Int J Clin Exp Pathol 2015;8(10):12688-12697
www.ijcep.com /ISSN:1936-2625/IJCEP0014069

Original Article

Notch signaling affects biliary fibrosis via transcriptional regulation of RBP-jκ in an animal model of chronic liver disease

Sun-Jae Lee[1], Kyung-Hyun Kim[1], Sok Cheon Pak[2], Yu-Na Kang[3], Ghil-Suk Yoon[4], Kwan-Kyu Park[1]

[1]Department of Pathology, Catholic University of Daegu School of Medicine, 3056-6 Daemyung-4 Dong, Nam-Gu, Daegu, Republic of Korea; [2]School of Biomedical Sciences, Charles Sturt University, Bathurst, NSW 2795, Australia; [3]Department of Pathology, Keimyung University School of Medicine, 2800, Dalgubeoldaero, Dalseo-Gu, Daegu, Republic of Korea; [4]Department of Pathology, Kyungpook National University School of Medicine, 680 Gukchaebosang-Ro, Jung-Gu, Daegu, 700-842, Republic of Korea

Received August 6, 2015; Accepted September 25, 2015; Epub October 1, 2015; Published October 15, 2015

Abstract: Liver repair in patients with a chronic liver disease requires the orchestrated action of epithelial, mesenchymal, and inflammatory cells. Notch components are expressed in both the epithelial and mesenchymal compartments of the adult liver and are differentially regulated after injury. However, the functional role of Notch signaling in regulating epithelial/mesenchymal cross-talk during fibrogenic pathologic repair remains unknown. The aim of this study was to investigate how proliferation of the bile duct influences biliary fibrosis and to recognize the effect of inhibiting Notch signaling in biliary fibrotic tissue of the injured liver. We designed a synthetic decoy oligodeoxynucleotide (ODN) for recombination signal binding protein immunoglobulin kappa J (RBP-jκ), which is a common DNA-binding partner of Notch receptors. The effect of blocking RBP-jκ on fibrogenesis was assessed in the 3,5-Diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet mouse model. We observed the reduced fibrosis and decreased expression of associated signaling molecules after the RBP-jκ decoy ODN treatment. These data demonstrate that Notch signaling may play an important role in progression of ductular reaction and fibrosis. Further studies are required to unveil how ductular cells interact with other liver cell types, such as hepatic stellate cells or Kupffer cells,in patients with cholestatic liver diseases based on Notch signaling. These results suggest that controlling the ductular reaction using a synthetic ring type decoy RBP-jκ ODN will help develop a novel therapeutic approach targeting biliary fibrosis in patients with chronic liver diseases.

Keywords: Liver fibrosis, Notch, RBP-jκ, decoy ODN

Introduction

Chronic liver injury, including chronic hepatitis and cirrhosis, triggers a repair response, and fibrosis occurs with atypical proliferation of the dysfunctional bile duct when repair becomes deregulated. This biliary fibrous tissue gradually replaces the hepatic parenchyma and causes a gradual decrease in the number of mature hepatocytes in the hepatic lobules. The bile duct basement membranes undergo degradation in patients with fibrogenic liver diseases and cholangiocytes assume fibroblast-like, non-cuboidal shapes. It is well established that proliferating cholangiocytes detectable in all types of chronic liver disease express a variety of pro-fibrogenic growth factors and cytokines that likely contribute to fibrosis and inflammation by promoting activation, proliferation, and collagen synthesis in the surrounding pro-fibrogenic cells [1-7]. However, the distinct pathogenesis of intrahepatic bile duct regeneration in patients with chronic liver diseases is poorly understood.

Transforming growth factor (TGF)-β1 is a major profibrogenic cytokine and a potent inducer of collagen production [8]. Activation of the TGF-β1 pathway results in nuclear translocation of phosphorylated Smad2 and Smad3, which form hetero-oligomers with Smad4, where they regulate transcription of target genes [9].

Overexpression of TGF-β1 is linked to liver fibrosis in diverse animal models [10] and in human patients with chronic liver diseases [11]. TGF-β1 regulates deposition of the extracellular matrix by controlling expression of vimentin, α-smooth muscle actin (α-SMA), and fibronectin. Participation of TGF-β1 in the regulation of Notch signaling during fibrogenesis has been reported previously in mammary glands, kidney tubules, and epidermis [12].

