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Nephroblastoma overexpressed gene (NOV) expression in rat hepatic stellate cells

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Abstract

Using the expression-profiling method, we identified nephroblastoma overexpressed gene (NOV) mRNA as one member of the mRNA population that was upregulated in cultured activated hepatic stellate cell (HSC). Northern analysis showed that NOV mRNA was increasingly expressed during progressive activation of cultured rat HSCs, and a significant increase was observed in both the carbon tetrachloride-induced and bile duct ligation/scission rat models of liver fibrosis. RT-PCR showed human NOV mRNA was increased in most fibrotic livers compared with normal livers. The expression of NOV protein in fibrotic rat and human livers was predominantly located in areas of ductular proliferation and HSC of the fibrous septa. HSCs stimulated with transforming growth factor β1 showed increased expression of NOV protein without changing its mRNA levels. Dexamethasone stimulated the expression of NOV mRNA and protein. Furthermore, we demonstrated that bile acids have a modulating effect on the induction of NOV mRNA expression. In conclusion, this study suggests that NOV is expressed during liver fibrogenesis and HSCs may be an important source of hepatic NOV.

Keywords: Rat hepatic stellate cell; Expression profile; Nephroblastoma overexpressed gene; Transforming growth factor-β1; Dexamethasone; Bile acids

1. Introduction

In liver fibrosis, the synthesis and deposition of several extracellular matrix (ECM) proteins are increased. Increased ECM production alters the normal architecture of the liver and inhibits its functions. The perisinusoidal hepatic stellate cell (HSC) is largely responsible for this increase in ECM deposition. After a fibrogenic stimulus, the HSC is transformed from a quiescent vitamin A storing cell type to that of an activated cell type [1–3]. Several key features of HSC activation can be recapitulated by culturing the cells on plastic, and this offers a convenient model system to study HSC activation [3]. Accompanying HSC activation are numerous changes in cellular morphology and in the pattern of gene expression [4–6]. Thus, analysis of the differences in gene expression between quiescent and activated HSCs has provided insights into the mechanisms of cell activation. Furthermore, knowledge of the molecular mechanisms underlying HSC activation is essential for the development of effective therapies against liver fibrosis.

We have undertaken a systematic survey of quiescent and activated HSCs to identify new genes associated with HSC activation [7] using an expression-profiling method [8,9] based on quantitative analysis of mRNA populations. This is performed by using 3′-directed cDNA libraries [10,11] that faithfully represent the mRNA population, and by obtaining short base sequences just upstream of the poly A, called gene signatures. We previously, demonstrated that the expression of *O*-acetyl disialoganglioside synthase is induced after activation of the HSC, both in vitro and in vivo [12].

Using the expression-profiling method as the initial screen, we identified nephroblastoma overexpressed gene

Abbreviations: ECM, extracellular matrix; NOV, nephroblastoma overexpressed gene; CTGF, connective tissue growth factor; TGF-β1, transforming growth factor β1; DEX, dexamethasone; BDL/S, bile duct ligation/scission; CCl_4, carbon tetrachloride; CHX, cycloheximide.

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(NOV) mRNA as one member of the mRNA population that was upregulated in cultured activated HSCs. NOV was first identified as an overexpressing gene in virus-induced avian nephroblastoma, and its orthologs were later isolated from *Xenopus laevis* ovary [13], rat lung [14], mouse embryonic kidney [15], and human embryonic kidney cell line 293 [14]. NOV belongs to the CCN gene family [16], which is constituted by connective tissue growth factor (CTGF) [17,18], cysteine-rich 61 (Cyr61) [19,20], NOV [21,22], and other related genes. Each of the proteins possesses an amino-terminal signal peptide, indicating that they are secreted proteins [23]. In the present study, we demonstrate the expression of an activated HSC-predominant gene, NOV, which is upregulated in both in vitro HSC activation and in vivo models of experimentally induced liver fibrosis; and we also examine its regulation by transforming growth factor β1 (TGF-β1), dexamethasone (DEX), and bile acids in this cell type.

2. Materials and Methods

2.1. HSC isolation and culture

Rat HSCs were isolated from the livers of Sprague-Dawley rats as described previously [12]. Stellate cells were plated for 3 h in William's Medium E (WME, Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL) and antibiotics (0.035 g/l penicillin and 100 mg/l streptomycin), and the cultures were subsequently washed to remove dead cells and cell debris. In the present study, freshly isolated HSCs from normal liver were referred to as quiescent HSCs. Likewise, HSCs isolated from normal liver and cultured for 14 days were referred to as in vitro activated HSCs.

After reaching confluency (about 14 days after plating), activated HSCs were detached by incubation with trypsin (0.025% trypsin/0.5 mM EDTA), split in a 1:3 ratio, and subcultured. Experimental manipulations were performed on cells between the third and fifth serial passages. The HSC were treated prior to the collection of conditioned media and cell lysates, as stated in the appropriate figures.

2.2. Library construction and sequencing

Construction of the 3′-directed cDNA library and transformation into *Escherichia coli* were carried out as described previously [7,12,24]. The transformant colonies were randomly selected and cultured in 96-well plates, and the inserted cDNAs amplified with flanking primers and subjected to cycle sequencing. Sequences of 3′-directed cDNA clones were compared with each other by using a computer program, DNASIS™ (Hitachi). Sequence similarities between cDNA clones, and between cDNA clones and GenBank entries, were analyzed using the BLAST nucleotide program (blastn) in the National Center for Biology Information.

