with additional costs, inconvenience, and potential side effects. Taribavirin (Viramidine), a liver-targeted prodrug of ribavirin, is associated with a lower incidence of anemia [17]. In the Phase II randomized study of taribavirin versus ribavirin combined with peginterferon alpha-2a in treatment-naïve patients with chronic hepatitis C, the rate of SVR did not differ significantly between the taribavirin (23%, 37% and 29% at 800, 1200, and 1600 mg) and ribavirin (44% at 1000 and 1200 mg) groups. Fewer patients receiving taribavirin (4%) than ribavirin (27%) had severe anemia (hemoglobin <10 g/dL).

NS3/4A SERINE PROTEASE INHIBITORS

BILN-2061

BILN-2061 was the first NS3/4A protease inhibitor to be examined. Administration of 200 mg BILN-2061 twice daily for 2 days resulted in an impressive effect that corresponds to a reduction in plasma HCV RNA levels of 2 to 3 $\log_{10}$ or greater for all treated patients with genotype 1 infection [18]. However, development was halted because of cardiac toxicity in laboratory animals.

Telaprevir (VX-950)

Telaprevir is an inhibitor specific to the HCV NS3/4A serine protease. In a Phase I randomized trial of 750 mg telaprevir 3 times daily with and without peginterferon alpha-2a in previously untreated patients with genotype 1 hepatitis C, the median change in HCV RNA from baseline to day 15 was –1.09 $\log_{10}$ in the placebo group, –3.99 $\log_{10}$ in the telaprevir group, and –5.49 $\log_{10}$ in the telaprevir and peginterferon alpha-2a group [19].

In Phase II randomized clinical trials known as the PROVE 1 and 2 Studies, conducted in patients with chronic HCV genotype 1 infection who had not been treated previously, the triple-therapy group that received telaprevir (1250 mg on day 1 and 750 mg every 8 hours thereafter), peginterferon alpha-2a, and ribavirin for 12 weeks followed by peginterferon alpha-2a and ribavirin for 12 more weeks (total 24 weeks) had significantly higher rates of SVR (61–69%) than did the standard-therapy group that received peginterferon alpha-2a and ribavirin for 48 weeks (41–46%) [20, 21]. However, viral breakthrough occurred in 7% of patients who received telaprevir. The rate of discontinuation because of adverse events, including pruritus, rash, and anemia, was higher in the groups who received telaprevir.

The interim analysis of the Phase II clinical trial PROVE3, conducted in patients with HCV genotype 1 infection who previously failed treatment with peginterferon alpha and ribavirin, showed that the rates of SVR in the triple-therapy group (telaprevir, peginterferon alpha-2a, and ribavirin for 12 weeks followed by peginterferon alpha-2a and ribavirin for 12 more weeks for a total of 24 weeks) was 51% compared with 14% in the standard-therapy group (peginterferon alpha-2a and ribavirin for 48 weeks) [22].

Boceprevir (SCH 503034)

Boceprevir is another oral HCV NS3/4A protease inhibitor. In the Phase I study, boceprevir plus peginterferon alpha-2b was well tolerated in patients with HCV genotype 1 infection who were previously nonresponders to peginterferon alpha-2b with or without ribavirin [23]. Mean maximum $\log_{10}$ changes in HCV RNA were –2.88 by treatment with peginterferon alpha-2b plus 400 mg boceprevir 3 times daily for 2 weeks compared with –1.08 to –1.26 by treatment with peginterferon alpha-2b alone for 2 weeks.

In the Phase II clinical trial known as HCV SPRINT-1, the triple-therapy group that received peginterferon alpha-2b and ribavirin for 4 weeks (lead-in) followed by the addition of 400 mg boceprevir 3 times daily to the combination for 24 or 44 weeks (total 28 or 48 weeks) had significantly higher rates of SVR (56–75%) than did the 48-week standard-therapy group (38%), which consisted of treatment-naïve patients with chronic HCV genotype 1 infection [24]. Of interest, introduction of a 4-week lead-in with the standard therapy before the addition of boceprevir reduced the incidence of viral breakthrough. The incidence of rash-related adverse events was similar with the boceprevir-containing regimens and the control. Treatment discontinuations due to adverse events, including anemia, nausea, and headache, were 9–19% in the boceprevir arms compared with 8% in the control arm.

TMC435 (TMC435350-C201)

Interim results from the Phase II clinical trial OPERA-1 showed that 75 or 200 mg of the protease inhibitor TMC435 once daily, in combination with peginterferon alpha-2a and ribavirin, decreased HCV RNA levels to below the lower limit of quantification (<25 IU/mL) on day 28 in all treatment-naïve patients with HCV genotype 1 who were assigned to the triple-therapy arms [25]. The trial is ongoing for treatment-experienced patients.

R7227/ITMN-191

In the Phase I multiple ascending dose study, the protease inhibitor R7227/ITMN-191 reduced HCV RNA in a dose dependent manner through day 14 [26]. Treatment with 200 mg of R7227/ITMN-191 3 times daily resulted in rapid and sustained reductions in HCV RNA, with a median reduction of 3.8 $\log_{10}$ at day 14. The rate of treatment response was lower in prior non-responders than in treatment-naïve HCV genotype 1 patients.

NUCLEOSIDE POLYMERASE INHIBITORS

R1626

R1626 is a prodrug of the nucleoside inhibitor of HCV NS5B polymerase R1479. In the Phase II clinical trial in HCV genotype 1–infected treatment-naïve patients, triple therapy with peginterferon alpha-2a and ribavirin plus 1500 mg R1626 twice daily resulted in a rapid virologic response (defined as an undetectable HCV RNA level at week 4) in 74% of patients compared with 5% of patients receiving standard therapy with peginterferon alpha-2a and ribavirin [27]. However, development was recently discontinued because of hematological toxicity issues and a high relapse rate [28].

R7128

R7128 is a prodrug of the nucleoside inhibitor of HCV NS5B polymerase PSI-6130. The interim results of the Phase I clinical trial showed that R7128 delivered a rapid virologic response in 85–88% of treatment-naïve patients with HCV genotype 1 infection, compared with 10% with the standard of care, when administered at doses of 1000 to 1500 mg twice daily in combination with peginterferon alpha-2a and ribavirin for 28 days [29]. The high rate of rapid virologic response (≥86%) was also reported in HCV genotype 2/3 non-responders [30].

NON-NUCLEOSIDE POLYMERASE INHIBITORS

Filibuvir (PF-00868554)

Filibuvir is a non-nucleoside inhibitor of HCV NS5B polymerase. Interim 4-week results of the Phase I clinical trial in treatment-naïve patients with chronic hepatitis C genotypes 1 showed that the addition of 200, 300, and 500 mg Filibuvir twice daily for the first 4 weeks of peginterferon alpha-2a and ribavirin treatment resulted in a rapid virologic response in 60%, 75% and 63% of patients, respectively, compared with 0% with peginterferon alpha-2a and ribavirin [31].

GS-9190

GS-9190 is also a non-nucleoside HCV NS5B polymerase inhibitor. In the Phase I dose-escalation trial in treatment-naïve patients with chronic HCV genotypes 1, 40–240 mg GS-9190 was well tolerated, and reductions in individual HCV RNA levels ranged from 0.19 to 2.54 $\log_{10}$ after single-dose exposure [32]. In the multiple dose cohort, however, a possible but not confirmed QT elongation was observed. The trial to study the safety and effectiveness of GS-9190 in combination with peginterferon alpha-2a and ribavirin is ongoing.

