Prevention of CCl_4-induced liver cirrhosis by ribbon antisense to transforming growth factor-ß1

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Abstract. Transforming growth factor-ß1 (TGF-ß1) is an important mediator of tissue fibrosis, including liver cirrhosis. Ribbon-type antisense oligonucleotide to TGF-ß1 (TGF-ß1 RiAS) was designed and combined with cationic peptide derived from the nuclear localization signal of human immunodeficiency virus-1 Tat protein for enhanced cellular uptake. When Hepa1c1c7 cells were transfected with TGF-ß1 RiAS, the level of TGF-ß1 mRNA was reduced by >70%. TGF-ß1 RiAS, mismatched RiAS, and normal saline were each injected into mice via the tail vein, beginning the week after intraperitoneal CCl_4 injection and continuing for 7 weeks, in order to determine whether TGF-ß1 RiAS prevents the fibrotic changes induced by the CCl_4 injection. After 8 weeks of the experiment, all of the mice treated with TGF-ß1 RiAS survived, compared to 50% of the control group and 65% of the mismatched RiAS-treated group. Upon examining the biochemical effects on the liver, TGF-ß1 mRNA levels were reduced significantly only in the TGF-ß1 RiAS-treated group. Immunohistochemical studies showed a reduced accumulation of collagen and α-smooth muscle actin. Our experimental results suggest that ribbon antisense to TGF-ß1, with efficient uptake, effectively blocks the expression of TGF-ß1 and prevents fibrosis of the liver.

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Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; DP complex, DNA/peptide complex; ECM, extracellular matrix; RiAS, ribbon-type antisense; TGF-ß1, transforming growth factor-ß1; NLS, nuclear localization signal; HIV, human immunodeficiency virus

Key words: transforming growth factor-ß1, liver cirrhosis, ribbon antisense, cationic peptide

Introduction

Liver fibrosis and cirrhosis are the common pathological consequences of chronic liver injury caused by a variety of agents, including viruses, alcohol, hepatotoxins, and autoimmune disorders (1). Fibrogenesis is characterized by an excessive accumulation of extracellular matrix (ECM) as the result of an imbalance of its synthesis versus degradation (2). 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-ß (TGF-ß) has been particularly well-studied and is recognized as being profibrogenic in the case of liver injury (3-5). TGF-ß1 is involved in the accumulation of ECMs as a response to tissue injury for normal repair, and is also responsible for fibrous changes due to aberrant overproduction of ECMs, including proteoglycans, collagens, fibronectin, and glycoproteins. TGF-ß1 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 (6). Thus, the effective blockade of TGF-ß1 synthesis or action appears to constitute a promising approach for the prevention of fibrous conditions, as suggested by previous reports (7-9).

Antisense oligonucleotides (AS oligos) have proven valuable in the functional study of gene products as they reduce the expression of genes in a sequence-specific manner. However, the use of oligos is still hindered by several critical problems, including an instability to nuclease, sequence nonspecificity, and inadequate cellular uptake (10,11). 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 suffers from its own problems, such as lack of sequence specificity, insensitivity to *RNase* H, and the prolongation of partial thrombosis time. We reported previously that ribbon-type antisense (RiAS) oligos possessing a covalently closed structure were quite stable and effective in the specific ablation of target mRNA and were associated with few of the problems of other modified AS oligos (12-14). We also previously reported that

ribbon antisense to TGF-ß1, when coupled with efficient uptake, effectively blocks the expression of TGF-ß1 and preserves tissue integrity in kidneys with unilateral ureteric obstructions (15).

The cellular uptake of AS oligos can be enhanced via the formation of complexes with liposomes. Although liposomes exhibit several advantages, such as low toxicity, lack of immunogenicity, and simple production, liposomes tend to manifest relatively poor cellular uptake. 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* (16,17). These peptides are derived from the HIV Tat protein (16,18-20), SV40 large T antigen (21,22), *Drosophila Antennapedia* (23), protamine sulfate (24,25) and histone H1 (26,27). In this study, we modified the nuclear localization signal (NLS) of human immunodeficiency virus (HIV)-1 Tat protein and used this as a vehicle both *in vitro* and *in vivo*.