2.3. Experimental fibrotic models and human liver samples

Liver fibrosis was induced in pathogen-free male Sprague-Dawley rats (200–250 g). Biliary cirrhosis was induced by ligation and excision of the bile duct (BDL/S) as described [12,24]. Rats ($n = 8$) were anesthetized with Ketamine/Rompun and double ligatures were performed on the common bile duct and the scission was made into the bile duct between the two ligatures. In sham operated rat ($n = 4$), only an incision was made in the abdomen and it was closed without any damage to bile duct. The animals were sacrificed after 28 days of obstruction. The carbon tetrachloride (CCl_4) group ($n = 6$) was treated with an oral administration of CCl_4 (Sigma Chemical) diluted 50% (v/v) with corn oil (Sigma) at a dose of 1 ml/kg body weight for each rat. CCl_4 was administrated twice per week for six weeks. Three days after the last CCl_4 treatment, the rats were sacrificed; control animals ($n = 6$) were treated with corn oil for six weeks. All animals received humane care according to National Institute of Health guidelines.

The fresh frozen tissue from cirrhotic liver was obtained from individuals undergoing transplantation for end-stage cirrhosis, which included a case of primary biliary cirrhosis, two cases of biliary atresia, two cases of alcoholic cirrhosis, and three cases of hepatitis B cirrhosis. The patients consisted of four males and four females, with ages ranging from 2 to 60 years (37.8 ± 22.80 years, mean ± S.D.). As controls, three samples of normal liver were obtained from the resected livers of benign lesions including hemangioma and focal nodular hyperplasia. These livers contained no hepatitis B or C, and showed normal histology. The patients in the normal group consisted of one male and two females, with ages ranging from 44 to 74 years (55.0 ± 16.52 years, mean ± S.D.). The fresh tissue from each lesion was snap-frozen in liquid nitrogen and stored at −70 °C until required. Representative sections were submitted for a histological examination and hematoxylin-eosin stained sections from paraffin blocks were used for the histological examination.

2.4. Northern analysis

Total RNA was extracted from HSCs using the RNeasy Mini kit (Qiagen) and quantified by assessing absorbance at 260 nm using UV spectrometry. Twenty micrograms of total RNA was electrophoresed on a 1% agarose gel containing 5.4% formaldehyde, transferred to nylon membranes (Hybond-N; Amersham Biosciences) by electroblotting, and fixed by UV irradiation. The RNA was hybridized with random primed [^{32}P]-labeled cDNA specific for NOV and GAPDH, and using a portion of CTGF cDNA amplified by RT-PCR and corresponding to bases

433–577, of the CTGF sequence (GenBank Accession No. NM_022266) [25]. Purified PCR product was sequenced for gene identification. Prehybridization and hybridization were performed in a solution of 50 mM PIPES, 100 mM NaCl, 50 mM sodium phosphate, 1 mM EDTA, and 5% SDS at 65 °C overnight. After hybridization, the nylon membrane was rinsed twice in 1 × SSC containing 5% SDS at room temperature for 10 min, and subsequently washed in 0.1 × SSC containing 5% SDS at 65 °C for 30 min. Reactive bands were detected by autoradiography using X-ray film (Agfa).

2.5. Reverse-transcription polymerase chain reaction (RT-PCR)

Five micrograms of total RNA were reverse-transcribed using the Moloney Murine Leukemia Virus reverse transcriptase (Gibco BRL) and the resultant cDNA was diluted 10-fold for PCR. Oligonucleotide primers to rat NOV were: (sense, 5′-ctg aga tga gac cct gcg ac-3′ corresponding to nt 235–254: antisense, 5′-gtg caa ttc ttg aac tgt agg tgg-3′ corresponding to nt 830–853, according to GenBank Accession No. NM_030868), human NOV [26] were: (sense, 5′-ctg tgt gtt cga tgg ggt ca-3′ corresponding to nt 399–418: antisense, 5′-cac agc tct tgg agc atg c-3′ corresponding to 715–733, according to GenBank Accession No. X96584), and rat egr 2 were: (sense, 5′-tct gcc tcc ccc aac cca ct-3′ corresponding to nt 755–774: antisense, 5′-tgg tcc tcc aat ggc gct gt-3′ corresponding to 1701–1720, according to GenBank Accession No. NM_053633). PCR reactions were carried out in the presence of 1.5 mM $MgCl_2$ at the following temperatures and times: 94 °C, 30 s; 57 °C, 30 s for rat NOV, 56 °C, 30 s for human NOV, and 62 °C, 30 s for egr 2; 72 °C, 1 min; for 30 cycles and a final extension at 72 °C for 10 min.

The amplified PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. The integrity of cDNA samples used was confirmed using primers for rat GAPDH [12] and human GAPDH (sense, 5′-cgg agt caa cgg att tgg tcg tat-3′ corresponding to nt 888–911: antisense, 5′-agc ctt ctc cat ggt ggt gaa gac-3′ corresponding to nt 1171–1194, according to GenBank accession No. BC014085). Purified PCR products were sequenced using an autosequencer (ABI 377 DNA sequencer, Perkin-Elmer) and the obtained sequence was compared to the DNA databases for gene identification using the BLAST nucleotide program (blastn).

2.6. NOV antibody

The NOV antibody was manufactured by Takara Korea Biomedical Inc. A synthetic peptides (Takara Korea Biomedical Inc.) corresponding to the 21 C-terminal residues (334–354) of mouse NOV [27] was used as an immunogen and immunized in rabbit. This region bears little sequence homology to other CCN family proteins.

