Prevention of Liver Fibrosis by Triple Helix-Forming Oligodeoxyribonucleotides Targeted to the Promoter Region of Type I Collagen Gene

Subramaniyan Koilan,1 David Hamilton,2 Narina Baburyan,1 Mythili K. Padala,1 Karl T. Weber,3 and Ramareddy V. Guntaka1

Hepatic fibrosis leading to cirrhosis remains a global health problem. The most common etiologies are alcoholism and viral infections. Liver fibrosis is associated with major changes in both quantity and composition of extracellular matrix and leads to disorganization of the liver architecture and irreversible damage to the liver function. As of now there is no effective therapy to control fibrosis. The end product of fibrosis is abnormal synthesis and accumulation of type I collagen in the extracellular matrix, which is produced by activated stellate or Ito cells in the damaged liver. Therefore, inhibition of transcription of type I collagen should in principle inhibit its production and accumulation in liver. Normally, DNA exists in a duplex form. However, under some circumstances, DNA can assume triple helical (triplex) structures. Intermolecular triplexes, formed by the addition of a sequence-specific third strand to the major groove of the duplex DNA, have the potential to serve as selective gene regulators. Earlier, we demonstrated efficient triplex formation between the exogenously added triplex-forming oligodeoxyribonucleotides (TFOs) and a specific sequence in the promoter region of the COL1A1 gene. In this study we used a rat model of liver fibrosis, induced by dimethylnitrosamine, to test whether these TFOs prevent liver fibrosis. Our results indicate that both the 25-mer and 18-mer TFOs, specific for the upstream nucleotide sequence from −141 to −165 (relative to the transcription start site) in the 5′ end of collagen gene promoter, effectively prevented accumulation of liver collagen and fibrosis. We also observed improvement in liver function tests. However, mutations in the TFO that eliminated formation of triplexes are ineffective in preventing fibrosis. We believe that these TFOs can be used as potential antifibrotic therapeutic molecules.

Introduction

Hepatic fibrosis or cirrhosis remains a formidable disease. More than 400,000 people are affected in the United States and millions worldwide (Wells, 2006). The most common etiologies are alcoholism and viral infections. More than 2 billion people are currently infected by either Hepatitis B or C (Hu, 2008). Regardless of its etiology, cirrhosis is progressive with disorganization of the liver architecture and irreversible damage to the liver function. Upon injury to any tissue in the body, damage to the blood vessels triggers an inflammatory response, which is an adaptive response to traumatic, infectious, toxic, or autoimmune injury and involves a complex set of interactions between various soluble factors and cells (Friedman, 2008; Medzhitov, 2008). Liver fibrosis is associated with major changes in both quantity and composition of extracellular matrix (Bataller and Brenner, 2005). Normal liver contains hepatocytes, Kupffer cells, an endothelial lining, and the sub-endothelial space of Disse harboring stellate cells. Stellate cells represent 5%–8% of all human liver cells and about 15% in rat liver (Reynaert et al., 2002; Friedman, 2008). Activated stellate cells are involved in collagen synthesis and subsequent remodeling or liver fibrosis (Friedman, 2008).

As of now there is no effective therapy to control fibrosis. Some of these, targeting various pathways, are in development (Rockey, 2008; Zardi et al., 2008). Since the end product of fibrosis is abnormal synthesis and accumulation of type I collagen, a major component of the extracellular matrix, our goal is to inhibit transcription of type I collagen in stellate cells. Type I collagen consists of 2 α1(I) and 1 α2(I) polypeptide chains, synthesized in a 2:1 ratio. It is well known that the
tissue-specific expression of eukaryotic genes is governed not only by the presence of tissue-specific transcription factors, but also by their interaction with their cognate cis-acting elements in the promoters of these genes. Several lines of evidence suggest that the 5' sequences flanking the structural gene, as well as sequences located in the first intron of the COLIA1 and COLIA2 are required to attain expression levels comparable to the endogenous gene (Guntaka et al., 1995; Karsenty and Park, 1995).