ANA598

In the Phase I multiple ascending dose trial in treatment-naïve patients with chronic HCV genotypes 1, a non-nucleoside polymerase inhibitor ANA598 dosed 200 mg twice daily for 3 days as monotherapy demonstrated potent antiviral activity with median viral load decline of 2.4 $\log_{10}$ [33].

NS5A INHIBITOR

BMS790052

BMS790052 is a first-in-class and highly selective HCV NS5A inhibitor. The function of NS5A remains to be poorly understood, but increasing evidence implicates NS5A in multiple essential functions, including modifying the interferon response, facilitating RNA replication, and assembling the virus. In the Phase I ascending-dose study in treatment-naïve or experienced patients with genotype 1 chronic hepatitis C, mean decline in HCV RNA 24 hours after a single 1, 10 and 100 mg dose of BMS790052 was 1.8 $\log_{10}$, 3.2 $\log_{10}$ and 3.3 $\log_{10}$, respectively. Multiple dose trials are ongoing [34].

HOST-TARGETED DRUGS

Nitazoxanide

Nitazoxanide is a member of the thiazolide class of antiparasitic drugs and is licensed in the United States for the treatment of *Cryptosporidium parvum* and *Giardia lamblia*. HCV genotype 4 is the predominant type in the Middle East and Africa, particularly Egypt, and is insensitive to interferon therapy, as is genotype 1. In the Phase II trial conducted in Egypt [35], SVR rates were significantly higher in previously untreated patients with chronic hepatitis C genotype 4 infection given 500 mg nitazoxanide twice daily for 12 weeks followed by nitazoxanide plus peginterferon alpha-2a and ribavirin for 36 weeks (total 48 weeks) than in those treated with peginterferon alpha-2a and ribavirin for 48 weeks (79% compared with 50%; $P = 0.023$). Adverse events were similar across treatment groups. Although the exact mechanism of action remains unknown, *in vitro* nitazoxanide may result in the activation of the double-stranded RNA-activated protein kinase—a key mediator of host cell defenses [36].

Debio-025

Cyclosporin A inhibits the replication of HCV subgenomic replicons. This antiviral effect is not mediated by its immunosuppressive action, but by blockade of intracellular ligands of cyclosporin A, the cyclophilins [37]. Debio-025 is an oral, non-immunosuppressive cyclophilin inhibitor derived from cyclosporin A that has more potent anti-HCV activity than does cyclosporin A in *in vitro* subgenomic replicons and in *in vivo* chimeric mice models [38, 39]. The Phase II trial [40] showed, in treatment-naïve patients with chronic hepatitis C genotypes 1 and 4, that 600 and 1,000 mg Debio-025 per day in combination with peginterferon alpha-2a induced continuous decays in viral load at week 4 that reached –4.61 and –4.75 $\log_{10}$, respectively, compared with –2.49 $\log_{10}$ by peginterferon alpha-2a alone. Headache, nausea, fatigue, and hyperbilirubinemia were the most frequent treatment-emergent adverse events, but no serious events were reported.

CONCLUSIONS

Many novel antiviral agents for HCV are now under clinical investigation. However, any single agent cannot lead to sustained viral eradication. In particular, STAT-C agents cause the rapid selection of drug-resistant mutants when used alone. At present, combination therapy with peginterferon alpha and ribavirin is necessary to reduce the incidence of viral breakthrough and to raise the SVR rate. It is also important to establish measures to overcome safety issues, because the addition of some STAT-C agents to the current standard of care is sometimes associated with a higher rate of treatment discontinuation because of adverse events. In the future, the safety and efficacy of combining multiple direct antiviral agents from different classes should be established,

as for highly active antiretroviral treatment of human immunodeficiency virus infection. The Phase I trial INFORM-1 to study the safety, efficacy and pharmacokinetics of combination of protease inhibitor R7227/ITMN-191 and nucleoside polymerase inhibitor R7128 is currently underway [41].

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Suppression of type I collagen production by microRNA-29b in cultured human stellate cells

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ABSTRACT

MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene expression through imperfect base pairing with the 3′ untranslated region (3′UTR) of target mRNA. We studied the regulation of alpha 1 (I) collagen (Col1A1) expression by miRNAs in human stellate cells, which are involved in liver fibrogenesis. Among miR-29b, -143, and -218, whose expressions were altered in response to transforming growth factor-β1 or interferon-α stimulation, miR-29b was the most effective suppressor of type I collagen at the mRNA and protein level via its direct binding to Col1A1 3′UTR. miR-29b also had an effect on SP1 expression. These results suggested that miR-29b is involved in the regulation of type I collagen expression by interferon-α in hepatic stellate cells. It is anticipated that miR-29b will be used for the regulation of stellate cell activation and lead to antifibrotic therapy.

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Introduction

Hepatic stellate cells, which reside in the Disse's space outside the liver sinusoids, maintain a quiescent phenotype and store vitamin A under physiological conditions [1,2]. When liver injury occurs due to alcohol abuse, hepatitis viral infection, or obesity, stellate cells activate in response to inflammatory stimuli and become myofibroblastic cells that express smooth muscle α-actin as a representative marker [2]. Myofibroblastic cells secrete profibrogenic mediators, such as transforming growth factor-β (TGF-β), connective tissue growth factor, and tissue inhibitor of matrix metalloproteinases, and generate extracellular matrix materials including collagens, fibronectin, and laminin; thus, they play a pivotal role in liver fibrogenesis [3]. In particular, collagen production by activated stellate cells is regulated by TGF-β in an autocrine loop, which is accompanied by the induction of TGF-β receptors [4]. Suppression of hepatic stellate cell activation and collagen expression is thus a critical issue to establish therapeutic strategies for human liver fibrosis [1,5].

MicroRNAs (miRNAs) are endogenous small noncoding RNAs that modulate gene expression through imperfect base pairing with the 3′ untranslated region (UTR) of target mRNA, resulting in the inhibition of translation or the promotion of mRNA degradation [6,7]. miRNAs play roles in cell proliferation [8], development [9], and differentiation [10], and their contribution to human diseases such as cancer, cardiomyopathies, and schizophrenia have been reported [11–13]. miR-122 is also involved in the defense system against viral hepatitis C with regard to interferon (IFN)-β therapy [14], and miR-26 expression status is associated with survival and response to adjuvant IFNα therapy in patients with hepatocellular carcinoma [15]. Some miRNAs are involved in liver development and hepatocyte lipid metabolism [16–18].

Recent studies have shown that miRNAs are additionally involved in the alteration of hepatic stellate cell phenotypes; down-regulation of miR-27a and -27b allows culture-activated rat stellate cells to return to a quiescent phenotype with abundant vitamin A storage and decreased cell proliferation [19]; miR-15b and -16, which target the Bcl-2 and caspase signaling pathways, may affect stellate cell activation and liver fibrosis [20]. However, the function of miRNAs in hepatic stellate cell activation and their collagen production is largely unknown.