2.7. Immunohistochemistry procedure

Immunohistochemistry was performed either on paraffin-embedded sections for rat liver specimens or fixed HSCs. For rat liver specimens, immunohistochemistry was performed using the LSAB® 2kit (Dako), based on the strepavidin-biotin-peroxidase complex method. Following endogenous peroxidase blocking, the sections were incubated at room temperature for 2 h with rabbit anti-NOV (1:250 dilution). For cultured HSCs, the same immunohistochemical procedure was run manually on cells fixed in acetone/methanol (1:1) for 10 min at 4 °C.

For human liver sample, immunohistochemical studies were performed on frozen sections of liver with a catalyzed signal amplification system (Dako) as described previously [12]. Anti-NOV antibody was used at a 1:100 dilution.

2.8. Western blot detection of NOV in HSC

The production of NOV protein was examined in HSC-conditioned media; equal volumes of conditioned media from HSCs were concentrated 10-fold in 10 kDa microcentrifuge-concentrators (Millipore). Protein concentration was then determined using a Bio-Rad DC protein assay kit (Bio-Rad Laboratories). The proteins (20 μg) were separated by 10% (w/v) SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were treated with a blocking buffer comprising 5% (w/v) non-fat milk in phosphate-buffered saline containing 0.5% Tween-20, and then incubated with polyclonal anti-NOV antibody (diluted 1:500) and anti-CTGF antibody (Abcam; diluted 1:5000) for 2 h at room temperature. After vigorous washing, the membrane was then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology; diluted 1:2000) for 1 h at room temperature. Immunoreactive bands were visualized on X-ray film (Agfa) using the enhanced chemiluminescence western blotting detection system kit (Amersham Biosciences) according to the manufacturer's recommended protocol. The developed films were subjected to densitometric analysis (JX-330P, Pharmacia).

2.9. Statistical analysis

Data were analyzed with Student's *t*-test when appropriate or one-way ANOVA and Tukey's multiple comparison tests when comparing means of more than three. Calculations were performed with the Graphpad Prism program (GraphPad Software).

3. Results

3.1. Expression of NOV mRNA in rat HSCs

Using the 3′-directed cDNA library technique, we investigated the pattern of overall mRNA that altered their

expression levels during the activation process [7]. This result included the upregulation of thrombospondin [28], dystroglycan [29], α-B-crystallin [5,30], and *O*-acetyl disialoganglioside synthase [12] in activated HSC. In addition, NOV was found to be upregulated; this represents a new finding.

To confirm that NOV is differentially expressed after HSC activation, we performed northern blot analyses using total RNA obtained from quiescent and culture-activated HSCs. NOV mRNA was virtually undetectable in cellular RNA prepared from freshly isolated HSC, but increased dramatically following HSC activation (Fig. 1). NOV mRNA was detected as a major transcript of 2.5 kb and a minor transcript of 2.0 kb.

3.2. Expression of NOV mRNA in experimental fibrotic models and human liver

Northern blot analysis was used to determine if NOV gene expression could be induced following a fibrogenic stimulus in vivo. Fig. 2A reveals negligible expression in the sham and CCl_4-control livers, whereas significantly expression is seen in both the BDL/S-operated and CCl_4-induced fibrosis livers.

We also examined NOV mRNA expression in human liver explants assessed by RT-PCR (Fig. 2B). To visualize this result, densitometry analysis of the upper panel is demonstrated graphically in the lower panel. This assay demonstrated that transcripts for human NOV mRNA were readily detectable in each of the diseased liver groups but were virtually absent in histologically normal liver.

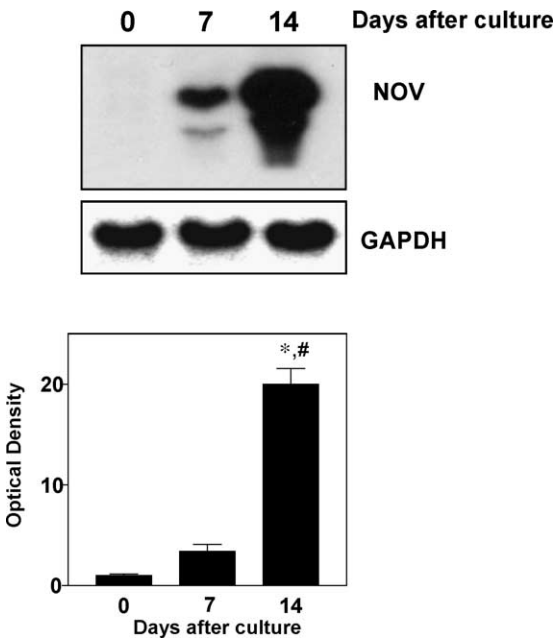

Fig. 1. NOV mRNA expression was determined by northern blot using GAPDH as the quantity control. Total cellular RNA was prepared from freshly isolated HSCs, or from HSCs that had been cultured in 10% FBS-containing media on plastic for the times shown. A representative Northern blot is shown. Results are mean ± S.D. from three independent experiments. The NOV mRNA densitometry values were normalized to their respective GAPDH densitometry values. Statistical significance: $^{*}P < 0.001$ vs. 0 day; $^{\#}P < 0.001$ vs. 7 days, as analyzed by one-way ANOVA and Tukey's multiple comparison tests.

3.3. Immunohistochemical detection of NOV

NOV expression by HSCs was supported by immunohistochemistry of primary HSCs 10 days after isolation. NOV immunostaining was strong and diffuse in the cytoplasm (Fig. 3A). No staining was observed when immunostaining was performed after omitting the primary antibody (Fig. 3B).