Several studies have indicated that the Notch pathway plays a key role maintaining liver homeostasis during post-natal life and is involved in the reparative reaction to biliary damage, as well as in liver carcinogenic, metabolic and inflammatory responses [13]. Ligand-activated Notch receptors are cleaved at the Notch intracellular domain (NICD). The NICD translocates to the nucleus where it binds the recombination signal binding protein immunoglobulin kappa J (RBP-jκ) transcription factor [14-17] and initiates transcription of Notch target genes. Liver repair in patients with chronic liver diseases requires the orchestrated action of epithelial, mesenchymal, and inflammatory cells. Notch components are expressed in both the epithelial and mesenchymal compartments in adult liver and are differentially regulated after injury [13]. However, the functional role of Notch signaling in regulating epithelial/mesenchymal cross-talk during fibrogenic pathologic repair remains unknown [13].

Studies to control the proliferation of reactive cholangiocytes associated with fibrosis at the molecular level remain limited in adult patients with chronic liver disease. Therefore, we attempted to suppress biliary fibrosis by regulating bile duct proliferation at the transcription level via blocking the transcription factor RBP-jκ. Several attempts have been made previously to inhibit specific targeted gene expression to control the pathologic condition by modulating transcription factor function. Decoy technology uses synthetic double-stranded oligodeoxynucleotide (ODN) containing the consensus binding site sequence of a targeted transcription factor. The decoy ODN blocks the activity of specific transcription factor and inhibits specific gene expression at the DNA level [18, 19]. This decoy ODN strategy is an effective approach to suppress specific gene expression *in vitro* and *in vivo* [20, 21]. It has been proposed as an effective therapeutic tool for inhibiting specific gene expression to treat several disorders [22-24]. Several studies have reported that introducing a decoy ODN with high affinity for target transcription factors into specific cells leads to selective down regulation and expression of the genes regulated by those transcription factors [25-27]. In addition, we previously demonstrated that several decoy ODNs effectively suppressed the expression of fibrosis-related genes *in vivo* and *in vitro* [28, 29].

The aim of this study was to investigate how proliferation of the bile duct influences biliary fibrosis and to identify the effect of inhibiting Notch signaling in biliary fibrotic tissue of the injured liver. The current data demonstrate that liver cell plasticity could contribute to regeneration of the intrahepatic bile duct in mice by disrupting Notch signaling and regulating transcription factor RBP-jκ activity. These results suggest that Notch signaling may play an important role in biliary fibrosis seen in chronic diseases. Novel approaches to treat chronic liver diseases could be developed using the synthetic decoy ODN strategy with transcription factor RBP-jκ to regulate biliary fibrosis.

Materials and methods

ODN synthesis (ring type decoy)

We designed a synthetic decoy ODN for RBP-jκ, the common DNA-binding partner of Notch receptors, as described previously [18, 30]. The sequence of each decoy ODN was as follows (consensus sequence for RBP-jκ is underlined):

RBP-jκODN: 5'-GAAATTTCA**TTCCCAC**TTCAAGAAAACTTGAA**GTGGGAA**T-3'; Scrambled ODN: 5'-GAATTCAATTCAGGGTACGGCAAAAAATTGCCGTACCCTGAATT-3'.

The decoy ODN was purchased as a high performance liquid chromatography-purified product from Bionics (Seoul, Korea). These ODNs were annealed for 8 h, as temperature was decreased from 80 to 25°C. Both ODNs were predicted to form a stem-ring-type. Following the addition of T4 DNA ligase (1 U; Takara Bio, Shiga, Japan), the mixture was incubated for 16 h at 16°C to obtain covalently ligated ring-type decoy ODN molecules.

Table 1. List of antibodies used for the Western blot analysis

Antibody	Source/Clonality	Supplier
TGF-β1*	mouse monoclonal	R&D Systems
p-Smad2*	rabbit polyclonal	Novus Biologicals
Smad7	rabbit polyclonal	Santa Cruz Biotechnology
Smad2/3	mouse monoclonal	Santa Cruz Biotechnology
Vimentin	mouse monoclonal	Santa Cruz Biotechnology
α-SMA*	mouse monoclonal	Sigma-Aldrich Inc.
Fibronectin	mouse monoclonal	BD Transduction Laboratories
TNF-α*	mouse monoclonal	Abcam
p-Stat3	rabbit monoclonal	Cell Signaling Technology

*Abbreviations: TGF-β1, transforming growth factor-β1; p-Smad2, phosphorylated-Smad2; α-SMA, α-smooth muscle actin; TNF-α, tumor necrosis factor-α.

