Published in final edited form as:

Crit Rev Ther Drug Carrier Syst. 2007 ; 24(2): 93–146.

Gene Modulation for Treating Liver Fibrosis

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Abstract

Despite tremendous progress in our understanding of fibrogenesis, injury stimuli process, inflammation, and hepatic stellate cells (HSC) activation, there is still no standard treatment for liver fibrosis. Delivery of small molecular weight drug, proteins and nucleic acids to specific liver cell types remains a challenge due to the overexpression of extra cellular matrix (ECM) and consequent closure of sinusoidal gaps. In addition, activation of HSCs and subsequent release of inflammatory cytokines and infiltration of immune cells are other major obstacles to the treatment of liver fibrosis. To overcome these barriers, different therapeutic approaches are being investigated. Among them, modulation of certain aberrant protein production is quite promising for treating liver fibrosis. In this review, we will describe the mechanism of antisense, antigene and RNA interference (RNAi) therapies, and will discuss how the backbone modification of oligonucleotides affects their in vivo stability, biodistribution and bioactivity. Strategies for delivering these nucleic acids to specific cell types will be discussed. This review will critically address various insights developed in each individual strategy and for multipronged approaches, which will be helpful in achieving better outcomes.

Keywords

Liver fibrosis; Gene modulation; Hepatic stellate cells; Antisense; Antigene; siRNA; Triplex forming oligonucleotide

I INTRODUCTION

Fibrosis is an inappropriate repair by connective tissue. It can affect every organ and it is estimated that 45% of deaths in the United States are attributed to fibrotic disorders.(1) Fibrotic tissue is characterized by loss of normal structure, replacement of blood vessels and other parenchymal structures by increasingly stable extracellular matrix (ECM), in which type I fibrillar collagen is the major component. Fibrosis has been attributed to phenotypically transformed fibroblast like cells, often termed myofibroblasts in myocardium, hepatic stellate cells (HSCs) in the liver, mesangial cells in the kidney, and lung fibroblasts in the lung.(2–4)

Liver fibrosis is a wound healing process that results from chronic liver damage, such as alcohol abuse, chronic hepatitis, nonalcoholic steatohepatitis and overload of metal ions, such as copper and iron. The overall prevalence of cirrhosis is estimated to be 0.36% of the total population in the United States.(5) In general, liver fibrosis is an imbalance between the synthesis and degradation of ECM. Liver fibrosis is reversible before turning into cirrhosis, which is the end stage and irreversible, leading to liver failure. In the presence of hepatic injury, HSCs become

activated and transform into proliferative myofibroblast-like cells, which are the major source of ECM.

Despite significant progress in our understanding of fibrogenesis, injury stimuli process, inflammation, HSC activation and ECM expression, there is still no standard treatment for liver fibrosis. This chapter will highlight the evaluation process of liver fibrosis, and critically discuss different barriers and therapeutic strategies of liver fibrosis.

II EVALUATION OF LIVER FIBROSIS

Proper diagnosis of liver fibrosis is important for prognosis, stratification for treatment, monitoring and treatment efficacy. It has traditionally been relied on liver biopsy, which is believed to be the “gold standard”. However, recent studies revealed that this method is far from being perfect and there are up to 30% error in the diagnosis.(6) In addition, liver biopsy is invasive and sampling site and size also restricts the accuracy of this method. Therefore, physicians have to rely on many other noninvasive techniques for accurately identifying liver fibrosis.

- *Needle Liver Biopsy.* It is the primary tool for the diagnosis and staging of liver fibrosis regardless of its potential morbidity and mortality. Standardized staging systems were used to evaluate biopsy and quantification of liver fibrosis can be achieved by computer-aided image analysis. However, it only removes 1 in 50,000 of the liver and therefore has a substantial sampling error. In addition, liver biopsy only provides static results, but not dynamic information reflecting ongoing change.(5)
- *Immunohistochemical Studies.* Progression of liver fibrosis can be evaluated by immunohistochemical analysis. Vimentin,(7) α -smooth muscle actin,(8) desmin,(9) and nestin(10) are used to identify activated HSCs which produce excessive collagens. Connective tissue growth factor (CTGF), a down-stream regulator of TGF- β , and fibroblast activating protein can also be used as markers for matrix deposition.
- *Liver imaging techniques.* Liver imaging techniques, such as ultrasound, computed tomography (CT) scan and magnetic resonance imaging (MRI) are attractive approaches because they are non-invasive and can detect the liver structural changes. (11) These noninvasive imaging techniques have limited sensitivity but high specificity and thus can only be used for diagnosis of advanced liver fibrosis. In addition, it cannot provide information about delineation of fibrotic stage. However, the further improvement in imaging techniques will allow more reliable assessment of hepatic structure in the future.(12)
- *Serological Assays.* Both direct and indirect markers have been applied for evaluating liver fibrosis. Indirect markers reflect the alterations of liver function instead of the ECM metabolism. Aspartate aminotransferase (AST)/Alanine aminotransferase (ALT) ratio, platelet count, prothrombin index, PGA index (combines Prothrombin time, GGT, and Apolipoprotein A1) and PGAA index (combines Prothrombin time, GGT, Apolipoprotein A1 and Alpha-2-macroglobulin) have been adopted for diagnosis. Among them, AST/ALT ratio has attracted the most attention, since it provides a high diagnostic accuracy. With progressive liver fibrosis, AST and ALT are moderately elevated, with AST > ALT, probably due to the impaired hepatic sinusoidal cells.(13,14) AST/ALT ratio of greater than 1 is suggestive of liver fibrosis. However, the usage of AST/ALT ratio alone should be used prudently for diagnosis. Deposition or removal of ECM can be determined by serological assays using direct markers, such as procollagen I/III, which reveals the matrix deposition and remodeling. Glycoproteins and polysaccharides (hyaluronan, laminin, tenascin, YKL-40) associate with regions of matrix deposition. Matrix metalloproteinases

(MMPs) are major enzymes for degradation of collagen and the tissue inhibitors of metalloproteinases (TIMPs) can inhibit MMPs' effect. Therefore, levels of MMPs and TIMPs reflect the alternation of collagen. Transforming growth factor (TGF)- β is one of the best studied cytokines involved in liver fibrosis. An international multicenter effort developed a panel of sensitive automated immunoassays to detect liver fibrosis. This panel include multiple serum markers, which provides a sensitive, specific, and reproducible method to distinguish between early and late stages of liver fibrosis.(15)

III EXPERIMENTAL MODELS OF LIVER FIBROSIS

The following *in vitro* and *in vivo* fibrosis models are used to determine the bioactivity of potential antifibrotic compounds.

III. A. Cell Culture Model

Since HSCs are the major cell type responsible for liver fibrosis, HSC cell lines or isolated primary HSCs are widely used for evaluating antifibrotic agents *in vitro*. Scott Friedman and associates developed immortalized rat liver stellate cell line (HSC-T6)(16) and human HSC cell lines (LX-1 and LX-2)(17) to evaluate anti-fibrotic effect *in vitro*. These cell lines retain major features of cytokine signaling, retinoid metabolism, neuronal gene expression and fibrogenesis. Among them, LX-2 cell lines have high transfectability and can withstand serum free condition, making it more flexible tool in the discovery of antifibrotic approaches.(17) In addition, there are additional three established human stellate cell lines, such as LI90, GREF-X and hTERT-HSC.(18–20) LI90 is the first established human stellate cell line derived from human hepatic mesenchymal tumor. It has well developed α -SMA filaments in cytoplasm and shows polygonal cell shape.(18) GREF-X cells were obtained from human liver explants and immortalized with polyoma virus large T antigen. They stain positively for myofibroblasts markers, express collagens, fibronectin and laminin. Additionally, GREF-X cells respond to TGF- β , a key mediator of liver fibrogenesis, and therefore can be used for studying the pathophysiology and pharmacology of liver fibrosis.(19) hTERT-HSC was developed from isolated HSCs from surgical specimens of normal liver and immortalized by introducing human telomerase reverse transcriptase (hTERT) gene. Morphologic and functional characteristics of activated HSCs were maintained after introduction of hTERT.(20)

III. B. Animal Models

Once *in vitro* studies show promising effect, *in vivo* animal model should be used to further evaluate anti-fibrotic effect. Experimental animal models of liver fibrosis have been well developed and various models including hepatotoxins, nutritional, immunologic and biliary were reviewed by Tsukamoto et al.(21) and Wasser et al.(22) However, there is no model that can perfectly mimics a human disease. Each model has its limitations and antifibrotic effect obtained in animal model should be confirmed in human diseases.(23) Commonly used fibrotic models include carbon tetrachloride (CCl₄), dimethylnitrosamine (DMN) and common bile duct ligation (CBDL).

CCl₄ is the oldest and most widely used toxin for inducing experimental liver fibrosis and it requires activation by mixed function of oxidases. Any diet or xenobiotic which decreases the enzyme activity will inhibit fibrosis. Combination of phenobarbital with CCl₄ can induce cirrhosis in 4 to 6 weeks, while CCL₄ alone needs more than 12 to 15 weeks. Compared to inhalation and subcutaneous injection, intragastrical administration of CCl₄ is more efficient because it enters the liver via portal vein and is selectively concentrated in the liver.(22) Degree of fibrosis depends on many factors, such as level of oxidases, weight of the animal, dose, route and duration of administration.(23) High mortality and less consistent induction are the major

weaknesses of this method. Another disadvantage is that the fibrosis may diminish after discontinuation of CCl₄ if the duration of treatment is less than 7–8 weeks.(23)

DMN is a commonly used carcinogen for inducing liver fibrosis. DMN-induced liver fibrotic Sprague-Dawley rat is a good and reproducible model accompanied with many decompensating features of human alcoholic fibrosis. Generally, rats are given intraperitoneal injection of DMN at a dose of 10mg/kg for 4 weeks. This is well characterized and can provide consistent and stable fibrotic model even for several months after discontinuation of induction. (21) High doses of DMN leads to necrosis of the liver and subsequent liver fibrosis, while low doses of DMN are carcinogenic. Metabolism of DMN can cause hepatocellular necrosis in both centrilobular and periportal areas within 1 to 2 weeks after administration.

Common bile duct ligated (CBDL) animal model has low degree of cell damage and inflammation. After ligation of bile duct, numerous myofibroblasts expressing α -SMA appeared in parallel with many proliferating connective tissue cells. These myofibroblasts secreted high amount of elastic fiber components, elastin and fibrillin-1. Therefore, it represents an invaluable model to study myofibroblastic differentiation and extracellular matrix adaptation.(24) Moreover, this animal model simulates many histological features of human chronic biliary fibrosis and cirrhosis. Bile duct ligation has several advantages over CCl₄ and DMN models in i) individual animal response to CCl₄ and DMN are variable, ii) it takes longer time for CCl₄ and DMN to induce persistent fibrosis.(25) However, technical problems, such as recanalization of the ligated ductal system, and high mortality make it less common method than CCl₄ and DMN.

IV. BARRIERS TO TREATING LIVER FIBROSIS

Liver is the major organ responsible for the uptake, metabolism, detoxification and storage of macromolecules, and production of bile for digestion.(26) It also plays an important role in the clearance of pathogens and antigens entering the body via gastrointestinal tract. Liver consists of four cell types, such as hepatocyte, Kupffer, endothelial and stellate cells (Fig. 1 and Table 1).(27,28) Hepatocytes are the liver parenchymal cells and have metabolic, endocrine and secretory functions.(29) Liver endothelial cells form the discontinuous lining of the sinusoids and have fenestrations of 0.1 μ m in diameter, which are grouped together in so called sieve plates.(30) The pore size plays an important role in the filtration of particles between blood with parenchymal cells.(30) Kupffer cells are the largest group of tissue macrophages and their main function is to phagocytose foreign particles like bacterial or colloids. HSCs (also known as Ito cells, vitamin A-storing cells, lipocytes or fat-storing cells) localize within the space of Disse in close proximity of both hepatocytes and endothelial cells (Fig. 1). HSCs are the main storage of retinol (vitamin A) and are involved in the development of liver fibrosis.