To assess the location and dynamics of NOV expression in liver fibrosis, we studied the expression of NOV in rats treated with CCl_4 and in BDL/S-operated rats. In normal liver, only mild immunostaining was detected in the portal tracts (Fig. 3C). As fibrosis progressed, the reactivity increased, particularly in the fibrotic septa of the liver in CCl_4-treated rats (Fig. 3D). In the BDL/S liver, the reaction was also strong along the septa and showed a significant increase in areas of ductular proliferation (Fig. 3E). Also in normal and fibrotic rat liver, no staining was observed in the negative control (data not shown).

In human liver, NOV was also detected by immunohistochemistry. In biliary cirrhosis due to biliary atresia, a positive reaction was observed in the proliferated duct with cholestasis (Fig. 4B), in contrast to negative reaction in normal liver (data not shown). Additionally, in macronodular cirrhosis due to hepatitis B, NOV immunoreactivity was strong in the ductules and stellate cells of the fibrous septa (Fig. 4D).

3.4. The effects of exogenous TGF-β1 on NOV expression

As TGF-β1 is the major profibrogenic cytokine in the liver, we examined the effect of exposing cultured HSC to TGF-β1. To determine the kinetics of NOV expression under TGF-β1 stimulation, HSCs were treated for varying time periods with 10 ng/ml TGF-β1. Consistent with the previous results [25], CTGF mRNA expression increased at 1 h, reaching a maximum at 4 h. Under the same conditions, however, no differences were observed in the levels of NOV mRNA between the control and TGF-β1-treated groups (Fig. 5A). The effect of TGF-β1 on NOV expression was also studied at the protein level. Western blot analysis was performed on concentrated conditioned media. The conditioned media resulted in specific binding of anti-NOV antibody, whereas the cell lysates resulted in non-specific binding pattern (data not shown). Interestingly, a significant increase in the NOV expression band was observed in the TGF-β1-treated conditioned medium by comparison with control (Fig. 5B). These data indicate that TGF-β1 induced NOV by post-transcriptional activation without changing its mRNA level.

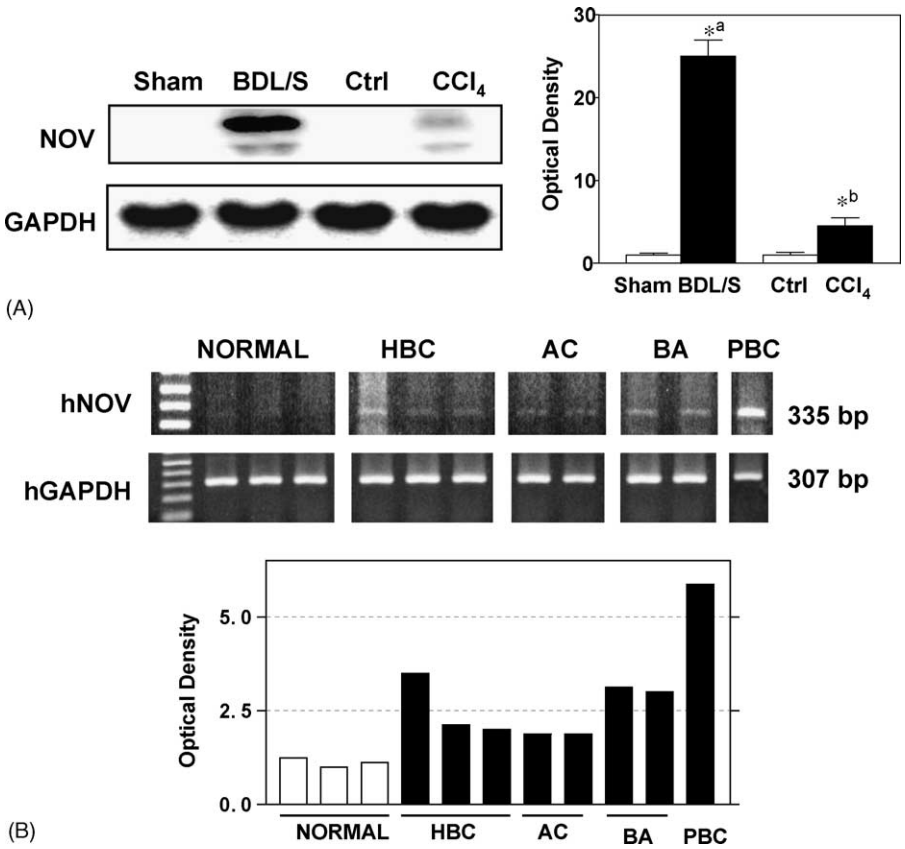

Fig. 2. (A) Northern blot analysis of NOV mRNA in BDL/S (28 days after operation) and CCl_4-induced (for six weeks) liver fibrosis. Sham: sham operated; Ctrl: CCl_4-control (treated with corn oil for six weeks). A representative Northern blot is shown. Results are mean ± S.D. from three independent experiments. The NOV mRNA densitometry values were normalized to their respective GAPDH densitometry values. Statistical significance: $^*P < 0.05$; a, vs. sham; b, vs. CCl_4-control, as analyzed by Student's t-test. (B) Human NOV (hNOV) mRNA expression in histologically normal and fibrotic human liver. Total RNA isolated from three normal and eight fibrotic livers (HBC: Hepatitis B cirrhosis, n = 3; AC: Alcoholic cirrhosis, n = 2; BA: Biliary atresia, n = 2; and PBC: Primary biliary cirrhosis, n = 1) was subjected to RT-PCR analysis. The hNOV mRNA densitometry values were normalized to their respective human GAPDH (hGAPDH) densitometry values.

