Inhibitory Effect of Nuclear Factor- κ B Decoy Oligodeoxynucleotide on Liver Fibrosis Through Regulation of the Epithelial–Mesenchymal Transition

Kyung-Hyun Kim,¹ Woo-Ram Lee,¹ Yu-Na Kang,² Young-Chae Chang,¹ and Kwan-Kyu Park¹

Abstract

The epithelial–mesenchymal transition (EMT) has been recognized to occur during embryonic development, fibrosis, and tumor metastasis. Nuclear factor (NF)- κ B plays a central role in mediating the inflammation and wound-healing responses during liver fibrogenesis. However, the involvement of NF- κ B during EMT in liver cells remains unidentified. To develop a therapeutic approach to EMT during liver fibrosis, we examined the inhibition of transcription factor NF- κ B, using a decoy oligodeoxynucleotide (ODN) strategy in liver fibrosis *in vitro* and *in vivo*. NF- κ B decoy ODN contains consensus binding sequences of the NF- κ B-binding site. NF- κ B decoy ODN effectively suppresses transforming growth factor- β_1 -induced EMT in AML12 murine hepatocytes. Liver fibrosis induced by CCl₄ administration was suppressed by NF- κ B decoy ODN. Furthermore, NF- κ B decoy ODN was shown to inhibit the EMT process in fibrotic liver *in vivo*. This study demonstrates the feasibility of NF- κ B decoy ODN treatment for preventing liver fibrosis via EMT processes.

Introduction

LIVER FIBROSIS IS A CONSEQUENCE of sustained wound-healing responses, which can be caused by chronic liver injuries including viral, alcoholic, and autoimmune hepatitis (Friedman, 2000). Fibrosis in the liver is described as excessive deposition of extracellular matrix (ECM), leading to destruction of organ structures and impairment of organ functions. Activated fibroblasts were believed to be the key contributors to organ fibrosis. In the liver, several studies have demonstrated that resident hepatic stellate cells (HSCs) can respond to many different forms of liver insults by converting or contributing to activated fibroblasts seen in the injured liver. Activated HSCs are traditionally known as the principal, but not the only, source of ECM-producing myofibroblasts in the injured liver (Brenner *et al.*, 2000; Gabele *et al.*, 2003; Mehal, 2006). The epithelial-to-mesenchymal transition (EMT), during which epithelial cells acquire typical features of mesenchymal cells such as fibroblasts, is claimed to be another significant source of fibrosis in liver and several other organs (Zeisberg *et al.*, 2007). Indeed, EMT has been identified as a major mechanism in the excessive accumulation of ECM in renal and pulmonary fibrosis injury models (Iwano *et al.*, 2002; Willis *et al.*, 2005).

Most liver injuries (viral, alcoholic, and autoimmune hepatitis) activate acute inflammation and wound-healing responses. When the injury is repetitive or chronic, liver tissue is

exposed to continuously elevated expression of proinflammatory cytokines and chemokines. Hepatic nuclear factor (NF)- κ B is increased by elevated inflammatory changes in various nonparenchymal and parenchymal liver cells. In particular, the induction of NF- κ B in hepatocytes (liver parenchymal cells) during liver injury contributes to a chronic inflammatory state (Knittel *et al.*, 1999; Lv *et al.*, 2007). However, whether NF- κ B participates in the progression of EMT during liver fibrogenesis still remains unclear.

In general, gene transcription is regulated by two regulatory pathways: chromatin rearrangement and transcription factors. Transcription factors are controlled by direct binding to their binding sites. To inhibit the binding of transcription factors, a decoy strategy is employed to block transcription factor activity via the use of a synthetic double-stranded oligodeoxynucleotide (ODN), which contains a consensus binding sequence. The decoy ODN strategy has been shown to be an effective approach for suppressing specific gene expression both *in vitro* and *in vivo* (Morishita *et al.*, 1998; Tomita *et al.*, 2007). In abdominal aortic aneurysms and renal ischemia–reperfusion, the decoy ODN strategy has been reported as a novel therapeutic application for treating these disorders (Cao *et al.*, 2004; Miyake *et al.*, 2006). In addition, we previously demonstrated that Sp1 and chimeric decoy ODN effectively suppressed the expression of fibrosis-related genes *in vivo* and *in vitro* (Park *et al.*, 2009; Kim *et al.*, 2013).