In the last few years there has been considerable progress in the application of triplex-forming oligodeoxyribonucleotides (TFOs) as (1) site-directed mutagens, (2) agents that cleave target DNA sequences, and (3) antigenic therapeutic molecules that specifically and selectively downregulate target genes (Guntaka et al., 2003; Kalota et al., 2004; Kalish and Glazer, 2005; Mahato et al., 2005). Normally, DNA exists in a duplex form. However, under some circumstances, DNA can assume triple helical (triplex) structures, which are either intramolecular or intermolecular (Duca et al., 2008). Intramolecular triplexes, formed by the addition of a sequence-specific third strand to the major groove of the duplex DNA, have the potential to serve as selective gene regulators. The polypyrrimidine/polypurine sequence (C1) of the COLIA1 gene can form triplexes if the third strand is added. Depending on the relative orientation of the third strand, 2 types of triplex structures can be formed. The polypyrrimidine third strand binds parallel to the polypurine strand of the duplex DNA by Hoogsteen hydrogen bonding, whereas the polypurine third strand, which is typically G-rich and antiparallel to the polypurine tract, binds to the purine strand of the duplex DNA via reverse-Hoogsteen hydrogen bonds (Duca et al., 2008). We have previously demonstrated both polypyrrimidine and polypurine types of triplex formation with the C1 region of the COLIA1 promoter (Kovacs et al., 1996; Joseph et al., 1997; Nakanishi et al., 1998). This triplex structure is formed by the antiparallel polypurine TFO with the duplex C1 sequence (Fig. 1) and that the antiparallel polypurine C1-specific TFO appears to be more efficient in forming triplexes than either the parallel purine I TFO or polypyrrimidine type of TFO.

Earlier, we demonstrated efficient triplex formation with all the 3 TFOs (25-mer, 158TFO, and 164TFO; sequences are shown in Fig. 1). The 158TFO and the 25-mer TFO, both of which contain a stretch of 6 guanine (G) residues at the 3' end, formed triplexes more efficiently than the 164TFO, which lacked this hexanucleotide sequence. Further, we showed that these triplexes, once formed, are very stable and the kinetics of triplex formation indicated that at a molar ratio of 50:1 of the TFO:duplex, triplexes formed in less than half an hour at 37°C (Joseph et al., 1997; Nakanishi et al., 1998). Mutations in the C1 sequence completely eliminated triplex formation, indicating the specificity of triplex formation of these TFOs (Nakanishi et al., 1998). When these TFOs are injected intravenously into rats, we found that after initial plasma clearance, most of the TFO was distributed in liver and kidney and that more than 70% of the TFO taken up by the liver was found in the Kupffer cells, endothelial cells, and stellate cells (Cheng et al., 2005). A significant amount of the TFO, taken up by the stellate cells, is translocated to the nuclei, the site of collagen gene transcription (Cheng et al., 2005). We also demonstrated triplex formation with native DNA in cultured stellate cells (Ye et al., 2007). These results provide evidence for the proof of concept that the TFO can enter into the target cells, form triplexes, and inhibit transcription of the COLIA1 gene. In this study we show that these TFOs prevent liver fibrosis in a rat model of liver fibrosis induced by dimethyl-nitrosamine (DMN).

![FIG. 1. Nucleotide sequence of the rat COLIA1 gene promoter. The triplex-forming sequence (marked as C1), corresponding to -170 to -141 (upstream from transcription start site), containing all pyrimidines (green) in the coding strand and the complementary purines in the noncoding strand (blue), is underlined. The antiparallel phosphorothioate TFOs are shown in red. C2 corresponds to the sequence immediately upstream of C1. In this sequence the polypurines are located in the noncoding strand. ICAT: inverted CCAAT sequence. Shaded sequence is TATA box. +1 indicates transcription start site. The G-quartet-forming sequence 6 G's at the 3' end of C-1 and in the TFOs is underlined. TFO, triplex-forming oligodeoxyribonucleotide. Color images available online at www.liebertonline.com/oli.](image-url)
CONTROL OF FIBROSIS BY COLLAGEN GENE-SPECIFIC TFO

Experimental Procedures

Animals

Male Harlan Sprague-Dawley rats (Madison, WI) were used in all these experiments. Animals weighing 110–120 g were obtained and housed under controlled temperature, humidity, and light. Initially, 4 rats per cage were maintained and then shifted to 3 per cage. After 4–5 days acclimatization, rats were injected with DMN as previously reported (Yasuda et al., 1999; George et al., 2001). All experiments were performed using protocols approved by the IACUC following the guidelines for the use of animals in research administered by the University of Tennessee Health Science Center.