To further study the mechanism of post-transcriptional activation of NOV by TGF-β1, HSCs were pretreated with the mRNA translational inhibitor, cycloheximide (CHX) [31]. In a eukaryotic cell, CHX inhibits protein synthesis on ribosomes in the cytosol. As shown in Fig. 5C, NOV protein was increased after a single treatment of TGF-β1, while the inducing effect of TGF-β1 was completely blocked by CHX pretreatment. However, TGF-β1-dependent CTGF protein expression was not altered by CHX pretreatment, as described previously [25]. These data indicate that NOV activation by TGF-β1 results primarily from an increase in the efficiency of NOV mRNA translation, not from post-translational mechanisms such as increasing NOV protein stability.

3.5. The effect of DEX on NOV mRNA expression

DEX has been demonstrated to be a potent inducer of CCN family gene expression [14]. To determine whether DEX also affects the expression of NOV in this system, we treated cultured HSCs with 1 μM DEX for different time periods and analyzed NOV mRNA and protein expression. In comparison with untreated controls, DEX treatment resulted in a strong induction of NOV mRNA expression. A significant induction was seen 10 h after addition of the DEX, and increased NOV mRNA levels were still observed after 12 h (Fig. 6A). This DEX-mediated induction was dose-dependent, whereby 1 μM DEX was sufficient to elicit a maximal response (data not shown). DEX-induced NOV expression was also studied at the protein level. Western blot analysis was performed on concentrated conditioned media from untreated and DEX-treated HSC. An increase in the specific NOV protein band was observed in response to treatment with DEX (1 μM) for 10 h and 12 h compared with the unstimulated control (Fig. 6B).

3.6. The effect of bile acids on NOV mRNA expression

Bile acids have been demonstrated to be co-stimulatory activating molecules of HSC perpetuation [32]. Moreover, in this study we observed a marked increase of NOV mRNA expression in the BDL/S-induced fibrogenesis compared with CCl_4-induced fibrogenesis (Fig. 2A) and

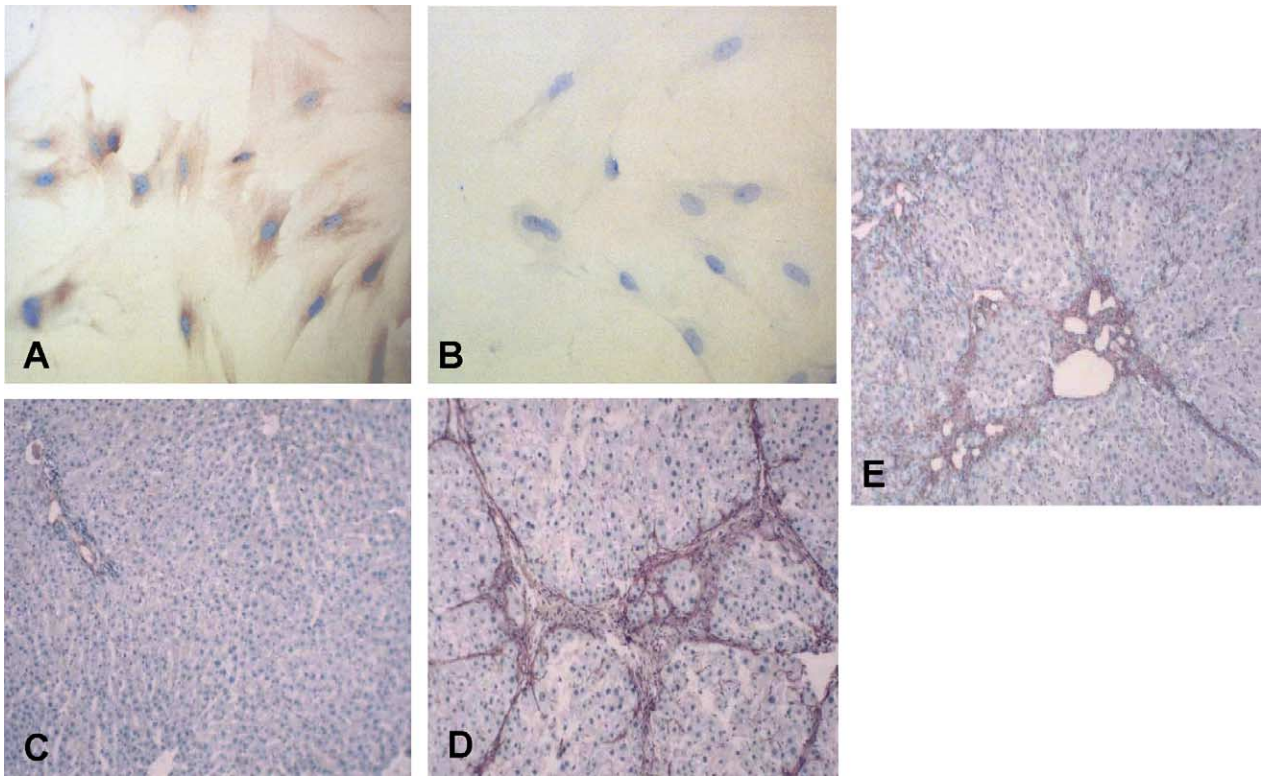

Fig. 3. Detection of NOV expression by immunohistochemistry. (A, B) NOV immunostaining in cultured HSCs for 10 days (A). No staining was observed in the negative control (B). Immunohistochemistry of normal liver (C). CCl_4-induced (for six weeks) fibrotic liver (D). And BDL/S (28 days post-operation) fibrotic liver (E). Original magnification: 100×.