Experimental animal model and transfer of decoy ODN

Experiments were performed with mice to induce chronic liver injuries using a 3,5-Diethoxycarbonyl-1,4-dihydrocollidine (DDC) containing diet. Male C57BL/6 mice (6 weeks old, 20-22 g; Orient Bio, Seongnam, South Korea) were housed in a room with controlled humidity of 60 ± 5% and temperature of 25 ± 1°C, and a 12:12 h light/dark cycle. Mice were fed a 0.1% DDC-supplemented diet for 4 weeks, and permitted *ad libitum* consumption of water. Controls were fed a standard mouse diet (Sniff, Soest, Germany). Mice were divided randomly into five groups of five mice per group: A normal control group (NC), the RBP-jκ decoy ODN treated group (NC+RBP-jκ), the DDC diet with or without Scr decoy ODN group (DDC+Scr and DDC, respectively), and the DDC diet with RBP-jκ decoy ODN treated group (DDC+RBP-jκ). One week after first feeding the DDC diet, the RBP-jκ decoy ODN (10 μg) was injected biweekly through the mouse tail vein, using an *in vivo* gene delivery system (Mirus Bio Corp., Madison, WI, USA). All mice were sacrificed 4 weeks after the first DDC supplement, and serum and whole liver tissues were harvested. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Catholic University of Daegu (Daegu, South Korea) in accordance with criteria outlined in the Institutional Guidelines for Animal Research.

Histopathological investigation

For conventional light microscopy, livers were fixed in 10% formalin solution and embedded in paraffin. A 4 μm thick cross-section was taken from the blocks and stained with hematoxylin and eosin (H&E) and Masson's trichrome according to standard protocols.

Computer-assisted morphometry

Measurements were made to quantify the extent of fibrosis using a semiautomatic image analysis system. Masson's trichrome stained regions were analyzed with a microscope (Nikon Eclipse 80i; Nikon, Osaka, Japan) supplied with a motorized stage system (NIS-Elements BR 3.00). Portal field images were collected at 200X magnification using a digital camera (Nikon DS-Ri1; Nikon) and analyzed using an image-analysis software (i-Solution; IMT i-Solution Inc., Vancouver, BC, Canada). Biliary fibrosis was calculated as the percentage of Masson's trichrome positive pixels above the threshold value with respect to the total pixels per lobule area. The extent of fibrosis was determined using 10 different fields in each liver. Obvious artifacts, such as nonspecific staining of necrotic areas or incompletely cut portal fields at the edges of the specimens, were excluded from the analysis.

Western blot analysis

Frozen liver tissues were homogenized in RIPA buffer (Cell Signaling Technology, Danvers, MA, USA) and centrifuged at 12,000 × g for 30 min after a 30 min incubation on ice. Protein concentrations were measured using the Bradford protein assay (BioRad, Hercules, CA, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed at 100 V for 3 h. The resolved proteins were transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA) and probed with primary antibodies followed by incubation with a secondary antibody conjugated to horseradish peroxidase. Finally, the membranes were washed and developed with an enhanced chemiluminescence detection system (Amersham Pharmacia, Piscataway, NJ, USA). Signal intensity was quantified with an image analyzer (LAS-3000; Fuji, Tokyo, Japan). Antibodies and conditions for the Western blot analysis are presented in **Table 1**.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts of liver tissue were obtained with an NE-PER nuclear and cytoplasmic extrac-