Protocol for liver injury and fibrosis

Animals were divided into various groups (n = 6–8 per group). Liver fibrosis was induced by repeated intraperitoneal injections of DMN, given on the first 3 consecutive days of every week for a period of 4 weeks at a dose of 1 mL of 1% solution made in saline (0.9% or 0.09%) per gram body weight. Control rats received equal volume of saline (0.9%). The antiparallel phosphorothioate TFOs were injected every alternate or third day starting on the 4th day of after first week DMN treatment. The TFOs, dissolved in 0.9% or 0.09% saline, were injected intraperitoneally at 4 mg per kg body weight. All TFOs were custom-synthesized by Invitrogen (Carlsbad, CA). The dose of DMN and the concentration of TFOs administered were adjusted for body weight each week.

At the end of 4 weeks all rats were anesthetized under inhaled isofluorane. Blood was collected from each rat at the end of the experiment with sera prepared and assayed for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. Livers were excised, and fragments either snap-frozen in liquid nitrogen for biochemical analysis or fixed in 10% buffered formalin for histological studies.

Estimation of collagen content by hydroxyproline assay

The total collagen content in the liver tissue was determined by estimating hydroxyproline, a characteristic amino acid present in collagen, as described (Edwards and O’Brien, 1980; George et al., 2001) with some modifications. Approximately 150–200 mg of liver tissue was incubated in 6 N HCl in sealed tubes at 110°C for 10–15 minutes, homogenized with a tissue homogenizer, made up to a known volume (50 mg of tissue/mL) with 6 N, and hydrolyzed for 16–18 hours. The digests were filtered through 0.2 μm filters and the clear filtrate was used for further steps. Hydroxyproline was estimated by the method described by Edwards and O’Brien (1980) with some modifications. About 60 μL of the acid lysate was neutralized with 57 μL of 6 N NaOH and dried at 50–55°C overnight, and the residue dissolved in 0.6–0.8 mL of isopropanol-sodium acetate buffer pH 6–6.5. Samples were then oxidized for 15–20 minutes at room temperature with 30–40 mM chloramine-T (Sigma, St. Louis, MO) followed by the addition of 1–1.2 mL of Ehrlich’ reagent (6% dimethylaminobenzaldehyde dissolved in perchloric acid-isopropanol). Samples were incubated at 50°C for 75 minutes or 25 minutes at 68°C, and cooled to room temperature, and the absorbance was measured at 558 nM in a spectrophotometer. Hydroxyproline (Sigma) dissolved in 0.1 M HCl was used as standard. This method is highly sensitive detecting hydroxyproline in the range of 0–1 μg in the 60 μL aliquot.

The total collagen content in the liver tissue was calculated by multiplying by a factor of 8, assuming that 12% of collagen is hydroxyproline in rat liver tissue (Edwards and O’Brien, 1980).

Histological examinations

Livers were isolated from sacrificed rats at the end of 28–29 days and stored in buffered formalin. Five-micron sections were stained with standard Massons trichrome stain.

Statistical analysis

All data were analyzed and expressed as mean ± SD. Comparisons were performed by Student’s t-test to detect differences between the groups. P values < 0.05 were considered statistically significant.

Results

Having carried out extensive studies on the efficiency and specificity of triplex formation, we wanted to test whether these TFOs are effective in preventing liver fibrosis. We tested this in the DMN model of liver fibrosis (Yasuda et al., 1999; George et al., 2001). Results presented in Fig. 2 indicate that the animals treated with the wild-type TFO, but not the mutant TFO, significantly inhibited accumulation of collagen in the liver, suggesting that triplex formation is required for this inhibitory effect. The mutant TFO does not form triplexes even at 1000-fold higher concentrations compared with the wild type (Nakanishi et al., 1998). Since the mutant TFO has no inhibitory effect, in all the subsequent experiments we used only the 3 TFOs (TFO 25-mer, 158TFO, and the 164TFO). The sequences of these 3 TFOs are shown in Fig. 1.

