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Effects of TGF-β1 Ribbon Antisense on CCl_4-induced Liver Fibrosis

Kyung-Oh Doh

Department of Physiology, College of Medicine, Dongguk University, Gyeongju 780-714, Korea

Ribbon-type antisense oligonucleotide to TGF-β1 (TGF-β1 RiAS) was designed and tested to prevent or resolve the fibrotic changes induced by CCl_4 injection. When Hepa1c1c7 cells were transfected with TGF-β1 RiAS, the level of TGF-β1 mRNA was effectively reduced. TGF-β1 RiAS, mismatched RiAS, and normal saline were each injected to mice via tail veins. When examined for the biochemical effects on the liver, TGF-β1 mRNA levels were significantly reduced only in the TGF-β1 RiAS-treated group. The results of immunohistochemical studies showed that TGF-β1 RiAS prevented the accumulation of collagen and α-smooth muscle actin, but could not resolve established fibrosis. These results indicate that ribbon antisense to TGF-β1 with efficient uptake can effectively prevent fibrosis of the liver.

Key Words: Transforming growth factor-β1, Liver cirrhosis, Ribbon antisense, Cationic peptide

INTRODUCTION

Liver cirrhosis is the common pathological consequences of chronic liver injury caused by a variety of agents, including viruses, alcohol, hepatotoxins, and autoimmune disorders (Friedman, 2000). Fibrogenesis is characterized by an excessive accumulation of extracellular matrix (ECM), as the result of an imbalance of its synthesis versus degradation (Arthur, 2000). Although the mechanisms underlying the progression of liver cirrhosis have yet to be fully elucidated, cytokines have been implicated as mediators of fibrosis in the liver. Among these cytokines, transforming growth factor-β1 (TGF-β1) is particularly well-studied, and has been recognized as pro-fibrogenic in the case of liver injury (Kanzler et al, 1999; Bissell et al, 2001; Gressner et al, 2002). TGF-β1 is involved in the accumulation of ECMs for normal repair as a response to tissue injury, and is also responsible for fibrous changes due to aberrant overproduction of ECMs, including proteoglycans, collagens, fibronectin, and glycoproteins. TGF-β1 also inhibits the degradation of newly synthesized matrix protein via an upregulation of the synthesis of protease inhibitors and a downregulation of the synthesis of matrix-degrading proteases (Knittel et al, 1999). Thus, an effective blockade of TGF-β1 synthesis or action appears to constitute a promising approach for the prevention of fibrous conditions, as suggested by previous reports (George et al, 1999; Qi et al, 1999; Ueno et al, 2000).

Antisense oligonucleotides (AS oligos) have proven to be valuable in the functional study of gene products, as they reduce the expression of genes in a sequence-specific manner. However, the use of AS oligos is still hindered by several critical problems, including instability to nuclease, sequence nonspecificity, and inadequate cellular uptake (Wagner et al, 1993; Gryaznov et al, 1996). A variety of chemically modified oligos, including phosphorothioate and methylphosphonate oligos, have been developed as a measure to augment stability against nucleases. However, each of these modified oligonucleotides suffered from its own problems, which included a lack of sequence specificity, insensitivity to *RNase* H, and the prolongation of partial thrombosis time (Henry et al, 1997). Recently, it was reported that ribbon-type antisense (RiAS) oligos, which possess a covalently closed structure, are quite stable and effective in the specific ablation of target mRNA, and that they are associated with few of the problems of other modified AS oligos (Moon et al, 2000a; Moon et al, 2000b; Bajpai et al, 2005).

The cellular uptake of AS oligos can be enhanced via the formation of complexes such as liposomes. Although liposomes exhibit several advantages, including low toxicity, lack of immunogenicity, and simple production, they tend to manifest relatively poor cellular uptake. Recent studies have demonstrated the utility of the cationic peptide as a delivery vehicle for biologically active drugs, including antisense oligonucleotides, both in cell cultures and *in vivo* (Schwarze et al, 1999; Fulda et al, 2002). These peptides are derived from the HIV Tat protein (Vives et al, 1997; Schwarze et al, 1999; Eguchi et al, 2001; Futaki et al, 2001), SV40 large T antigen (Zanta et al, 1999; Torchilin et al, 2001), *Drosophila Antennapedia* (Derossi et al, 1996), and histone H1 (Sorgi et al, 1997; Bharath et al, 2002). In this study, the modified nuclear localization signal (NLS) of human immunodeficiency virus (HIV)-1 Tat protein (Moon et al, 2007) was used as a vehicle both *in vitro* and *in vivo*. Thus, a RiAS to TGF-β1 was designed and tested to prevent or resolve CCl_4-induced liver fibrosis and tissue damage. In