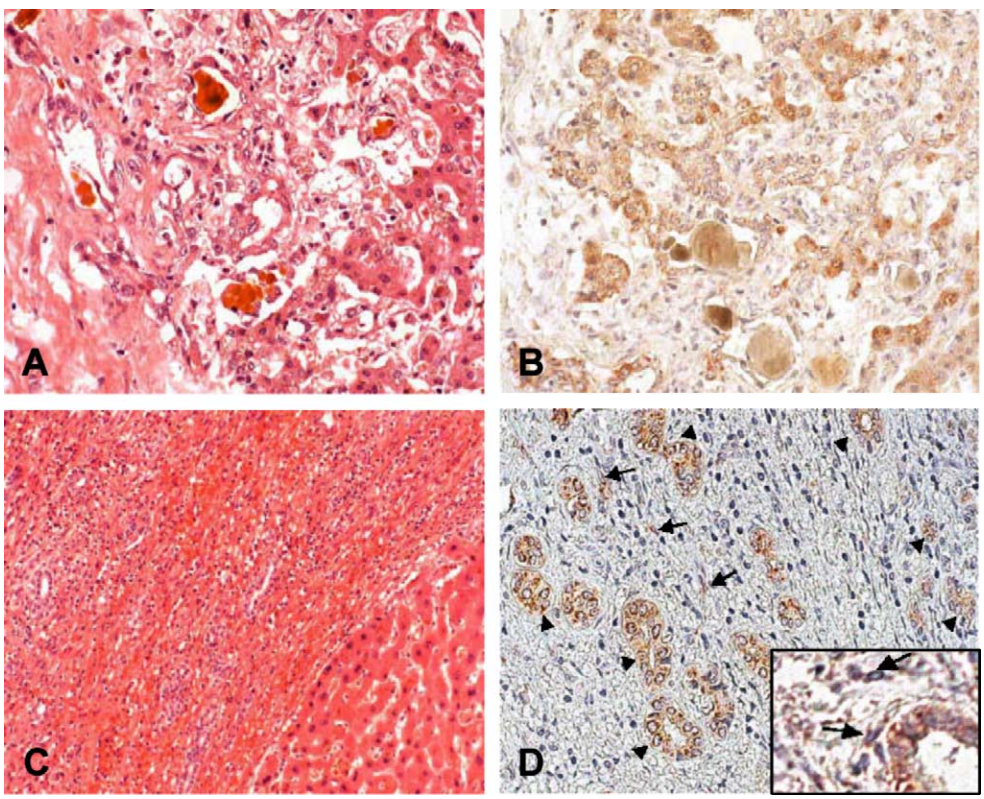

Fig. 4. Expression of NOV in cirrhotic human liver. (A, B) Human biliary atresia showed positive reaction in proliferated bile duct. (C, D) Hepatitis B cirrhosis showed a positive reaction in ductules (arrow heads) and HSCs (arrows) of the fibrous septa. A and C showed Hematoxylin-eosin staining; B and D showed immunohistochemical stain for NOV. In D, the inset (original magnification 400×) shows positive staining of NOV in HSCs. Original magnification: 200× (A, B, D) and 100× (C).