FIG. 2. Effect of the TFO on collagen accumulation. Rats were treated with saline (control), DMN, DMN +25-mer TFO or the mutant (TFO-M), and collagen content was estimated by hydroxyproline assays. The wild-type TFO, GCGAAG GAAAGGGAGGAGGGGGGAG, forms efficient triplexes, whereas the mutant (TFO-M, GAGGAAG GAAAGGGAGGAGGGGGGAG) does not form triplexes. Only the phosphorothioate TFOs were injected into rats. Note that the wild-type TFO significantly inhibited collagen accumulation compared with the mutant. The inhibition by TFO but not by the mutant is highly significant (P < 0.05) compared with the mutant. DMN, dimethylnitrosamine.
Earlier, we demonstrated triplex formation not only with the 25-mer but also with 2 other overlapping 18-nucleotide long TFOs, which are referred to as 158TFO (−158 to −141, relative to the transcription start site) and 164TFO (−164 to −147) (Joseph et al., 1997). Triplex formation was efficient with all the 3 TFOs with a Kd $\approx 5 \times 10^{-8}$ to $5 \times 10^{-7}$ for the rodent sequence and $\approx 10^{-8}$ for the human sequence (Joseph et al., 1997; Nakanishi et al., 1998). Earlier, we demonstrated G-quartet formation by gel retardation assay with the 25-mer TFO, which contains the G-quartet-forming sequence (Cheng et al., 2005). Here we repeated the experiment with the 158TFO and showed that it too forms G-quartets because of the presence of 6 consecutive G residues in a salt-dependent reaction (Fig. 3A). We have also determined plasma clearance rate of the antiparallel phosphorothioate TFOs in rats and found to be cleared with a clearing time of about 34 minutes. Tissue uptake studies indicated that maximum accumulation occurred in the liver and kidney, and in the liver as much as 50% of the injected dose was found to be accumulated in about 100 minutes, with about 28% of the TFO remained in the liver after 24 hours (Cheng et al., 2005).

If triplex formation with the COL1A1 gene promoter is required for inhibition of transcription, then this ability should be abrogated by G-quartet (quadruplex) formation, because in the G-quartet form the 158TFOs do not form triplexes. We also found that the preformed G-quartets could be disrupted by boiling in water or in 0.09% saline (Fig. 3A). The boiled (monomeric form) and the control nonheated (quadruplex form) were injected at 4 mg/kg body weight by intraperitoneal route every third day after the first 3 DMN injections. At the end of 4 weeks, rats were sacrificed, livers were isolated, and collagen content was determined by hydroxyproline assay. It is clear from Fig. 3B that the TFO (158TFO) under G-quartet forming conditions (158TFO-G) has no effect on collagen, whereas the same TFO dramatically inhibited collagen levels, suggesting that triplex formation is necessary for the observed inhibition of collagen synthesis and accumulation. Visual inspection on the hardness, texture, and color of the livers indicated that all 8 out of 8 rat livers injected with DMN+158TFO-G appeared fibrotic-like DMN alone (Table 1), whereas 6 out of 8 livers of rats treated with DMN + 158TFO and 5 out of 8 treated with the 25-mer appear to be soft and less fibrotic (Table 1). Histological examination of liver tissues using Masson’s trichrome staining to detect fibrillar collagen confirmed that the TFO effectively inhibited accumulation of fibrous tissue (Fig. 3C). Again, the mutant TFO and the wild-type 158TFO under G-quartet-forming conditions had no

---

**FIG. 3.** Effect of the G-quartet-forming TFO on collagen accumulation in rat livers. (A) Effect of boiling on G-quartet formation in different concentrations of saline. (B) 158TFO-G refers to G-quartet. Note that 158TFO significantly reduced collagen accumulation ($P < 0.5$) compared with DMN or DMN rats treated with the same 158TFO injected without boiling (favors G-quartet formation), suggesting that triplex formation is required for this effect. Inhibition of collagen accumulation by the 158TFO is highly significant at $P < 0.05$. (C) Effect of the TFOs on collagen accumulation. Liver sections were stained with standard Masson’s trichrome stain. 1, Control rat liver; 2 and 3, DMN-treated livers; 4 and 5, DMN+158TFO-G treated livers; 6, DMN+158TFO-treated liver. Magnification, 20x.
effect on preventing hepatic fibrosis. From these results we conclude that these 3 TFOs (25-mer TFO, 158TFO, and 164TFO) prevent fibrosis and that triplex formation is essential for the observed inhibitory effect.