¹Department of Pathology, Catholic University of Daegu, College of Medicine, 3056-6 Daemyung 4-dong, Nam-gu, Daegu 705-718, Republic of Korea.

²Department of Pathology, Keimyung University, School of Medicine, Dalseong-ro 56, Jung-gu, Daegu 700-712, Republic of Korea.

In this study, we examined the effect of ring-type NF- κ B decoy ODN transforming growth factor (TGF)- β_1 -induced EMT *in vitro* and further characterized the inhibitory mechanisms *in vivo*, using CCl₄-induced liver fibrosis.

Materials and Methods

Synthesis of ring-type NF- κ B decoy ODN

Decoy ODN was purchased as an HPLC-purified product from Bionics (Seoul, Korea). NF- κ B and scrambled decoy ODN sequences were used as follows (the consensus binding sequence is underlined):

NF- κ B decoy ODN: 5'-GAAATTCAGGGAAATCCC
TTCAAGAAACTTGAAGGGATTTCCT-3'

Scrambled (Scr) decoy ODN: 5'-GAATTCATTTCAG
GGTACGGCAAAAATTGCCGTACCCTGAATT-3'

NF- κ B and Scr decoy ODNs were annealed for 8 hr, while the temperature was decreased from 80 to 25°C. These decoy

ODNs were predicted to form a stem-loop structure (Fig. 1A). To obtain a covalent ligation for ring-type decoy ODN, each decoy ODN was mixed with T4 ligase (Takara Bio, Otsu, Japan) and incubated for 18 hr at 16°C.

Cell culture

AML12 (CRL-2254), the murine hepatocyte cell line, was purchased from the American Type Culture Collection (ATCC, Manassas, VA). AML12 cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium containing 10% fetal bovine serum supplemented with insulin-transferrin-selenium-X, dexamethasone (40 ng/ml), penicillin (100 U/ml), and streptomycin (100 μ g/ml) (Gibco BRL; Life Technologies, Bethesda, MD).

Transfection of NF- κ B decoy ODN and transfection efficiency

The transfection efficiency of decoy ODN was examined by fluorescence microscopy (Eclipse 80i; Nikon, Tokyo, Japan)

FIG. 1. Structure of ring-type nuclear factor (NF)- κ B decoy oligodeoxynucleotide (ODN) and effect of NF- κ B decoy ODN on transforming growth factor (TGF)- β_1 -induced hepatocyte differentiation. **(A)** Structure of ring-type NF- κ B and scrambled (Scr) decoy ODNs. GGGATTTCCT is the consensus sequence for the NF- κ B-binding site. **(B)** Transfection efficiency of NF- κ B decoy ODN in AML12 hepatocytes. Cells were cultured in 6-well plates and transfected with 2 μ g of fluorescein isothiocyanate (FITC)-labeled NF- κ B ODN or nonlabeled NF- κ B decoy ODN. Fluorescence was detected in both the nuclei and cytoplasm with FITC-labeled NF- κ B decoy ODN (bottom), whereas fluorescence was not detected with the nonlabeled NF- κ B decoy ODN (top). Arrows (bottom right) indicate nuclear transfer of FITC-labeled NF- κ B decoy ODN. Original magnification: left, $\times 200$; right, $\times 400$. **(C)** The percentage of FITC/DNA-positive cells and the mean fluorescence intensity were determined by flow cytometry. **(D)** Morphological changes in TGF- β_1 -treated AML12 hepatocytes are shown. AML12 hepatocytes were treated with TGF- β_1 (2.5 ng/ml) in the presence of Scr or NF- κ B decoy ODN as indicated. NC, normal control. **(E)** Nuclear extracts of AML12 cells were subjected to electrophoretic mobility shift assay (EMSA) to determine NF- κ B DNA-binding activity. Color images available online at www.liebertpub.com/hum

and flow cytometry (Navios; Beckman Coulter, Miami, FL). Before the transfection, NF- κ B decoy ODN was labeled with fluorescein isothiocyanate (FITC), using a *Label IT* nucleic acid-labeling kit (Mirus Bio, Madison, WI). AML12 cells were cultured in 6-well plates (0.8×10^5 cells/ml) and transfected with 2 μ g of FITC-labeled NF- κ B or nonlabeled NF- κ B decoy ODN, using FuGENE HD (DNA:lipid molar ratio, 2:3) (Roche, Mannheim, Germany). For flow cytometric analysis, cells were washed, trypsinized, dispersed, and then transferred into 500 μ l of phosphate-buffered saline (PBS). Samples were analyzed with Navios (Beckman Coulter) and calculations were done with Kaluza software (Beckman Coulter).