Since liver has the high ability to regenerate, treating liver fibrosis is more attractive than treating other organs' fibrosis.(31) However, there are several challenges to designing effective anti-fibrotic therapeutics.

IV. A. Insufficient Delivery of Anti-fibrotic Drugs in Fibrotic Liver

The first barrier to developing anti-fibrotic therapy is the insufficient drug delivery to the fibrotic liver. Fibrotic tissue is characterized by the loss of normal architecture and replacement of blood vessels by deposition of excessive ECM proteins in the space of Disse, leading to less perfusion than the normal liver. This, in turn, results in decreased free exchange of flow between hepatocytes and sinusoidal blood.(32) In addition, sinusoidal endothelial fenestra disappears during liver fibrogenesis, which further inhibits the hepatic uptake of anti-fibrotic agents. Alterations in the liver microcirculation contribute to the progressive limitation of blood-liver exchange and to the reduced elimination of endogenous and exogenous substances.

Collagenization of the space of Disse with the formation of a basement membrane and intrahepatic, protohepatic shunts are the two major anatomical alterations in long-standing human cirrhosis. Decreased space of Disse with collagenization of the extravascular space limits the exchange of large molecules such as albumin, while the development of small channels with poorly permeable walls limits the diffusion of small molecules. (33)

In liver fibrosis, accumulation of scar matrix contributes to the loss of hepatocyte microvilli and sinusoidal fenestrae. Thus, lipid and polymeric particulate carrier systems cannot efficiently deliver drugs, oligonucleotides and genes to fibrotic liver. Besides, how to specifically deliver anti-fibrotic agents to specific liver cell types is an even more challenge in fibrotic liver. In fact, we have shown significant decrease in triplex forming oligonucleotide (TFO) delivery to the liver when injected into the tail vein of the liver fibrotic rats. In the DMN induced liver fibrotic rats, hepatic uptake of TFO decreased from 43% to 33% at 30 min and from 38% to 27% at 4h postinjection (Table 2).(34)

IV. B. Alteration of Extracellular Matrix in Fibrogenesis

Liver fibrosis is the excessive accumulation of extracellular matrix (ECM) proteins including collagens, elastin, fibronectin, laminin, structural glycoproteins, proteoglycans and pure carbohydrates.(35) Therefore, how to remove accumulated excessive ECM is a challenge for any anti-fibrotic agent whose target is not the ECM, but other pathways of fibrogenesis.

Liver fibrosis has the mechanism similar to the normal wound-healing process in the initial stages.(36) For normal wound-healing process, the scaffold/ECM is dismantled by matrix proteinases in the final stage of healing. However, in the liver fibrosis, multiple repair processes induced by repeated injuries cause incomplete degradation of scaffold/ECM. Meanwhile, HSCs activate or transdifferentiate into myofibroblast-like cells, migrate into the sites of liver repairing and secrete large amount of ECM in the injured liver.

Normal liver contains approximately 5mg of collagen per gram of fresh tissue. Although there are 12 different types of collagen in the liver, type I and III account for 70% of total liver collagen and the ratio of type I to III is 1:1. In liver fibrosis, the synthesis of collagen is increased while its degradation is normal or decreased. Most of the increased collagen belongs to type I and the ratio of type I to III is increased to 4:1. Type I collagen content in the fibrotic liver is approximately four folds compared to the normal liver.(29,30) The half-life of $\alpha 1(I)$ collagen mRNA is 20-fold longer in activated HSCs than that of quiescent HSCs and it contributes to the main mechanism for upregulation of collagen.(37) In addition, the chondroitin sulfate/heparin sulfate ratio is also elevated in fibrogenesis.(35)

ECM could influence profoundly the phenotype of HSCs and proteolytic degradation of the normal hepatic ECM leads to the activation of HSCs (Fig. 2). During liver injuries, Kupffer cells secrete matrix metalloproteinase-9 (MMP-9) and leukocytes secrete matrix degrading proteinase to degrade the normal ECM. HSCs themselves can secrete MMP-2, MMP-3, and MMP-1/13 in the early stage of liver injury. After prolonged liver injury, degradation of abnormal ECM is inhibited by the expression of tissue inhibitor of metalloproteinase (TIMP) by HSCs. However, removal of the injury stimuli could decrease the TIMP expression effectively.(29)

IV. C. Activation of Hepatic Stellate Cells

Both parenchymal and non-parenchymal cells can proliferate during wound-healing process. However, proliferation of HSCs (Fig. 3) plays an important role in the liver fibrosis. Regardless of the underlying diseases, proliferating activated HSCs display a high level of $\alpha 1(I)$ collagen gene expression.(38) In addition, the degree of fibrogenesis is affected by the increased

population of HSCs, which results from their proliferation. In the response to injuries, activated HSCs migrate to the wound-healing area and secrete large amount of ECMs including fibril forming collagens, fibronectin, and proteoglycans, resulting in the formation of septa in chronically damaged liver. Upon liver injury, biliary cells, resident Kupffer cells and infiltrating macrophages, platelets, and sinusoidal endothelial cells secrete growth factors, such as TGF- β and platelet-derived growth factor (PDGF) at the sites of inflammation. These growth factors in turn trigger the proliferation and secretion of ECMs by HSCs. Also, activated HSCs themselves secrete PDGF and further increased the proliferation of HSCs. On the other hand, sinusoidal endothelium and hepatocytes secrete endothelin-1 (ET-1) and insulin-like growth factor-1 (IGF-1), respectively to induce the proliferation of HSCs.(28,29)

During activation, HSCs acquire contractility, which is the major determinant of early and late increases in portal resistance during liver fibrosis. Activated HSCs impede portal blood flow by both constricting individual sinusoids and contracting the cirrhotic liver. The major contractile stimulus towards HSCs is endothelin-1 (ET-1).(29) There are two types of ET receptors, ET_A and ET_B, expressed on both quiescent and activated HSCs. As the HSCs activate, ET_B receptor increase relative to ET_A receptor. ET-1 stimulates proliferation by interacting with ET_A, while interacting with ET_B suppresses the proliferation of HSCs.(28)

IV. D. Inflammation and Activation of Liver Fibrogenic Cells

Liver fibrosis is initiated from chronic liver injuries in response to hepatitis B or C, excessive alcohol ingestion, nonalcoholic steatohepatitis and metal ions over load. After injuries, leukocytes become tethered to vessel through labile adhesions which allow them rolling in the direction of the blood flow and then were recruited to inflammation sites with the help of several adhesion molecules.(39) Proinflammatory cytokines (IFN- γ , TNF- α) released by natural killer (NK) cells and Kupffers alter the expression of cell adhesion molecules on sinusoidal endothelial cells, recruiting inflammatory cells at inflammation sites.(40) Three pathways, such as portal tract, hepatic vein and sinusoid, were involved in the recruitment and migration of lymphocyte to the inflammation sites. Inflammatory infiltrate includes T lymphocytes, B lymphocytes, plasma cells, histocytes, eosinophils, neutrophils, macrophages, NK cells and mast cells.(39,40) Infiltrated leukocytes (neutrophils, lymphocytes and monocytes) along with resident macrophages (Kupffer cells) release various inflammatory cytokines and growth factors, leading to activation of quiescent HSCs into activated proliferating α -smooth muscle actin (α -SMA) positive myofibroblast-like cells.(41) Monocytes and macrophages produce large amounts of nitric oxide (NO) and inflammatory cytokines, such as TNF- α . Kupffer cells stimulate ECM synthesis and cell proliferation through cytokines, such as TGF- β , platelet-derived growth factor (PDGF), epithelial growth factor (EGF) and reactive oxygen intermediates. In addition, Kupffer cells also express TNF- α , TNF related apoptosis inducing ligand (TRAIL) and Fas ligand.(39)

Although activated HSCs are the principal fibroblastic cell type responsible for liver fibrosis, other liver cell type of fibroblast lineage, such as portal, centrolobular and periductular fibroblasts have also fibrogenic potential.(42,43) These fibroblast-like cells may proliferate, migrate and contribute to ECM production in response to biliary injury. Origin of liver injuries decides the relative importance of different liver cell types on fibrogenesis. Portal myofibroblasts play an important role when liver injury occurs around portal tracts, while HSCs are the main fibrogenic cells in pericentral areas.(36) Recently, bone marrow was found to be another important source of fibrogenic cells.(44) Cross-sex transplantation demonstrated the contribution of bone marrow to tissue fibrosis.(45) Forbes et al. analyzed the origin of myofibroblasts within fibrotic liver in two scenarios. These include i) seven male patients received liver transplants from female donors and subsequently developed liver fibrosis, and ii) one female patient who received bone marrow transplant from a male donor and

subsequently developed cirrhosis. For male patients, significant number of α -SMA positive myofibroblasts contained the Y chromosome, indicating extrahepatically derived myofibroblasts. In the female patient, 12.4% of the myofibroblasts were Y chromosome positive, indicating a bone marrow origin.(45) Furthermore, bone marrow contributes to myofibroblasts in multiple organs including lung, stomach, skin, kidney, adrenal gland, colon and small intestine.(46)

Activated HSCs increase the synthesis of ECM and release of PDGF, TNF- α , TGF- β , chemokines and reactive oxygen intermediates (ROI).(37) Among all fibrogenic cytokines, TGF- β is the key mediator in the liver fibrosis and acquired high attention.(36) TGF- β contains three isoforms, TGF- β 1, TGF- β 2 and TGF- β 3. TGF- β 1 is the dominant stimulus to ECM production from HSCs and its content increases in liver fibrosis.(28) TGF- β 1 can be secreted by Kupffer cells, biliary cells and recruited inflammatory cells, but the autocrine expression of TGF- β 1 by HSCs is the most important.(47) Compared to quiescent HSCs, fully activated HSCs are relatively unresponsive to TGF- β , due to the down-regulation of TGF- β receptor. TGF- β 1 knockout mice had reduced collagen accumulation in response to liver injury compared to that of normal mice.(48) Disrupting TGF- β 1 synthesis or signaling pathways significantly decreased fibrosis in experimental models.(49,50) However, due to its multiple actions, TGF- β represents an important link between neoplasia and fibrosis in the liver. Mutation of TGF- β receptor or signaling intermediates was found in various epithelial cancers.(51) Therefore, the application of TGF- β 1 as a target in liver fibrosis should be prudent.

Testing of antifibrotic drugs in clinical trials is another challenge. Since fibrogenesis is a very long process and anti-fibrotic efficacy can only be observed after a prolonged period of treatment. Additionally, diagnosis of liver fibrosis also limits the frequency to monitor the liver fibrosis accurately and timely.

V. CURRENT THERAPEUTIC APPROACHES OF LIVER FIBROSIS

Although there is still no standard treatment for liver fibrosis,(36) the following therapeutic approaches are currently being investigated.

