Targeted RNA interference for hepatic fibrosis

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Hepatic fibrosis is a common consequence in patients with chronic liver damage. To date, no agent has been approved for the treatment of hepatic fibrosis. RNA interference (RNAi) is known to be a powerful tool for post-transcriptional gene silencing and has opened new avenues in gene therapy. The problems of lack of cell specificity in vivo and subsequently the occurrence of side effects has hampered the development of hepatic fibrosis treatment. To overcome these shortcomings, several targeted strategies have been developed, such as hydrodynamics-based approaches, local administration, cell-type-selective ligands and cell-type-specific promoters or enhancers, etc. Here, we provide an overview of targeted strategies for the treatment of hepatic fibrosis, and particularly, targeted RNAi for hepatic fibrosis.

Keywords: hepatic fibrosis, hepatic stellate cell (HSC), hepatocyte, RNA interference (RNAi), small interfering RNA (siRNA), targeted therapy

1. Introduction

Hepatic fibrosis, a wound-healing response to chronic liver injury, is characterized by excess production and deposition of extracellular matrix (ECM), regardless of the underlying etiology [1]. The activation of hepatic stellate cells (HSCs) remains the dominant event in hepatic fibrogenesis [2]. Normally, these cells reside in the subendothelial space of Disse. Following chronic liver injury, HSCs proliferate and acquire a myofibroblast-like phenotype (activated HSCs). In this activated fibrogenic state, HSCs become contractile and secrete a wide variety of ECM proteins and bioactive mediators, including TGF-β1, platelet-derived growth factor (PDGF) and connective transforming growth factor (CTGF), which in turn, accelerate HSCs proliferation and collagen synthesis. The development of fibrosis, and particularly cirrhosis, is associated with a significant morbidity and mortality [3]. Rational treatments for hepatic fibrosis include curing the primary disease, suppressing hepatic inflammation, inhibiting HSC activation, stimulating HSC apoptosis and facilitating ECM degradation [4,5]. In addition, enhancing hepatocytes regeneration and prevention of liver function loss are another essential strategy for the treatment of hepatic fibrosis.

RNA interference (RNAi) is triggered by double-stranded RNAs that cause sequence-specific degradation of the homologous mRNAs [6]. From a practical perspective, RNAi is known to be a powerful tool for post-transcriptional gene silencing. Compared with other antisense strategies such as antisense oligonucleotides, ribozymes or DNAzymes, RNAi has been proved to be more specific and far more potent in terms of gene knockdown [7,8]. There has been considerable interest in harnessing the power of RNAi to treat liver fibrosis in recent years (Table 1) [9-14]. Most of the targeted genes are those critical for HSC activation, proliferation and/or collagen synthesis and deposition which are usually markedly
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Table 1. Summary of studies regarding RNAi therapy of hepatic fibrosis in vivo.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Vector/promoter</th>
<th>Method of delivery</th>
<th>Animal model</th>
<th>Target tissues or cells</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGFR-β</td>
<td>Plasmid/CMV</td>
<td>HTV</td>
<td>DMN and BDL</td>
<td>Hepatocytes and activated HSCs</td>
<td>[10]</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Plasmid/U6</td>
<td>Tail vein injection</td>
<td>CCl₄</td>
<td>Not mentioned</td>
<td>[12]</td>
</tr>
<tr>
<td>gp46</td>
<td>vitamin A–coupled liposomes</td>
<td>Intravenous injection</td>
<td>DMN, CCl₄ and BDL</td>
<td>HSCs</td>
<td>[13]</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>Chemically synthetic siRNA</td>
<td>Tail vein injection</td>
<td>CCl₄</td>
<td>Not mentioned</td>
<td>[14]</td>
</tr>
<tr>
<td>PDGFR-β</td>
<td>Plasmid/GFAP</td>
<td>HTV</td>
<td>BDL</td>
<td>HSCs</td>
<td>[67]</td>
</tr>
<tr>
<td>Fas</td>
<td>Chemically synthetic siRNA</td>
<td>HTV</td>
<td>ConA</td>
<td>88 ± 6% of hepatocytes</td>
<td>[24]</td>
</tr>
<tr>
<td>CTGF</td>
<td>Chemically synthetic siRNA</td>
<td>Intraportal vein injection</td>
<td>CCl₄</td>
<td>67.2 ± 6.3% of HSCs</td>
<td>[27]</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Adenovirus/Ad1</td>
<td>Tail vein injection</td>
<td>DMN and BDL</td>
<td>Over 80% of liver cells</td>
<td>[76]</td>
</tr>
</tbody>
</table>