For transfection analysis, AML12 cells were incubated with serum starvation for 24 hr before the experiment. Cells were then transfected with 2 μ g of NF- κ B or Scr decoy ODN, using FuGENE HD. After incubation at 37°C for 8 hr, AML12 cells were cultured in serum-free medium containing TGF- β_1 (2.5 ng/ml; Santa Cruz Biotechnology, Santa Cruz, CA) for 48 hr.

Immunofluorescence staining

AML12 cells were collected by Cytospin centrifugation (Thermo Fisher Scientific, Waltham, MA) and fixed with 3.7% paraformaldehyde in PBS for 30 min at room temperature. Cells were permeated with 0.5% Triton for 15 min and incubated with primary antibodies against vimentin (BD Biosciences, San Jose, CA) and E-cadherin (Cell Signaling Technology, Beverly, MA) for 1 hr at room temperature. After washing with the PBS, cells were incubated with secondary antibodies (Alexa Fluor 488 and/or Alexa Fluor 594) for 30 min at room temperature. Cells were then counterstained with Hoechst 33342 for 10 min at 37°C. Immunofluorescence labeling was examined with an Eclipse 80i microscope (Nikon).

Animal model and NF- κ B decoy ODN transfer

Animal protocols were approved by the Institutional Animal Care and Use Committee of the Catholic University of Daegu (Daegu, South Korea). Male C57BL/6 mice (6 weeks old, 20–22 g; Orient Bio, Seongnam, South Korea) were housed in a room with controlled humidity and temperature, and a 12-hr light–dark cycle. To examine the *in vivo* transfection efficiency of NF- κ B decoy ODN, FITC-labeled NF- κ B decoy ODN was injected into mice via the tail vein. The mice were killed 24 hr after injection. Liver tissues were frozen with O.C.T. compound (Sakura Finetek Japan, Tokyo, Japan). Cryosections of liver, which were transfected with FITC-labeled NF- κ B decoy ODN, were examined by fluorescence microscopy.

Mice were randomly divided into three groups as follows: (1) untreated group (normal control, NC), (2) group treated with CCl₄ and Scr decoy ODN (CCl₄ + Scr), and (3) group treated with CCl₄ and NF- κ B decoy ODN (CCl₄ + NF- κ B). Chronic liver injuries were induced by intraperitoneal injection of CCl₄ (2 ml/kg, dissolved in corn oil [1:3 ratio]) three times a week.

One week after the first CCl₄ injection, Scr or NF- κ B decoy ODN (10 μ g) was transferred biweekly via the mouse tail vein, using an *in vivo* gene delivery system (Mirus Bio). Mice were killed 4 and 8 weeks after the first CCl₄ injection.

Histopathology and immunohistochemistry

Paraffin-embedded liver tissues were sectioned and stained with Masson's trichrome according to standard protocol. Immunohistochemical staining of liver sections was performed as described previously (Park *et al.*, 2009). Primary antibodies used were as follows: type I collagen and fibronectin (Santa Cruz Biotechnology), TGF- β_1 (R&D Systems, Minneapolis, MN), and α -smooth muscle actin (SMA; Sigma-Aldrich, St. Louis). For immunofluorescence staining, the sections were incubated with primary antibodies against E-cadherin and vimentin. The slides were examined with an Eclipse 80i microscope (Nikon).

Measurement of alanine aminotransferase and aspartate aminotransferase

The blood of each mouse was collected and serum was separated to analyze the activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), using an automated analyzer.

ELISA

The blood of each mouse was collected and serum was separated. Serum tumor necrosis factor (TNF)- α and interleukin (IL)-1 β concentrations (pg/ml) were measured with Quantikine mouse TNF- α and IL-1 β kits (R&D Systems).