In the present study, a RiAS to TGF-ß1 was designed and tested with regard to its antisense activity for the prevention of CCl_4-induced liver fibrosis and tissue damage. In an attempt to enhance cellular uptake, the TGF-ß1 RiAS was added to the cationic peptide, thereby forming a DNA/peptide (DP) complex. The DP complex harboring TGF-ß1 RiAS was determined to be very effective in the blockage of TGF-ß1 expression and in preserving the tissue integrity of the liver.

Materials and 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 male ICR mice, weighing 30-35 g, were supplied by Japan SLC Inc. (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. RiAS to mouse TGF-ß1 was also intravenously administered twice per week to the RiAS group (n=20), at a dosage of 100 μg/ 30 g body weight. Mismatched RiAS was intravenously administered twice per week at an identical dosage to the mismatched group (n=20). Normal saline was intravenously administered twice per week at an equal volume to 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 of the mice was collected in order to measure the serum parameters of liver cirrhosis. Serum parameters were determined via standard spectrometric methods.

Construction of mouse TGF-ß1 RiAS. Target sites for RiAS were selected via the sequential overlap simulation of secondary structures using the DNAsis program (Hitachi Software, San Bruno, CA). The antisense sequence was 5'-gatccagggccacatgttgctccacacttgattttaatctctgaaccctg-3' (from 50 bp on the TGF-ß1 sequence), and the mismatched sequence was 5'-gatccagggccacatattactgcatacatgcttatattctctgcaaccctg-3'. Oligonucleotides were synthesized as previously described (12) via standard phosphoramidite chemistry using an automated DNA synthesizer, Expedite™ M8909 (Applied Biosystems, Foster City, CA). Two molecules of the TGF-ß1 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.

Peptide synthesis and modifications. Peptide used in the present study was derived from the Tat protein of HIV-1. The Tat peptide corresponds to the nuclear localization signal sequence of 9 amino acids (49-57: Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg). The peptide was modified at the C-terminus by the addition of cysteine residue. Peptides were prepared in a solid phase synthesis using a peptide synthesizer, purified by preparative LC, and characterized using an analytical HPLC system (Shimadzu, Japan) comprised of a C18 column and a MALDI-TOF mass spectrometer (Applied Biosystems). Purified peptides were re-suspended at a concentration of 10 μg/μl in ddH_2O and kept at -70˚C prior to further use.

Transfection efficiency of RiAS in vitro and in vivo. FITC-labeled TGF-ß1 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-ß1 RiAS (0.3 μg) in a 200 μl volume. The DP complex containing FITC-labeled TGF-ß1 RiAS (0.3 μg) in 600 μl saline was infused into the tail veins of the mice for *in vivo* study. The liver was removed after 16 h, 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-ß1 expression. After the transfection of the TGF-ß1 RiAS, TGF-ß1 expression was assessed via RT-PCR. RNA was prepared with Welprep™ RNA isolation reagent (WelGENE). The purified RNA was subjected to RT-PCR using the Access RT-PCR kit (Promega, Madison, WI) and a thermal cycler (MJ Research, Watertown, MA). The following primer pairs were used: left 5-ggactctccacctgcaa gac-3 and right 5-gactggcgagccttagtttg-3 for mouse TGF-ß1, and left 5-agtgtgacgttgacatccgta-3 and right 5-gccagagcagtaa tctccttct-3 for mouse ß-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).

Histological analysis. The fixation and embedding of liver tissues were conducted as previously described (15). The tissue sections were incubated with anti-type I collagen antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and α-smooth muscle actin antibody (Dako) in PBS containing 0.5% BSA and 2% FCS at 4˚C overnight. The next day, the tissue sections were incubated with anti-rabbit HRP conjugates for 1 h at room temperature. The fibrous lesion areas were