BDL: Bile duct ligation; CMV: Cytomegalovirus; CTGF: Connective transforming growth factor; DMN: Dimethylnitrosamine; GFAP: Glial fibrillary acidic protein; HSCs: Hepatic stellate cells; HTV: Hydrodynamic tail vein; PAI-1: Plasminogen activator inhibitor-1; PDGFR-β: Platelet-derived growth factor receptor-β; TIMP-2: Tissue inhibitor of metalloproteinases-2.

upregulated during hepatic fibrogenesis, including CTGF, TGF-β1, PDGF, tissue inhibitor of metalloproteinases (TIMPs) and plasminogen activator inhibitor-1 (PAI-1). However, these molecules may also play a role in many physiological processes and their inhibition may lead to some lethal side effects. In addition, there are many obstacles to in vivo delivery of siRNAs [15,16], such as rapid excretion via the kidneys, degradation by serum and tissue nucleases, inefficient endocytosis by tissue cells and release from endosomes [17]. Santel et al. investigated the biodistribution characteristics of naked siRNAs after systemic administration and demonstrated that the naked siRNAs were predominantly found in the kidney 20 min post-injection, with no detectable signals in other organs (pancreas, heart, lung, liver and spleen), indicating a rapid renal excretion of siRNA molecules and suggesting that the naked siRNAs could not reach any tissues other than kidney after systemic administration [18]. Therefore, systemic administration of naked siRNA seems to be inefficient and unpredictable for in vivo study.

To date, the most effective way available for hepatic fibrosis is to remove the primary cause and no agent has been approved for hepatic fibrosis therapy. One of the reasons is a lack of cell specificity in vivo and consequently the occurrence of deleterious effects on other tissues. Therefore, targeted delivery of the agents to the liver or specific cells especially HSCs and hepatocytes may provide a solution for reducing the adverse reactions and optimizing the efficacy as well. In past years, substantial progress has been made on the targeted delivery systems. Here, we provide an overview of the advances in the targeted strategies for hepatic fibrosis therapy and targeted RNAi for hepatic fibrosis.

2. Liver-tissue targeted strategies

2.1 Hydrodynamics-based approaches

Hydrodynamic tail vein (HTV) delivery is a simple and highly effective delivery method for transferring various agents to the liver in mice [19]. It was reported by Liu et al. for the first time that more than 1000-fold higher transgene expression in liver could be achieved by rapid injection of a large volume of DNA solution into animals via the tail vein. A ratio of injection volume to animal weight of approximately 8 – 12 ml per 100 g and delivery at a high velocity (in about 5 – 7 s) were required for an optimal level of transgene expression [20]. Using this HTV delivery procedure, Yang et al. administered naked plasmid containing hepatocyte growth factor (HGF) cDNA driven by a cytomegalovirus promoter (pCMV-HGF) into mice, which produced a remarkable level of human HGF protein in the circulation, beginning to appear at 4 h and peaking at 12 h following injection. Tissue distribution studies identified the liver as the organ with the highest level of transgene expression. Through weekly repeated injections of plasmid vector, it displayed sustained, long-term and high levels of exogenous HGF expression in mice for 8 weeks [21]. Moreover, Mei et al. administered the C/EBP-α gene into mice by the HTV procedure and found that in vivo transfection of the C/EBP-α gene could ameliorate carbon tetrachloride (CCl₄)-induced hepatic fibrosis. Surprisingly, they found that the transgene expression mainly located in non-parenchymal cells within and around fibrotic septa, as well as at the vasculature [22].