V. A. Elimination or Suppression of Injury Stimuli

The first and most effective way to reverse liver fibrosis is to eliminate or suppress the initial stimuli, i.e. the factors that cause the chronic injury to liver cells that in turn triggers the activation of HSCs and their production of ECM. Alcohol causes liver injury by blocking the normal metabolism of proteins, fats and carbohydrates. Abstinence can reverse liver injury and fibrosis in alcoholic liver disease. Infection with hepatitis B and C viruses causes liver inflammation and injury that over several decades can lead to cirrhosis. Reversed fibrogenesis was demonstrated in chronic hepatitis B patients after treatment with antiviral drug lamivudine. Liver biopsies from hepatitis B patients treated with lamivudine showed significant decrease in α -SMA and C-terminal procollagen alpha1 propeptide (PIIICP) levels compared to placebo group.(52) Poynard et al. conducted a clinical trial to determine the effect of interferon and ribavirin on liver fibrosis. Significant reduction in the progression rates of liver fibrosis was observed when tested in 3010 patients with hepatitis C.(53) In addition, one clinic case report showed a woman with autoimmune hepatitis and cirrhosis in 1985 was found normal in liver biopsy performed in 1999 after corticosteroid therapy for hepatitis.(54) However, many cases factors causing liver injury cannot be adequately removed and thus other means to treat liver fibrosis are required.

V. B. Immunomodulatory Agents for Anti-inflammation

Liver fibrosis is often accompanied by inflammation, which promotes the progression of the fibrotic process. Thus, immunomodulatory agents that inhibit inflammatory response are regarded as potential antifibrotic agents. These agents may down-regulate the stimuli to HSC activation. Corticosteroids have been used for many years for treating various liver diseases. (55) In addition, tumor necrosis factor- α (TNF- α), Fas, (56) Bcl-xL (57) and caspase 8 (58) pathway were investigated and showed promising anti-fibrotic effect. In addition, rennin-angiotensin system plays an important role in liver fibrogenesis. Both culture-activated and in vivo activated HSCs highly express active rennin and angiotensin-converting enzyme (ACE) and secrete angiotensin II, which can promote inflammation and ECM synthesis. (59) Fas mediated apoptosis of hepatocytes was shown to induce liver fibrosis when wild-type and Fas-deficient lymphoproliferating (lpr) bile duct ligated mice were compared for their liver injuries. (60) Furthermore, Song et al. demonstrated anti-fibrotic effect of Fas specific siRNA in concanavalin A (Con A)-induced hepatitis mouse models for the first time. A small number of people develop a very severe, life-threatening form of acute hepatitis called fulminant hepatitis. When fulminant hepatitis was induced in mice by injecting agonistic Fas antibody, 82% of mice treated with Fas siRNA survived for 10 days of observation, whereas all control mice died within 3 days after induction of fulminant hepatitis. Treatment with *Fas* siRNA also abrogated hepatocyte necrosis and infiltration of inflammatory immune cells, and markedly reduced serum concentrations of transaminases. (56)

V. C. Inhibitors of Stellate Cell Activation

In response to liver injury, the normally quiescent vitamin A storing HSC undergoes a dramatic phenotypic transformation termed “activation” or “trans-differentiation”. (61) The process of HSC activation generates an α -smooth muscle actin (α -SMA) positive myofibroblast-like cell that is proliferative and is responsible for deposition of the majority of ECM (predominantly type I and III collagen) that forms scar tissue in the fibrotic liver. Therefore, blockade of HSC activation is the most attractive therapeutic approach. TGF- β 1 signaling pathway, (25,62–64) PDGF- β , (65–67) IFN- γ , (68,69) hepatocyte growth factor (HGF) (70,71) and peroxisome proliferator activated nuclear receptors (PPAR) (72,73) were all targeted as antifibrotic approaches to inhibit HSC activation. PPAR- γ nuclear receptors are expressed in HSCs. Synthetic PPAR- γ ligands, such as thiazolidinediones, down-regulated HSC activation. Endothelin and angiotensin II signaling pathways have also been reported as potential targets to inhibit activation or proliferation of HSCs. Endothelin receptor antagonists, angiotensin-converting enzyme inhibitors, and angiotensin II receptor antagonists have been proven useful in treating liver fibrosis. (74–76)

Additionally, leptin, which is a 16kDa hormone mainly produced by adipocytes, appears to be profibrogenic in activated HSCs and therefore is a potential target for treating liver fibrosis. Only activated HSCs, but not quiescent or freshly isolated HSCs, express leptin. Immunohistochemical studies also showed upregulation of leptin receptors in activated HSCs. (77) Injection of leptin enhanced inflammatory and profibrogenic responses in the fibrotic mouse liver induced by CCL₄. (78)

Interleukin-1 β (IL-1 β) is another cytokine which can enhance collagen synthesis by increasing the proliferative activity of HSCs. Consequently, interleukin-1 receptor antagonist (IL-1Ra) was explored as antifibrotic approach via blocking the binding of IL-1 to its receptors in fibrotic animal model induced by DMN. In animals given DMN and IL-1Ra, less deposition of laminin and collagen, as well as decreased number of α -SMA reactive cells were found compared with animals receiving DMN alone. (79)

V. D. Inducing Apoptosis of Activated Stellate Cells

Apoptosis of activated HSCs makes important contribution to the resolution of liver fibrosis. (8) Inducing apoptosis of activated HSCs is therefore another potential anti-fibrotic approach. Gliotoxin is an effective drug to induce apoptosis of activated HSCs and has been shown to reverse liver fibrosis in rats.(80) However, the mechanism is poorly defined. Generally, gliotoxin induces HSCs apoptosis via inducing membrane permeability transition (MPT), cytochrome c release and caspase-3 activation under low concentration and induce necrosis under high concentration.(81) Orr et al. reported that gliotoxin induces HSC apoptosis through a thio redox-dependent interaction with the adenine nucleotide transporter (ANT).(82)

Cell matrix adhesion is an important control factor in the regulation of apoptosis in nearly all cell types. Integrins are the major class of adhesion receptors present on the surface of HSCs. Therefore, modulation of HSCs adhesion capability represents an approach to activate apoptosis in these cells. Iwamoto et al. demonstrated that integrin antagonist, Gly-Arg-Gly-Asp-Ser (GRGDS), induced apoptosis of activated HSCs.(83) In addition, Arg-Gly-Asp (RGD) was targeted to HSCs upon conjugation with mannose-6-phosphate (M6P), leading to significant decrease in the proliferation rate of HSCs.(84)

V. E. Inducing Resolution of Excessively Produced ECM Component

Removal of existed ECM is an excellent approach in addition to prevent accumulation of new ECM after starting treatment of liver fibrosis. As the major enzyme in remodeling of ECM, MMPs can be directly delivered via gene expression vector to degrade ECM.(85,86) Iimuro et al. delivered adenovirus encoding human matrix metalloproteinase-1 (Ad5MMP-1), which degrades type I and type III collagen, into fibrotic rats induced by thioacetamide or bile duct ligation. The fibrosis was dramatically attenuated 2 weeks after administration of Ad5MMP-1. Interestingly, the number of activated HSCs was also decreased in Ad5MMP-1 treated rats. Furthermore, decrease in type I collagen was found in fibrotic rats treated with Ad5MMP-1. Since TIMP-1 is the major inhibitor of MMPs and inhibits ECM degradation, gene therapy approaches were also applied to inhibit TIMP-1 expression for treating liver fibrosis.(87,88) For an example, Nie et al. demonstrated that after injection of antisense phosphorothioate ODNs into fibrotic rats through coccygeal vein, the gene and protein expression of TIMP-1 was significantly decreased.(88) Instead of targeting MMPs and TIMP-1 directly, TGF- β 1 antagonist attracts more attention, since it can stimulate ECM degradation by up-regulating MMPs and down-regulating TIMP simultaneously.(25,62–64) Type I collagen itself could also be targeted for degradation by TFO, which form triple helix with the promoter region of type I collagen gene and shut down its gene expression.(34,89,90)

V. F. Using Free Radical Scavengers to Remove Oxidative Stress

Reactive oxygen species and oxidative stress are important fibrogenic stimuli for HSC activation and responsible for apoptosis/necrosis of hepatocytes as well as the activation of Kupffer cells which can secrete cytokines upon liver fibrogenesis. Therefore, antioxidative treatment is an effective approach for attenuating liver fibrosis.(91) Antioxidant as vitamin E (92) and free radical scavengers such as superoxide dismutase (SOD) (91,93,94) were evaluated for preventing various liver injuries including liver fibrosis. Zhong et al.(94) accessed antifibrotic effect of SOD using bile duct ligation animal model. Introducing SOD using adenovirus vector significantly reduced liver injury and minimized fibrosis. Meanwhile, SOD treatment also prevented increases of fibrogenic cytokines, such as NF- κ B, TNF- α and TGF- β .

V. G. Herbal medicines

Traditional herbal medicine may open a new era for treating liver fibrosis. Several Chinese herbal medicines including Qinggan Huoxuefang (QGHXF), Danshao huaxian capsule and crude astragalosides fraction, have been reported promising effect in patients with liver fibrosis. (95–97) Ji et al. compared antifibrotic effect of two Chinese medicines, QGHXF and Xiaochaihu Chongji (XCH), at different doses in fibrotic rats induced by CCl₄. Both QGHXF and XCH treatments reduced the level of ALT and AST compared to controlled group, while QGHXF was better than XCH group. High dose QGHXF reduced liver inflammation and collagen expression significantly. In addition, QGHXF induced apoptosis of activated HSCs and inhibited the proliferation of HSCs.(95) However, most of the herbal medicines contain multiple active components which make difficult to explain their mechanisms of action.

VI. OLIGONUCLEOTIDE-BASED THERAPY OF LIVER FIBROSIS

Significant progress has been made in oligonucleotide (ODN) technology and there is already one FDA approved antisense ODN product (Vitravene™) in the market for treating cytomegalovirus-induced retinitis in AIDS patients. Moreover, antisense ODNs have been shown to inhibit type $\alpha 1(I)$ collagen gene expression in NIH 373 cells. Another FDA approved oligonucleotide based (aptamer) drug is Macugen®, derived from a modified 2'fluoro pyrimidine RNA inhibitor to vascular endothelial growth factor (VEGF). It is used for treating the wet form of age-related macular degeneration.(98)

VI. A. Mechanism of Action

1. Antisense Oligodeoxynucleotide—Antisense ODNs mostly work at post-transcription levels. The length of ODNs is determined based on antisense mechanism and other practical considerations. The minimum ODN size needed to recognize a specific gene is between 12 and 15 bases in length. Consequently, antisense ODNs are typically synthesized in lengths of 13 to 35 nucleotides. It is estimated that at least 12 bp need to form a stable hybrid with a phosphodiester backbone at physiologic conditions.

Normally, transcription of any given gene is carried out by the RNA polymerase II from a transcription start site in the DNA to give rise to heteronuclear RNA, which is subsequently processed by splicing and polyadenylation to mature mRNA that is trafficked to the cytoplasm where they get translated into proteins. Antisense ODNs are designed to bind to their target sense RNA sequence through the formation of reverse complementary (antisense) strands with the mRNA. The two most likely mechanisms of inhibition of gene expression by antisense ODNs appear to be i) steric blocking in pre-mRNA and/or mRNA of sequences important for processing or translation, and ii) degradation of the mRNA by RNase H activated at the site of ODN binding (Fig. 4). (99,100) RNase H can cleave the RNA component of RNA:DNA hybrids and it is abundant in the cytoplasm and nucleus. Nevertheless, the mechanism appears to be more complex than originally thought. Events that are triggered as a result of heteroduplex formation are dependent on the nature of the antisense molecules used for mRNA targeting. ODNs of many, but not all, types support the binding of endogenous RNase H at sites of RNA-DNA duplex formation.(101,102) Such binding is thought to be an important effect of antisense actions because once bound, RNase H functions as an endonuclease to recognize and cleave the RNA moiety in the hybrid (Fig. 4). Of significant interest also is the fact that the DNA comprising the duplex is undamaged by the enzymatic attack. Therefore, it is free to hybridize with multiple RNA molecules, leading to their destruction in a catalytic manner.