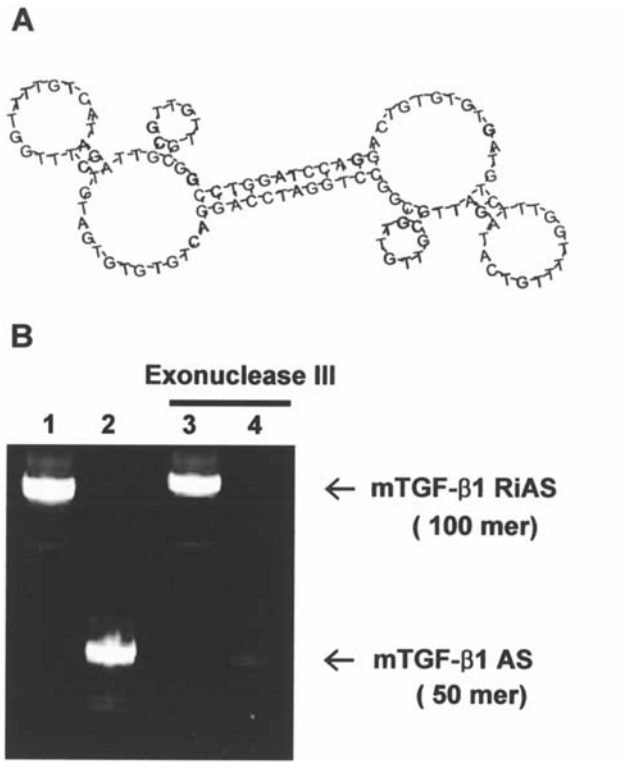

Figure 1. (A) Schematic representation of ribbon-type antisense to TGF-ß1 (TGF-ß1 RiAS). The stem is formed by complementary sequences at both ends of each oligo. The 5' terminus of the stem has 4 bases of a single-stranded overhang of 5'-GATC-3'. Two TGF-ß1 monomer molecules were ligated to generate 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-ß1. (B) Resistance of TGF-ß1 RiAS oligos to exonuclease III. Oligos were analyzed on a 15% denaturing polyacrylamide gel. Lane 1, 100 mer TGF-ß1 RiAS; lane 2, 50 mer TGF-ß1 AS oligos; lane 3, 100 mer TGF-ß1 RiAS treated with exonuclease III; lane 4, 50 mer TGF-ß1 AS oligos treated with exonuclease III.

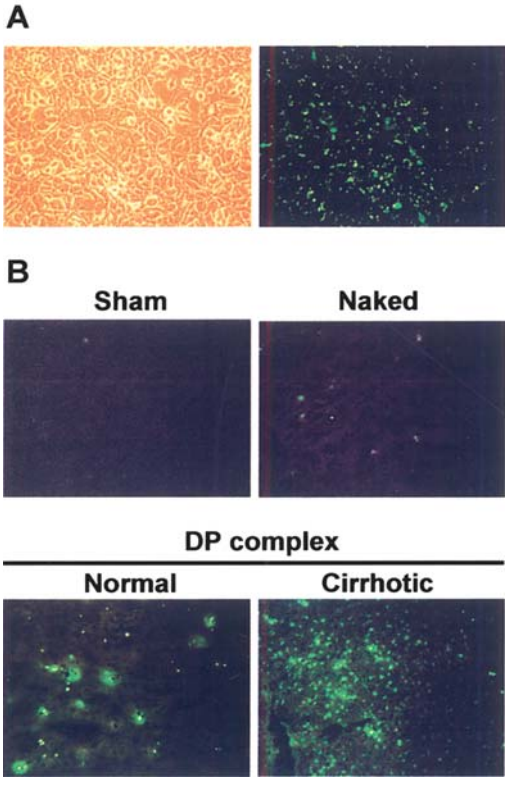

Figure 2. (A) The DP complex mediated transfection in Hepa1c1c7 mouse hepatoma cells. Transfection of FITC-labeled TGF-ß1 RiAS was conducted using cationic peptide. The DP complex was added to Hepa1c1c7 cells for 24 h. Fluorescence signals are shown in the right panel. (B) Normal saline (sham) or 10 μg of FITC-labeled TGF-ß1 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 mouse liver were observed under a fluorescence microscope (x200).

determined via Masson's trichrome method, which is used to stain collagen fibers.