Here, we show that miR-29b, which is induced in human stellate cells (LX-2) treated with IFNα, is a potential regulator of type I collagen mRNA and protein expression. Although the primary action of IFNs is to eradicate viruses, i.e., hepatitis B and C viruses in

Abbreviations: Col1A1, alpha 1 (I) collagen; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; IFN, interferon; miRNAs, microRNAs; TGF-β, transforming growth factor-β; UTR, untranslated region.

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the case of the liver, IFNs also exhibit an antifibrotic action in human chronic hepatitis [21,22] and rodent liver fibrosis models [23]. Our data suggest that miR-29b may be a novel regulator of type I collagen expression in addition to its involvement in the well-known Smad cascade. Moreover, miR-29b upregulation may play a partial role in the antifibrotic action of IFNs.

Materials and methods

Materials. Recombinant human TGF-β1 was purchased from PeproTech (London, UK). Human natural IFNα was obtained from Otsuka Pharmaceutical Co. (Tokushima, Japan). Precursors of miR-29b, -143, and -218, and the negative control were purchased from Ambion (Austin, TX, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Rabbit monoclonal antibodies against Smad2 and phospho-Smad2 were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). The mouse monoclonal antibody against SP1 was purchased from Bio Matrix Research Inc. (Chiba, Japan). Rabbit polyclonal antibody against type I collagen was purchased from Rockland Immunochemicals, Inc. (Gilbertsville, PA, USA). Mouse monoclonal antibody against GAPDH was purchased from Chemicon International Inc. (Temecula, CA, USA). Enhanced Chemiluminescence plus detection reagent was purchased from GE Healthcare (Buckinghamshire, UK). Immobilon P membranes were purchased from Millipore Corp. (Bedford, MA, USA). All other reagents were purchased from Sigma Chemical Co. or Wako Pure Chemical Co. (Osaka, Japan).

Preparation of the human hepatic stellate cell line LX-2. The human hepatic stellate cell line (LX-2, donated by Dr. Scott Friedman), which was spontaneously immortalized by growth in low serum, was established as reported previously [24]. Characterizations of the cells are described in detail elsewhere. The cells were maintained on plastic culture plates in DMEM supplemented with 10% FBS. After the culture had continued for the indicated number of days, the medium was replaced with DMEM supplemented with 0.1% FBS plus test agents, and the culture was continued for another 24 h.

Quantitative real-time PCR. Total RNA was extracted from human stellate cells using the miRNeasy Mini Kit (Qiagen, Valencia, CA, USA). cDNAs were synthesized using 0.5 μg of total RNA, ReverTra Ace (Toyobo, Osaka, Japan), and oligo(dT)$_{12-18}$ primers according to the manufacturer's instructions [25]. Gene expression was measured by real-time PCR using cDNA, real-time PCR Master Mix Reagents (Toyobo), and a set of gene-specific oligonucleotide primers (alpha 1 (I) collagen [Col1A1]: Forward 5′-CCCGGGTTTCAGAGACA ACTTC-3′, Reverse 5′-TCCACATGCTTTATTCCAGCAATC-3′; TGF-β1: Forward 5′-AGCGACTCGCCAGAGTGGTTA-3′, Reverse 5′-GCAGTG TGTTATCCCTGCTGTCA-3′; SP1: Forward 5′-TCGGATGAGCTACA GAGGCACAA-3′, Reverse 5′-GTCACTCCTCATGAAGCGCTTAGG-3′; and GAPDH: Forward 5′-GCACCGTCAAGGCTGAGAAC-3′, Reverse 5′-TGGTGAAGACGCCAGTGGA-3′) with an Applied Biosystems Prism 7500 (Applied Biosystems, Foster City, CA, USA). To detect miRNA expression, the RT reaction was performed using the TaqMan MicroRNA Assay (Applied Biosystems) according to the manufacturer's instructions. The GAPDH level was measured and used to normalize the relative abundance of mRNAs and miRNAs.

Immunoblot. Proteins (20–50 μg) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred onto Immobilon P membranes. After blocking, the membranes were treated with primary antibodies, followed by peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized by the enhanced chemiluminescence system using the Fujifilm Image Reader LAS-3000 (Fuji Medical Systems, Stamford, CT, USA).

Transient transfection of miRNA precursors. Precursors of miR-29b, -143, and -218, and the negative control were transfected into human stellate cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) at a final concentration of 50 nM. Briefly, the cells were plated in DMEM supplemented with 10% FBS at a density of 1–2 × 10^5 cells/ml 24 h prior to the transfection. miRNA precursors and Lipofectamine 2000 were mixed at a ratio of 25 (pmol):1 (μl) in Opti-MEM I Reduced Medium (Invitrogen) and incubated for 20–30 min at room temperature. The miRNA precursor–Lipofectamine 2000 complexes were then added to stellate cell culture medium. After 6 h, the culture medium was changed, and TGF-β1 was added at a concentration of 2 ng/ml.

Luciferase reporter assay. 3′UTRs containing putative miRNA target regions of the Col1A1 and SP1 genes were obtained by PCR using human stellate cell cDNA as a template and primer sets as follows: Col1A1–miR-29: Forward 5′-TTCTCGAGGTTCTTGTCTTG ATGTGTCACC-3′, Reverse 5′-TTTCTAGAGAGAGCAGAGGCCTGAGA AG-3′; Col1A1–miR-143: Forward 5′-CTCGAGACTCCCTCCATCCCAA CCT-3′, Reverse 5′-TCTAGAATTGCTGGGCAGACAATAC-3′; Col1A1–miR-218: Forward 5′-CTCGAGGTGGATGGGGACTTGTGAAT-3′, Reverse 5′-TCTAGATTATGTTTGGGTCATTTCCAC-3′; SP1–miR-29: Forward 5′-TTCTCGAGTGGGTGCTACACAGAATGC-3′, Reverse 5′-TTTC TAGAAGACTGTCCTTATTTCCTTGGTA-3′; and SP1–miR-218: Forward 5′-CTCGAGGATGTTTTCCCTTAACTTTTCCT-3′, Reverse 5′-TCT AGACTAAAAGCTTATATCCTCAGCATC-3′. Each of the forward and reverse primers carried the *Xho*I and *Xba*I sites at their 5′-ends. The obtained DNA fragments were inserted into the pmirGLO Vector (Promega, San Luis Obispo, CA, USA). The resulting vectors were dubbed pCol1A1–miR-29/mirGLO, pCol1A1–miR-143/mirGLO, pCol1A1–miR-218/mirGLO, pSP1–miR-29/mirGLO, and pSP1–miR-218/mirGLO. Human stellate cells were seeded on 96-well plates (Microtest 96-well Assay Plate; Becton Dickinson, Franklin Lakes, NJ, USA) in DMEM supplemented with 10% FBS at a density of 2 × 10^4 cells/well. The following day, they were transfected with 200 ng of reporter plasmid along with miRNA precursors using Lipofectamine 2000 as described above and incubated for an additional 24 h. After incubation, the medium was removed from the wells, and 20 μl of phosphate-buffered saline was added. The Dual-Glo Luciferase Assay System (Promega) was used to analyze luciferase expression according to the manufacturer's protocol. Firefly luciferase activity was normalized to *Renilla* luciferase activity to adjust for variations in transfection efficiency among experiments.

Statistical analysis. Data presented as bar graphs are the means ± SD of at least three independent experiments. Statistical analysis was performed using Student's *t*-test, and $P < 0.05$ was considered significant.