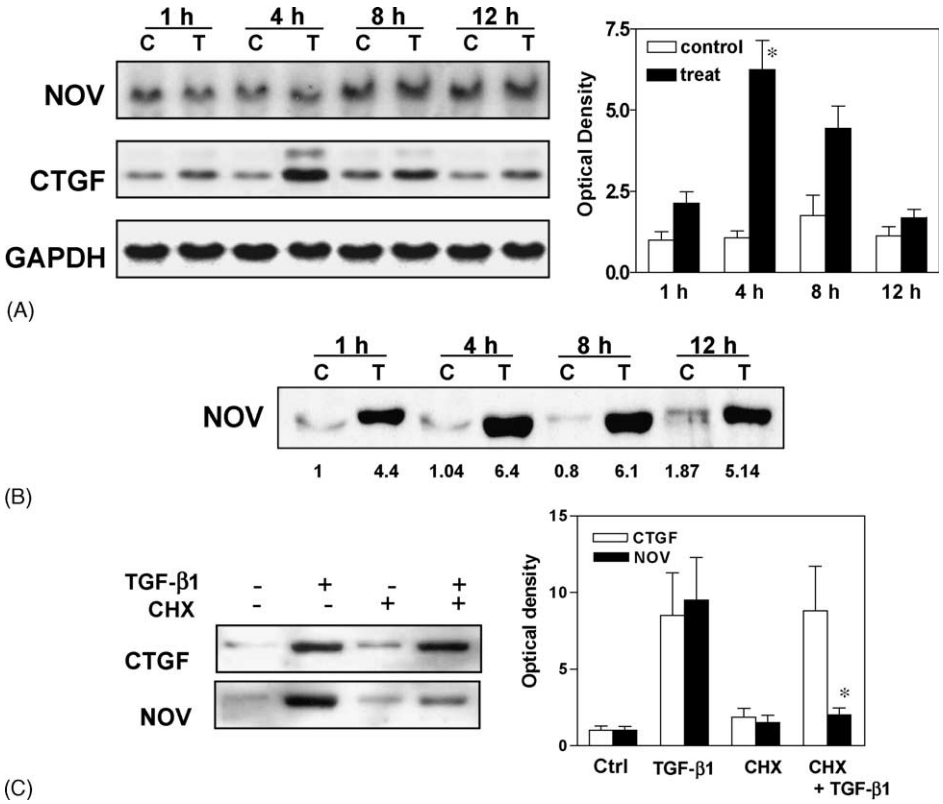

Fig. 5. The effect of exogenous TGF-β1 on NOV expression in cultured HSC. HSC cultured in serum containing media were washed three times in serum-free WME and left in this medium for 1 h following the final wash. The cells were then incubated in fresh media containing 0.01% FBS for 24 h. TGF-β1 was added to the cells to a final concentration of 10 ng/ml. Untreated cells were used as controls. HSC were incubated in the presence of TGF-β1 (10 ng/ml) for various periods of time (1, 4, 8, and 12 h). (A) Northern blot analyses of NOV and CTGF mRNA were performed using total RNA. C: control; T: TGF-β1-treated. The right panel represents the results of Northern blot analysis. The CTGF mRNA densitometry values were normalized to their respective GAPDH densitometry values. Results are mean ± S.D. from three independent experiments. Statistical significance: $^{*}P < 0.01$ vs. 1 h-TGF-β1-treated groups, as analyzed by one-way ANOVA and Tukey's multiple comparison tests. (B) Western blot of NOV after TGF-β1 stimulation of HSCs was performed on concentrated conditioned media from untreated and TGF-β1-treated HSC for the times shown after addition of the TGF-β1. Two independent experiments gave the same results. C: control; T: TGF-β1-treated. The optical density were obtained from densitometry analysis and shown beneath each lane. (C) Regulation of the expression of NOV and CTGF by TGF-β1 following treatment with CHX. Western blot analysis was performed on concentrated conditioned media. Cells were pretreated for 30 min with CHX (10 μg/ml) before the addition of TGF-β1 for 4 h. A representative immunoblot from three independent experiments is shown. Results are the mean ± S.D. of three independent experiments. Ctrl: control. Statistical significance: $^{*}P < 0.05$ vs. TGF-β1-treated group, as analyzed by Student's t-test.

in primary biliary cirrhosis compared with other diseased liver groups (Fig. 2B); and in biliary atresia patient liver, NOV protein expression was observed in the proliferating duct with cholestasis (Fig. 4B). This result suggests that bile acids may be having a modulating effect on the induction of NOV expression. Therefore, in an additional experiment we determined if NOV mRNA expression might mediate through direct interaction between bile acids and cultured HSCs.

Cholic acid represents a major class of hydrophobic bile acids that increase in concentration during liver injury [33,34]. As shown in Fig. 7, cholic acid treatment resulted in a significant induction of NOV mRNA expression. Maximal induction was seen at 60 min after addition of cholic acid, where it was correlated temporally with egr 2 expression, a gene rapidly induced by bile acids in HSCs [32].

To explore the possibility that the response was specific for cholic acid, HSCs were exposed to other major bile acids believed to play significant roles during liver disease. All of the bile acids were studied at a concentration of 50 μM and to the 60 min time point, as these appeared to be the optimal conditions for cholic acid stimulation. Chenodeoxycholic acid and ursodeoxycholic acid treatment also resulted in the induction of NOV mRNA expression, which correlated temporally with egr 2 expression.

4. Discussion

Stellate cell activation is a broad phenotypic conversion resulting from a highly coordinated and sequential expression of several gene families. To gain a better understanding of the molecular events that take place after HSC activation, we have been using expression-profiling methods to isolate the genes that are either activated or suppressed following HSC activation in culture. These genes are identified by their recurrences. The resulting list of the

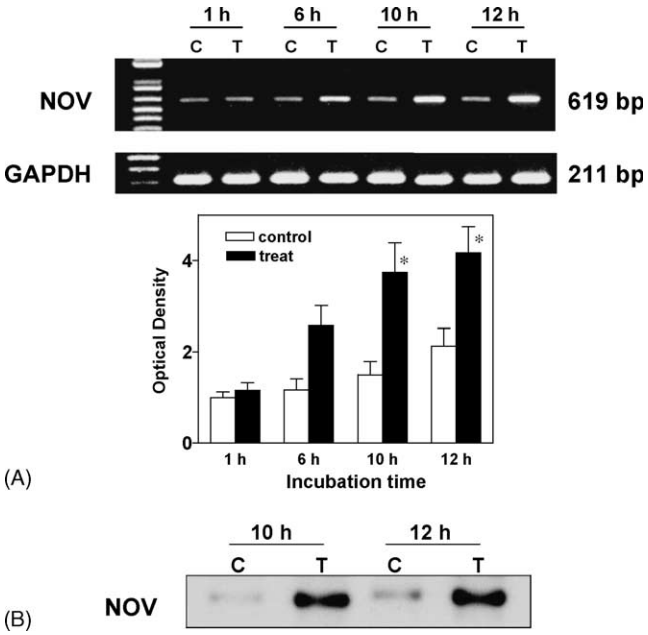

Fig. 6. DEX induces NOV expression in HSC. HSCs cultured in serum containing media were washed three times in serum-free WME and left in this medium for 1 h following the final wash. The cells were then incubated in fresh media containing 0.01% FBS for 24 h. DEX was added to the cells to a final concentration of 1 μM. Untreated cells were used as controls. Aliquots of cells and media were harvested at the indicated time points after addition of DEX. (A) RT-PCR analysis was performed using total RNA. C: control; T: DEX-treated. A representative RT-PCR result is shown. Results are mean ± S.D. from three independent experiments. The NOV mRNA densitometry values were normalized to their respective GAPDH densitometry values. Statistical significance: $^{*}P < 0.05$ vs. 1 h-DEX-treated groups, as analyzed by one-way ANOVA and Tukey's multiple comparison tests. (B) Western blot analysis was performed on concentrated conditioned media from untreated and DEX-treated HSC for the times shown after addition of the DEX. C: control; T: DEX-treated.

expressed gene species and the abundance of their transcripts is called an expression profile; this profile identifies the cellular phenotype by its gene products. By comparing the expression profiles between quiescent and activated HSCs, we identified NOV as one of the mRNAs with a significantly upregulated expression in cultured activated HSCs. Previous studies suggest that CTGF, a member of the CCN family, contributes to liver fibrosis and that activated HSC are a source of CTGF [25,35,36]. However, NOV expression in activated HSCs has not been studied.