Measurement of liver hydroxyproline contents and collagen synthesis levels. Samples (100 g) of snap-frozen liver were weighed and then hydrolyzed in 6 M HCl. Each sample was incubated with chloramine-T (2.5 mM) for 5 min and with Ehrlich's reagent (410 mM) for 30 min at 60˚C. Absorption was determined at 560 nm. The degree of liver cirrhosis was determined via measurements of total soluble collagen using the Sircol Collagen Assay kit (Biocolor, Belfast, Ireland). This method measures newly synthesized collagen which has not been extensively cross-linked.

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

Results

Construction of ribbon antisense oligos to TGF-ß1. We reported previously that RiAS is stable to nucleases and effective in the specific ablation of target mRNA and exhibits few of the problems associated with chemically modified antisense oligos (12,13). The entire length of the mouse TGF-ß1 cDNA was assessed to find antisense target sites that were readily accessible to an antisense molecule. The antisense molecule harbors an overhang GATC sequence at the 5' terminus. Two identical AS oligos possessing the stem-loop structure were covalently ligated to form a ribbon-antisense molecule, termed TGF-ß1 RiAS (Fig. 1A). As expected, the TGF-ß1 RiAS oligo was retarded on a denaturing polyacrylamide gel when compared with the linear precursor (Fig. 1B). These results indicate that the TGF-ß1 RiAS molecules possess a ribbon-type closed structure, without an open end that would be attackable by exonucleases.

Efficient cellular uptake of TGF-ß1 RiAS when delivered as a DP complex. Nucleic acid including antisense oligomers shows poor cellular uptake largely due to the charged polymeric backbone. The TGF-ß1 RiAS was labeled with FITC-11dUTP incorporation during chemical synthesis, and was used for both *in vitro* and *in vivo* uptake. When the FITC-labeled antisense oligos were used to form the DP complex and added to the Hepa1c1c7 cells, the cells exhibited strong fluorescence signals (Fig. 2A). Thus, we attempted to determine whether the DP complex could also be employed

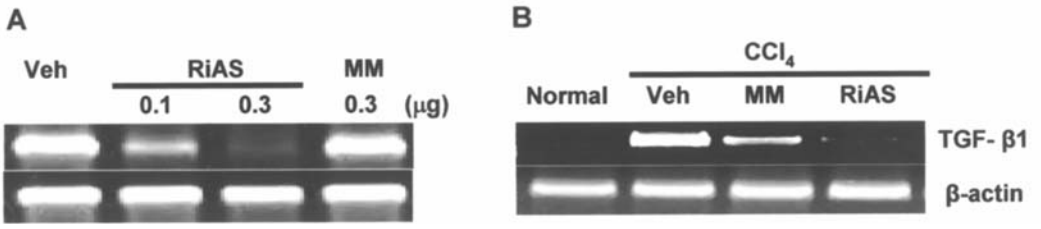

Figure 3. (A) Specific reduction of TGF-ß1 mRNA by TGF-ß1 RiAS. Hepa1c1c7 cells were transfected with DP complex, and RT-PCR was conducted in order to determine the antisense activity of TGF-ß1 RiAS. Transfection of TGF-ß1 RiAS reduced TGF-ß1 expression in Hepa1c1c7 cells. (B) After 8 weeks of treatment, the livers of each group were harvested and analyzed for TGF-ß1 expression using RT-PCR. Normal, no CCl_4 treatment; Veh, vehicle; RiAS, TGF-ß1 RiAS; MM, mismatched RiAS.

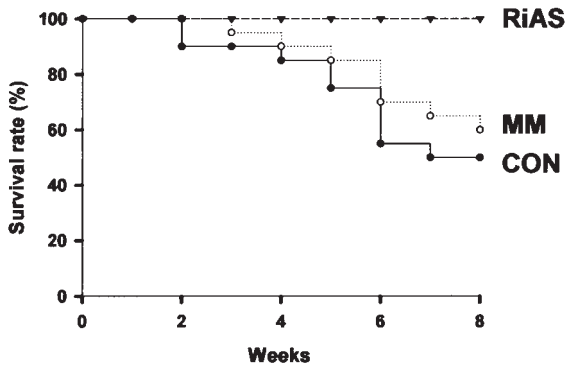

Figure 4. Survival curve during the 8 weeks of the experiment. In order to induce liver cirrhosis, 1 ml/kg body weight of CCl_4 was intraperitoneally administered twice per week. Mice were treated twice per week with normal saline (CON), mismatched RiAS (MM), and TGF-ß1 RiAS (RiAS) via the tail vein. The dose of RiAS was 100 mg/30 g body weight. n=20.