Results and discussion

Regulation of Col1A1 expression by TGF-β1 and IFNα in human stellate cells

Immortalized human stellate cells, LX-2, are classified as an activated phenotype that expresses mRNAs for Col1A1 and other fibrogenetic molecules and are reported to be highly gene-transfectable [24]. At first, we observed that Col1A1 mRNA expression increased dose-dependently by TGF-β1 (Fig. 1A), whereas this upregulation was significantly inhibited by the presence of 100 IU/ml of human IFNα (Fig. 1B).

Extraction of miR-29b, -143, and -218 as candidates interacting with Col1A1 3′UTR

To determine the role of miRNAs in human stellate cell collagen expression, we searched for predictable miRNAs that could interact

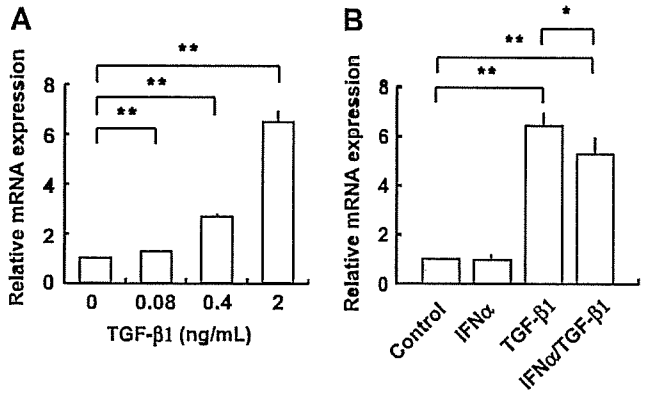

Fig. 1. Regulation of alpha 1 (I) collagen (Col1A1) expression in human stellate cells. (A) Dose-dependent effect of TGF-β1 on Col1A1 mRNA expression. Human stellate cells, LX-2, were treated with TGF-β1 (0, 0.08, 0.4, and 2 ng/ml) for 24 h in DMEM containing 0.1% FBS. (B) Effect of IFNα on Col1A1 mRNA expression in human stellate cells stimulated with TGF-β1. The cells were treated with IFNα (100 IU/ml), TGF-β1 (2 ng/ml), or IFNα (100 IU/ml) + TGF-β1 (2 ng/ml) for 24 h in DMEM containing 0.1% FBS. Control: human stellate cells were cultured for 24 h in DMEM containing 0.1% FBS. mRNA expression was analyzed by real-time PCR. The results are expressed as relative expression against control expression without treatment. $^{*}P < 0.05$; $^{**}P < 0.01$.

with 3′UTR of human Col1A1 mRNA using TargetScan Human Release 5.1 (http://www.targetscan.org/). As a result, miR-29, -98, -129, -133, -143, -196, -218, and let-7 were extracted as candidates. Because further *in silico* analyses among the eight candidates indicated that miR-29b, -143, and -218 were highly homologous to the Col1A1 3′UTR, we checked the expression levels of these miRNAs in human stellate cells by real-time PCR. As a result, miR-143 and -218 expressions were up and downregulated dose-dependently by TGF-β1, respectively, (Fig. 2A and B). Although miR-29b expression was unaffected by TGF-β1, it increased in

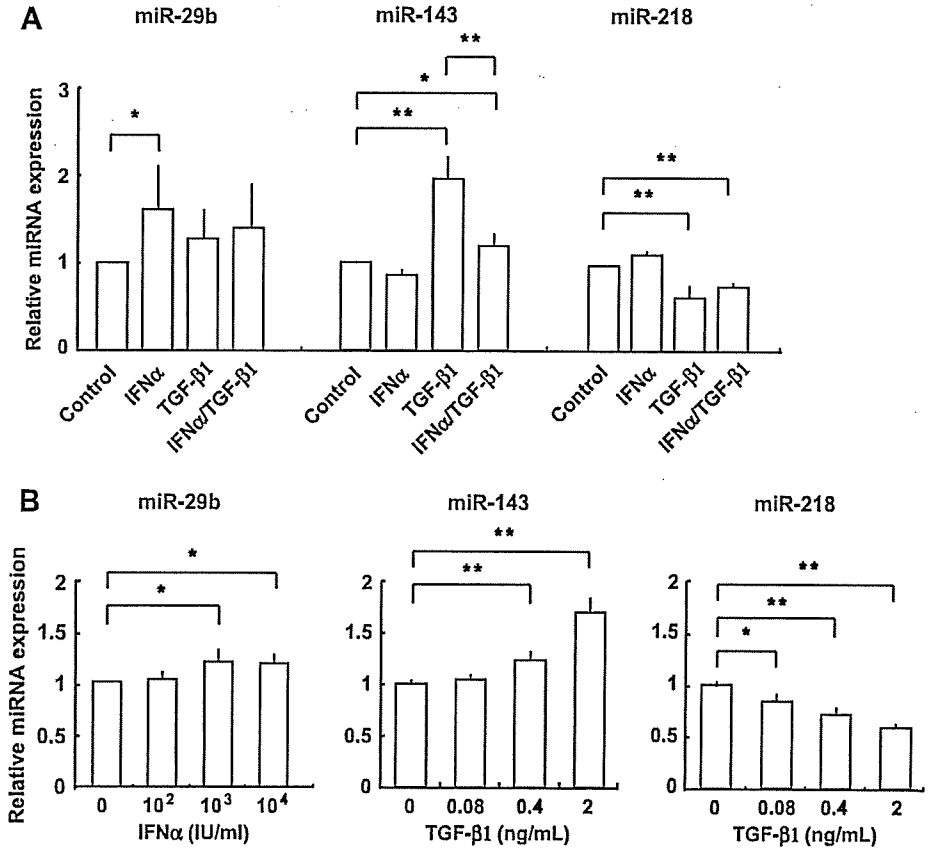

Fig. 2. Expression of miR-29b, -143, and -218 in human stellate cells. (A) Expression of miR-29b, -143, and -218 in human stellate cells, LX-2. The cells were treated with IFNα (100 IU/ml), TGF-β1 (2 ng/ml), or IFNα (100 IU/ml) + TGF-β1 (2 ng/ml) for 24 h in DMEM containing 0.1% FBS. Control: human stellate cells were cultured for 24 h in DMEM containing 0.1% FBS. (B) Dose-dependent effect of IFNα or TGF-β1 on the expression of miR-29b, -143, and -218 in human stellate cells. miRNA expression was analyzed by real-time PCR. The results are expressed as relative expression against control expression without treatment. $^{*}P < 0.05$; $^{**}P < 0.01$.