Phosphorothioate (PS) ODNs are thought to activate RNase H efficiently, while methylphosphonate (MP) ODNs do not support the activity of this enzyme at all. Phosphodiester (PO), PS and MP ODNs hybridize to pre-mRNA in a sequence specific manner.

(100) Duplexes formed with PO and PS ODNs are susceptible to cleavage by RNase H, while RNA in the duplexes formed with an MP ODNs is resistant to cleavage by RNase H. The resistance of MP ODNs to RNase H decreases with the number of methylphosphonate deoxynucleotides in the MP ODNs. (100) Eukaryotic RNase H generally requires the DNA portion of the duplex to have five or six consecutive internucleotide linkages that can be recognized by RNase H and is highly active against duplexes of 9–10 consecutive internucleotide linkages. (99,103,104) Keeping this in mind, ODNs are often synthesized to contain with nuclease resistant modifications at the 3' and 5' ends of the oligo, and six to eight unmodified or phosphorothioate modified linkages in the central portion. These chimeric ODNs inhibit 3' and 5' exonuclease degradation, while still serving as a substrate for RNase H. (99,103) However, RNase H may produce unanticipated, non-sequence-dependent effects by cleaving transiently formed duplexes, or with sites of partial complementarity. Therefore, this “non-targeted” cleavage can compromise the sequence specificity of RNase H-competent ODNs.

Antisense ODNs containing four contiguous guanosine residues should not be employed as they may lead to G-quartet formation via Hoogsteen base-pairing that can decrease the available ODN concentration and might result in undesired side-effects. Modified guanosines (for example 7-deazaguanosine, which cannot form Hoogsteen base pairs) may be used to overcome this problem. (105) In addition, the ODN may show reduced water solubility if it contains more than 7 total guanines or more than 3 contiguous guanines in a 25-mer ODN. (106)

2. Antigenic Therapy—In contrast to antisense ODNs, TFOs inhibit gene transcription by forming DNA triple helices in a sequence-specific manner in the major groove. (107) Triplex formation is favored at polypurine:polypyrimidine tracts of DNA. (108,109) Targeting TFOs to the gene itself presents several advantages as compared to *antisense ODNs* which are directed to mRNAs. (110,111) There are only two copies (two alleles) of the targeted gene whereas there are thousands of copies of an mRNA. Blocking mRNA translation even by inducing sequence-targeted cleavage of the RNA does not prevent the corresponding gene from being transcribed, thereby repopulating the RNA pool. In contrast, prevention of gene transcription is expected to bring down the mRNA concentration in a more efficient and long-lasting way.

Inhibition of gene transcription depends on the residence time of the TFO on its target sequence as well as its nuclease sensitivity. One of the difficulties in designing a TFO is its accessibility to the target sequence in the chromatin structure of the cell nuclei. DNA normally exists in a duplex form, but under some circumstances, DNA can assume triple helical (triplex) structures, which are either intramolecular or intermolecular. Intermolecular triplexes are formed by the addition of a sequence-specific third strand to the major groove of the duplex DNA. (112) Triplex formation may then prevent the interaction of various transcription factors, or it may physically block the initiation or elongation of the transcription complex.

TFOs are of 10–30 nucleotides in length and require runs of purines on one strand and pyrimidines on the other for stable hybridization. TFOs may consist of either pyrimidine bases to form the Py-Pu*Py triplex or predominantly Purine bases to form Py-Pu*Pu triplexes, depending on the nature of the target sequences (Fig. 5). TFO bases form reverse Hoogsteen hydrogen bonds only with adenines and guanines, thus forming base triads. An A or a T in the TFO can bond with the A of an A-T pair in the DNA duplex, while G can bond with the G of a G-C pair. C can also bond with the G of a G-C pair after protonation at the N3 position in slightly acidic media (pH < 6). Depending on the relative orientation of the third strand (R), two types of triplex structures can be formed. In the pyrimidine (or Y:R:Y) motif, a homopyrimidine ODNs binds in a direction *parallel* to the purine strand by Hoogsteen hydrogen bond, with canonical base triplets of T:A:T and C:G:C. In the alternate purine motif

(R.R:Y), a homopurine strand binds *antiparallel* to the purine strand by reverse Hoogsteen hydrogen bonds, with base triplets of A.A:T and G.G:C.(107,113)

A (G, T)–motif TFO is also permitted, whose orientation depends on both the number of GpT or TpG steps and the length of G and T tracts. Some TFOs have been designed to “switch” strands when blocks of homopurine sequences alternate between strands at the duplex DNA target sites. Based on the studies of TFOs performed to date, it appears that in the absence of chemical modification, a TFO sequence should contain at least 20 bases in order to bind to its target site with sufficient affinity so as to achieve biochemical effects. Guanine-rich TFOs can form intra- or inter-molecular four-stranded structures involving G-quartets; these structures are favored when the sequences containing repeats of consecutive guanines. (G, A)-TFO can form other intermolecular structures, such as parallel homoduplexes involving A-A, G-G and G-A base pairs. Monovalent cations, such as K^+ present under physiological conditions, enhance the formation of quadruplexes, but decrease the formation of a parallel homoduplex in the presence of divalent cations.(114) To overcome this K^+ effect, several strategies have been employed. In one case, some of the G residues within a G-rich TFO were replaced with 6-thioguanine. Although this modification reduces G-quartet formation, it also lessens the overall binding affinity of the third strand. Similarly, the replacement of the N-7 of guanine with carbon, creating 7-deazaguanine, eliminates the ability of the TFO to form G-quartets but also decreases the capacity of the ODNs to form triple helices.(115) Cytosine-rich TFO can form, under acidic pH conditions, four-stranded structures called i-DNA involving hydrogen-bonded pairs between cytosine and protonated cytosine ($C\cdot C^+$).

VI. B. Backbone and Chemical Modification for Enhanced Stability

ODNs must reach their targets intact to modulate gene expression by binding to mRNA or genomic DNA in the cells. However, endonucleases and exonucleases attack natural ODN molecules at the phosphodiester bridges and break them down to mononucleotides. To improve the stability of ODNs, several modifications have been reported in all the relevant subunits, such as sugar, base and phosphate moieties.

The *first-generation* ODNs were designed to make the internucleotide linkages more resistant to this attack. This is accomplished primarily by replacing one of the nonbridging oxygen atoms in the phosphate group with either sulfur or a methyl group. The former modified forms are called phosphorothioate oligonucleotides (PS ODNs) and the latter as methylphosphonate ODNs (MP ODNs), respectively.(116) MP ODNs are neutral in charge and lipophilic in nature. In addition to being nuclease resistant, MP ODNs are usually taken up by cells in a more efficient manner.

PS ODNs are by far the most extensively studied and significant progress has been made in this modification, which resulted in one commercial product (Vitravene[®], treatment of cytomegalovirus retinitis in people with AIDS)(117) and several promising clinical trials. (118) PS ODNs have a half-life of approximately 9–10 h in human serum compared to ~1h for unmodified ODNs.(119,120) In addition to nuclease resistance, PS ODNs form regular Watson-Crick base pairs, activate RNase H in the DNA-RNA hybrid duplex, and display attractive pharmacokinetic profiles.(121) However, the substitution of non-bridging oxygen by sulfur brings several limitations. PS ODNs have chiral centers at the internucleoside phosphorothioates and thus have slightly reduced affinity towards complementary RNA molecules in comparison to the corresponding PO ODNs. On the other hand, they also bind to some key structural proteins, enzymes, receptors, growth factors, and transcription factors via ionic or hydrophobic interactions with their modified backbones which leads to several side effects.(122,123)

A number of strategies have been introduced to overcome the limitations of PS ODNs, while preserving their useful properties. Using end-capped PS, where both 3' and 5' ends are phosphorothioated, appears to be a reasonable compromise in that exonuclease stability is conferred on the molecules and side effects associated with fully phosphorothioated molecules are significantly reduced.(124) The *second generation ODNs* contain nucleotides with the alkyl modification at the 2' position of the ribose. These ODNs are less toxic than PS ODNs and have enhanced affinity towards their complementary RNAs.(125)

PS ODNs are often combined with other modifications, especially phosphorothioate 2'-O-methyl-ODNs and MP ODNs to develop mixed-backbone oligonucleotides (MBOs).(126) These MBOs have all the required properties for antisense activity with minimal polyanion-related effects.(127) End-modified MBOs, in which other modifications are incorporated at the 3'-end or at both the 3'- and 5'-ends of PS ODNs, have shown improved specificity, biological activity, *in vivo* stability, and reduced protein binding. Centrally modified MBOs, in which other modifications are in the center of the PS ODNs, show increased binding affinity, increased RNase H activation and consequently rapid degradation of RNA compared with end-modified MBOs.(127)

In addition to modifications to the internucleoside bridge, the 2'-deoxy- β -D-ribofuranose unit of the DNA backbone is another site for ODN modification. Various sugar modifications have been introduced into antisense ODNs to enhance binding affinity and nuclease resistance. Changing the sugar's glycosidic linkage from the naturally occurring β form to the α anomeric form increases the nuclease stability and compromises hybridization stability and ability to activate RNase H. Sugars are also typically modified at the 2' position with O-methyl, fluoro, O-propyl, O-allyl or other groups.(128,129) These modifications have been shown to increase affinity for RNA and impart some nuclease stability. Nevertheless, these molecules do not support RNase H activity and, for this reason, do not appear to have significant antisense activity in some assays.

DNA and RNA analogues with modified phosphate linkages or riboses as well as nucleotides with a completely chemical moiety substituting the furanose ring have been developed as *third generation antisense ODNs*.(105) N3'→P5' phosphoramidates is an example of third generation antisense ODNs, in which the 3'-hydroxyl group of the 2'-deoxyribose ring has been replaced with 3'-amino group. It has a very high binding affinity to complementary DNA or RNA with nuclease resistance. Unlike PS ODNs, N3'→P5' modified ODNs do not activate RNase H when bind to complementary RNA. (130) Despite inability to activate RNase H, N3'→P5' phosphoramidate ODNs have shown more potent sequence-specific antisense activity than phosphorothioate derivatives *in vitro* (130) and *in vivo*.(131)

VI. C. Biodistribution of Oligonucleotides

The biodistribution of ODNs have been studied extensively in rodents and non-human primates. For their action at a target site within the body, there are many biological barriers to be overcome: moving from the bloodstream into tissues, from the extracellular space, across the plasma membrane and into the cytoplasm, and from the cytoplasm into the nucleus. To construct strategies of developing efficient and safe delivery systems of ODNs, a thorough understanding of their *in vivo* disposition characteristics is necessary.

Gross tissue distribution of PS ODNs is independent of its sequence. Plasma clearance rates are largely species independent in rat, rabbit, dog and monkey and has been estimated to be between 1 and 3 ml/min/kg.(132,133) DeLong et al. (1997) compared the pharmacokinetics and tissue distribution of PS, phosphorodithioate (PS₂), MP ODN analogs, which were 15-nt ODN complimentary to the AUG region of K-ras.(134) These ODNs were injected intravenously as a single dose used in nude mice bearing a K-ras-dependent human pancreatic

tumor. There was a rapid distribution phase with $t_{1/2\alpha}$ values of 1 min and an elimination phase with average $t_{1/2\beta}$ values of 24 or 35 minutes. Volumes of distribution (V_d) were 3.2, 4.8, and 6.3 ml for PS₂, MP, and PS, respectively, in comparison to 3.6 ml for sucrose, a fluid-phase marker. In general agreement with previous studies, relative tissue drug levels obtained at 1 and 24 hours after administration were kidney > liver > spleen > tumor > muscle. Total kidney and liver ODN accumulation was approximately 7% to 15% of the initial dose, with tumor accumulating 2% to 3%. Intact compound was recovered from all tissues, including tumor. Importantly, integrity of the ODNs was 73% in the blood, 43% in the kidney, and 46% in the liver, which appear to be the primary sites of metabolism.

