoptosis
Takahashi *et al*[50], 2009	50 nmol	NF-κB decoy ODN	90% PH, mice, PV	↑Survival, ↓apoptosis

AAF: Acetylaminofluorene; ALT: Alanine transaminase; AST: Aspartate transaminase; ABIN-1: A20 binding inhibitor of nuclear factor κB; ALR: Augmenter of liver regeneration; BCG: Bacille Calmette-Guerin; CAG: Chicken β-actin promoter and cytomegalovirus enhancer; CBA: Chicken β-actin; D-Gal: D-galactosamine; TNF: Tumor necrosis factor; HVJ: Hemagglutinating virus of Japan; DMN: Dimethylnitrosamine; EP: Electroporation; Gal: Galactosamine; h: Human; h-HIL-6: Human hyper-interleukin-6 (IL-6) cDNA gene coding the human sIL-6R (amino acid residues 1-323) and human IL-6 (amino acid residues 29-212) fused by a synthetic DNA linker; HNF6: Hepatocyte nuclear factor 6; IA: Intra-arterial injection (hepatic artery); IM: Intramuscular injection; IP: Intraperitoneal injection; IV: Intravenous injection; M: Murine; HGF: Hepatocyte growth factor; MIF: Macrophage migration inhibitory factor; MIP-2: Macrophage inflammatory protein-2; VEGF: Vascular endothelial growth factor; CMV: Cytomegalovirus; ODN: Oligodeoxynucleotides; PH: Partial hepatectomy; LPS: Lipopolysaccharide; SECs: Sinusoidal endothelial cells; *P. acnes*: *Propionibacterium acnes*; PV: Portal vein injection; r: Rat; sPV: Selective portal vein injection; SRα: Simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat; TAA: Thioacetamide; TGFβ2R: Truncated transforming growth factor β type 2 receptor; TPO: Thrombopoietin; TU: Transducing units (1 vp = 25 TU); vp: Viral particles (1 vp = 100 pfu).

ture studies as a preparation for clinical trials. An overview of the different elements of gene therapy for liver regeneration studies are given below.

Vector type

Given the merits of adenoviruses as a powerful vector that has the highest transduction rate for liver cells, it is not surprising that two-thirds of all reviewed studies used it to prove the effect of the therapeutic gene (Table 2). It was the only viral vector used. The non-viral vectors employed in the rest of the reviewed studies are divided between naked DNA and liposomes. Despite the lower transfection rate of the non-viral vectors, their safety makes them suitable candidates for preclinical studies.

Vector dose

The administered adenoviral dose ranged between 1×10^8 pfu and 4×10^9 pfu with a dose of 1×10^9 pfu used in 80% of the studies[39,44-47,80-85,97]. The average vector dose for mice was no different from that for rats despite considerable differences in their body weights. Phaneuf *et al*[46] have examined the effect on liver regeneration of increasing doses (1×10^9 to 4×10^9 pfu) of adenoviral vector encoding for human HGF. They have found that DNA synthesis of hepatocytes and liver weight increased in a dose-dependent fashion, such that the maximal effect was seen after the infusion of 3×10^9 pfu, which resulted at day 5 in a > 130% increase in relative liver mass, with little cytopathic effect. The average single dose of naked DNA was 10-50 μg and that of liposomes was 50 nmol[50,86-91,98].

Therapeutic genes

By far the most studied therapeutic gene was HGF, which is not surprising given the fact that it is the single most important growth factor implicated in liver regeneration. It has been used in about two-thirds of studies, either alone

or in combination with other growth factors (Table 2). Other genetic materials used include growth factors, cytokines or transcription factors involved in direct liver cell proliferation, e.g. VEGF, HB-EGF, C/EBPβ, and IL-6. Two studies have reported the use of antisense ODN to NF-κB (NF-κB decoy ODN) encapsulated in hemagglutinating virus of Japan (HVJ) liposomes to prevent endotoxin- or massive hepatectomy-induced liver failure[50,92]. Antisense ODNs are not natural genes, and they are short (15-20 bases in length) synthetic oligonucleotides that are designed to hybridize to RNA through Watson-Crick base pairing. Upon binding to the target RNA, ODNs prevent expression of the encoded gene product. Although stimulation of the transcription factor NF-κB in Kupffer cells, with production of inflammatory cytokines, has been shown to be involved in liver proliferation, excessive production of cytokines is thought to be responsible for liver failure following excessive hepatectomy[50].