Western blot analysis

Cells and liver tissue were homogenized in radioimmuno-precipitation assay (RIPA) buffer (Cell Signaling Technology) for 15 min on ice and centrifuged at 6000 $\times g$ for 15 min. The supernatant was collected and analyzed by Western blot as described previously (Park *et al.*, 2009). Primary antibodies used were against E-cadherin, vimentin, TGF- β_1 , α -SMA, fibronectin, type I collagen, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Signal intensity was quantified with an image analyzer (LAS-3000; Fuji, Tokyo, Japan).

Electrophoretic mobility shift assay

Nuclear extracts of cells and liver tissues were made with an NE-PER nuclear and cytoplasmic extraction kit (Thermo Fisher Scientific). A DIG (digoxigenin) gel shift kit (Roche) was used for electrophoretic mobility shift assay (EMSA) analysis, according to the manufacturer's protocol. The probe for NF- κ B was as follows: 5'-CTT GAA GGG ATT TCC CTG GCT TGA AGG GAT TTC CCT GG-3'; only the sense strand is shown, and consensus binding sequences for NF- κ B are underlined.

Statistical analyses

Results were analyzed with Duncan's test and expressed as means $\pm$ SD. All experiments were performed at least three times.

Results

Transfection efficiency of NF- κ B decoy ODN in AML12 cells

To assess the successful transfer of NF- κ B decoy ODN, the transfection efficiency of the FITC-labeled ODN was

initially analyzed by fluorescence microscopy. Cells transfected with nonlabeled NF- κ B decoy ODN did not show any specific fluorescence (Fig. 1B, top). Twenty-four hours after transfection of the FITC-labeled NF- κ B decoy ODN, fluorescence was detected in both the cytoplasm and nuclei of AML12 cells (Fig. 1B, bottom). To quantify the proportion of FITC-labeled NF- κ B decoy ODN in AML12 cells, flow cytometric analysis was performed. Flow cytometric analysis showed a high positive rate (83.3%) of fluorescence activity compared with nonlabeled NF- κ B decoy ODN in AML12 cells (Fig. 1C). These results indicated that NF- κ B decoy ODN was effectively transfected into AML12 cells.

Effect of NF- κ B decoy ODN in AML12 cells treated with TGF- β_1

To induce EMT, AML12 cells were incubated for 48 hr with TGF- β_1 (2.5 ng/ml), which is the most important profibrogenic cytokine in the process of liver EMT. In response to

TGF- β_1 , the epithelial cobblestone appearance of the AML12 cells changed to a fibroblastic spindle-shaped morphology after 48 hr of treatment (Fig. 1D). To examine the effect of NF- κ B decoy ODN in TGF- β_1 -treated hepatocytes, NF- κ B DNA-binding activity was analyzed by EMSA. The DNA-binding activity of NF- κ B was increased after treatment with TGF- β_1 and transfection with Scr decoy ODN, whereas the increase was greatly suppressed by the NF- κ B decoy ODN (Fig. 1E). E-cadherin is a prototypical epithelial cell marker that plays an important role in the maintenance of cellular integrity at the cell-cell junction. In addition, vimentin is an intermediate filament used to identify mesenchymal cells in EMT (Zeisberg and Kalluri, 2004). The changes in AML12 cellular morphology were accompanied by an increase in the labeling intensity of vimentin after TGF- β_1 treatment. NF- κ B decoy ODN treatment attenuated the expression of vimentin compared with Scr decoy ODN treatment. In contrast, the decreased labeling of E-cadherin after TGF- β_1 treatment was reversed by treatment with NF- κ B decoy ODN (Fig. 2A). Consistent with the immunofluorescence staining, NF- κ B

FIG. 2. NF- κ B decoy ODN suppresses TGF- β_1 -induced epithelial-mesenchymal transition (EMT) in AML12 murine hepatocytes. AML12 hepatocytes were treated with TGF- β_1 (2.5 ng/ml) in the presence of Scr or NF- κ B decoy ODN as indicated. (A) Typical example of immunofluorescence in hepatocytes. Immunofluorescence double staining for E-cadherin (green) and vimentin (red) in TGF- β_1 -treated AML12 hepatocytes after treatment with decoy ODNs. Cells were counterstained with Hoechst 33342 (blue). (B) Western blot analysis of E-cadherin, vimentin, fibronectin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in TGF- β_1 -treated AML12 hepatocytes after treatment with decoy ODNs. (C) Graphical presentation of the ratio of E-cadherin, vimentin, and fibronectin to GAPDH in various groups. * p < 0.05 versus TGF- β_1 + Scr group. NC, normal control group; TGF- β_1 , TGF- β_1 -treated group; TGF- β_1 + Scr, TGF- β_1 treated with Scr decoy ODN; TGF- β_1 + NF- κ B, TGF- β_1 treated with NF- κ B decoy ODN. Results are representative of three independent experiments. Color images available online at www.liebertpub.com/hum