Studies have also been performed on patients with leukemia and acquired immunodeficiency syndrome (AIDS). In six HIV patients, after 2 hours of intravenous infusions (0.1mg/kg) of PS, plasma disappearance curves could be described by the sum of two exponential, with half-lives of 0.18 ± 0.04 and 26.71 ± 1.67 hours. Urinary excretion represented the major pathway of elimination, with ~50% of the administered dose excreted within 24 hours and ~70% eliminated over 96 hours after dosing. Intact and degraded material was found in the urine. The half-lives of the ODNs were shorter than those observed in experimental animals.

Unmodified PO ODNs is rapidly degraded by serum and cellular nucleases. Following intravenous administration, ODNs are rapidly eliminated from the circulation and are widely distributed to most peripheral tissues, with the liver, kidney, bone marrow and muscle accumulating the most.(135,136) PS ODNs, possibly due to the extensive binding to the plasma proteins, accumulate much less in the kidney compared to PO ODNs.

Although there are many reports about the biodistribution of antisense ODNs at whole body and organ (liver and kidney) levels, little information is available on ODNs' delivery to HSCs. Moreover, most biodistribution studies used PS ODNs, which are not G-rich. PS ODNs containing four or more consecutive guanidine residues forms G-quartet structure and have a tendency to show non-specific antiproliferative activity due to their binding to cellular protein molecules.(137,138) Therefore, we determined the pharmacokinetics and biodistribution of 25-mer antiparallel phosphorothioate triplex-forming oligonucleotide (TFO) after intravenous injection into normal and fibrotic rats. TFOs distributed to all the major organs, with higher uptake in the liver, kidney and spleen. The plasma concentration versus time profile of the ³³P-TFO was biphasic, with 4.36 min at $t_{1/2\alpha}$ of distribution and 34.6 min as $t_{1/2\beta}$ of elimination. TFO concentration in the liver increased nonlinearly with increase in its dose from 0.2 to 50mg/Kg.

Fibrosis results in scar formation and changes in the subendothelial space of Disse and sinusoid, which may affect the hepatic uptake of the TFO. Therefore, we determined the biodistribution of TFO in DMN-induced liver fibrotic rats at 30min post systemic injection. The hepatic uptake of TFO decreased from 43% to 33%, while the plasma concentration increases from 15% to 26% at 30 min postinjection. Similarly, the liver uptake of TFO decreased from 38% to 27% and the plasma concentration increased from 0.78% to 1.75% at 4h postinjection.(34) Compared to the normal rats, a significant increase in TFO concentration was observed in the kidney, heart, spleen, and lung at 30 min and 4h post-injection of ³³P-TFO in the liver fibrotic rats.

Takakura et al. examined the hepatic disposition characteristics of 20 mer PO ODNs and their partially (PS₃ ODNs, in which three internucleotide linkage at the 3'-end are phosphorothioated) and fully phosphorothioated (PS) derivatives in the isolated rat liver perfusion system after bolus injection into the portal vein.(136) The magnitude of the hepatic interaction of ODNs increased as the extent of PS modification in the molecules increased: About 20, 36 and 52 of the injected dose was taken up by the liver during a single passage after

bolus injection of PO, PS₃ and PS, respectively.(106,136,139) PS are mainly taken up by the liver endothelial cells, and to some extent by hepatocyte and Kupffer cells.(140,141) We recently also determined the intrahepatic cellular distribution of TFO and demonstrated that the uptake by Kupffer, endothelial and HSCs accounted for almost 70% of the liver uptake of ³³P-TFO, while only 30% was associated with hepatocytes. Competition studies with polyinosinic acid and dextran sulfate suggest the involvement of scavenger receptor in the hepatic uptake of the TFO. Furthermore, we determined the subcellular distribution of TFO in the liver and found nuclei-associated TFO was much lower relative to that found in the cytoplasm at 2 and 4 h postinjection.(34)

Sawai et al. determined the renal disposition characteristics of ³²P-labeled 20 mer ODNs using the isolated rat kidney perfusion experimental system.(135) Binding of the ODNs to bovine serum albumin (BSA) in the perfusate prior to kidney perfusion was in the following order of magnitudes: PS>PS₃>PO. ODNs showed a sulfur-atom dependent interaction with renal vasculature and hence the volume of distribution (V_d) increased as the extent of PS modification increased. A significant amount of the ODNs filtered through glomeruli underwent tubular reabsorption, which might be mediated by the interactions with specific proteins in the brush border membrane. PS ODNs are accumulated in a non-filtering kidney, suggesting that there is also uptake from the basal side.(142)

VI. D. Delivery Strategies of ODNs

Both macromolecular conjugates and particulate systems are being developed to enhance the stability, control the pharmacokinetic profiles and facilitate site-specific delivery of ODNs. ODNs can be linked directly to a carrier protein or targeting ligand via a covalent bond or non-covalently via polycation-carrier conjugates. The choice of a carrier is dependent on its known ability to bind to specific cell membrane receptors and accumulate in the cell via endocytosis.

1. Conjugation of Lipophilic Molecules—Because ODNs are polyanions and do not readily penetrate biological membranes, various strategies have been developed to increase their cellular uptake. Complex formation with cationic lipids or polycations (such as polyethylene imine) is presently commonly applied to facilitate their uptake by cells in culture, but it is not well suited for use in vivo.(143,144) An approach that was found effective, both in vitro and in vivo, is conjugation of ODNs with cholesterol. Conjugation of ODNs to hydrophobic compounds such as cholesterol, alkyl chain, or lipid have been shown to have higher cellular uptake than their unconjugated counterparts.(145) Among them, cholesterol is perhaps the best characterized. Biophysical and pharmacokinetic properties of 20 mer PS ODNs and its cholesterol analogs have been evaluated. Cholesterol conjugation to PS ODNs resulted in increased retention in plasma as well as accumulation in various liver cell types, with up to 5-fold increase in the uptake by Kupffer cells.(146,147) Cholesterol conjugated PS ODNs (ISIS-8005) showed greater affinity to serum proteins than free PS ODNs (ISIS-3082). This difference in serum protein binding is responsible for the difference in pharmacokinetics. The concentration in liver was correlated with the therapeutic effect of cholesterol conjugated PS ODNs as measured by ICAM-1 mRNA levels in mouse liver *in vivo*, whereas free PS ODNs showed little effect.(148) Moreover, the presence of cholesterol moiety at the 3' end of an ODN did not affect hybridization with target sequences. However, this 5'-cholesterol conjugate of PS ODNs (ISIS-8005) did not change the stability of ODNs significantly, whereas the 3' cholesterol conjugate (ISIS-9388) of ISIS-3082 was much more stable.(147) This is because the 3'-hydroxyl group, which is involved in the nucleophilic attack of the adjacent phosphate bond when the exonuclease enzyme makes a complex with the nucleic acid, is unavailable in ISIS-9388. Many other studies (149,150) have shown similar effects in increasing the cellular uptake of ODNs when cholesterol was conjugated with ODNs. Cellular uptake of 3'-cholesterol-conjugated ODNs has been examined with a real-time confocal laser microscopy. Cytosolic

uptake of cholesterol conjugate was five times as rapid as that of PS ODNs and nuclear uptake of cholesterol conjugate was twice as fast as that of unmodified PS ODN. The effects of cholesterol conjugation to either or both ends of a PS ODN were compared in terms of cellular uptake and antisense efficacy against the p75 nerve growth factor receptor (p75) in differentiated PC12 cells.(151) The addition of a single cholesterol group to the 5' end significantly increased cellular uptake and improved p75 mRNA down-regulation compared with the unmodified ODN. While the 3'-cholesterol analogue was still active, bis-cholesteryl (5'- and 3'-) conjugated ODN was even more potent. LeDoan et al.(152) reported on the interactions of PO ODNs linked to the cholesterol group at an internal position. The conjugates were assessed for their capacity to bind, penetrate, and partition in the cytoplasmic compartment of murine macrophages and showed similar effects.

To increase the liver uptake of TFO, we conjugated cholesterol to the 3' end of TFO via disulfide bond, which can be broken in the cells (Fig. 6). Compared to commonly used liposomal and nanoparticulate delivery systems, conjugation of TFO with cholesterol does not increase the size of delivered TFO, which can be easily taken up by fibrotic liver because of the disappearance of sinusoidal gap in the fibrotic liver. The hepatic uptake of TFO-Chol after systemic administration was 72.22% of the dose compared with 45.8% of unmodified TFO. There was significant increase in the uptake of TFO-Chol by HSCs and hepatocytes. More importantly, the nuclear uptake of TFO-Chol was higher than TFO in cell culture system and in vivo studies.(90) We also showed both TFO and TFO-Chol can silence the expression of primary mRNA of type $\alpha 1(I)$ collagen in rat hepatic stellate cell lines. Moreover, TFO-Chol had higher inhibition on type $\alpha 1(I)$ collagen at low concentration.

In addition to effects on the cellular uptake, cholesterol modulates ODN-mRNA hybrid stability via hydrophobic interactions.(153) An increase in the T_m of up to 13.3°C was observed. Stabilization of triplexes by up to 30°C due to intercholesteryl interaction using 5', 3'-bis-cholesterol-containing ODNs was also observed.

2. PEGylation of Oligonucleotides—PEGylation of ODNs plays an important role in the uptake and biological properties of ODNs. Manoharan et al. showed that when an ODN that targets human ICAM-1 has been conjugated to a series of poly(ethylene glycol) (PEG) esters of average molecular weight 550, 2200, and 5000, the cellular permeation of ODNs *in vitro* can be interfered.(147) The effect of the different structures of high-molecular-weight PEG chains on the biological properties of the conjugated antisense ODNs have been investigated by Bonora et al.(154) Two different conjugates of an anti-HIV 12-mer ODN have been tested for antisense activity in MT-4 cells. Only the ODN conjugated to the linear monomethoxy PEG showed anti-HIV activity. A 20-mer ODN targeting mouse β -globin mRNA has been conjugated at the 5'-terminus to bis-aminoalkyl PEG. At 15 μ M the conjugate selectively inhibited Hb synthesis in cultured Friend murine erythroleukemia cells by 95%. They also conjugated PEG to ODNs at the 5', 3' or both 5' and 3' termini. The number and attachment sites (3'-terminal, 5'-terminal and internal positions) of coupled ethylene glycol units had great influence on the hydrophobicity and electrophoretic mobility of the conjugates. PEGylation had little effect on the hybridization behavior of ODNs. Conjugates with PEG coupled to both 3'- and 5'-terminal positions showed a more than 10-fold increase in exonuclease stability of the ODNs.(154)