Correspondence to: Kyung-Oh Doh, Department of Physiology, College of Medicine, Dongguk University, 707, Seokjang-dong, Gyeongju 780-714 Korea. (Tel) 82-54-770-2413, (Fax) 82-54-770-2440, (E-mail) kodoh@dongguk.ac.kr

ABBREVIATIONS: ALT, alanine aminotransferase; AST, aspartate aminotransferase; DP complex, DNA/peptide complex; ECM, extracellular matrix; RiAS, ribbon-type antisense; TGF-β1, transforming growth factor-β1; NLS, nuclear localization signal; HIV, human immunodeficiency virus.

an attempt to enhance cellular uptake, the TGF-β1 RiAS was added to the cationic peptide, thereby forming a DNA / peptide (DP) complex.

METHODS

Cell line and animals

Hepa1c1c7 (mouse hepatoma cell line) was obtained from the American Type Culture Collection (ATCC) and maintained in α-MEM containing 10% heat-inactivated fetal bovine serum (Welgene, Daegu, Korea) in a humidified 5% CO_2 incubator at 37°C. Sixty ICR male mice, weighing 30~35 g, were supplied by SLC (Hamamatsu, Shizuoka, Japan). All animals received humane care. Animal experiments were performed according to international guidelines concerning the conduct of animal experimentation. In order to induce liver cirrhosis, 1 ml/kg (body weight) of CCl_4 was intraperitoneally administered twice per week (Fig. 1). RiAS to mouse TGF-β1 (100 μg/30 g body weight) was also intravenously administered twice per week from the second week of experiment in the prevention group (n=20), and 5th week of experiment in the treatment group (n=20). Mismatched RiAS was intravenously administered twice per week at an identical dosage in the mismatched group (n=20). Normal saline was intravenously administered twice per week at an equal volume in the control group (n=20). At the administration of the drugs, RiAS was mixed with cationic peptide (DP complex) at a ratio of 1 : 3. Prior to sacrifice, the blood was collected in order to measure the serum parameters. Serum biochemical parameters were determined via standard spectrometric methods.

Construction of mouse TGF-β1 RiAS

Target sites for RiAS were selected via the sequential overlap simulation of secondary structures using the DNAsis program (Hitachi Software, San Bruno, CA, USA). The antisense sequence was 5'-gatccaggccacatgttgctccacacttgattttaatctctgcaacctg-3' (from 49 bp on the TGF-β1 sequence), and the mismatched sequence was 5'-gatccaggccacatattactgcatacatgcttatattctctgcaacctg-3'. Oligonucleotides were synthesized as previously described (Moon et al, 2000a) via standard phosphoramidite chemistry using an automated DNA synthesizer, Expedite^{TM}8909 (Applied Biosystems, Foster City, CA, USA). Two molecules of the TGF-β1 antisense of the stem-loop structure were ligated in order to form a ribbon-type antisense molecule by the complementary 4 base sequences at the 5' ends of the molecules at 16°C with T4 DNA ligase overnight.

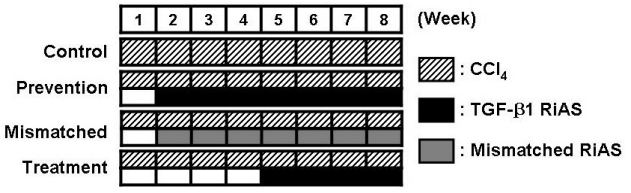

Fig. 1. Experimental schedule. For 8 weeks of experiment, 1 ml/kg (body weight) of CCl_4 was intraperitoneally administered twice per week. RiAS to mouse TGF-β1 or mismatched RiAS (100 μg/30 g body weight) was also intravenously administered twice per week from the second week of experiment in the prevention group and mismatched group, and from the 5th week of experiment in the treatment group.