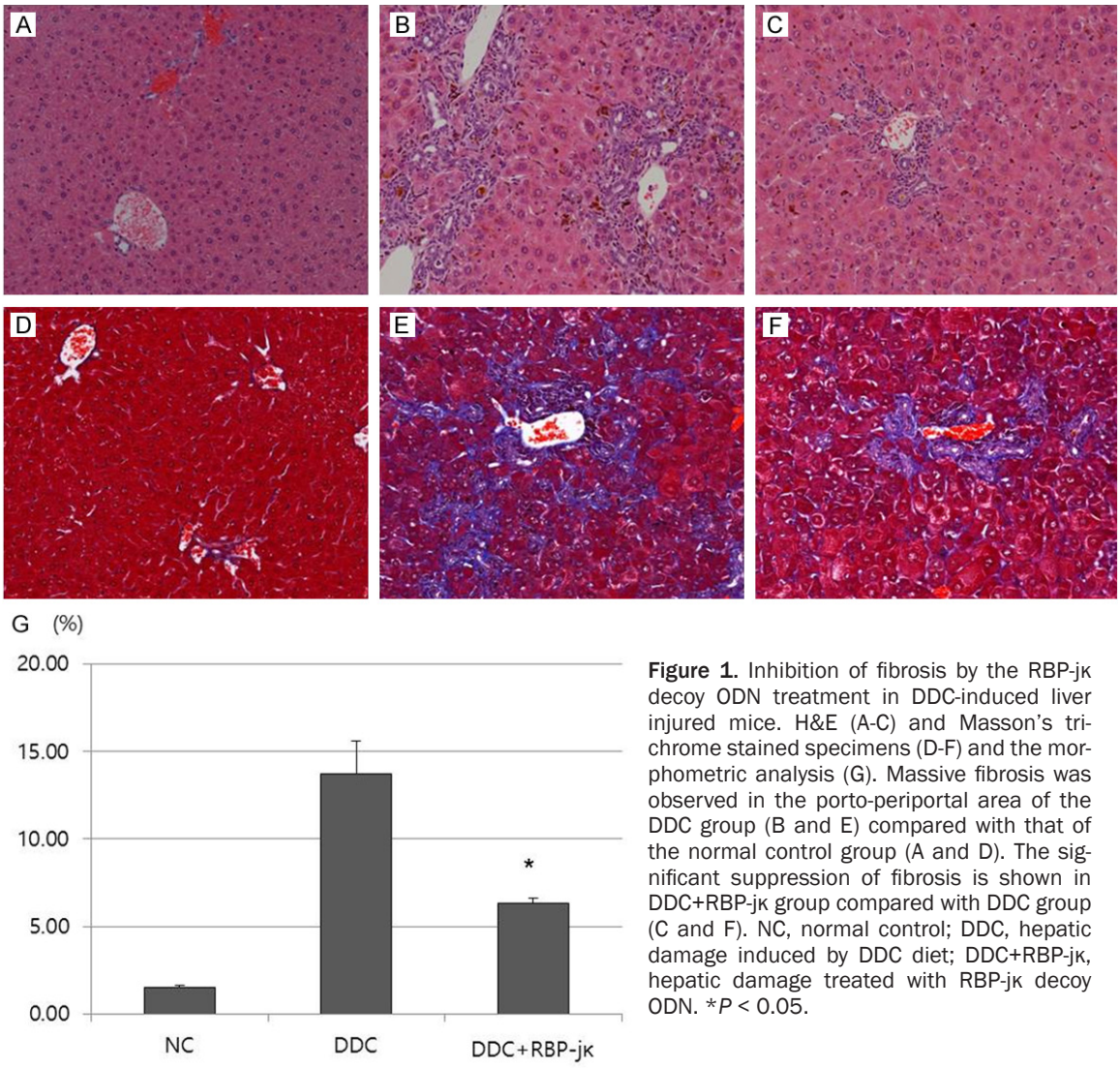

Figure 1. Inhibition of fibrosis by the RBP-jκ decoy ODN treatment in DDC-induced liver injured mice. H&E (A-C) and Masson's trichrome stained specimens (D-F) and the morphometric analysis (G). Massive fibrosis was observed in the porto-periportal area of the DDC group (B and E) compared with that of the normal control group (A and D). The significant suppression of fibrosis is shown in DDC+RBP-jκ group compared with DDC group (C and F). NC, normal control; DDC, hepatic damage induced by DDC diet; DDC+RBP-jκ, hepatic damage treated with RBP-jκ decoy ODN. *$P < 0.05$.