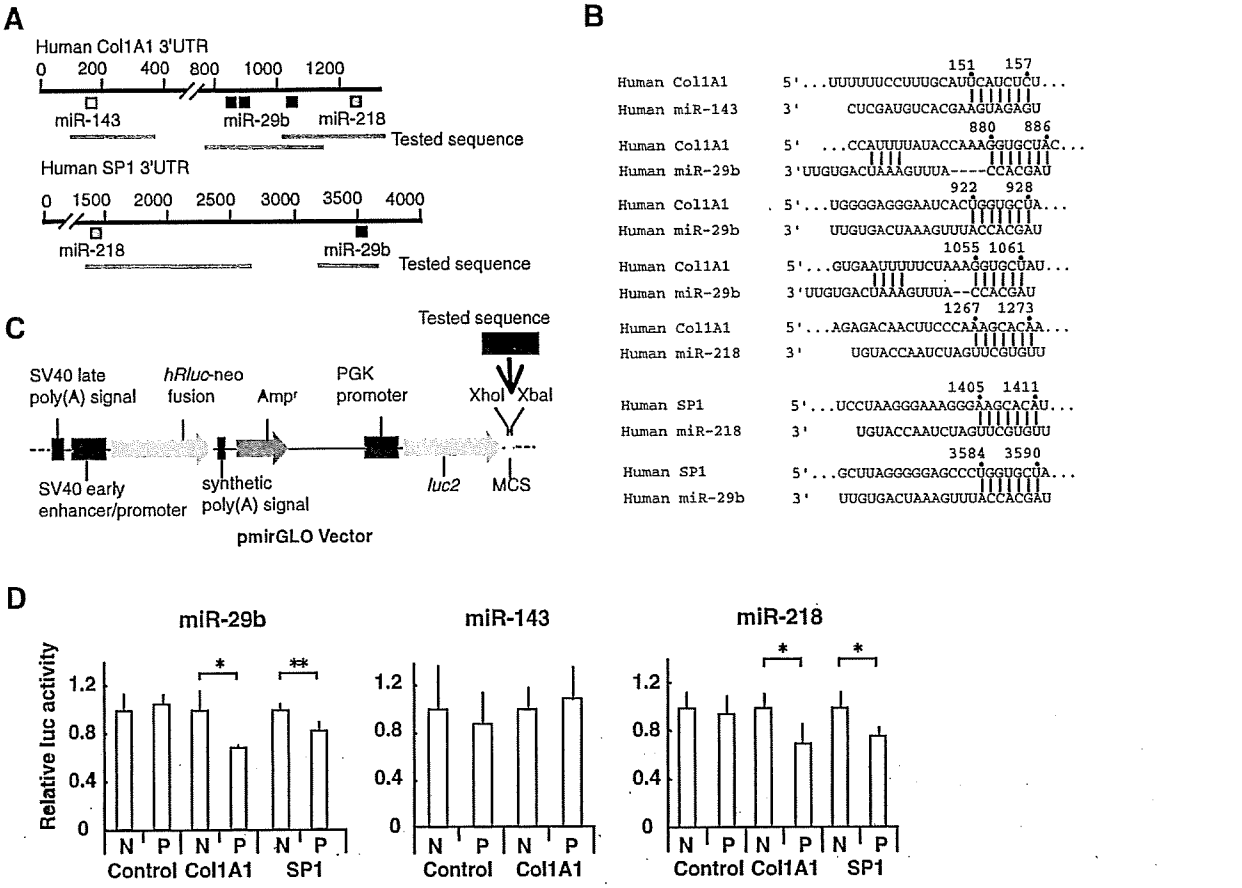

Fig. 3. Interaction of miR-29b, -143, and -218 with the 3′UTRs of alpha 1 (I) collagen (Col1A1) and SP1 mRNAs. (A) Schematic indication of the miRNA binding sites in the 3′UTRs of Col1A1 and SP1 mRNAs based on TargetScan Human Release 5.1 (http://www.targetscan.org/). Each black, white, and gray box indicates miR-29b, -143, and -218, respectively. Tested sequences indicate the regions that were inserted into the luciferase reporter vector. (B) Predicted consequential pairing of the target region and miRNAs. Arabic numerals above indicate the positions relative to the 3′UTR start sites. (C) Luciferase reporter vector structure. The vector contained two expression units; one for the *Renilla* luciferase gene (*hRluc*-neo fusion) expression. This unit was driven by an SV40 early promoter. The other was for the firefly luciferase gene (*luc2*). This unit was driven by a human phosphoglycerate kinase (PGK) promoter and contained multiple cloning sites (MCS) downstream of the *luc2* sequence. Each Col1A1 and SP1 3′UTR containing a putative miRNA target region (tested sequence) was cloned into the MCS. Arrows indicate the gene directions. Amp^R indicates an ampicillin-resistant plasmid gene. (D) Interaction of miR-29b, -143, and -218 with the 3′UTRs of Col1A1 and SP1 mRNAs in human stellate cells. Relative luciferase activity derived from pCol1A1–miR-29/mirGLO and pSP1–miR-29/mirGLO in the presence of miR-29b precursors (left panel), pCol1A1–miR-143/mirGLO in the presence of miR-143 precursors (center panel), and pCol1A1–miR-218/mirGLO and pSP1–miR-218/mirGLO in the presence of miR-218 precursors (right panel). The pmirGLO vector was used as a negative control reporter vector (control). N: cotransfection of reporter vectors along with negative control precursors, which have a scrambled sequence. P: cotransfection of reporter vectors along with miRNA precursors. Firefly and *Renilla* luciferase activities were determined, and firefly luciferase was normalized to *Renilla* luciferase activity. Results are expressed as relative activities against the activity in the presence of negative control precursors. $^{*}P < 0.05$ and $^{**}P < 0.01$.

the presence of IFNα (Fig. 2A and B). Thus, we assumed that these miRNAs might affect type I collagen expression via their interaction with Col1A1 3′UTR in human stellate cells.

Interaction of miR-29b, -143, and -218 with 3′UTRs of Col1A1 and SP1 mRNAs

The prediction of miRNA target regions on Col1A1 3′UTR by TargetScan indicated that Col1A1 3′UTR has three target regions for miR-29b, one for miR-143, and one for miR-218 (Fig. 3A and B). Because collagen gene expression is regulated by miR-192 via an interaction with the transcriptional repressor E-box [26], we additionally considered SP1, which is a transcriptional regulator of Col1A1 expression induced by TGF-β1 [27,28]. The predicted miRNA target regions of SP1 3′UTR contained one target region for miR-29b and one for miR-218 (Fig. 3A and B).

To investigate the direct targeting of Col1A1 by miR-29b, -143, and -218 and that of SP1 by miR-29b and -218, the sequence of each target region was cloned and inserted into the downstream region of the firefly luciferase reporter gene (Fig. 3C). The resulting vectors were dubbed pCol1A1–miR-29/mirGLO, pCol1A1–miR-143/mirGLO, pCol1A1–miR-218/mirGLO, pSP1–miR-29/mirGLO, and pSP1–miR-218/mirGLO. These vectors were cotransfected into human stellate cells with miRNA precursors. As a result, the miR-29b and -218 precursors inhibited luciferase activity derived from the vectors carrying Col1A1 or SP1 3′UTRs (Fig. 3D). In contrast, the miR-143 precursors had no effect on luciferase activity of the vector carrying Col1A1 3′UTR (Fig. 3D). According to these observations, we assumed that the Col1A1 and SP1 3′UTR sequences could be targeted by miR-29b and -218, whereas miR-143, which was induced by TGF-β1 (Fig. 2A and B), had a negligible effect on Col1A1 expression in human stellate cells.