Poly(L-lysine)-PEG block copolymer(155) and PEG-polyethylenimine (PEI)copolymer(156) can be used to form soluble polyion complexes with ODN and the complex is self-assembled through electrostatic interaction. The ODN is entrapped in the core of the complex, the serum protein binding is prevented and the nuclease resistance is improved. Furthermore, complement activation decreases with increase in the number and chain length of PEG.(157) On the other hand, Jeong et al.(158,159) conjugated ODN to PEG via a noncleavable amide linkage or acid-

cleavage linkage which can be cleaved in the acidic endosome, then form polyion complex micelles with various cationic peptides (fusogenic KALA peptide) and polymers such as poly (L-lysine), PEI and protamine. The ODN is complexed with cationic polymers to form an inner core, surrounded by PEG on the surface of the complexes. The hydrated PEG chains on the surface minimized the adsorption of serum proteins and prolonged the circulation in the blood. In one study, therapeutic antisense *c-raf* ODN was conjugated to PEG and form complex micelles with PEI. The complex showed a higher antiproliferative activity against ovarian cancer cells both *in vitro* and *in vivo*.(158)

3. Conjugation of Ligands Specific for Different Liver Cell Types—As mentioned before, the liver contains hepatocytes, endothelial cells, Kupffer cells, HSCs, biliary epithelial cells and “pit” cells. Hepatocytes express asialoglycoprotein receptors, Kupffer and endothelial cells express mannose specific receptors and activated HSCs express mannose 6-phosphate/insulin like growth factor II (M6P/IGF-II). Complex formation with galactosylated poly(L-lysine) (Gal-PLL) has been shown to enhance ODN uptake by the hepatocyte via galactose receptor mediated endocytosis, which was influenced by the particle size, zeta potential, sugar substitution level, molecular weight of both polycations and ODNs, and types of ODNs. (160) To avoid the use of polycations, Rajur et al. covalently conjugated ODNs to asialoglycoprotein via a disulfide bond. These conjugates showed comparable biological active to ODNs complexed with PLL.(161)

Carbohydrate cluster conjugates may also be used for targeted delivery of ODNs to different liver cells. When a MP ODN was conjugated to the triantennary, N-acetylgalactosamine neoglycopeptide, Tyr-Glu-Glu-(aminohexyl GalNAc)₃, YEE(ahGalNAc)₃, there were 20–40 fold enhancement in the uptake of the ODNs by HepG2 cells.(162) These carbohydrate clusters are known to bind to Gal/GalNAc receptor sites on hepatocytes with high affinity (K_d ~7 nM). Similarly, when a PS oligomer was conjugated to this glycotriptide, there was sequence-specific suppression of the integrated HBV viral expression in hepatoma cells (greater than 90% inhibition) in a dose-dependent concentration range of 1–20 μM of ODNs.(163)

Galactose is the ligand for the asialoglycoprotein receptor, which is expressed on the surface of liver parenchymal cells. Biessen et al.(164,165) constructed four molecules of galactoses on a oligolysine (N2-(N2-(N2, N6-bis[N-[p-(β-D-galactopyranosyloxy)-anilino]thiocarbamyl]-L-lysyl)-N6-(N-[p-(β-D-galactopyranosyloxy)-anilino]thiocarbamyl)]-L-lysyl)-N6-(N-[p-(β-D-galactopyranosyloxy)-anilino]thiocarbamyl)]-L-lysine, (L3G4)), and attached it to a 20-mer PO ODN 3'-capped with an amine. The ligand has a K_d of 6.5 nM. This L3G4-conjugated ODN was far more efficiently taken up by parenchymal liver cells than underivatized ODN. Studies in rats showed that hepatic uptake was greatly enhanced from 19% for unconjugated ODN to 77% of the injected does after glycoconjugation. Accumulation into liver parenchymal cells was increased by 60-fold.

M6P/IGF II receptor is expressed on HSCs, and its expression is upregulated upon activation of these cells due to acute or chronic liver injuries.(166) Beljaars et al. demonstrated a preferential distribution of M6P conjugated human serum albumin to HSCs after systemic administration into rats.(167) Ye et al. also conjugated TFOs with M6P-BSA via disulfide bond and demonstrated higher uptake by HSCs in rat liver after systemic injection. Almost 66% of the injected M6P-TFO accumulated in the liver at 30min postinjection, which is significantly higher than that deposited after injection of unmodified TFO. In addition, almost 82% of the total liver uptake in fibrotic rats was contributed by HSCs.(168,169)

4. Conjugation of Polyamines and Polypeptides—Polyamine-conjugated ODNs form amphipathic molecules and reduce the net negative charge on ODNs. As these modified ODNs may serve as ligands for polyamine receptors present on certain cells, polyamine conjugation

may improve the antisense activity of ODNs. Since the monoalkylamines and at least some of the amino groups of the polyamines are protonated at the physiological conditions, they can also improve the hybridization rates of antisense ODNs to the target RNA. Corey (170) demonstrated 48,000-fold increase in hybridization of ODNs conjugated to cationic peptides derived from lysines. Cationic polypeptides (polylysine, polyornithine, polyhistidine, and polyarginine), due to their positive charges, when conjugated with ODNs, which are negatively charged, can enhance hybridization of ODNs.

A fusogenic peptide derived from the influenza hemagglutinin envelope protein has been conjugated to antisense ODNs. This peptide changes its conformation at acidic pH and destabilized the endosomal membrane resulting in an increased cytoplasmic delivery of the antisense ODNs. A similar fusogenic peptide conjugated to an antisense ODNs via a disulfide or thioether bond resulted in 5- to 10-fold improvement of the anti HIV activity on de novo infected CEM-SS lymphocytes in serum free media. However, no sequence specificity was observed and the fusogenic peptide also possessed some antiviral activities on its own.

Although the sequence specificity, biostability and low toxicity of PMOs make them good antisense agents, they are poorly taken up by the cells. Conjugation to positively charged arginine-rich TAT-like peptide has been shown to significantly enhance the cellular uptake of the PMOs. PMO delivery to the cell nucleus and cytosol required conjugation rather than complex formation of peptides to the PMO. Conjugation of $\text{NH}_2\text{-RRR RRR RRR-FFC-CONH}_2$, designated R9F2 peptide, to the PMO resulted in the strongest antisense effect. This particular peptide-conjugated PMO was more effective than the PMO conjugated to the transmembrane transport peptides of HIV-1 TAT protein.(171) Moreover, antiviral effect of PMO was significantly increased upon conjugation to R9F2 peptide. In contrast, peptide conjugate did not result in antiviral function of the nonsense PMOs.(172) However, R9F2-PMO conjugate was ineffective in the presence of culture medium containing 10% FBS, raising a question about the utility of intravenous or orally administered R9F2-PMO conjugate in vivo. (172)

VI. E. Therapeutic Applications of Oligonucleotides

1. Application of Antisense Therapy

TGF- β 1: TGF- β 1 is the key cytokine of the fibrotic response to chronic liver injuries. It not only enhances synthesis of ECM but also inhibits ECM degradation by down-regulating expression of matrix-degrading enzymes and inducing tissue inhibitor of metalloproteinases-1 (TIMP-1).(173) Therefore, blockade of TGF- β 1 synthesis or signaling pathway is considered the most potent therapeutic strategy for treating liver fibrosis and attracted the most attention for developing anti-fibrotic approaches.

TGF- β 1 can be silenced directly by antisense ODNs. Uchio et al. tested antisense ODNs against TGF- β 1 in fibrotic mice induced by CCl_4 . TGF- β 1 mRNA was decreased by 50% and type I collagen mRNA was also decreased by about 40% in the preventive experiment.(174) Arias et al. constructed an adenovirus expressing antisense mRNA complementary to the 3' end of TGF- β 1 coding sequence (Ad5-CMV-AS-TGF- β 1).(64) Isolated rat HSCs were infected with the recombinant adenovirus and high expression of the transgene was observed. Northern blot and western blot showed inhibition of TGF- β 1 as well as TGF- β 2 and TGF- β 3 since they have a high degree of sequence identity. Accordingly, suppression of fibrogenic markers (e.g., α -SMA, type I collagen) were also demonstrated. These in vitro data suggested that blockade of TGF- β 1 is a possible candidate for treating liver fibrosis.(64) In their subsequent study, Ad5-CMV-AS- TGF- β 1 was tested in fibrotic rat model, induced by common bile duct ligation. (25) Treatment with the adenoviral vector inhibited the expression of type I collagen and α -SMA. In addition, α -SMA positive cells around the bile ductular structures were also reduced

after treatment. Consistent with their in vitro studies, these in vivo data provided strong evidence that the antisense strategy for TGF- β 1 was able to specifically block its effect on liver fibrogenesis.(25)

Another possible option to inhibit TGF- β 1 signaling pathway is to interfere with post-receptor signaling. Signaling of TGF- β 1 is through two types of transmembrane protein kinase receptors, known as receptor I (T β R I) and receptor II (T β R II). Therefore, blocking expression of T β R I and T β R II is believed to have anti-fibrotic effect.(63,175,176) Jiang et al. constructed rat antisense T β R I and T β R II plasmid separately using pcDNA3 vector, encapsulated with glycosylated poly (L-lysine) and then delivered into rats with induced liver fibrosis. The plasmid treated group showed low expression of T β R I and T β R II in mRNA and protein levels. Furthermore, it reduced the levels of TGF- β 1 from 32.960 ng/ml to 23.998 ng/ml (T β R I group) and 23.156 ng/ml (T β R II group). Compared with control group, type I collagen index was also decreased from 1209 to 669 (T β R I group) and 661 (T β R II group) using immunohistochemistry and image analyzing software.(63) Combined with another studies from the same group,(175,176) antisense T β R I and T β R II strategy was demonstrated the reverse effects on liver fibrosis.

In the TGF- β 1 signaling pathway, TGF- β 1 binds to its receptor TGF β RII. TGF β RII then forms a heterotetrameric type I/type II receptor complex with TGF β RI and TGF β RII kinase phosphorylates TGF β RI. Activated TGF β RI phosphorylates the TGF β -specific signal transducer Smad2 or Smad3. Phosphorylated Smads then form heteromeric complexed with the co-Smad, Smad4, to move into the nucleus, where they modulate transcription of TGF β 1 responsive genes.(177,178) Therefore, as the common mediator, blockade of Smad4 was explored as therapeutics for liver fibrosis. Xu et al. constructed antisense Smad4 in adenoviral vector and infused into fibrotic rats via portal vein.(179) The expression of both Smad4 and type I collagen was both inhibited compared to non-treated group. Pathological study also showed the reduced fibrosis degree in the antisense treated group.(179)

2. Roles of TIMP-1, PDGF, c-myb and Type III collagen in Liver Fibrosis—Liver fibrosis is associated with increase in both TIMP-1 and TIMP-2, leading to decrease in MMP and consequent increase in ECM accumulation. During fibrogenesis, TIMP-1 increased more significantly than TIMP-2 and high expression of TIMP-1 indicated the severity of liver fibrosis.(180) The promoting effect of TIMP-1 is more important than that of TIMP-2 in liver fibrosis. Blocking the gene expression of TIMP-1 gene expression is therefore considered as a good strategy to reverse liver fibrosis. Nie et. al. designed four phosphorothioate antisense ODNs based on the sequences of modulator, structural protein and coding region of TIMP-1 genome.(88) After induction of liver fibrosis in rats by human serum albumin, these ODNs were injected into rats via coccygeal vein. Both mRNA and protein levels of TIMP-1 were increased significantly after induction of liver fibrosis. ODNs treated group showed reduced mRNA and protein levels of TIMP-1, although these levels were still higher than that of normal rats. Immunohistochemical and electron microscopic examination also revealed that ODN treatment had some anti-fibrotic effect in this animal model.(88) Furthermore, Wei et al. constructed a plasmid expressing rat antisense TIMP-1 and tested antifibrotic effect in a fibrotic rat model.(87) Recombinant plasmid and empty vector were encapsulated with glycosylated PLL and administrated into fibrotic rats via the caudal vein. Antisense TIMP-1 was successfully expressed and it inhibited both gene and protein expression of TIMP-1. Interstitial collagenase activity was increased in the antisense TIMP-1 group, while there is no difference between empty vector and control group. Moreover, hepatic hydroxyproline assay showed lower accumulation of collagen in antisense treated group, demonstrating the inhibitory effect of antisense TIMP-1 plasmid on liver fibrosis.(87)