The advantage of the HTV procedure is high efficiency at delivering different kinds of agents including nucleic acids, peptides, proteins, inert polymers and small molecules into
liver cells independently of their size and charge [19,23]. This is especially appropriate for siRNA, as this procedure shortens the contact period with the blood and therefore reduces nuclease degradation. Following HTV delivery of fluorescently labeled siRNA, about 50% of hepatocytes demonstrated uptake of siRNA with cytoplasmic staining and nuclear accumulation. Furthermore, double stranded RNA oligonucleotides, smaller than 100 – 200 bp in size, will enter the nucleus more efficiently than larger DNA molecules [23]. Song et al. delivered Fas siRNA duplexes into mouse hepatocytes in vivo by HTV. The results revealed that almost 90% of hepatocytes had taken up the siRNA 24 h after the last injections. The higher transduction efficiency might be attributed to the repeated injection. Meanwhile, levels of both Fas mRNA and Fas protein were stably reduced up to 10 days after the last injection, and returned to normal 20 days later. More importantly, application of Fas siRNA protected mice from Fas antibody-mediated liver failure and attenuated liver fibrosis in ConA-mediated hepatitis in mice [24]. Moreover, Zender et al. reported that a single application of caspase 8 siRNAs by HTV resulted in transduction of approximately 70% of hepatocytes in the liver [25]. Taking these results together, the HTV procedure is appropriate for siRNA delivery into liver in animals with high efficiency. Nevertheless, this procedure is not applicable for clinical practice as it needs large injection volumes and the high pressure of injection may result in cellular toxicities [26].

2.2 Local administration

Obviously, local injection can induce a high level expression of target gene in tissues to achieve potent activity and reduce potential adverse effects compared with systemic delivery. The liver receives its dual blood supply from both the hepatic artery and the portal vein. Several studies have reported that injection via the portal vein could deliver the target genes to the liver, and it appears to be more efficient and safer than the systemic delivery methods [27,28]. Moreover, less drugs were needed to obtain the similar results [28,29]. Li et al. reported that a synthetic siRNA against CTGF delivered through portal vein injection was taken up by about 70% of HSCs. Moreover, the suppressive effect could maintain up to 3 days after siRNA injection [27]. Zender et al. administered siRNA against caspase 8 either by HTV or via portal vein injection into mice with acute liver failure. Intriguingly, they found that local injection achieved more efficacy compared with HTV delivery [25]. It has been reported that the HTV procedure may lead to a transient increase of serum ALT level and irreversibly damage approximately 5 – 15% of hepatocytes [30]. Therefore, local injection via the portal vein may be more appropriate for the treatment of acute liver failure compared with HTV delivery. Furthermore, George et al. reported for the first time that plasmid vector-encoded siRNAs could be delivered into liver through intra-peritoneal injection [11]. The siRNA-recombinant plasmid DNA vector was mixed with a suitable amount of lipophilic transfection reagent. After intraperitoneal injection, the siRNAs entered the circulatory system and reached the liver through hepatic portal veins. More interestingly, Sakai et al. reported that a more than 200,000-fold increase of transgene expression in the liver was achieved by in vivo electroporation applied to the left lateral lobe of the liver after intravenous injection of naked plasmid via the tail vein. The distribution of naked plasmid as well as transgene expression was largely restricted to hepatocytes in the electroporated lobe [31].

All together, local administration of siRNA could lead to a high transgene expression in liver. However, it is an invasive procedure, which is particularly inappropriate for cirrhosis as it would be difficult to perform and dangerous due to coagulopathy. More importantly, this strategy could not selectively direct the agents to the parenchymal cells (hepatocytes) or non-parenchymal cells (i.e., HSCs) in liver.

3. Hepatocyte specific strategies

Hepatocytes comprise approximately 80% of the total liver mass and are the key parenchymal cells in the liver. It has been believed that hepatocytes play an insignificant role in hepatic fibrogenesis in the past decades [32]. Nevertheless, it is known that hepatic fibrosis is associated with reduced volume and functional loss of hepatocytes. Additionally, more and more studies have proven that hepatocytes also contribute to the accumulation of activated fibroblasts via epithelial-mesenchymal transition (EMT) in fibrotic liver and the EMT involving hepatocytes plays a significant role in the development of hepatic fibrosis [33]. In this case, hepatocytes will emerge as a new target for the treatment of hepatic fibrosis.