decoy ODN treatment significantly inhibited the upregulation of vimentin and fibronectin as well as the downregulation of E-cadherin (Fig. 2B and C). These results indicate that NF- κ B decoy ODN effectively blocks mesenchymal changes and maintains the epithelial phenotype in TGF- β ₁-induced hepatocyte EMT.

Effect of NF- κ B decoy ODN in CCl₄-induced liver fibrosis

On the basis of the results of NF- κ B decoy ODN administration in AML12 hepatocytes, we determined the effect of NF- κ B decoy ODN on CCl₄-induced liver fibrosis *in vivo*. To

FIG. 3. NF- κ B decoy ODN effectively suppresses CCl₄-induced liver fibrosis through inhibition of inflammatory changes and NF- κ B-binding activity. (A) Typical example of immunofluorescence in liver of mice transfected with FITC-labeled NF- κ B decoy ODN. (B) Histological section of murine liver stained with Masson's trichrome 8 weeks after CCl₄ administration. Original magnification, $\times 200$. (C) Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were significantly decreased by NF- κ B decoy ODN treatment compared with Scr decoy ODN treatment. * $p < 0.05$ versus CCl₄ + Scr group. (D) Serum concentrations of tumor necrosis factor (TNF)- α and interleukin (IL)-1 β were decreased by NF- κ B decoy ODN treatment compared with Scr decoy ODN treatment. * $p < 0.05$ versus CCl₄ + Scr group. (E) Representative results of electrophoretic mobility shift assay for NF- κ B-binding sites 8 weeks after CCl₄ administration. NC, normal control group; CCl₄ + Scr, treated with CCl₄ and Scr decoy ODN; CCl₄ + NF- κ B, treated with CCl₄ and NF- κ B decoy ODN. Color images available online at www.liebertpub.com/hum

confirm the successful transfer of NF- κ B decoy ODN *in vivo*, we examined the distribution of FITC-labeled NF- κ B decoy ODN in mouse liver. The FITC-labeled NF- κ B decoy ODN exhibited apparent fluorescence in mouse liver (Fig. 3A).

We then investigated the effect of NF- κ B decoy ODN in CCl₄-induced liver fibrosis. NF- κ B decoy ODN was transferred biweekly during CCl₄ administration. According to Masson's trichrome staining, 8 weeks of treatment with CCl₄ established bridging fibrosis in the CCl₄+Scr mice, whereas NF- κ B decoy ODN was shown to markedly suppress fibrosis in CCl₄+NF- κ B mice (Fig. 3B). At 4 and 8 weeks after CCl₄ administration, AST and ALT were increased in CCl₄+Scr mice, whereas the increase was significantly smaller in CCl₄+NF- κ B mice (Fig. 3C). CCl₄ administration increased the serum concentrations of TNF- α

and IL-1 β in CCl₄+Scr mice. However, these increases were significantly inhibited in CCl₄+NF- κ B mice (Fig. 3D). To determine the effect of NF- κ B decoy ODN on NF- κ B DNA-binding activity in liver fibrosis, EMSA was performed to analyze the transcriptional activity of NF- κ B. NF- κ B was significantly increased in CCl₄+Scr mice. In contrast, transfection of NF- κ B decoy ODN significantly suppressed the activation of NF- κ B in CCl₄+NF- κ B mice (Fig. 3E).