for the efficient delivery of RiAS into the liver after intravenous infusion. For *in vivo* tissue uptake, 10 μg of FITC-labeled TGF-ß1 was infused into the tail vein of mice, and the livers were harvested 24 h 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 exhibited no fluorescent signals. We also determined that the DP complex could be employed for efficient RiAS uptake in cases of CCl_4–induced liver cirrhosis (Fig. 2B).

Specific reduction of TGF-ß1 mRNA and prevention of liver cirrhosis by TGF-ß1 RiAS. We attempted to determine whether TGF-ß1 RiAS is effective in the elimination of target mRNA in a sequence-specific manner when delivered as a DP complex. Hepa1c1c7 cells treated with TGF-ß1 RiAS showed a reduction of TGF-ß1 RNA by ~50% at 0.1 μg (lane 2), and by >80% at 0.3 μg (lane 3). By way of contrast, when Hepa1c1c7 cells were treated with mismatched RiAS, TGF-ß1 expression was not significantly affected (lane 4). ß-actin expression was not affected by RiAS treatment (Fig. 3A, lower bands; control). TGF-ß1 RiAS was assessed with regard to its efficacy in the prevention of liver fibrosis induced by intraperitoneal CCl_4 injection. After infusion of TGF-ß1 RiAS for seven weeks, the livers from each group were harvested. The livers of mice treated with TGF-ß1 RiAS showed a reduction of TGF-ß1 RNA by ~80% after the experiment. In contrast, in the livers of mice treated with mismatched RiAS, TGF-ß1 expression was not significantly affected (Fig. 3B). All mice treated with TGF-ß1 RiAS survived, whereas the control group and the mismatched RiAS-treated group showed survival rates of 50% and 65%, respectively (Fig. 4). At the end of the 8 weeks of the experiment, mice treated with TGF-ß1 RiAS manifested lower levels of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) than those observed 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 I).

Effect of TGF-ß1 RiAS treatment on histology and collagen synthesis. CCl_4 injected intraperitoneally induced a massive

Table I. Effect of TGF-ß1 RiAS on serum biochemical parameters.

	Control	Mismatched RiAS	TGF-ß1 RiAS
Aspartate aminotransferase (U/l)	605.0±199.9	578.1±217.4	147.4 ±50.2[a]
Alanine aminotransferase (U/l)	316.3±100.7	367.7±125.8	104.3±17.5[a]
Albumin (g/dl)	3.2±0.2	3.3±0.2	3.4 ±0.2
Total bilirubin (mg/dl)	0.3±0.2	0.2±0.2	0.2±0.1

After 8 weeks, blood was collected from each group and analyzed. Data are expressed as the mean ± SD, n=5 or 6 per group; [a]$p<0.05$ vs. control.