Regulation of type I collagen expression by miR-29b and -218

Next, we examined the effect of miR-29b and -218 overexpression on type I collagen mRNA and protein expression in human stellate cells. Transient transfection of miR-29b precursors signifi-

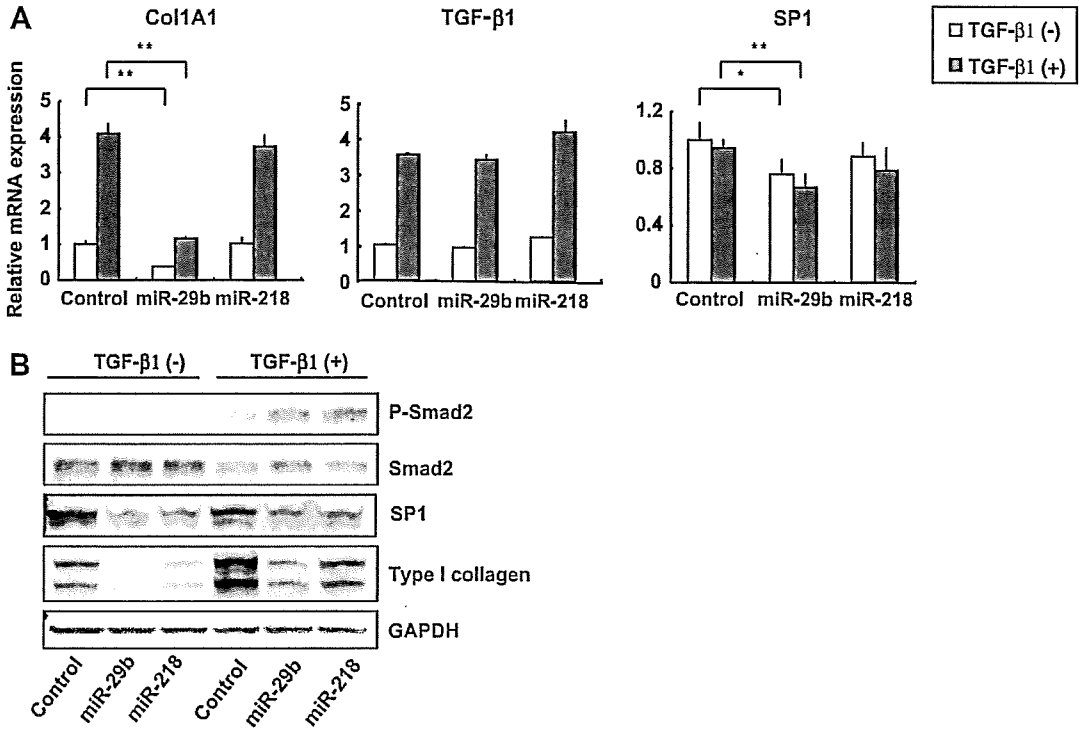

Fig. 4. Effect of miR-29b and -218 on type I collagen expression in human stellate cells. Human stellate cells were cultured in DMEM supplemented with 10% FBS and were transfected with 50 nM miR-29b, -218 precursors, or a negative control, which had a scrambled sequence (control) using Lipofectamine 2000. After 6 h, the medium was changed to DMEM containing 0.1% FBS with or without 2 ng/ml TGF-β1, and the culture was continued for another 24 h. (A) Effect of miR-29b and -218 precursors on the expression of Col1A1, TGF-β1, and SP1 mRNAs in human stellate cells with (gray column) or without (white column) TGF-β1. mRNA expression was analyzed by real-time PCR. The results are expressed as relative expression against control expression. $^{*}P < 0.05$; $^{**}P < 0.01$. (B) Effect of miR-29b and -218 precursors on the protein expression of phospho-Smad2 (P-Smad2), Smad2, SP1, type I collagen, and GAPDH in human stellate cells in the presence (+) or absence (−) of TGF-β1.

cantly inhibited type I collagen mRNA and protein expression (Fig. 4A, left panel, and B) in unstimulated human stellate cells. Additionally, transfection of miR-29b precursors completely suppressed the upregulation of type I collagen mRNA and protein under TGF-β1 stimulation. TGF-β1 stimulation induces Col1A1 mRNA expression through a pathway that includes SP1 and phosphorylated Smad2/3 [29]. In our results, upregulation of TGF-β1 mRNA (Fig. 4A, center panel) and phosphorylation of Smad2 (Fig. 4B) under TGF-β1 stimulation were unaffected by the transfection of miR-29b precursors. These results suggested that miR-29b may affect the downstream of phosphorylated Smad2. Moreover, the transfection of miR-29b precursors decreased SP1 mRNA and protein expression (Fig. 4A, right panel, and B). Thus, the miR-29b-induced repression of type I collagen expression could be caused by its direct interaction with Col1A1 3′UTR and additionally by its interaction with SP1 expression in human stellate cells. These observations agree with a report showing the role of miR-29 in collagen expression and cardiac fibrosis after cardiac infarction [30]. In contrast, transfection of miR-218 precursors triggered a negligible change in Col1A1 and SP1 mRNA expression (Fig. 4A, left and right panels) but slightly reduced their protein level (Fig. 4B). Taken together, these results imply that miR-29b is the most potent miRNA with regard to collagen production in human stellate cells.

Conclusions

We found a potent repression of collagen production by miR-29b in human stellate cells. IFNs attenuate and may regress liver fibrosis caused by hepatitis C viral infection [21–23], although the precise molecular mechanism has yet to be demonstrated. The present study using human stellate cells demonstrated that IFNα upregulates miR-29b (Fig. 2B and C), which is a negative regulator of type I collagen production via the interaction with Col1A1 and SP1 3′UTRs. This observation implies the contribution of miR-29b to antifibrotic IFN actions. Targeted delivery of miR-29b to activated stellate cells in the liver could become a new therapeutic strategy for human liver fibrosis in the future.

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ORIGINAL ARTICLE

Effect of natural interferon α on proliferation and apoptosis of hepatic stellate cells

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Abstract Inhibition of the proliferation of hepatic stellate cells (HSC) is clinically important for the control of liver fibrosis and cirrhosis. Interferons are now frequently used for chronic viral hepatitis because of their anti-viral activity. However, patients treated with interferons exhibit a regression of liver fibrosis even if viral eradication is not achieved, indicating that interferon itself has anti-fibrotic activity. Herein, we show the anti-proliferation and pro-apoptotic activity of natural interferon α against HSC. We found that interferon α inhibited serum-stimulated [^{3}H]thymidine incorporation of HSC in a dose-dependent manner, with a significant reduction at more than 100 U/ml. Interferon α also attenuated PDGF-BB-stimulated DNA synthesis of HSC. Although the molecular mechanism behind these phenomena has not been defined, we found that interferon α triggers the apoptosis of HSC treated with low-dose tumor necrosis factor α, as determined by the Alamar blue assay, morphology, and DNA ladder formation. Furthermore, interferon α decreased inhibitor of caspase-activated DNase (ICAD) levels, which may augment tumor necrosis factor α-induced cell death signals. Thus, interferon α regulates the number of myofibroblastic hepatic stellate cells and may clinically contribute to the regression of human liver fibrosis.

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Keywords Tumor necrosis factor α · Caspase · Cyclin · Cytochrome c · Caspase-activated DNase

Introduction

Hepatic stellate cells (HSC), which reside in Disse's space outside sinusoids, maintain a quiescent phenotype, and store vitamin A under physiologic conditions. They undergo activation in response to inflammatory stimuli and become myofibroblastic cells [1, 2]. The latter phenotype secretes profibrogenic mediators, generates extracellular matrix materials, and thus plays a pivotal role in the fibrogenesis of the liver [3–5]. One of the features of activated HSC is their proliferation. An increase in the number of activated myofibroblasts together with the deposited extracellular matrix materials contributes to the formation of fibrotic septa forming C–C and P–C bridges. The suppression of HSC activation and cell number is thus a possible mechanism which can be exploited to establish therapeutic strategies against human liver fibrosis [6, 7].