Effect of NF- κ B decoy ODN during EMT in liver fibrogenesis

To assess the molecular mechanism of NF- κ B decoy ODN, we examined its effects on liver fibrosis and matrix accumulation. An immunohistochemical study demonstrated that accumulation of cells positive for TGF- β ₁, fibronectin, α -SMA,

FIG. 4. Inhibition of fibrosis-related gene expression by NF- κ B decoy ODN treatment in CCl₄-induced liver fibrosis. **(A)** Typical example of immunohistochemical staining of TGF- β ₁, fibronectin, α -smooth muscle actin (α -SMA), and type I collagen 8 weeks after CCl₄ administration. Original magnification, $\times 200$. **(B)** Representative Western blot and RT-PCR analysis of TGF- β ₁, fibronectin, α -SMA, type I collagen, and GAPDH 4 and 8 weeks after CCl₄ administration. NC, normal control group; CCl₄+Scr, treated with CCl₄ and Scr decoy ODN; CCl₄+NF- κ B, treated with CCl₄ and NF- κ B decoy ODN. Color images available online at www.liebertpub.com/hum

and type I collagen within the liver sinusoid was increased in CCl₄+Scr mice compared with NC mice. However, NF- κ B decoy ODN effectively suppressed the expression of TGF- β ₁, fibronectin, α -SMA, and type I collagen in CCl₄+NF- κ B mice (Fig. 4A). Consistent with the immunohistochemical staining, Western blotting results showed that NF- κ B decoy ODN significantly inhibited the expression of TGF- β ₁, fibronectin, α -SMA, and type I collagen (Fig. 4B). These results collectively suggest that inhibition of NF- κ B is able to ameliorate liver fibrotic lesions through the suppression of TGF- β ₁ and ECM-related proteins in liver fibrosis.

During liver fibrogenesis, hepatocytes lose their epithelial phenotype and acquire features of mesenchyme via EMT processes. We next examined the effect of NF- κ B decoy ODN on the expression of E-cadherin and vimentin, which are essential markers for epithelial cells and mesenchymal cells, respectively. Consistent with a previous report (Zeisberg *et al.*, 2007), immunofluorescence staining revealed that administration of CCl₄ induced suppression of E-cadherin as well as dramatic induction of vimentin, a shift that is in agreement with hepatocyte EMT. Interestingly, NF- κ B decoy ODN treatment resulted in restoration of E-cadherin and reduction of vimentin expression in fibrotic liver (Fig. 5A). In addition, Western blotting results showed that NF- κ B decoy ODN treatment preserved E-cadherin expression and inhibited vimentin induction in CCl₄+NF- κ B mice (Fig. 5B). Therefore, blocking NF- κ B activity effectively inhibits EMT during fibrotic changes in the liver.

Discussion

NF- κ B regulates the transcription of many genes, such as interleukins and adhesion molecules, that are primarily related to inflammation. The present study has investigated how NF- κ B acts on the EMT process during liver fibrogenesis.

TGF- β ₁-induced EMT has been implicated in tissue and organ fibrosis (Zeisberg and Kalluri, 2004; Willis and Borok, 2007). Inflammation is closely related to the progression of organ fibrosis and cancer (Cordon-Cardo and Prives, 1999; Mantovani *et al.*, 2008). However, the detailed molecular mechanisms of inflammation for organ fibrosis have only begun to be elucidated. Most liver injuries activate acute inflammatory and wound-healing responses. NF- κ B has been closely linked to the induction of proinflammatory gene expression and has been focused on as a therapeutic target for inflammatory diseases. Persistent hepatic damage activates the inflammatory cascade through the activation of NF- κ B in various nonparenchymal and parenchymal liver cells (Elsharkawy and Mann, 2007). In particular, it has been reported that the exposure of various types of injury (alcohol, endotoxin, TNF- α , and cholestasis) induces the expression of NF- κ B in hepatocytes (Miyoshi *et al.*, 2001). However, whether the inhibition of NF- κ B plays a role in TGF- β ₁-induced EMT in hepatocytes remains unanswered. Here, we provide evidence that the inhibition of NF- κ B, using a decoy ODN strategy, attenuates liver fibrogenesis through inhibition of the EMT process.