Platelet-derived growth factor B-chain (PDGF-B) is a potent mitogen for HSC activation and overexpressed during hepatic fibrogenesis. Therefore, blocking PDGF expression in the liver during the fibrogenesis process provides an interesting approach to treat liver fibrosis. Borkhan-Kamphorst et al. constructed an adenoviral vector Ad5-CMV-asPDGF, expressing an antisense mRNA corresponding to the 5'-coding sequence of the PDGF.(65) After treatment with Ad5-CMV-asPDGF, both culture-activated HSC and rat livers showed down-regulation of PDGF B-Chain. Fibrotic rats treated with Ad5-CMV-asPDGF also showed reduced levels of type I collagen and α -SMA, demonstrating attenuation of liver fibrogenesis.

c-myc gene expression is another critical factor for HSC activation and proliferation in livers affected by chronic injuries. To investigate the effect of c-myc antisense RNA on cell proliferation and expression of TGF- β 1 and type I collagen in HSCs, Ma et al. constructed retroviral vector of c-myc antisense gene (pDOR-myc). After treatment with pDOR-myc, cell proliferation, expression of type I collagen and TGF- β 1 were all repressed significantly, suggesting blocking of c-myc gene is a promising treatment for liver fibrosis.(181)

Since overaccumulation of collagen is the end stage during liver fibrogenesis, Horie et al. designed PS ODN complementary to different regions of procollagen type III gene and studied anti-fibrotic effect using HSCs.(182) Antisense strand complementary to the AUG start site was most effective in inhibiting procollagen type III gene expression. Although the effect was transient and large amount of antisense ODN was required to induce inhibition, the proof of concept study showed promising effect in attenuating liver fibrosis.

3. Regulation of Type I Collagen Gene Transcription—The major structural protein of extracellular matrix (ECM) is type I collagen, which is the heterotrimer product of two α 1(I) and one α 2(I) polypeptide chains encoded by the α 1(I) genes, found in human chromosomes 17 and 7, respectively.(183–185) The entire region spanning from –140 to –200 bp exists as an asymmetric polypurine-polypyrimidine tract in which the polypyrimidine sequence at –141 to –170 is called C1 region which is localized on the non-coding strand, whereas the adjacent polypurine sequence from –171 to –200 is called C2 region, which is present on the coding strand.(186) The cis-acting elements in the C1 and C2 region play a key role in collagen transcription, and hence makes it an ideal target for developing antigene-based antifibrotic agents.(187),(188)

a. Triplex formation with Antiparallel TFO Specific for C1 Region: Using parallel (P) and antiparallel (AP) TFO of 18–30 nucleotides in length corresponding to the C1 sequence, Nakanishi et al.(89) found that TFOs specific for C1 form triplexes much more efficiently than C2 and that the antiparallel polypurine TFOs form triplexes 100 to 1000 times more efficiently than parallel TFOs. The antiparallel 18 mer TFOs (–158 to –141 or –164 to –147) form triplexes at a K_d similar to the 30 mer TFOs and that the effective K_d is at least 100x lower for antiparallel compared with parallel TFOs. In contrast to the polypurine TFOs, there was no appreciable triplex formation for 18 mer polypyrimidine TFOs at physiological or slightly acidic pH in the presence of polyvalent cations. They also found that the efficiency of triplex formation with phosphorothioate TFOs is comparable with that with phosphodiester TFOs. Of the three TFOs used corresponding to the C1 region, 158 APS appears to form a triplex at a lower concentration than 164 APS, whereas 170 APS formed a triplex at a slightly higher concentration than 164 APS.

The physiological milieu that exists within a cell has been reported to be unfavorable for purine motif triplex formation, possibly due to the high potassium concentration (140 mM), which promotes aggregation of guanine-rich ODNs into guanine quartets.(114,189) Hence, Joseph et al. tested the ability of 158 APS TFO to form triplex under conditions closely simulating those

in the living cells.(186) C1 TFOs formed stable complexes even under physiological conditions (1 to 5 mM Mg^{2+} , 140 mM KCl).

It is important to determine whether the TFO is specific for C1 region of collagen $\alpha 1(I)$ promoter and not for other sequences present in the rodent and human genomes. The overall sequence organization of C1 and C2 regions of the human $\alpha 1(I)$ procollagen promoter is similar to that of rat and mouse promoters, however, there are 8 nucleotide changes in the human $\alpha 1(I)$ sequence compared with rat or mouse. These nucleotides are -141, -142, -145, -150, -153 to -155 and -165. Out of these 8 nucleotides, alteration of nucleotides -141 to -143 resulted in the association constant as evidenced by C1 AP or C1 APS. Most of the nucleotide differences in the human C1 sequence are A→G, which could be responsible for triplex formation with higher efficiency. Antiparallel TFO to rat C1 forms stable triplexes with a K_d of ~100 nM and TFOs as short as 18 nucleotides could form stable triplexes. Similar analysis with human C1 indicated the formation of triplexes with a K_d of ~10–20 nM (Fig. 7A). Antiparallel TFO to human C2 formed triplexes C2, but very inefficiently at extremely high concentrations (>8000 nM) (Fig. 7B). Since the human C1 sequence is different from rat or mouse sequence, we tested the formation of triplexes by these heterologous TFOs. The human C1 double-stranded probe was incubated with rat C1 TFO and vice versa. Our results indicate that while rat TFO formed less stable triplexes at very high concentrations (>1000 nM) (Fig. 7C), human C1 APS formed stable triplexes with rat C1 duplex DNA with a K_d of ~200 nM (Fig. 7D), suggesting that the TFO rather than the duplex DNA is important to drive stable triplex formation. Therefore, the sequence organization and composition are important in determining the specificity of triplex formation.(89)

b. Inhibition of Collagen Promoter Activity In Vitro: The C1-specific (-141 to -170) antiparallel TFOs ranging in size from 18 to 30 nucleotides significantly inhibited transcription of the $\alpha 1(I)$ collagen promoter activity in in vitro cell-free transcription systems as well as in rat transfecting fibroblasts in culture. pCOL-CAT 220, containing the reporter gene chloramphenicol acetyltransferase (CAT) driven by the rat $\alpha 1(I)$ collagen promoter from -220 to +100, was transfected into rat 2tk⁻ fibroblast cells, which express high levels of type I collagen.(186) This was followed by transfection with specific antiparallel phosphorothioate (APS) TFO and cellular extracts were assayed for CAT activity. The results clearly indicated that both 158 and 164 APS TFOs form triplexes and significantly inhibit transcription in rat cells (~50% of control) at a TFO to plasmid ratio of 125:1. 164 APS TFO showed a more pronounced effect. CAT activity was reduced by 70% compared with the control ODN (5'-CAT GGA GCC ACA TTC ATG-3') at a TFO to plasmid ratio of 125:1, with a further reduction in activity (80%) on increasing the ratio to 250:1. These results clearly demonstrate significant inhibition of collagen promoter function on triplex formation of the target region with 158 APS and 164 APS, with 164 APS showing a more dramatic effect.(186)

VII. siRNA THERAPY

RNA interference (RNAi) is the phenomenon in which small interfering RNA (siRNA of 21–23 nucleotides in length) silences target gene by binding to its complementary mRNA and triggers their elimination. It was initially discovered by Fire et al. in *Caenorhabditis elegans* in 1998 (190) that small double-stranded RNAs can lead to potent targeted degradation of complementary mRNA. This finding generated huge interest in the application of siRNA for biomedical research community.(191) Potent knockdown of the targeted gene with high sequence specificity make RNAi a powerful tool for studying gene function and a promising therapeutic strategy for some diseases.

VII. A. Mechanism of siRNA

The silencing process by siRNA occurs post-transcriptionally in the cytoplasm (192) and it is an ATP-dependent and translation-independent event. The current RNA-induced silencing complex (RISC) model is shown in Fig. 8. The first step is the production of dsRNA directed against an mRNA.(193) The second step is the cleavage of the dsRNA by the endogenous enzyme Dicer into fragments of 21–23 nucleotide siRNAs. The third step is the duplex siRNA unwound using an ATP-dependent helicase. Antisense strand of siRNA is incorporated into a multiprotein RNA-inducing silencing complex (RISC), which is the key step in RNA interference.(194,195) Subsequently, the incorporated siRNA strand guides RISC to its homologous target mRNA for endonucleolytic cleavage.(196) Only one of the strands from siRNA is incorporated into RISC, whereas biologists previously believed that both of the siRNA strands were incorporated. The RISC assembly is governed by an enzyme that selects which strand of a siRNA is loaded into RISC. The stability at the 5' ends of the two siRNA strands determines which one enters RISC (197) and the strand with less stable 5' end is more likely to be incorporated. To be functional against target mRNA, the antisense strand must be the one incorporated into RISC, whereas the sense siRNA strand directs cleavage of an antisense target.(197)

There are several strategies to introduce siRNA into mammalian cells. The most commonly used strategy is to chemically synthesize double-strand siRNAs of 21–23 nucleotides for transfection into cells. There are five advantages of synthesized siRNAs: i) providing precise control of the amount and purity of siRNA used in the experiment; ii) siRNA can be characterized; iii) modifications can be introduced into the siRNA to enhance its target specificity; iv) high transfection efficiency into the cells of interest; and v) the initiation of siRNA-transfected silencing is immediate.(196,198) With the help of rapidly evolved siRNA synthetic chemistry and siRNA sequence design software, most current protocols for RNAi in mammalian systems use chemically synthesized siRNAs.

Since there is no indication of siRNA replication in mammals,(192) the transduction of siRNA into cells leads to only a transient knockdown of the target gene which limit its application in *Drosophila* and mammals.(196) Consequently, plasmid DNA and viral vectors are developed to express shRNA that can be converted into siRNA *in vivo*. Compared to synthesized siRNAs, expression vectors can produce siRNAs continuously in cells. In addition, viral vectors have been shown to have higher transfection efficiency, especially in non-dividing cells.(112)

In the plasmid-based expression vector, hairpin RNA can be expressed in cells under the control of an RNA polymerase II (cytomegalovirus, CMV) (199) or polymerase III (U6 or H1).(200, 201) Polymerase II (pol II) promoters can induce tissue specific RNA expression. However, pol II expresses long hairpin RNA which can induce the interferon response in many mammalian cells.(196) Therefore, the application of pol II promoter was limited and most of the current plasmid-based expression systems use polymerase III (pol III) promoter. Pol III expresses short hairpin RNA species and does not induce interferon responses as pol II. Typically, a single promoter is used to express hairpin precursor transcript. However, the hairpin vectors have some limitations: i) hairpin is hard to synthesize in bacteria; ii) difficulty in sequencing; iii) length and sequence of hairpin can affect the gene silencing ability of siRNA. (202) To overcome these limitations of hairpin vectors, dual promoter siRNA expression vectors have been developed.(202,203)

Although plasmid-based siRNA expression vectors have been successfully used in different cell types, these vectors usually have low transfection efficiency in non-dividing and primary cells.(196) To overcome the problem of poor transfection efficiency of plasmid-based siRNA expression vectors, viral vectors for siRNA delivery are being developed. Adeno-associated viral (AAV) vector,(204) adenoviral vector,(205) retroviral vector (206) and lentiviral vector

(112,207–209) have been reported for siRNA delivery. Lentiviral vector can express integrated siRNA efficiently in a wide variety of actively dividing, non-dividing and primary cells, both *in vitro* and *in vivo*.