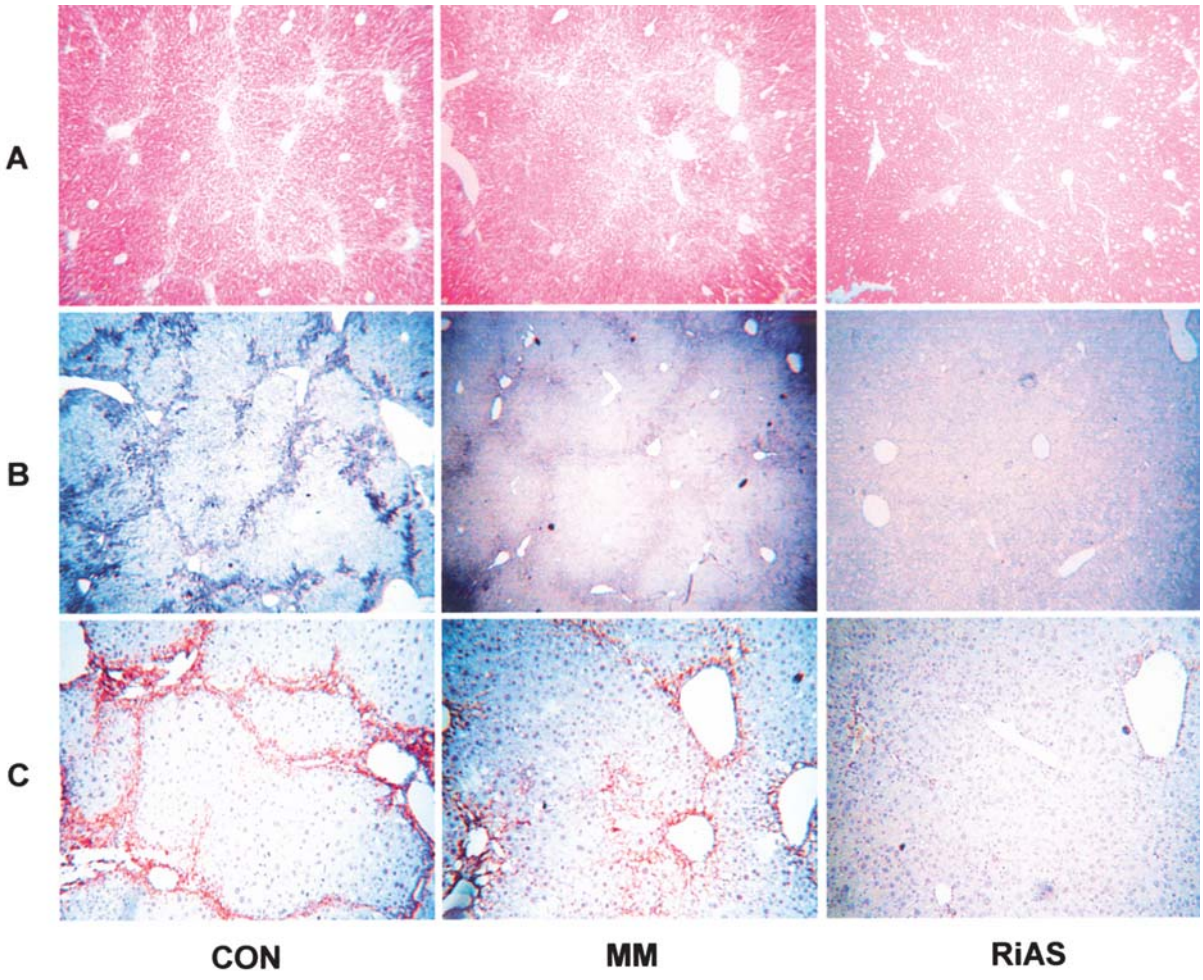

Figure 5. Histological observation of the liver. Collagen deposition was detected as blue staining on Masson's trichrome staining (A). Immunohistochemistry for type I collagen (B) and α-smooth muscle actin (C). Staining was conducted with fixed and dehydrated tissues from mice treated with normal saline (CON), mismatched RiAS (MM), and TGF-ß1 RiAS (RiAS). Stained tissues were mounted with a synthetic mounting solution for microscopic observation. Gray and brown staining indicates the presence of type I collagen and α-smooth muscle actin, respectively.

Table II. Effect of TGF-ß1 RiAS on collagen accumulation and synthesis.

	Normal	Control	Mismatched RiAS	TGF-ß1 RiAS
Hydroxyproline (μg/g liver)	94.1±3.5	165.3±16.9[a]	141.1±7.2[a]	94.1±21.4
Soluble collagen (% normal)	100.0±9.7	133.1±12.0[a]	127.0±15.2[a]	104.8±14.1

After 8 weeks, the livers of each group were harvested and analyzed for hydroxyproline and soluble collagen. Data are expressed as the mean ± SD; n=5 or 6 per group; [a]p<0.05 vs. normal.