FIG. 5. Inhibition of epithelial-mesenchymal transition by NF- κ B decoy ODN treatment in CCl₄-induced liver fibrosis. (A) Typical example of immunofluorescence double staining for E-cadherin (green) and α -SMA (red) 8 weeks after CCl₄ administration. Counterstained with Hoechst 33342 (blue). (B) Representative Western blot analysis of E-cadherin, vimentin, and GAPDH 8 weeks after CCl₄ administration. NC, normal control group; CCl₄+Scr, treated with CCl₄ and Scr decoy ODN; CCl₄+NF- κ B, treated with CCl₄ and NF- κ B decoy ODN. Color images available online at www.liebertpub.com/hum

In this study, we demonstrated that NF- κ B inactivation works to attenuate hepatocyte EMT during liver fibrogenesis. To assess the effectiveness of NF- κ B inactivation, we designed a ring-type NF- κ B decoy ODN, which has two consensus NF- κ B-binding sites in a single decoy molecule without an open end. In our previous reports, we developed a ring-type NF- κ B decoy ODN constructed by enzymatic ligation with two hairpin overhangs at both ends to prevent degradation by nucleases. This ring-type NF- κ B decoy ODN was more stable than conventional phosphorothiolated double-strand ODNs in the presence of serum or nucleases (Kim *et al.*, 2009). We observed that NF- κ B decoy ODN treatment significantly inhibited TGF- β ₁-induced hepatocyte EMT *in vitro*. Direct inhibition of NF- κ B suppresses chronic inflammation and extracellular matrix expression in CCl₄-induced liver fibrogenesis. Treatment with NF- κ B decoy ODN was also shown to effectively inhibit the EMT processes *in vivo* in the current study. Disruption of tight junctions and adherens junctions is the critical process of EMT. Downregulation of E-cadherin, which is the main component of adherens junctions, is widely known to be a marker of EMT in epithelial cells. This study demonstrated that NF- κ B decoy ODN treatment restored the expression of E-cadherin *in vivo* and *in vitro*. Several studies have reported that NF- κ B is a potent activator of Snail, Twist, and Zeb, which directly activate EMT at the transcriptional level (Peinado *et al.*, 2004). In addition, one study has shown that NF- κ B stabilizes the expression of Snail, which is involved in inflammation-induced cell migration and invasion (Wu *et al.*, 2009). These findings indicate that NF- κ B decoy ODN treatment regulates not only inflammation-related gene expression, but also the expression of EMT-related genes, which leads to potent therapeutic effects on the degeneration of hepatocyte integrity during liver fibrogenesis. Therefore, a possible advantage of NF- κ B decoy ODN is the inhibition of transcription of several genes including EMT-related genes through the regulation of NF- κ B function.

In summary, our data collectively show that NF- κ B plays an important role in EMT during liver fibrogenesis. Inhibition of NF- κ B by the decoy ODN strategy led to attenuation of liver fibrosis and improvement of liver function. Our findings may open new avenues for the suppression of EMT in liver fibrogenesis, thus leading to new therapeutic options for liver fibrosis.

Acknowledgments

This work was supported by the National Research Foundation of Korea Grant funded by the Korean Government [NRF-2009-353-E00022]. This work was supported by the National Research Foundation of Korea Grant funded by the Korean Government [2012R1A1A401015639].

Author Disclosure Statement

There are no conflicts of interest to disclose for any of the authors.