As a hepatocyte specific promoter, the albumin promoter has been used to target trantere expression in hepatocytes [34,35]. Moreover, to improve the antitumor efficacy with fewer potential side effects, many studies have reported the hepatoma-specific gene therapy using the AFP promoter or enhancer [36,37]. On the other hand, membrane receptors specifically expressed on the surface of hepatocytes can be used for targeted delivery via the receptor-mediated endocytotic pathway as well. Lots of studies revealed that galactose was a promising targeting ligand to hepatocytes because the cells possess a large number of asialoglycoprotein receptors that recognize the galactose units on the oligosaccharide chains of glycoproteins or on the chemically synthesized galactosylated carriers [38–42]. Sato et al. reported that galactosylated liposomes/siRNA complex exhibited a higher stability than naked siRNA in plasma. After intravenous administration of a galactosylated liposomes/siRNA complex, the siRNA did not undergo nuclease digestion or urinary excretion and was delivered efficiently to the hepatocytes and caused hepatocyte-selective gene silencing [41]. Additionally, Rozema et al. developed a siRNA Dynamic PolyConjugates for targeted delivery of siRNA to hepatocytes both in vitro and in vivo. A key feature of the Dynamic PolyConjugates was the use of
hepatocytes targeting ligand N-acetylgalactosamine (NAG). When NAG was attached, the polyconjugates preferentially accumulates in hepatocytes. Conversely, substituting mannose for NAG redirected the polyconjugates to nonparenchymal cells in the liver. [43]. In addition, apolipoprotein A-I, a protein component of high-density lipoprotein that guides the transport of cholesterol from cells of the arterial wall to the liver, has been used for specific delivery of siRNA to the liver as well [44]. In their study, synthetic siRNAs against hepatitis B virus were formulated into complexes of apo A-I and 1,2-dioleoyl-3-trimethylammonium-propane/cholesterol (DTC-Apo) and injected intravenously into a mouse model carrying replicating HBV. This strategy allowed the effective delivery of siRNAs selectively to the hepatocytes and significantly reduced viral protein expression by receptor-mediated endocytosis. However, there is no report on the application of a hepatocyte-targeted strategy to treat liver fibrosis to date.

4. HSC-specific strategies

HSCs comprise approximately one-third of the nonparenchymal cell population in the liver [45]. And they are the primary source of ECM in normal and fibrotic liver [4]. It has been generally accepted that activation of HSCs plays a pivotal role in hepatic fibrogenesis. Consequently, it has been a major focus for hepatic fibrosis therapy by repressing activation, proliferation and migration of HSCs as well as inducing their apoptosis in the past decades [46].

4.1 HSC specific ligands

Ligands bound to the receptor are subject to receptor-mediated endocytosis. It has been reported that the expression of mannose 6-phosphate/IGF-II (M6P/IGF-II) receptor was increased on activated HSCs, particularly during fibrosis [47-49]. And M6P-modified human serum albumin (M6PHSA) has been shown to accumulate preferentially in HSCs via binding to M6P/IGF-II receptors [58]. Greupink et al. coupled the antiproliferative drug doxorubicin (DOX) with M6PHSA and found that after injection with M6P-HSA-DOX the conjugate rapidly and selectively accumulated in the liver. Within the liver, the uptake of M6PHSA-DOX was confined to the nonparenchymal cells, mainly in HSCs. Moreover, treatment with M6PHSA-DOX reduced the number of activated HSCs in the fibrotic liver and attenuated the degree of fibrosis induced by bile duct ligation (BDL) [50]. Furthermore, Gonzalez et al. conjugated the PDGF tyrosine kinase inhibitor with this HSC-directed carrier M6PHSA using a novel type of platinum linker, which resulted in selective accumulation of this inhibitor in HSCs and prevented the side effects in the other tissues [51]. In addition, the 10 cyclic peptide moieties-modified HSA (C1-3 scAb), in which C* denotes the cyclizing cysteine residues, pCVI-HSA), which recognize collagen type VI receptors, has been reported to be preferentially taken up by activated HSCs in vitro and in vivo, indicating that pCVI-HSA might be used as a homing device to HSCs in fibrotic livers as well [52]. On the other hand, Sato et al. encapsulated the siRNAs against collagen-specific chaperone molecule (gp46) in vitamin A-coupled liposomes. Notably, the siRNAs was mainly observed in areas with HSCs but not in parenchymal areas in the cirrhotic liver of rats that received intravenous injections of the vitamin-A-coupled liposomes, suggesting a specific uptake by HSCs. Moreover, it did not trigger immune reactions and the suppression effect was sustained for at least three days. After five treatments with the siRNA-bearing vitamin-A-coupled liposomes, it almost completely resolved hepatic collagen deposition and prolonged survival in rats exposed to normally lethal dimethylnitrosamine (DMN) treatment in a dose- and duration-dependent manner [13]. This is the first study, to our knowledge, successfully targeting siRNA to HSCs using an elegant vitamin-A-coupled liposomal delivery system [53].