tion kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). A DIG Gel Shift kit (Roche, Mannheim, Germany) was used for the EMSA assays, according to the manufacturer's protocol. The RBP-jκ oligonucleotide probe (5'-CTTGAA**GTGGGAA**TGAAATTTCA**TTCCCAC**TTCAAG-3'; only sense strands are shown, consensus sequence for RBP-jκ is underlined) containing the RBP-jκ binding motifs were end-labeled with DIG-ddUTP. A 10 μg aliquot of the sample protein was incubated at room temperature for 30 min with a DIG labeled probe for the binding reaction. The DNA-protein complexes were separated by 6% native polyacrylamide gels electrophoresis using 0.25× Tris-borate-EDTA as the running buffer. After electrophoresis, the gels were transferred to nylon membranes and detected by chemiluminescence. The membranes were exposed to X-ray film for 40 min. Signal intensity was quantified by an image analyzer (Las3000).

Statistical analysis

Results were presented as mean ± standard deviation and analyzed with Duncan's test. All experiments were performed at least three times. A p-value < 0.05 was considered significant.

Results

Histopathological features

Well-formed portal triads consisted of vein (portal vein), artery (hepatic artery) and biliary tract

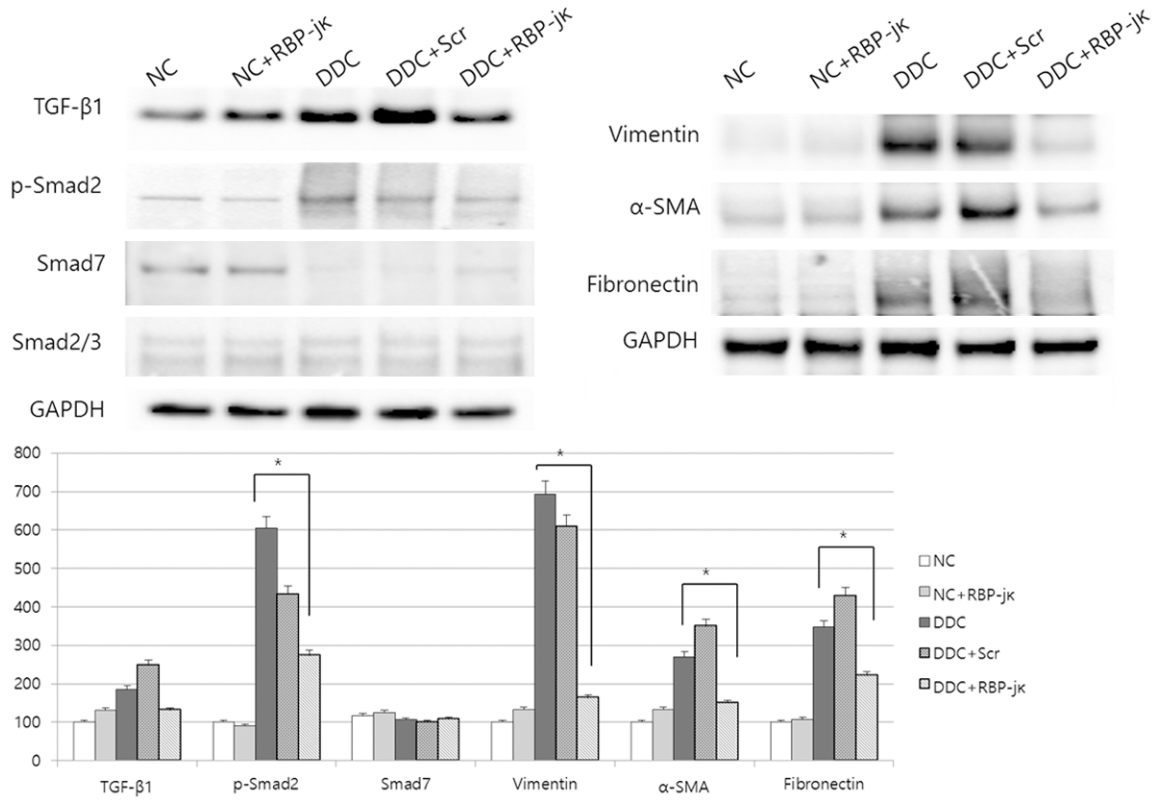

Figure 2. Inhibition of fibrosis by the RBP-jκ decoy ODN treatment in DDC-induced liver injured mice. The levels of TGF-β1, p-smad2, Smad7, α-SMA and fibronectin were analyzed by Western blot. TGF-β1, p-smad2, vimentin, fibronectin, and α-SMA were upregulated and smad7 was downregulated in the DDC-fed groups compared to those in the NC group. In contrast, expression of TGF-β1, p-smad, vimentin, fibronectin, and α-SMA was significantly inhibited and smad7 was overexpressed in the DDC+RBP-jκ group compared with DDC and DDC+Scr groups. NC, normal control; NC+RBP-jκ, normal control treated with RBP-jκ decoy ODN; DDC, hepatic damage induced by DDC diet; DDC+Scr, hepatic damage treated with Scr decoy ODN; DDC+RBP-jκ, hepatic damage treated with RBP-jκ decoy ODN. *$P < 0.05$.