Route of administration

The liver is an attractive target for *in vivo* gene transfer studies because hepatocytes are readily accessible *via* the blood stream. The endothelium of hepatic sinusoids displays fenestrations that are 100 nm wide and that allow macromolecules such as viral particles to cross the endothelium and reach hepatocytes. Moreover, the hepatic blood flow represents one-fifth of the cardiac output. Thus, any particle injected into the blood circulation can quickly reach the liver[54]. For this reason, the vascular route constitutes the most commonly used in 80% of the reviewed studies. The intravenous route is the commonest among the vascular routes not only because it is the easiest route compared with intra-arterial or portal vein administration, but also due to the enhanced transduction rate following the recent modification of the hydrodynamic technique mentioned above.

Duration of transgene expression

Few of the reviewed studies have reported the duration of expression of the transduced gene or its protein[39,45,81,83,85,87,88,93-95]. Those studies that had extended observation periods have shown that the duration of transgene expression does not extend beyond 1 wk following vector administration[39,81,83,87,88,94]. These data agree with the accumulated knowledge that gene therapy using adenoviral vectors or non-viral naked DNA and liposomes confers a limited duration of gene expression. Moreover, it should be noted that the efficiency of gene transduction, which directly affects the duration of gene expression, is lower in cirrhotic liver than in normal liver due to capillarization of sinusoidal endothelial cells as a result of the decreased size or loss of the fenestrae of sinusoidal endothelial cells[99]. Nishino *et al*[87] have demonstrated that only 5%-6% of hepatocytes in cirrhotic rat livers were successfully transfected with human HGF plasmid enveloped in HVJ liposomes.

Non-hepatic gene transfection

There was a tendency towards excluding gene therapy studies for liver regeneration in which gene transduction involved organs other than the liver, e.g. skeletal muscles. Although this could be appropriate for the sake of presenting a homogeneous group of investigations, it was felt however that this would have omitted an important cluster of studies that represented an emerging direction in gene therapy for liver regeneration. In this regard, two studies used liposomes and naked plasmid to transduce skeletal muscles with HGF in animals with liver cirrhosis. They demonstrated expression of the transduced HGF gene and elevation of its plasma levels that exerted proliferative and antifibrotic effects on the liver[88,89].

FUTURE PERSPECTIVES

In 20 years of gene therapy research, there have been few studies that have aimed at enhancing liver regeneration. However, the accumulated knowledge from these studies has allowed the validation of proof of principle gene therapy investigations for promoting liver regeneration in different animal models of liver diseases. Future progress in this field is expected to tackle several points.

First, determination of the combination of gene therapy that works better for a specific disease condition. As mentioned above, enhancing liver regeneration is seldom the only goal of therapy. Treating associated liver fibrosis/cirrhosis or toxic injury requires the combined effects of genetic materials such as growth factor genes and antisense ODN. This should be based on the outcomes drawn from experimental comparative studies of different combinations of therapeutic genes for each defined disease. An example of such comparative studies is that of Ozawa *et al*[47]. In rats with liver cirrhosis, combination gene therapy of HGF, a powerful liver mitogen, and truncated type Ⅱ TGF-β receptor that specifically inhibits TGF-β signaling that is responsible for progression of liver fibrosis[100], resulted in decreased liver fibrosis and improved liver function, compared with monotherapy with either gene alone. These studies provide an opportunity to shed light on how the administered genes influence the pathogenesis of the multifactorial disease process. Also, it could identify synergistic combinations that could enhance regeneration, disease resolution and reduce the amount of transferred genetic material. An example of such studies would make use of HGF and NF-κB decoy ODN, which prevents excessive cytokine production, to prevent hepatocyte apoptosis and enhance regeneration after massive resection or liver injury[50,92].

Secondly, evaluation of the trade-off of risk against the benefits of viral *vs* non-viral gene therapy. Unlike gene therapy for liver genetic diseases that require a high rate of liver transduction to express the therapeutic protein efficiently in the systemic circulation, at a clinically relevant concentration, gene therapy for liver regeneration or resolution of fibrosis aims at locally expressing the desired proteins, which act in an autocrine or paracrine fashion[93]. Thus, despite non-viral systems having a lower transfection rate, they are safer, easy to produce in large quantities, and can be repeatedly administered, which can

aid in gauging the amount and duration of gene expression. Moreover, hydrodynamic injection in murine models and its clinically relevant retrodynamic hepatic vein gene delivery in large animals have dramatically increased transfection efficiency of non-viral systems.