References

- Brenner, D.A., Waterboer, T., Choi, S.K., *et al.* (2000). New aspects of hepatic fibrosis. *J. Hepatol.* 32, 32–38.
- Cao, C.C., Ding, X.Q., Ou, Z.L., *et al.* (2004). *In vivo* transfection of NF- κ B decoy oligodeoxynucleotides attenuate renal ischemia/reperfusion injury in rats. *Kidney Int.* 65, 834–845.
- Cordon-Cardo, C., and Prives, C. (1999). At the crossroads of inflammation and tumorigenesis. *J. Exp. Med.* 190, 1367–1370.
- Elsharkawy, A.M., and Mann, D.A. (2007). Nuclear factor- κ B and the hepatic inflammation–fibrosis–cancer axis. *Hepatology* 46, 590–597.
- Friedman, S.L. (2000). Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. *J. Biol. Chem.* 275, 2247–2250.
- Gabele, E., Brenner, D.A., and Rippe, R.A. (2003). Liver fibrosis: Signals leading to the amplification of the fibrogenic hepatic stellate cell. *Front. Biosci.* 8, d69–d77.
- Iwano, M., Plieth, D., Danoff, T.M., *et al.* (2002). Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J. Clin. Invest.* 110, 341–350.
- Kim, K.H., Lee, E.S., Cha, S.H., *et al.* (2009). Transcriptional regulation of NF- κ B by ring type decoy oligodeoxynucleotide in an animal model of nephropathy. *Exp. Mol. Pathol.* 86, 114–120.
- Kim, K.H., Park, J.H., Lee, W.R., *et al.* (2013). The inhibitory effect of chimeric decoy oligodeoxynucleotide against NF- κ B and Sp1 in renal interstitial fibrosis. *J. Mol. Med. (Berl)* 91, 573–586.
- Knittel, T., Dinter, C., Kobold, D., *et al.* (1999). Expression and regulation of cell adhesion molecules by hepatic stellate cells (HSC) of rat liver: Involvement of HSC in recruitment of inflammatory cells during hepatic tissue repair. *Am. J. Pathol.* 154, 153–167.
- Lv, P., Luo, H.S., Zhou, X.P., *et al.* (2007). Reversal effect of thalidomide on established hepatic cirrhosis in rats via inhibition of nuclear factor- κ B/inhibitor of nuclear factor- κ B pathway. *Arch. Med. Res.* 38, 15–27.
- Mantovani, A., Allavena, P., Sica, A., and Balkwill, F. (2008). Cancer-related inflammation. *Nature* 454, 436–444.
- Mehal, W.Z. (2006). Activation-induced cell death of hepatic stellate cells by the innate immune system. *Gastroenterology* 130, 600–603.
- Miyake, T., Aoki, M., Nakashima, H., *et al.* (2006). Prevention of abdominal aortic aneurysms by simultaneous inhibition of NF- κ B and ETS using chimeric decoy oligonucleotides in a rabbit model. *Gene Ther.* 13, 695–704.
- Miyoshi, H.R., Rust, C., Guicciardi, M.E., and Gores, G.J. (2001). NF- κ B is activated in cholestasis and functions to reduce liver injury. *Am. J. Pathol.* 158, 967–975.
- Morishita, R., Higaki, J., Tomita, N., and Ogihara, T. (1998). Application of transcription factor “decoy” strategy as means of gene therapy and study of gene expression in cardiovascular disease. *Circ. Res.* 82, 1023–1028.
- Park, J.H., Jo, J.H., Kim, K.H., *et al.* (2009). Antifibrotic effect through the regulation of transcription factor using ring type-Sp1 decoy oligodeoxynucleotide in carbon tetrachloride-induced liver fibrosis. *J. Gene Med.* 11, 824–833.
- Peinado, H., Ballestar, E., Esteller, M., and Cano, A. (2004). Snail mediates E-cadherin repression by the recruitment of the Sin3A/histone deacetylase 1 (HDAC1)/HDAC2 complex. *Mol. Cell. Biol.* 24, 306–319.
- Tomita, N., Kashiwara, N., and Morishita, R. (2007). Transcription factor decoy oligonucleotide-based therapeutic strategy for renal disease. *Clin. Exp. Nephrol.* 11, 7–17.
- Willis, B.C., and Borok, Z. (2007). TGF- β -induced EMT: Mechanisms and implications for fibrotic lung disease. *Am J. Physiol. Lung Cell. Mol. Physiol.* 293, L525–L534.

- Willis, B.C., Liebler, J.M., Luby-Phelps, K., *et al.* (2005). Induction of epithelial–mesenchymal transition in alveolar epithelial cells by transforming growth factor- β_1 : Potential role in idiopathic pulmonary fibrosis. *Am. J. Pathol.* 166, 1321–1332.
- Wu, Y., Deng, J., Rychahou, P.G., *et al.* (2009). Stabilization of snail by NF- κ B is required for inflammation-induced cell migration and invasion. *Cancer Cell* 15, 416–428.
- Zeisberg, M., and Kalluri, R. (2004). The role of epithelial-to-mesenchymal transition in renal fibrosis. *J. Mol. Med. (Berl)* 82, 175–181.
- Zeisberg, M., Yang, C., Martino, M., *et al.* (2007). Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition. *J. Biol. Chem.* 282, 23337–23347.

Address correspondence to:

Dr. Kwan-Kyu Park

Department of Pathology

Catholic University of Daegu, College of Medicine

3056-6 Daemyung 4-Dong, Nam-Gu

Daegu, 705-718

Republic of Korea

E-mail: kkpark@cu.ac.kr

Received for publication May 22, 2013;

accepted after revision June 20, 2014.

Published online: June 24, 2014.