in normal control cases (**Figure 1A**). Two or three bile ductules existed in one portal area. The ductular system formed an even round to oval luminal space. No morphologic differences were detected between the NC group and the RBP-jκ decoy treated group (data not shown).

In contrast, all liver tissues obtained from DDC-fed mice showed massive fibrosis with marked bile duct proliferation in the porto-periportal area, as revealed by Masson's trichrome stained specimens (**Figure 1B**, **1E**, and **1G**). The proliferated bile duct cells were flattened with or without luminal space and observed in the periportal or intralobular area, which was called a ductular reaction (**Figure 1B**). Biliary fibrosis in DDC-fed mice was paralleled by pronounced proliferation of the bile duct (**Figure 1E**).

In contrast, the scrambled decoy treated group (DDC+Scr) reduced fibrosis and an expanded lobular areas were found in the DDC+RBP-jκ group (**Figure 1C** and **1F**). The Masson's trichrome stain results were correlated with these findings (**Figure 1G**). No significant microscopic changes were detected in the morphology of hepatocytes with or without decoy treatment in the DDC-fed groups (**Figure 1A-C**).

Expression of fibrogenic and inflammatory markers

The effects of RBP-jκ decoy ODN on liver fibrosis and matrix accumulation were examined by Western blot analysis of TGF-β1, p-smad, vimentin, fibronectin, and α-SMA. TGF-β1, p-smad2, vimentin, fibronectin, and α-SMA were upregulated, whereas smad7 was downregulated in the DDC-fed groups compared with

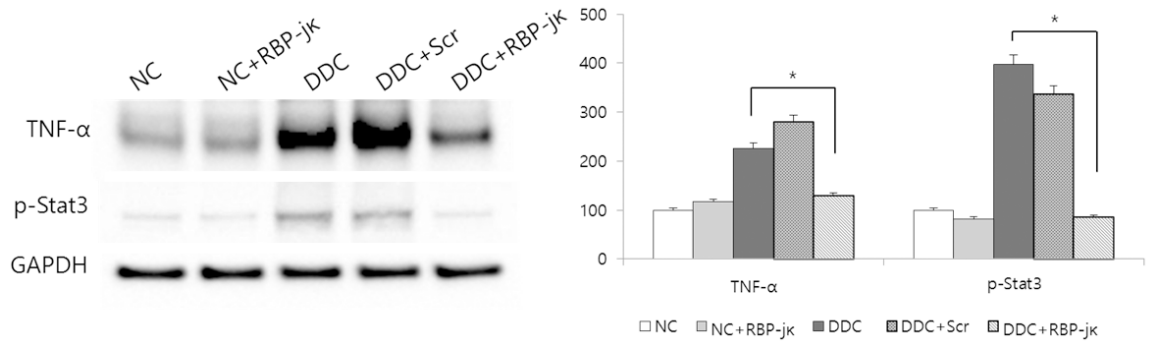

Figure 3. Inhibition of inflammation by the RBP-jκ decoy ODN treatment in DDC-induced liver injured mice. The levels of TNF-α and p-stat3, which are major inflammation-related genes, were analyzed by Western blot. The overexpressed TNF-α and p-stat3 in the DDC-fed mice were markedly downregulated after treatment with the RBP-jκ decoy ODN. NC, normal control; NC+RBP-jκ, normal control treated with RBP-jκ decoy ODN; DDC, hepatic damage induced by DDC diet; DDC+Scr, hepatic damage treated with Scr decoy ODN; DDC+RBP-jκ, hepatic damage treated with RBP-jκ decoy ODN. *$P < 0.05$.