Thirdly, employing the recently developed vectors that target specific liver cell types, and promoters that are capable of liver-specific sustained transgene expression in gene therapy studies to augment liver regeneration and treat associated liver injury. These new developments can be summarized as follows: (1) Cell-specific expression of therapeutic genes of interest is an extremely attractive strategy in gene therapy. Several investigators have developed selective hepatic cell delivery systems using receptors that are unique to and highly expressed by different liver cell types: (A) The asialoglycoprotein receptor (ASGPR) on the hepatocyte membrane is a specific targeting marker for gene and drug delivery. Studies have targeted the hepatocyte ASGPR using its natural ligand, asialoorosomucoid[101,102]. Chiba *et al*[103] recently have developed cationically modified biocompatible phospholipid polymer conjugated with hepatitis B surface antigen for the specific transfer of genes into human hepatocytes; (B) Quiescent HSCs lack specific receptors or motifs on their cell surface, thus, attempts to target HSCs have been a challenging task[104]. (a) The mannose 6-phosphate/insulin-like growth factor Ⅱ (M6P/IGF-Ⅱ) receptor expression is increased on activated HSCs, particularly during fibrosis. The receptor has binding sites for IGF-Ⅱ and M6P-containing ligands[105]. Beljaars *et al* have developed a carrier system that consists of human serum albumin modified with M6P, which binds to the M6P/IGF-Ⅱ receptors on HSCs[104-107]; (b) Vitamin A receptors on HSCs have been used to deliver siRNA against collagen-specific chaperone heat shock protein 47 *via* vitamin A-coupled liposomes[108]; and (c) Liposomes labeled with a cyclic RGD-peptide that recognizes the collagen type Ⅵ receptors[109,110]; (C) Sinusoidal endothelial cells (SECs) possess unique hyaluronan receptors that recognize and internalize hyaluronic acid (HA). SECs have been targeted using HA, the endogenous ligand for the HA receptor for endocytosis[111,112]; and (D) Kupffer cells possess receptors that recognize galactose and N-acetylgalactosamine. Studies have shown that galactosylation can target various DNA preparations including liposomes, low-density lipoprotein and chitosan polymer to Kupffer cells[113-115]; and (2) Liver-specific sustained transgene expression can be obtained at very high levels from optimized promoters[116]. Many experimental gene therapy vectors described in this review express transgenes under the control of non-specific promoters such as CMV, Rous sarcoma virus, simian virus 40 (SV40) and mammalian elongation factor 1α (EF1α) (Table 2). These promoters direct strong gene expression but are shut off rapidly *in vivo*[117,118]. A tissue-specific promoter is a promoter that has activity in only certain cell types. Use of a tissue-specific promoter in the expression cassette can restrict unwanted transgene expression as well as facilitate persistent transgene expression[119]. Ongoing developments are based on two liver-specific promoters, the albumin promoter and the α1 antitrypsin promoter. Wooddell *et al*[116] have demonstrated that when using a plasmid vector that contains albumin promoter combined with an α-fetoprotein (AFP) MERⅡ enhancer, 5' intron from the factor Ⅸ gene, and the 3'UTR from the albumin gene, including intron 14, the reporter gene expression levels remained high for 1 year, at levels comparable to those obtained from the CMV promoter on day 1. Ziegler *et al*[120] have shown that intravenous administration of a recombinant AAV2 vector encoding human α-galactosidase A under the transcriptional control of a liver-restricted enhancer/promoter consisted of human serum albumin promoter (nucleotides -486 to +20), to which were appended two copies of the human prothrombin enhancer (nucleotides -940 to -860). The enhancers were placed 5' of the promoter in the forward orientation. This vector mediated sustained hepatic expression of α-galactosidase A for 12 mo and was associated with a significantly reduced immune response to the expressed enzyme. Several investigators have reported encouraging long expression of transgenes using different modifications of α1 antitrypsin promoter[117,121-123]. Jacobs and his colleagues have compared 22 hepatocyte-specific expression cassettes and have found that a promoter that consists of an 890-bp human α1-antitrypsin promoter and two copies of the 160-bp α1-microglobulin enhancer results in the highest expression levels[124]. Comparisons between different liver-specific promoters have shown that α1-antitrypsin promoters induce higher levels and prolonged expression of transgenes than other liver-specific promoters such as AFP and albumin promoter[125-127]. The most recent investigations have shown the unlimited possibilities for gene therapy modifications. Li *et al*[128] have developed a small DNA fragment (347 bp) from the AAV chromosome 19 integration site that is capable of providing efficient and enhanced liver-specific transcription when used in recombinant AAV vectors. Previously described tissue-specific promoters for gene therapy are typically too big for AAV vectors. Wolff *et al*[129], in an effort to increase long-term expression of transgene products, have designed a plasmid DNA vector under the control of a tissue-specific promoter and have included microRNA target sites in the transcripts, in order to silence expression in antigen-presenting cells.

CONCLUSION

The success of several proof of principle studies of gene therapy for liver regeneration, coupled with the recent extensive search for the mechanisms of selective targeting of specific liver cells, should pave the way towards future clinical trials. As liver regeneration is usually an integral part of the therapeutic goals of many liver diseases, gene therapy to enhance liver regeneration needs to be combined with gene therapy for associated liver disease. Consequently, clinically relevant gene transfer protocols should be developed to address specific goals of such combined gene therapy trials.

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