Although RNAi provides a powerful tool for inhibiting targeted gene, there are several concerns and limitations in the practical application of this technology.(210) The transfection methods used for ODNs and plasmid can also be applied for siRNA delivery. However, the transfection conditions should be optimized for different cell types to get maximum gene silencing. Milhavet et al.(211) summarized several parameters which could affect transfection and gene silencing efficacy: i) cell culture conditions, cell density and medium composition; ii) amount and type of transfection agent; iii) quality and amount of siRNA and iv) exposing time of the cells to siRNA.

VII. B. siRNA for Liver Fibrosis

Connective tissue growth factor (CTGF) is a highly profibrogenic molecule which is overexpressed in liver fibrosis. It is transcriptionally activated by TGF- β and mediates some of the extracellular matrix inducing properties. Jin et al. designed chemically synthetic siRNA targeting CTGF and investigated the effect on the expression of ECM using HSCs.(212) Cells transfected with siRNA showed significant inhibition of CTGF expression, as well as secretion of ECM including type I and III collagen. Moreover, they determined the anti-fibrotic effect of the siRNA using a fibrotic animal model.(213) siRNA was injected into fibrotic rats via intraportal vein at a dose of 0.1mg/kg. At 4 to 6 weeks after administration of CTGF siRNA, inductions of CEGF, type I, III collagen, TIMP-1 and TGF- β 1 were all attenuated. In addition, the number of activated stellate cells were also decreased.(213)

Apoptosis of activated HSCs is expected to attenuate liver fibrosis. Therefore, Anan et al. studied the mechanism of apoptosis induction in HSCs by proteasome inhibitor and found that HSC activation is associated with activation of nuclear factor kappa B (NF- κ B). Survival of activated HSCs is dependent on the NF- κ B target gene A1, an anti-apoptotic Bcl-2 family members, which played an important role in the apoptosis process.(214) Thereafter, they designed siRNA to silence A1 gene and subsequently induce apoptosis of primary rat HSCs and immortalized human HSC line, LX-2. As suggested in this study, siRNA target A1 gene could be a potential antifibrotic agent.(214)

Activation of HSCs is the key step in fibrogenesis and the inhibition of its activation is therefore of therapeutic interest. Galectin-3 is a β -galactoside-binding animal lectin and its expression is upregulated in liver fibrosis. Henderson et al. investigated the mechanism of Galectin-3 in liver fibrosis and found that disruption of Galectin-3 inhibited myofibroblast activation and collagen expression *in vivo* and *in vitro*.(215) Galectin-3 siRNA was designed and administered to rats via portal vein at 2, 24, and 48 h after intraperitoneal injection of CCl₄, which is a standard method to induce liver fibrosis. Immunohistochemistry and real-time PCR results showed significant decrease in Galectin-3 and α -SMA expression, demonstrating Galectin-3 siRNA can block HSCs activation in fibrogenesis.(215)

Benyon's group provided the first evidence that $\alpha_v\beta_3$ integrin was involved in the activation and apoptosis of HSCs. siRNA which targets α_v subunit of $\alpha_v\beta_3$ inhibited HSCs proliferation and induced the apoptosis of activated HSCs.(216) Although this study focused on the elucidation of $\alpha_v\beta_3$'s role in the HSCs proliferation and apoptosis, it provides a potential therapeutic target for liver fibrosis.

During liver fibrosis, the half-life of type I collagen mRNA increases from 1.5h in quiescent HSCs to more than 24h in activated HSCs. Studies showed that this increase was mediated by post-transcriptional mechanism, in which the RNA-binding protein α CP binds to the 3'

untranslated region of type I collagen mRNA and stabilize it in activated HSCs.(217) siRNA against α CP was synthesized and transfected with activated HSC cell lines. α CP binding activity was decreased by ~40% in HSCs treated with siRNA and the reduction resulted in decreased steady state type I collagen mRNA level. This is the first work that demonstrated α CP binding activity is essential for type I collagen mRNA stabilization during HSCs activation and blockade of α CP is a potential strategy for therapy of liver fibrosis.(217)

As the most important profibrogenic mediators in liver fibrosis, TGF- β 1 gene expression was silenced using siRNA and explored as a potential therapeutic strategy for liver fibrosis. Kim et al. constructed TGF- β 1 siRNA in pU6-shX vector and investigated the anti-fibrotic effect in murine model of liver fibrosis.(62) After inducing liver fibrosis using CCl₄ for 3 weeks, siRNA was injected into mice via tail vein. The ALT level was significantly increased in CCl₄ treated group compared to the control group, but only slight increase was observed in CCl₄/siRNA treated group. Both mRNA and protein levels of TGF- β 1 were decreased in CCl₄/siRNA treated group compared to CCl₄ treated mice. Moreover, expression of type I collagen and α -SMA were also decreased after treatment of siRNA. Furthermore, the histopathological evaluation confirmed that TGF- β 1 siRNA treatment moderated liver fibrosis. (62)

Fas-mediated apoptosis also plays an important role in various liver diseases since hepatocytes highly express Fas. Fas-deficient mice showed resistant to chronic hepatic insults which can induce fibrosis.(60) Song et al. (56) investigated the *in vivo* silencing effect of siRNA targeting the Fas gene to protect mice from liver failure and fibrosis. The synthetic siRNA was injected into mouse by hydrodynamic tail vein injection and 88% of hepatocytes took up the siRNA after 24 hours. *Fas* expression at mRNA and protein levels in mouse hepatocytes was reduced and the effects persisted for 10 days without diminution. Furthermore, the treatment of *Fas* siRNA protected mice from fulminant hepatitis and hepatic fibrosis in two animal models (Fig. 9). Figure 9(A) showed there was no hepatic fibrosis or necrosis in siRNA treated group, while controlled group exhibited bridging fibrosis in the liver parenchyma. Both hepatic hydroxyproline (chemical indicator of active fibrosis) and serum procollagen type III of siRNA treated groups were reduced significantly compared to controlled group (Figure 9B). The results suggested the therapeutic promise of siRNA to prevent liver injury by protecting hepatocytes from cytotoxicity.

VIII. PERSPECTIVE

Fibrogenesis is a complicated process involving many cytokines which are also involved in various important functions of other organs. Therefore, having a better understanding of fibrogenesis is necessary for treating fibrosis. The selection of the most effective target while avoiding toxicity is the most challenging task. Once the target is selected, antagonist or agonist of this target could be developed easily using gene modulation approach. Unfortunately, it is becoming evident that intervention of inflammation or immunization alone is generally ineffective.

Regardless of the underlying diseases, over-expression of ECM is the last step in fibrogenesis. Also, for any patient diagnosed with liver fibrosis, excessive ECM is already there which needed to be reversed. Therefore, for effective treatment of liver fibrosis, we believe the “cocktail” therapy (multiple treatment) is the best choice. First, stimuli for chronic liver injuries should be attenuated. Secondly, a potent cytokine involved in the inflammation or HSCs’ activation should be silenced. Additionally, excessive production of ECM should be reversed.

How to transfer effective antifibrotic gene medicine from animal to human is another major challenge since there is always difference in gene sequence between animal and human. Even there is a minor difference in the target gene sequence, gene medicine needs to be validated.

Other major concern regarding treatment of liver fibrosis is how to monitor antifibrotic effect in clinical trials since the treatment is a long process. Therefore, dynamic non-invasive methods for evaluating liver fibrosis are needed.

Acknowledgments

This work was supported by grants R01 DK064633, R01 EB-003922 and USPHS 47379 from the NIH.

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Fig. 1.

Sinusoidal events during liver injury. Changes in the subendothelial space of Disse and sinusoidal fenestrae as fibrosis develops in response to liver injury. Stellate cell activation leads to accumulation of ECM. This in turn contributes to the loss of hepatocyte microvilli and sinusoidal endothelial fenestrae, which result in deterioration of hepatic function. Kupffer cell (macrophage) activation accompanies liver injury and contributes to paracrine activation of stellate cells. Reproduced with permission from *J Biol Chem* **275**: 2247–50 (2000).

Fig. 2.
Regulation by ECM in liver fibrosis.

PDGF (Platelet-derived growth factor), ET-1 (Endothelin-1), IGF-1 (Insulin-like growth factor-1)

Fig. 3.
Proliferation of hepatic stellate cells in liver fibrosis.

Fig. 4.

Mechanisms of action of antisense oligodeoxynucleotides. Translational arrest by blocking the ribosome and degradation of mRNA by RNase H cleavage.

Fig. 5.
Pyrimidine and purine motifs for triple helix formation.

Fig. 6.

Synthesis of TFO-Chol. Reagent and conditions: (1) 5ml pyridine, stirring at room temperature for 2h; (2) 1500 μ l dimethylformamide (DMF), stirring under N_2 protection at 40°C for 24 h. Reproduced from Cheng et al., *J Pharmacol Exp Ther* **317**: 797–805 (2006).

Fig. 7.

Electrophoretic mobility shift assays demonstrating triplex formation with human and rat antiparallel phosphorothioate TFO (APS TFO). Either human [(A and C) hC1ds; (B) hC2ds] or rat [(D) rC1ds] double-stranded oligonucleotides were radiolabelled and aliquots were incubated with human (hC1 APS) or rat (rC1 APS) TFOs and analyzed on polyacrylamide gels. D: duplex; T: triplex. Reproduced from Nakanishi et al., *Nucleic Acid Res* 26: 5218–5222 (1998)

Fig. 8. Mechanism of RNA interference. Long double-stranded RNA, pre-miRNA and shRNA are cleaved by Dicer into fragments of 21–23 nucleotide siRNAs. ATP-dependent helicase unwind the duplex siRNA and one strand of siRNA is incorporated into a multiprotein RISC. Subsequently, the incorporated siRNA strand guide RISC to its homologous target mRNA for endonucleolytic cleavage.

Fig. 9.

Fas gene silencing protects mice from fulminant hepatitis and hepatic fibrosis. Liver histology of mice after six weekly injections of ConA and treatment of Fas siRNA or control solution. Fas siRNA-treated group protect mice from fibrotic damage whereas control treatment did not. Reproduced with permission from Song et al., *Nat Med* **9**:347–351 (2003)

Table 1

Composition of Rat Liver

Cell Type	No. of Cells (% of total)	Mass (% of total)
Hepatocytes	65	94
Endothelia	18	2
Kupffer	10	3
Stellate	7	1

Table 2

TFO deposition (% of dose) to major organs after intravenous injection into normal and fibrotic rats at a dose of 1mg/kg

	30 minutes		240 minutes	
	Normal rat	Fibrotic rat	Normal rat	Fibrotic rat
Liver	43.00 ± 2.46	32.55 ± 3.53	37.97 ± 1.17	27.05 ± 9.87
Kidney	4.66 ± 0.63	3.87 ± 0.19	1.19 ± 0.22	1.85 ± 0.25
Heart	0.45 ± 0.03	0.35 ± 0.02	0.29 ± 0.06	0.46 ± 0.05
Spleen	1.61 ± 0.11	3.97 ± 0.99	1.28 ± 0.16	2.90 ± 1.51
Lung	0.82 ± 0.13	0.98 ± 0.11	0.38 ± 0.05	0.72 ± 0.40
Plasma	15.52 ± 0.98	26.04 ± 1.53	0.78 ± 0.15	1.75 ± 0.17

(Mean ± SD, n=5). Reproduced with permission from Cheng et al. *Mol Pharm* **2**: 206–217 (2005).