Peptide synthesis and modifications

Peptide used in the present study was derived from the Tat protein of HIV-1 which has been reported as effective in DNA delivery (Moon et al, 2007). The Tat peptide corresponds to the nuclear localization signal (NLS) sequence of 9 amino acids (49-57: Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg). The peptide was modified at C-terminus by the addition of cysteine residue. Peptides were prepared in a solid phase synthesis by a peptide synthesizer ABI 433A (Applied Biosystems, Foster City, CA, USA), purified by preparative LC, and characterized using an analytical HPLC system (Shimadzu, Kyoto, Japan) comprised of a C18 column and a MALDI-TOF Mass spectrometer (Applied Biosystems, Foster City, CA, USA). Purified peptides were resuspended in ddH_2O at a concentration of 10 μg/ μl, and kept at −70°C prior to further use.

Transfection efficiency of RiAS in vitro and in vivo

FITC-labeled TGF-β1 RiAS was synthesized via the incorporation of fluorescein-11dUTP instead of TTP. Hepa1c1c7 cells seeded in each well of a 24-well plate were treated with the DP complex, containing FITC-labeled TGF-β1 RiAS (0.3 μg), in a 200 μl volume. The DP complex, containing FITC-labeled TGF-β1 RiAS (0.3 μg) in 600 μl saline, was infused into the tail veins of mice for *in vivo* study. The liver was removed after 16 hours, and the tissue blocks of the fixed liver were cryosectioned to a thickness of 10 μm for slide mounting. Gene transfer efficacy was evaluated via fluorescent microscopy.

RT-PCR for mouse TGF-β1 expression

After the transfection of the TGF-β1 RiAS, TGF-β1 expression was assessed via RT-PCR. RNA was prepared with Welprep^{TM} RNA isolation reagent (Welgene, Daegu, Korea). The purified RNA was subjected to RT-PCR using the Access RT-PCR kit (Promega, Madison, WI, USA) and a thermal cycler (MJ Research, Watertown, MA, USA). The following primer pairs were used: forward 5-ggactctccacctgcaagac-3 and reverse 5'-gactggcgagccttagtttg-3' for mouse TGF-β1, and forward 5'-agtgtgacgttgacatccgta-3' and reverse 5'-gccagagcagtaatctccttct-3' for mouse β-actin. The PCR products were confirmed on 1% agarose gel, and quantitative analysis of the amplified DNA was conducted using the AlphaImager 1220, a gel documentation apparatus (Alpha Innotech, San Leandro, CA, USA).

Histological analysis

The fixation and embedding of liver tissues were conducted as previously described (Choi et al, 2005). The tissue sections were incubated overnight with anti-type I collagen antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and (-smooth muscle actin antibody (Dako, Glostrup, Denmark) in PBS containing 0.5% BSA and 2% FCS at 4°C. The next day, the tissue sections were incubated with anti-rabbit HRP conjugates for 1 hour at room temperature. The fibrous lesion areas were also determined via Masson's trichrome method, which is used to stain collagen fibers.

Statistical analysis

Results are expressed as means±standard deviations (SD). Statistical significance was assessed via one-way ANOVA. p values of < 0.05 were considered to be significant.

RESULTS

Construction of ribbon antisense oligos to TGF-β1 and specific reduction of TGF-β1 mRNA

Two identical AS oligos possessing the stem-loop structure were covalently ligated to form a ribbon-antisense molecule, termed TGF-β1 RiAS (Fig. 2A). These TGF-β1 RiAS molecules possess a ribbon-type closed structure without an open end that would be attacked by exonucleases. We then determined whether TGF-β1 RiAS was effective in the elimination of target mRNA in a sequence-specific manner, when delivered as a DP complex as shown Fig. 2B. Hepa1c1c7 cells treated with 0.1 μg TGF-β1 RiAS showed a reduction of TGF-β1 RNA by 71% and by 97% at 0.3 μg. On the other hand, when Hepa1c1c7 cells were treated with mismatched RiAS, TGF-β1 expression was not significantly affected.