Our previous studies have shown that antioxidative compounds, such as resveratrol and *N*-acetyl-L-cysteine exert anti-fibrotic activity in the liver and have an anti-proliferative effect on cultured HSC. [8–10]. Resveratrol functions as an inhibitor of tyrosine kinase and inhibits the phosphorylation of platelet-derived growth factor-receptor β (PDGFRβ) under PDGF-BB stimulation, leading to attenuation of the activation of mitogen-activated protein kinase (MAPK). *N*-acetyl-L-cysteine also inhibited DNA synthesis of cultured rat HSC stimulated by PDGF-BB through the cathepsin B-dependent proteolytic degeneration of PDGFRβ.

Recently, several clinical reports have revealed that interferon α (IFNα), especially when used in combination

with ribavirin, provides an effective therapy for chronic hepatitis C. Although the primary action of IFNα is to eradicate viruses, it has also been suggested to suppress and even cause the regression of liver fibrosis, as revealed by repeated liver biopsies performed in IFNα-treated patients with chronic hepatitis C [11, 12]. These reports strongly indicate that IFNα may induce the deactivation of human HSC, thereby reducing the septum-forming fibroblastic cell lineage. Herein, we show evidence for the anti-proliferative and pro-apoptotic actions of IFNα against human HSC [13].

Materials and methods

Materials

Recombinant PDGF-BB and tumor necrosis factor α (TNFα) were obtained from R&D Systems (Minneapolis, MO, USA). Polyclonal antibodies against extracellular signal-regulated kinases 1 and 2 (ERK1/2), phospho-ERK1/2 (Thr 202/Tyr 204), mitogen-activated kinase/ERK kinase (MEK), phospho-MEK, Akt, phospho-Akt (Ser 473), cytochrome c, caspase-3, and cleaved caspase-3 were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA) and those against cyclin D1, cdk2, cdk4, cdk6, p21, p27, p53, caspase-activated DNase (CAD), and inhibitor of CAD (ICAD) were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Sigma Chemical Co. (Saint Louis, MO, USA). [^{3}H]Thymidine and enhanced chemiluminescence (ECL) detection reagent were purchased from Amersham Pharmacia Biotech (Buckinghamshire, England). Immobilon P membranes were purchased from Millipore Corp. (Bedford, MA, USA). Kodak XAR5 film was purchased from Eastman Kodak Co. (Rochester, NY, USA). Human natural IFNα was donated by Otsuka Pharmaceutical Co. (Tokushima, Japan). All other reagents were obtained from Sigma Chemical Co. or Wako Pure Chemical Co.

Preparation of human hepatic stellate cell line, LX-2

Human HSC-line, (LX-2, donated by Dr. Scott Friedman), a cell line spontaneously immortalized by growth in low serum, were established as previously reported. Characterizations of the cells were described in detail elsewhere [13]. Human HSC were maintained on plastic culture plates in DMEM supplemented with 10% fetal bovine serum. After the culture had continued for the indicated number of days, the medium was replaced by serum-free DMEM with test agents and the culture was continued for 48 h.

Immunoblot

Proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to Immobilon P membranes. After blocking, the membranes were treated with primary antibodies followed by peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized using the enhanced chemiluminescence system (Amersham Pharmacia Biotech) and Kodak XAR5 film.

Cell growth assay

Subconfluent Human HSC-line was cultured on plastic dishes for 3 days in 10% FBS/DMEM, and then maintained for 24 h in serum-free DMEM. These cells were successively stimulated with test agents for 24 h, and then were pulse-labeled with 1.0 μCi/ml of [^{3}H]thymidine during the last 24 h. The incorporated radioactivity was counted by liquid scintillation, as previously described [9].

Cell survival assay

Cell survival was measured using the Alamar blue assay (BIOSOURCE) according to the manufacturer's instructions. In brief, 20 μl of Alamar blue was added to 6-well plates containing DMEM. Absorbance at 530 nm was measured using a micro-plate reader (Wallac 1420 ARVOsx, P-E Applied Biosystems).

DNA fragmentation assay

Cell samples were homogenized and centrifuged. The pellet was resuspended in 1 ml of lysis buffer consisting of 10 mmol/l Tris-HCl, pH 7.4, 10 mmol/l NaCl, 10 mmol/l ethylenediaminetetraacetic acid (EDTA), 100 g/l proteinase K, and 0.5% SDS and incubated for 2 h at 50°C before being treated with ribonuclease overnight at 37°C. After extraction with phenol–chloroform twice and precipitation with ethanol, the DNA was dissolved in TE buffer (10 mmol/l Tris-HCl, pH 7.5, 1 mmol/l EDTA). The DNA was loaded onto 1.5% agarose gel containing ethidium bromide, electrophoresed in Tris acetate/EDTA buffer for 2 h at 50 V, and photographed under ultraviolet illumination.

Flow cytometric quantification of apoptotic and necrotic cells

For quantification of apoptotic cells, we used flow cytometry (FACS Calibur HG; BD, Franklin Lakes, NJ) after trypsination of human HSC treated with IFNα or TNFα, IFNα/TNFα under serum-reduced conditions (0.1% FBS)

for 48 h [14]. To detect early apoptotic changes, staining with Annexin V-FITC, which is known to have high affinity to phosphatidylserine, and propidiumiodide were used according to the manufacturer's instructions (Roche Diagnostics, Rotkreuz, Switzerland).

Mitochondria/cytosol fractionation

Human HSC were treated with mediums supplemented with TNFα and/or IFNα for 24 h. Then, the cells were collected using a cell scraper, and the collected cells were pelleted by centrifugation at 600×*g* for 5 min at 4°C. After washing with ice-cold PBS, fractions with enriched mitochondria and cytosol were obtained using a Mitochondria/Cytosol fractionation kit (Bio Vision Research Products, Mountain View, CA, USA) according to the manufacturer's instructions. Individual fractions were stored at −80°C until use.

Statistical analysis

Data presented as bar graphs are the means ± S.D. of at least three independent experiments. Statistical analysis was performed using Student's *t*-test ($P < 0.01$ was considered significant).

Results

Effect of IFNα on DNA synthesis of human HSC-line

As shown in Fig. 1, human HSC incorporated 11 ± 2 (DPM × 10^4) of [^{3}H]thymidine in the presence of serum. DNA synthesis dose dependently decreased in the presence of IFNα; it decreased significantly to 71 and 48% of the control in the presence of 10^2 and 10^3 IU/ml, respectively, of IFNα. PDGF-BB (10 ng/ml) augmented DNA synthesis of HSC at about 1.2 times. Even in this condition, IFNα at a concentration of more than 10^3 IU/ml significantly suppressed DNA synthesis of HSC (Fig. 2).