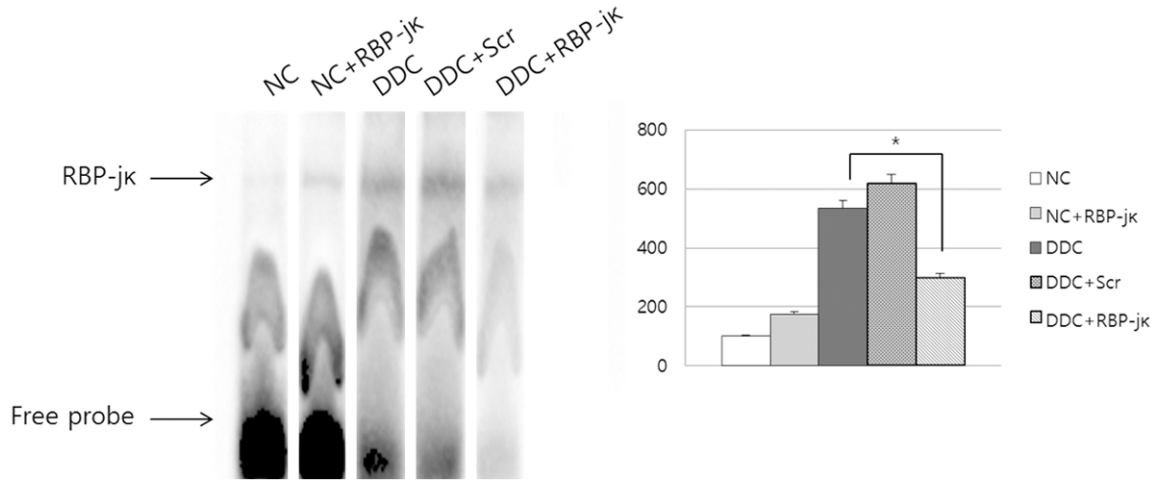

Figure 4. DNA binding activity according to the electrophoretic mobility shift assay (EMSA) results. The RBP-jκ decoy oligodeoxynucleotide (ODN)-treated livers showed decreased expression of the DNA-RBP-jκ complex compared with that in the scrambled decoy ODN treated group. NC, normal control; NC+RBP-jκ, normal control treated with RBP-jκ decoy ODN; DDC, hepatic damage induced by DDC diet; DDC+Scr, hepatic damage treated with Scr decoy ODN; DDC+RBP-jκ, hepatic damage treated with RBP-jκ decoy ODN. *$P < 0.05$.

those in the NC group (**Figure 2**). In contrast, expression of TGF-β1, p-smad, vimentin, fibronectin, and α-SMA was significantly inhibited and smad7 was overexpressed in the DDC+RBP-jκ group compared with those in the DDC and DDC+Scr groups (**Figure 2**).

The expression of tumor necrosis factor (TNF)-α and p-stat3, which are major inflammatory response markers, was examined with Western blot analysis to assess the molecular mechanism. The overexpressed TNF-α and p-stat3 in the DDC-fed mice were markedly downregulated after treatment with the RBP-jκ decoy ODN (**Figure 3**).

Suppressive effect of decoy ODN to RBP-jκ

An EMSA was performed to analyze RBP-jκ transcriptional activity and investigate the effect of RBP-jκ decoy ODN on the target DNA binding activity in liver specimen. This transcription factor increased significantly in the DDC and DDC+Scr mice. In contrast, the increase was significantly suppressed by RBP-jκ decoy ODN treatment (**Figure 4**).

Discussion

Chronic liver damage can be triggered by different mechanisms and is accompanied by changes in several key biochemical pathways involved in hepatic tissue homeostasis. One of the most important alterations is hepatic fibrosis, which is characterized by deposition of extracellular matrix components around the sinusoidal layer in the space of Disse, together with molecular reorganization of the matrix components resulting in altered composition. Chronic DDC feeding of mice is a well-established model to induce chronic cholestatic liver disease, cholangitis with pronounced ductular reaction, onion-skin-type periductal fibrosis, and biliary type liver fibrosis [31-33]. Therefore, we selected the DDC-induced cholangiopathy and biliary fibrosis mouse model as an atypical ductular reaction in patient with chronic liver diseases because it shares several specific pathological hallmarks with at least some human cholangiopathies associated with biliary type liver fibrosis.