4.2 Antibody-mediated delivery

Cell-type-specific antibodies have been explored for targeted delivery of siRNA as well. Synaptophysin expression is reported to be restricted to HSC-derived myofibroblasts in liver [54,55]. Recombinant monoclonal human single chain antibodies (C1-3 scAbs) were generated to a conserved peptide sequence present on an extracellular domain of synaptophysin. This antibody fragment interacted with HSCs, but not hepatocytes in vitro, suggesting that the C1-3 scAbs might be used to target therapeutics to HSCs in vivo [56]. Recently, glotoxin, which has been proven to stimulate myofibroblasts apoptosis [57], has been effectively targeted to myofibroblasts without affecting Kupffer cell numbers, when chemically conjugated to the C1-3 scAb [58]. Additionally, Song et al. reported for the first time, to our knowledge that a proteamine–antibody fusion protein could be used as a vehicle for receptor-directed delivery of siRNA [59]. In this study, a Fab antibody (F105) fragment against HIV-1 envelope fused to protamine (F105-P) was used for the targeted delivery of siRNAs, which induced gene silencing only in cells expressing HIV-1 envelope. Taken together, these studies demonstrated the potential for cell-type-specific antibody-mediated siRNA delivery.

4.3 HSC specific promoters

Delivery of transgenes under the control of specific promoters or enhancers is a widely used method to direct gene expression in specific tissues or cells. Gene targeting to HSCs is potentially possible by expressing transgenes under the control of promoters or enhancers exclusively expressed in HSCs or upregulated in the course of activation [60]. Type I collagen, which is comprised of 2 α1 chains and 1 α2 chain, is encoded by two distinct genes, COL1A1 and COL1A2, respectively. Increased transcription of those genes results in abnormal collagen deposition during the development of organ fibrosis [61]. Ikeda and his colleagues found that green fluorescent protein (GFP) expression driven by the tissue-specific enhancer COL1A2 was detected only on the activated HSCs in CCl4–induced fibrotic liver, but not in untreated normal liver. Moreover, no GFP fluorescence was observed...
in any other organs when the COL1A2 enhancer was used. The results validate a new concept of therapy for hepatic fibrosis to achieve cell-type-specific gene expression only in the fibrotic liver, with little damage to other organs [61]. Furthermore, using a Cre/loxP system and this cell-specific collagen promoter, they also achieved targeted and regulable expression of transgenes in HSCs and myofibroblasts both in vitro and in vivo [62]. A report by Herrmann et al. demonstrated that the promoter fragments of CSRP2, SM22α and TIMP-1 could direct transgene expression selectively in HSC in vitro, while none of these promoters exerted the ability to target a specific cell type gene expression in HSC/MFB in vivo [60].