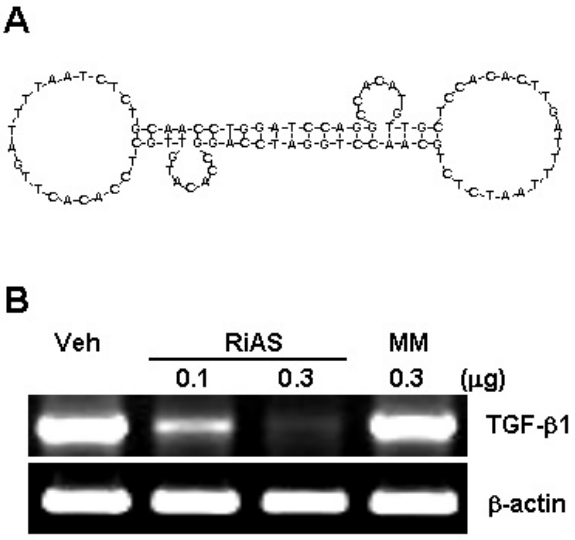

Fig. 2. (A) Schematic representation of ribbon-type antisense to TGF-β1 (TGF-β1 RiAS). The stem is formed by complementary sequences at both ends of each oligo. The 5' terminus of the stem has 4 bases of a single-stranded overhang of 5'-GATC-3'. Two TGF-β1 monomer molecules were ligated to generate a covalently closed molecule with diad symmetry. The RiAS oligos consist of two loops and an intervening stem. Each loop harbors an antisense sequence to TGF-β1. (B) Specific reduction of TGF-β1 mRNA by TGF-β1 RiAS. Hepa1c1c7 cells were transfected with DP complex and RT-PCR was conducted in order to determine the antisense activity of TGF-β1 RiAS. Transfection of TGF-β1 RiAS reduced TGF-β1 expression in Hepa1c1c7 cells. By way of contrast, however, when Hepa1c1c7 cells were treated with mismatched RiAS, TGF-β1 expression was not significantly affected. Mouse β-actin was as a control. Veh: vehicle, RiAS: TGF-β1 RiAS, MM: mismatched RiAS.

Efficient cellular uptake of TGF-β1 RiAS when delivered as a DP complex

Nucleic acid including antisense oligomers shows poor cellular uptake, largely due to the charged polymeric backbone. In order to study the cellular uptake, the TGF-β1 RiAS was labeled with FITC-11dUTP incorporation during chemical synthesis and used for both *in vitro* and *in vivo* uptake study. When the FITC-labeled antisense oligos were used to form the DP complex and added to the Hepa1c1c7 cells, the cells showed strong fluorescence signals (Fig. 3A). Thus, we attempted to determine whether the DP complex could also be employed for efficient delivery of RiAS into the liver after intravenous infusion. For *in vivo* tissue uptake, 10 μg of FITC-labeled TGF-β1 RiAS were infused into the tail vein of mouse, and the liver was harvested 24 hours after treatment. Whereas the intravenously infused DP complex showed strong fluorescent signals in the liver cells, indicating efficient cellular uptake, the fluorescent signals were quite weak in tissues when only the FITC-labeled 'naked' RiAS was infused. Control livers treated with sham treatment showed no fluorescent signals.