In the next experiment we studied whether the TFO itself has any effect on fibrosis and whether both 18-mer TFOs with (158TFO) or without (164TFO) G-quartet-forming ability have any effect on collagen accumulation. We injected the 158TFO TFOs at 4 or 10 mg/kg body weight after DMN treatment and the 164TFO at 10 mg/kg rat weight. The results indicate that the TFO itself has no adverse effect on rats, on liver-to-body weight ratio (Fig. 4A), alanine aminotransferase (ALT) levels (Fig. 4B), or on hepatic collagen content as determined by hydroxyproline assay (Fig. 4C). However, collagen accumulation was significantly abated even at 4 mg/kg by both 158TFO and 164TFOs in the rats treated with DMN+TFO (Fig. 4C). The 164TFO without the G-quartet-forming sequence, although has the ability to form efficient triplexes (17), is less effective in reducing collagen accumulation than 158TFO. Nevertheless, both these TFOs can be used to control fibrosis. The TFO by itself even at 10 mg/kg body weight has no effect on collagen synthesis, liver-to-body weight ratio, or ALT levels compared with saline-injected controls. DMN significantly affected the growth of the rats and the TFOs did not significantly improve these weights, although the overall

**FIG. 4.** Effect of 158TFO (10 mg/kg body weight) on collagen accumulation in rat livers. Rats were injected with TFO alone or DMN+158TFO (4 mg/kg or 10 mg/kg) and the 164TFO (10 mg/kg), and sacrificed on the 28th day. (A) Livers were excised on day 28 and analyzed for collagen content by the hydroxyproline assay. Liver weight-to-body weight ratio (LW/BW) multiplied by 100 (A) is plotted against different treatments. (B) Alanine aminotransferase (ALT) levels of rats treated with different TFOs. (C) Note that the liver-to-body ratio drastically reduced in DMN-treated rats but not in those treated with 158TFO alone. Also note that the TFO alone has no effect on liver weight/body weight ratio or on ALT levels. The TFO 158TFO at both 4 mg/kg and 10 mg/kg significantly improved liver weights ($P < 0.05$) and reduced ALT levels as well as collagen accumulation. The LW/BW ratio of rats treated with 164TFO, although showing improvement, appears not statistically significant compared with DMN alone.

---

### Table 1. Appearance of Livers in Dimethylnitrosamine and Dimethylnitrosamine + Triplex-Forming Oligodeoxyribonucleotide-Treated Rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Liver appearance</th>
<th>Rat livers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Normal</td>
<td>6/6</td>
</tr>
<tr>
<td>DMN</td>
<td>Fibrotic, hard</td>
<td>6/6</td>
</tr>
<tr>
<td>DMN + 158TFO</td>
<td>Mild to normal fibrotic</td>
<td>6/8/2/8</td>
</tr>
<tr>
<td>DMN + 158TFO-G</td>
<td>Fibrotic, hard</td>
<td>8/8</td>
</tr>
<tr>
<td>DMN + 25-merTFO</td>
<td>Mild to normal fibrotic</td>
<td>5/8/3/8</td>
</tr>
</tbody>
</table>

Color, morphology, and texture were assessed by a veterinarian. Note that the majority of livers of rats treated with 158TFO or the 25-mer TFO appear to be less fibrotic compared with the DMN or DMN+TFO-G-treated rats.