accumulation of connective tissues, principally in the centrilobular area and portal tract, upon examination of Masson's trichrome-stained sections of the livers from both the control group and the mismatched RiAS-treated group. By way of contrast, TGF-ß1 RiAS treatment significantly ameliorated the deposition of connective tissues (Fig. 5A). 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 greatly reduced in the TGF-ß1 RiAS-treated livers (Fig. 5B). Immunohistochemistry of the α-smooth muscle actin, a marker for hepatic stellate cells, showed marked reductions in the livers of the TGF-ß1 RiAS-treated group as compared with the control group and the mismatched RiAS-treated group (Fig. 5C). Determination of tissue hydroxyproline contents and collagen synthesis levels indicated that TGF-ß1 RiAS treatment induced a definitive diminution in collagen synthesis as compared with the vehicle and mismatched RiAS (Table II). These data showed

that TGF-ß1 RiAS treatment prevents the establishment of fibrosis.

Discussion

In this study, we evaluated the ability of RiAS to TGF-ß1 to eliminate target mRNA and to alleviate global tissue injuries in cases of liver cirrhosis. We introduced the FITC-labeled RiAS/cationic peptide complex to mice via the tail vein, and it was delivered effectively to the cirrhotic livers, as well as the normal livers. RiAS to TGF-ß1, efficiently delivered into the liver, suppressed hepatic TGF-ß1 expression and also blocked consequent fibrosis, including collagen accumulation. TGF-ß1 is the most potent profibrogenic factor in human fibrogenesis (5). Thus, a variety of studies concerning the blockage of TGF-ß1 synthesis or action have been conducted in order to develop a method for the prevention of liver cirrhosis. A variety of strategies, such as the use of adenoviral vectors expressing truncated TGF-ß type II receptor (8,9,28), chimeric IgG harboring the extracellular portion of the TGF-ß type II receptor (7), and adenoviral expression of a TGF-ß1 antisense (29,30), have all been identified as effective.

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. Certain cationic peptides such as HIV Tat protein, SV40 large T antigen, *Drosophila Antennapedia*, protamine sulfate and histone H1 exhibit nucleic acid condensation, membrane penetration, and nuclear localization activities (16,18-27). In this study, we modified the NLS of the 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 (31) and cells of bone marrow origin (32) have demonstrated fibrogenic potential, the activation of the hepatic stellate cells is the most important event in liver fibrogenesis (33). 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 (1,34,35). Our results indicated that TGF-ß1 RiAS reduced collagen and α–smooth muscle actin accumulation in immunohistochemical staining. We also detected reduced hydroxyproline content and reduced collagen synthesis.

Contrary to traditional theories, it is now believed that even advanced liver fibrosis is reversible. Therefore, many scientists have attempted to discover new antifibrotic drugs (36). However, it appears that a long time period is required to recover from fibrosis (37). We attempted to treat liver fibrosis after 6 weeks of intraperitoneal injection of CCl_4 with TGF-ß1 RiAS for 6 weeks in another experiment, but we were only marginally successful in the resolution of established fibrosis. This insufficient resolution may be attributable to ECM cross-linking or insufficient treatment time. However, the preventive effect of TGF-ß1 RiAS on fibrosis may be of clinical use as a prophylactic approach upon hepatic injury.

Although TGF-ß is the most important cytokine involved in liver fibrosis, other cytokines, such as platelet-derived growth factor (PDGF), are also involved (38). The antisense approach to PDGF was shown to attenuate liver fibrogenesis (39). The Smad signaling pathway is also a significant therapeutic target (40,41). It is fairly simple to develop RiAS to these candidates, and RiAS has the capacity to attack two targets simultaneously as the result of the inherent diad symmetrical structure in which two antisense oligo molecules of a stem-loop structure are joined together.

In conclusion, ribbon antisense to TGF-ß1 mRNA coupled with enhanced transfection using the antisense oligo/cationic peptide complex was determined to reduce levels of target mRNA in a sequence-specific manner, and was also shown to alleviate global tissue fibrosis in the livers of CCl_4-treated mice. As tissue fibrosis is a point of convergence in the progression of many human diseases of the liver, kidney and lung, the potent antisense activity of RiAS TGF-ß1 in the sequence-specific elimination of the target mRNA may prove useful in the development of antisense medicine to fibrous conditions in these human tissues.

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