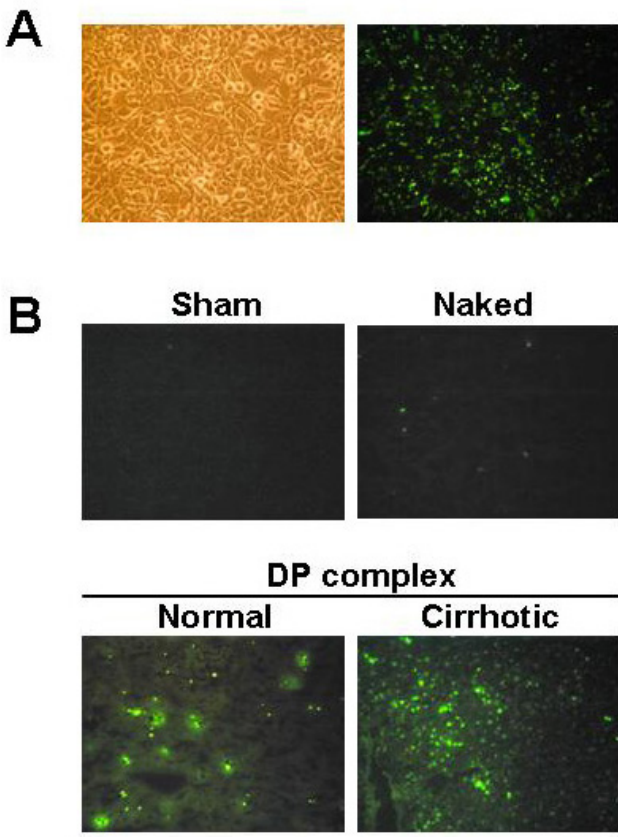

Fig. 3. (A) The DP complex mediated transfection in Hepa1c1c7 mouse hepatoma cells. Transfection of FITC-labeled TGF-β1 RiAS was conducted using cationic peptide. The DP complex was added to Hepa1c1c7 cells for 24 hours. Fluorescence signals are shown in the right panel. (B) Normal saline (sham) or 10 μg of FITC-labeled TGF-β1 RiAS as a form of naked DNA only and DP complex were injected through the tail veins of normal or cirrhotic mice. Tissue sections of mice liver were observed under a fluorescence microscope (×200).

Table 1. Effect of TGF-β1 RiAS on serum biochemical parameters

	Control	Mismatched	Prevention	Treatment
Aspartate aminotransferase (U/l)	605.0±199.9	578.1±217.4	147.4±50.2*	173.14±57.9*
Alanine aminotransferase (U/l)	316.3±100.7	367.7±125.8	104.3±17.5*	135.9±45.0*
Albumin (g/dl)	3.2±0.2	3.3±0.2	3.4±0.2	3.2±0.2
Total bilirubin (mg/dl)	0.3±0.2	0.2±0.2	0.2±0.1	0.2±0.1

After 8 weeks of experiments, blood was collected from each groups and analyzed. Data are expressed as mean±SD, n=5 to 8 per group (*$p < 0.05$ vs. control).

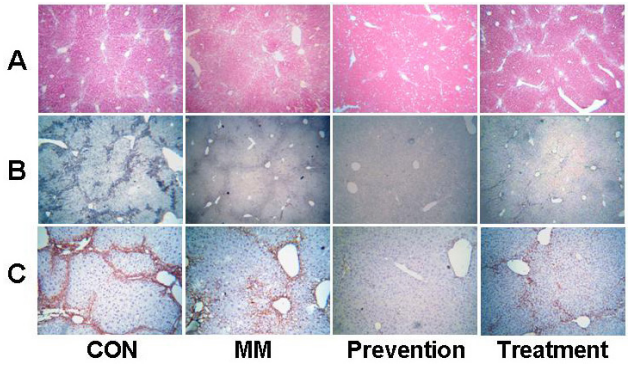

Fig. 4. Histological observation of liver. Collagen deposition was detected as blue staining on Masson's trichrome staining (A). Immunohistochemistry for type I collagen (B) and α-smooth muscle actin (C). Staining was conducted with fixed and dehydrated tissues from mice treated with normal saline (CON), mismatched RiAS (MM), and TGF-β1 RiAS (Prevention and Treatment group). Stained tissues were mounted with a synthetic mounting solution for microscopic observation (×200).

We also observed that the DP complex could be employed for efficient RiAS uptake in cases of CCl_4-induced liver cirrhosis (Fig. 3B).

Effects of TGF-β1 RiAS on liver cirrhosis

All mice in the prevention and treatment groups treated with TGF-β1 RiAS survived, whrereas the control group and the mismatched RiAS-treated group showed survival rates of 50% and 65%, respectively. At the end of 8 weeks of the experiment, TGF-β1 RiAS treated prevention and treatment groups showed lower levels of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) than those in the control mice and the mice treated with mismatched RiAS. However, no differences were noted among the groups in terms of albumin and total bilirubin levels (Table 1).

Effect of TGF-β1 RiAS treatment on histology and collagen synthesis

CCl_4 injected intraperitoneally induced massive accumulation of connective tissues, principally in the centrilobular area and portal tract, when examined by Masson's trichrome-stained sections of the livers from both the control group and the mismatched RiAS-treated group. However, TGF-β1 RiAS treatment significantly ameliorated the deposition of connective tissues (Fig. 4A). Positive collagen staining was detected at comparable levels in the livers treated with either normal saline or mismatched RiAS. In contrast, type I collagen was found to be greatly reduced in the TGF-β1 RiAS-treated livers (Fig. 4B). Immunohistochemistry of the (-smooth muscle actin, a marker for hepatic stellate cells, showed marked reductions in the livers of the TGF-β1 RiAS-treated group as compared with the control group and the mismatched RiAS-treated group (Fig. 4C). However, histological analysis showed no marked resolution of fibrosis in the treatment group. These data showed that TGF-β1 RiAS treatment can prevent the development of fibrosis.