A variety of profibrogenic growth factors and cytokines are expressed during the ductular reaction that likely contribute to fibrosis and inflammation by promoting activation, proliferation, and collagen synthesis in surrounding profibrogenic cells [1]. In this study, we observed that periportal fibrosis was accompanied by bile duct proliferation in DDC-induced liver. The fibrosis was suppressed by inhibiting the related pathway with the RBP-jκ decoy ODN. RBP-jκ is a co-transcription factor of Notch signaling [14-17], suggesting that the Notch signaling pathway has an inhibitory effect on fibrosis. Notch-1 and Notch-3 are both expressed by quiescent hepatic stellate cells (HSC) [34] and are respectively downregulated and upregulated during HSC transdifferentiation into myofibroblasts. The ligand Jag-1, has been detected on proliferating bile ductules [35, 36] and hepatocytes [34], as well as on activated HSCs [34] and is strongly upregulated in the injured liver. Notably, patients with Alagille syndrome, which is a human congenital biliary agenesis syndrome induced by mutations in the Notch ligand (Jagged-1) [37] or Notch receptor (Notch-2) [38], show limited deposition of fibrotic tissue, consistent with the slow progression to cirrhosis seen in these patients [39]. Thus, Jag-1, the defective protein in Alagille syndrome, may signal portal myofibroblasts and induce collagen production or proliferation [13]. Notch activation and upregulation of Notch-3 in myofibroblasts have been described in carbon tetrachloride-induced liver fibrosis experimental rat model. In this model, pharmacologically inhibiting Notch reduces the extent of liver fibrosis [34]. However, the functional role of Notch in regulating epithelial/mesenchymal cross-talk related with the TGF-β1 signaling pathway during fibrogenic pathologic repair remains to be fully understood [13].

A reduced inflammatory response is needed to suppress fibrosis and for the indirect effect of RBP-jκ decoy ODN treatment to be induced. It is typically seen in liver diseases associated with increased deposition of collagenous matrices produced by activated HSCs [40]. In such liver diseases, Kupffer cells are also activated to produce various inflammatory cytokines, such as TNF-α, which is thought to play important pathogenic roles [41]. Nishikawa et al showed that inflammatory cytokines exert distinct effects on hepatocyte differentiation, indicating that TNF-α is unique among these cytokines in its ability to suppress the hepatocytic phenotype [42]. Their results indicate that TNF-α also profoundly influences the differentiation status of hepatocytes [42]. They showed that TNF-α strongly enhances bile ductular transdifferentiation of hepatocytes within a collagen-rich matrix, particularly by suppressing hepatocytic differentiation and enhancing ductular morphogenesis [42]. The direct effects of TNF-α on hepatocyte differentiation might be involved in the pathogenesis of hepatic dysfunctional characteristic of the chronic liver injury associated with fibrosis [42]. In this study, the increased expression of TNF-α and p-stat3 induced by the DDC feeding was markedly downregulated by the RBP-jκ decoy ODN treatment, suggesting that DDC-induced inflammation and fibrosis is suppressed by inhibiting the Notch signaling pathway. Further studies are needed to establish the mechanisms of the interactions between Notch signaling and inflammatory responses.

In conclusion, we found that Notch signaling played an important part in the progression of the ductular reaction and fibrosis to induce differentiation and proliferation of bile duct epithelial cells. Further studies will be required to

unveil how ductular cells interact with other liver cell types, such as HSCs and Kupffer cells, during cholestatic liver diseases based on Notch signaling. Our result that the ductular reaction was controlled by the synthetic ring type decoy RBP-jκ ODN will help in the development of a novel therapeutic approach targeting biliary fibrosis in patients with chronic liver diseases.

Acknowledgements

This work was supported by the National Research Foundation of Korea Grant funded by the Korean Government [2014R1A1A2008955].

Disclosure of conflict of interest

None.

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. Tel: +82-53-650-4149; Fax: +82-53-650-4834; E-mail: kkpark@cu.ac.kr

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