DMN, dimethylnitrosamine; TFO, triplex-forming oligodeoxyribonucleotide.
Discussion

The results presented here clearly demonstrate the efficacy of these TFOs in preventing liver fibrosis. Several antisense oligodeoxyribonucleotides (ODNs) targeting various messenger RNAs are in various phases of clinical trials. It has been shown that these are well tolerated and not toxic even at 50 mg/kg body weight. In our previous experiments, using 33P-labeled TFO, we showed that the TFOs are rapidly cleared from plasma and are distributed into various tissues; we detected maximum uptake by liver in a concentration-dependent manner (Cheng et al., 2005). The TFOs must enter the activated stellate cells of fibrotic rat livers, translocate to the nucleus where they form triplexes, and inhibit transcription, as these are the cells involved in collagen synthesis and secretion into extracellular matrix. Indeed earlier, we demonstrated uptake of the TFO by activated HSC in fibrotic rats and that a significant amount also accumulated in the nuclei of stellate cells (Cheng et al., 2005; Ye et al., 2007). We also demonstrated formation of triplexes with the native collagen gene promoter in a stellate cell line, HSC-6, using psoralen cross-linked TFO (Ye et al. 2007). Although we have not directly demonstrated triplex formation and inhibition of transcription in rat livers, the fact that both the mutant TFO and the G-quartet TFO, which do not form triplexes, have no effect on collagen synthesis and accumulation (Figs. 2 and 3) strongly suggests that triplex formation is necessary for inhibition of transcription.

We could not detect any differences in the body weights, liver weights, and ALT levels in the TFO-treated rats compared to saline controls (Fig. 4), suggesting that the TFO at 10 mg/kg is not toxic to rats. Formation of triplexes with the native promoter of the COL1A1 gene should inhibit primary transcription. It was shown that upon activation of quiescent stellate cells into activated stellate cells z 1(I) mRNA levels have been shown to increase by more than 60–70-fold (Stefanovic et al., 1997). Also the half-life of collagen mRNA is increased by about 16-fold (24 hours in activated HSC compared to 1.5 hours in quiescent HSC), which is due to both transcriptional and posttranscriptional regulation (Stefanovic et al., 1997). Inhibition of any of these steps by the TFO could result in prevention of fibrosis. It is possible that, since both genes are coordinately regulated (Karsenty and Park, 1995; Canty and Kadler, 2005; Han et al., 2008), inhibition of COL1A1 transcription may also result in reduced synthesis of COL1A2 mRNA because some of the same transcription factors are involved in this coregulation. In addition, inhibition of transcription of the COL1A1 gene by the TFO could result in perturbing the stoichiometry of z1 (I) and z2 (I) polypeptide chains, thereby affecting intracellular processing of procollagen into the mature secreted forms. Indeed it was shown that in mild cases of osteogenesis imperfecta the abnormal pro-z1 (I) polypeptide chains are degraded as soon as they are synthesized. As a consequence, cells produce excess pro-z2 (I) chains, which cannot form trimers and they are not secreted (Gotkin et al., 2004). Targeting only the COL1A1 gene by the TFOs, which we are doing here, can result in changing the stoichiometry of z1(I) and z2(I) chains within stellate cells, and alter the rate of formation of fibrils as well as secretion into extracellular matrix. More experiments with stellate cells isolated from control and DMN or DMN + TFO-treated rat livers have to be carried out to show this abnormal processing. Many inhibitors, targeting various cytokines and enzymes involved in collagen synthesis, are being developed for controlling fibrosis (Rockey, 2008). Some success has been achieved in controlling fibrosis and cirrhosis by treating the underlying disorders. For example, it has been shown that inhibition of HBV or HCV by antiviral drugs could reverse cirrhosis in some patients (Mallet et al., 2007). Of several other compounds tried, interferon y appears to be very promising in preventing fibrosis (Weng et al., 2005; Pockros et al., 2007). However, as yet none of these agents is commercially available. The results presented here clearly indicate that this novel strategy, targeting type I collagen gene and tissue collagen content, may lead to the development of this TFO as a potential antifibrotic molecule.

Acknowledgments

This work was supported by a grant (DK-064366) from the NIH. We thank Drs. Ken Nishimoto and Liz Fitzpatrick for critically reading the article.

Author Disclosure Statement

No competing financial interests exist.

References


Address correspondence to:
Prof. Ramareddy V. Guntaka
Department of Molecular Sciences
The University of Tennessee Health Science Center
858 Madison Ave.
Memphis, TN 38163
E-mail: rguntaka@uthsc.edu

Received for publication May 28, 2010; accepted after revision July 21, 2010.