DISCUSSION

In this study, we evaluated the ability of RiAS to TGF-β1 to eliminate target mRNA and to alleviate global tissue injuries in cases of liver cirrhosis. We introduced the FITC labeled RiAS / cationic peptide complex via the tail vein and found that it was delivered effectively to the cirrhotic livers, as well as the normal livers. RiAS to TGF-β1 was also efficiently delivered into the liver, and blocked CCl_4-induced fibrosis, including collagen accumulation. TGF-β1 is the most potent profibrogenic factor in human fibrogenesis (Gressner et al, 2002). Thus, many studies on the blockage of TGF-β1 synthesis or action have been conducted in order to develop a method for the prevention of liver cirrhosis, and a variety of strategies, including the use of adenoviral vectors expressing truncated TGF-β type II receptor (Qi et al, 1999; Ueno et al, 2000; Nakamura et al, 2004), chimeric IgG harboring the extracellular portion of the TGF-β type II receptor (George et al, 1999), and adenoviral expression of a TGF-β1 antisense (Arias et al, 2002; Arias et al, 2003), have all been identified as effective strategies.

Antisense oligonucleotides in general show poor cellular uptake due to the charges on their polymeric backbone. Cellular uptake of oligonucleotides can be improved when complexed with cationic vehicle. However, non-viral delivery vehicles, including liposomes, do not exhibit uptake efficiency satisfactory for many types of cells, particularly cells in primary cultures. Thus, an improved transfection reagent would clearly benefit both *in vitro* cell-line studies and *in vivo* applications. Some cationic peptides such as HIV Tat protein, SV40 large T antigen, *Drosophila Antennapedia*, and histone H1 have been found to show nucleic acid condensation, membrane penetration, and nuclear localization activities (Derossi et al, 1996; Sorgi et al, 1997; Vives et al, 1997; Ludtke et al, 1999; Schwarze et al, 1999; Zanta et al, 1999; Dokka et al, 2000; Eguchi et al, 2001; Futaki et al, 2001; Torchilin et al, 2001; Bharath et al, 2002). In this study, we modified NLS of HIV-1 Tat protein and devised a simple DP complex composed of RiAS / cationic

peptide in an effort to augment cellular uptake. Although portal myofibroblasts (Ramadori and Saile, 2004) and cells from bone marrow origin (Forbes et al, 2004) have been demonstrated to have fibrogenic potential, the activation of the hepatic stellate cells is the most important event in liver fibrogenesis (Friedman et al, 1985). It has been shown that the quiescent hepatic stellate cells are transformed into active myofibroblasts expressing α-smooth muscle actin, and that these active hepatic stellate cells are the primary source of extracellular matrix proteins (Alcolado et al, 1997; Iredale et al, 1998; Friedman, 2000). Our present results indicated that TGF-β1 RiAS reduced the accumulation of collagen and α-smooth muscle actin, revealed by immunohistochemical staining. Contrary to what has traditionally been thought, it is now believed that even advanced liver fibrosis is reversible. Therefore, many scientists have attempted to discover new antifibrotic drugs (Hammel et al, 2001). In the present study, we attempted to treat liver fibrosis, which was formed after 4 weeks of intraperitoneal injection of CCl_4 with TGF-β1 RiAS for 4 weeks, however, there was only a marginally resolution of established fibrosis. This insufficient resolution may be attributable to ECM cross-linking or insufficient treatment time, because it takes a long time to recover from fibrosis (Issa et al, 2004).

In conclusion, ribbon antisense to TGF-β1 mRNA coupled with enhanced transfection using the antisense oligos / cationic peptide complex was found to reduce the levels of target mRNA in a sequence-specific manner, and was able to alleviate global tissue fibrosis in the livers of CCl_4-treated mice. As tissue fibrosis is a convergence point in the progression of many human diseases of the liver, kidney and lung, the potent antisense activity of RiAS TGF-β1 in the sequence-specific elimination of the target mRNA may prove to be useful in the development of antisense medicine against fibrous conditions in these human tissues.

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