Glia fibrillary acidic protein (GFAP), an intermediate filament protein, was traditionally considered to be a biomarker of astrocytes of the central nervous system [63,64]. Nevertheless, GFAP was also documented as a marker for activated and quiescent HSCs [65,66]. To avoid the nonspecific interference of PDGFR-β gene expression in other tissues or cells, we used GFAP promoter to drive the expression of PDGFR-β shRNA (short hairpin RNA) in HSCs. Our results indicated that the GFAP promoter could direct the transgene expression specifically in HSCs in vivo. Furthermore, this cell-specific PDGFR-β shRNA could alleviate liver injury and hepatic fibrosis in rats [67]. This study suggests that HSC-targeted RNAi might present as an effective therapy for hepatic fibrosis.

5. Adenovirus delivery systems

Because of their high titres and the ability to mediate a high level of transgene expression in a wide variety of cells and tissues, replication-deficient recombinant adenoviruses have gained widespread popularity in gene therapy in the past decades [68-73]. It was demonstrated that the transduction efficiency was 80% in cirrhotic livers with adenovirus carrying lacZ gene administered by injection, as compared with 40% in normal livers [74]. This greater transduction efficiency in fibrotic livers may be ascribed to the decreased portal flow velocity and deranged alignment structure of liver cells after hepatic fibrogenesis, which may provide more space and contact time between adenovirus and liver tissue. Furthermore, adenovirus-mediated gene transfer was safe and significant adverse effects were not observed in animal studies [74]. In addition, according to a study by Narvaiza et al., a single dose of adenovirus-mediated RNAi caused gene silencing from day 5 up to day 19 after vector administration, suggesting that adenovirus vector is highly efficient for the delivery of siRNA and further supporting the role of adenovirus vector in the treatment of liver diseases [75]. In our recent study, a recombinant adenovirus-mediated siRNA against PAI-1 (AdshPAI) was delivered into fibrotic liver induced by DMN or BDL. The results revealed that over 80% of liver cells were infected by the adenovirus. Moreover, both the mRNA and protein level of PAI-1 was significantly reduced up to 21 days after AdshPAI administration and AdshPAI treatment could significantly ameliorate liver fibrosis [76].

Our study suggests that adenovirus is an appropriate carrier to deliver siRNA for the hepatic fibrosis therapy.

6. Expert opinion and conclusions

Since it was first discovered in the nematode worm Caenorhabditis elegans [77], RNAi has become a powerful tool for functional genetic studies and gene therapy in mammals due to its superior potency and specificity [78]. The greatest challenge for the application of RNAi is the delivery of siRNAs into the target tissue or cells. Chemically synthesized siRNAs against Fas was first used to treat fulminant hepatitis by Song et al. in 2003 [24]. In their study, they administrated the siRNAs by HTV. Notwithstanding those exciting data, there are some important concerns as HTV is not an appropriate clinical option and chemically synthesized siRNAs are relatively expensive. Shortly thereafter, application of caspase 8 siRNAs together with lipiodol through the portal vein was reported to prevent acute liver failure in mice as well [25]. Local administration via portal vein could result in high transgene expression in liver. Nevertheless, as an invasive technique, it may present as an obstacle for cirrhosis, especially for patients. To date, several new strategies for the siRNA delivery have been applied for the treatment of hepatic fibrosis [13,67,76]. Cell-specific delivery holds the most interest as the potential damage to the non-target cells may counteract the beneficial effect of siRNA. Delivery of transgene with cell-specific ligands or driven by cell-specific promoters or transcriptional regulatory units is a well-established strategy to direct cell-specific gene expression, which has also obtained expected efficacy in the treatment of hepatic fibrosis [13,67]. The main advantages of adenoviral vectors are their high efficiency of transduction and relative liver tropism. However, the non-specific expression of the target gene in all of transduced cells remains a concern. Therefore, driven by the cell-specific promoter or enhancer, the adenoviruses may achieve more favorable efficacy with less side effects.

In conclusion, the application of targeted RNAi has opened a new door for the treatment of hepatic fibrosis. As each approach has its own advantages and disadvantages, we should choose the appropriate carriers according to our purposes, emphases and disease models. With the steady progress in further understanding the mechanism of fibrosis and the development of cellular and molecular techniques, it is anticipated that ultimately these approaches will lead to arrest of hepatic fibrosis with resolution to a normal architecture.

Declaration of interest

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