In our studies, we have demonstrated increasing NOV mRNA expression during progressive activation of HSC in culture, and have also provided evidence of an upregulation of NOV during liver fibrosis. Immunohistochemical detection of NOV showed that it was predominantly located in areas of ductular proliferation and fibrous septa.

Furthermore, we observed increased NOV mRNA in a variety of human fibrotic liver diseases. These results in human fibrotic liver therefore support our finding in the rat model of fibrosis. In addition, this suggests that NOV may be a ubiquitous mediator unrelated to any specific etiology. However, the diagnostic significance of NOV expression in chronic liver diseases must be further investigated with more samples. We also observed increased NOV protein in biliary atresia, with the most striking increases observed in proliferated bile ducts. From this result, in biliary fibrosis, we suggest that not solely HSCs but activated bile duct epithelial cells may be the source of NOV. Further investigations will be required to clarify this point.

TGF-β1-induced NOV expression was also studied. A significant increase in the NOV protein band was observed in the TGF-β1-treated conditioned medium, and its complete blockade by pretreatment with an inhibitor of mRNA translation, suggest that NOV induction by TGF-β1 occurs mainly by activating the translation of NOV mRNA to protein. These data confirm the regulatory role of TGF-β1 on NOV regulation in HSC activation.

In our studies, we have demonstrated the induction of NOV expression by DEX in HSC. The actual effect of the DEX-mediated increase in NOV expression on the extent of fibrosis is as yet unknown. However, because we have shown that elevated levels of NOV correlate with fibrosis (Figs. 1 and 2), our results provide one explanation for the limited clinical usefulness of this class of compounds in the treatment of liver fibrosis. Our data are consistent with the published study examining the effect of DEX on ECM expression and synthesis by HSC [37]. Niki et al. [37] found glucocorticoids do not possess a net suppressive effect on ECM synthesis by HSCs and the limited beneficial effects of glucocorticoids may be attributable to other mechanisms of action, such as their anti-inflammatory effect.

Additionally, we have demonstrated that bile acids have a modulating effect on the induction of NOV mRNA

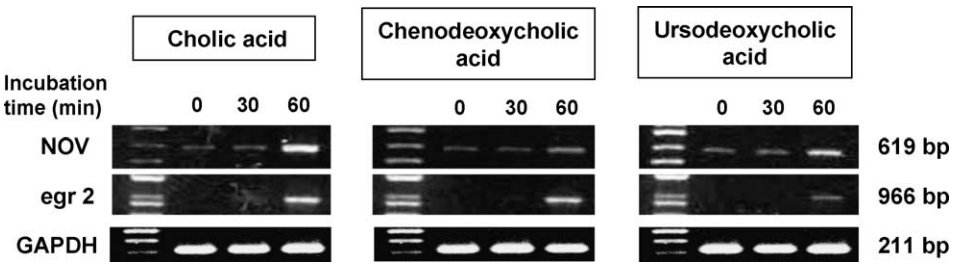

Fig. 7. Induction of NOV and egr 2 mRNA by bile acids. HSCs cultured in serum containing media were washed three times in serum-free WME and left in this medium for 1 h following the final wash. Cultured stellate cells were maintained in media containing 0.4% FBS for 24 h and then stimulated with 50 μM cholic acid, chenodeoxycholic acid, and ursodeoxycholic acid for the indicated time points. Integrity of the cDNA was confirmed by GAPDH.

expression. Since the elevated bile acid levels, which are a frequent counterpart of liver injury, NOV expression is induced by bile acids, suggesting a pathogenic role of this molecule in liver fibrogenesis.

Compared with Cyr61 and CTGF, NOV has been studied to a lesser degree and its biological role remains primarily unknown. However, there are several lines of evidence indicating that, in normal tissues, the expression of NOV is tightly regulated during the development of the central nervous system [38] and the skeletal and visceral muscles [39]. There are also several reports that have suggested that it up- or down-regulates cell proliferation, depending on the cell type [14,21,27]. Overexpression of NOV inhibits the proliferation of chicken embryonic fibroblasts, suggesting a negative effect on cell growth. To the contrary, human NOV protein stimulates the proliferation of fibroblasts and also induces protein tyrosine phosphorylation, implying a positive effect on cell proliferation. Therefore, the role and function of NOV in normal and pathological tissues is still controversial and intriguing. Based on our present findings, the overexpression of NOV in liver fibrosis, especially in perisinusoidal stellate cells, suggests that this novel factor may play an important role in the pathogenesis of hepatic fibrosis.

Additionally, it was recently shown that NOV is exhibited frequently in metastatic liver lesions [26] and acts directly upon endothelial cells to stimulate pro-angiogenic activity, and induces angiogenesis in vivo [40,41]. Therefore, it is possible that NOV may contribute to the progression from fibrosis to tumor in the liver. Further investigations will be required to clarify this point.

In conclusion, NOV is a novel gene expressed predominantly in the activated HSC. The expression levels of NOV parallel those found in liver fibrosis in vivo. If further studies confirm the function and the concept that NOV may be a downstream mediator of TGF-β1, the development of selective antagonists of NOV could specifically affect the fibrogenesis process. Indeed, the marked upregulation of NOV in activated HSCs suggests that NOV does play some role in the pathogenesis of liver fibrosis.

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