Effect of IFNα on cell cycle-related protein expression and PDGF-BB-stimulated signal transduction in HSC

Because IFNα suppressed DNA synthesis of human HSC, we hypothesized that IFNα regulates cell cycle-related protein expression, thereby hampering cell cycle transition from the G1 to S phase. Thus, the expression of several cell cycle-related proteins was determined by immunoblot. As shown in Fig. 3a, the amount of major cell cycle-related proteins such as cyclin D1, cdk2, cdk4, cdk6, p21, and p27 was not affected by IFNα. Although a previous report using hepatoma cell lines indicated that p53 is involved in growth suppression by IFNα [15], we failed to observe the suppression of p53 in HSC treated with IFNα. The PDGF-BB-activated MEK-MAPK cascade and Akt were also unaffected (Fig. 3b).

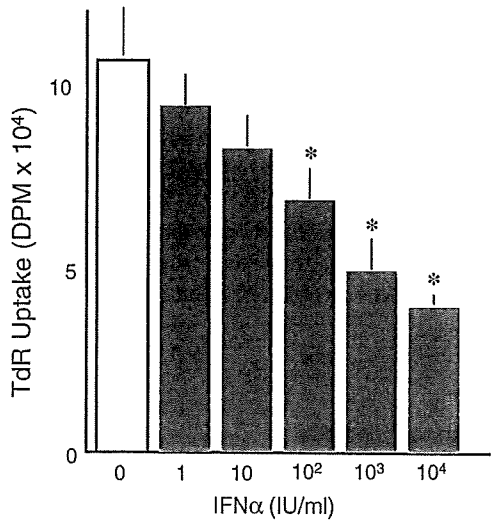

Fig. 1 Effect of IFNα on [^{3}H]thymidine incorporation in HSC. Sub-confluent HSC were cultured on plastic dishes for 3 days in 10% FBS/DMEM, and then maintained for 24 h in serum-free DMEM. These cells were successively stimulated with IFNα for 24 h, and then pulse-labeled with 1.0 μCi/ml of [^{3}H]thymidine during the last 24 h. The incorporated radioactivity was counted by liquid scintillation. *$P < 0.01$

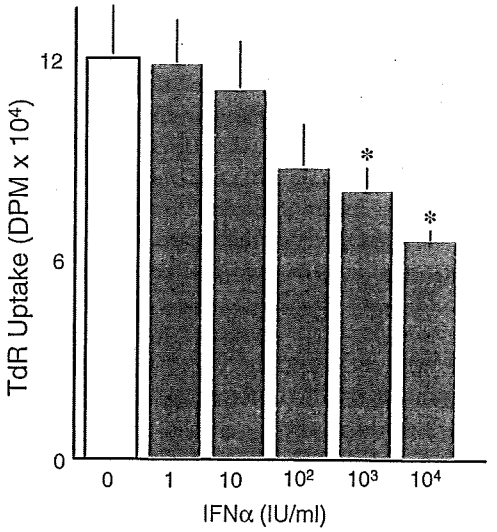

Fig. 2 Effect of IFNα on PDGF-BB-stimulated [^{3}H]thymidine incorporation in HSC. Sub-confluent HSC were cultured on plastic dishes for 3 days in 10% FBS/DMEM, and then maintained for 24 h in serum-free DMEM. These cells were successively stimulated with PDGF-BB (10 ng/ml) in the presence or absence of IFNα for 24 h, and then were pulse-labeled with 1.0 μCi/ml of [^{3}H]thymidine during the last 24 h. The incorporated radioactivity was counted by liquid scintillation. *$P < 0.01$

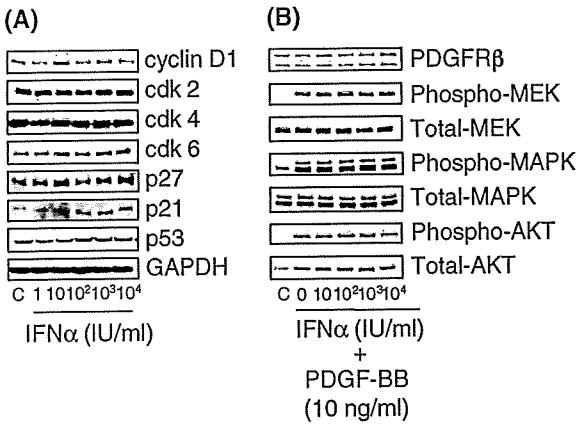

Fig. 3 Effects of IFNα on cell cycle-related protein expression and the activation of MEK, MAPK, and Akt stimulated with PDGF-BB in HSC. (a) Expression of cyclin D1, cdk2, cdk4, cdk6, p27, p21, and p53 was determined by immunoblot. (b) Expression of PDGFRβ, phospho-MEK, total MEK, phospho-MAPK, total MAPK, phospho-Akt, and total Akt in HSC under PDGF-BB (10 ng/ml) stimulation was analyzed by immunoblot

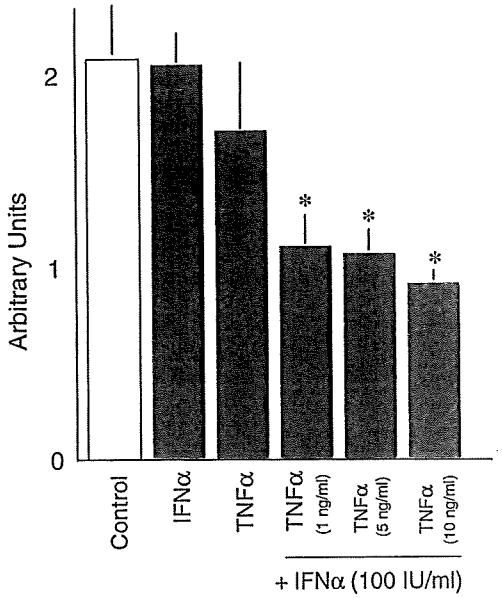

Fig. 4 Effect of IFNα and TNFα on number of HSC. HSC were maintained on plastic culture plates in DMEM supplemented with 10% fetal bovine serum. Then, the medium was replaced by serum-free DMEM with IFNα and/or TNFα and the culture was continued for another 48 h. Numbers of HSC were determined by the Alamar blue assay (BIOSOURCE) according to the manufacturer's instructions. $^*P < 0.01$

Induction of apoptosis of HSC by IFNα

Although the mechanism of IFNα-dependent DNA synthesis inhibition was not clear, we found that IFNα triggered the apoptosis of human HSC-line in the presence of a low dose of TNFα. As shown in Fig. 4, IFNα or TNFα alone failed to affect the number of HSC, as determined by the Alamar blue assay. However, the simultaneous addition of IFNα and TNFα significantly decreased the cell number to 54% of the control. In fact, when observed under a phase-contrast microscope, the cell density of human HSC became sparse when cells were treated with IFNα and TNFα for 48 h (Fig. 5). This finding indicates that cell death is actively stimulated by the combination of IFNα and TNFα. This observation was further supported by the fact that cell treatment with IFNα plus TNFα-induced DNA ladder formation (Fig. 6) and analysis using flow cytometric quantification of apoptosis of human HSC line (Fig. 7).

Role of IFNα on caspase cascade

Finally, we tested whether IFNα regulates the caspase cascade in human HSC-line. As shown in Fig. 8a, cytochrome c release from mitochondria into the cytosol was augmented by TNFα alone and TNFα/IFNα treatment. In accordance with this result, cleaved caspase 3 was enriched in cells treated with TNFα alone and TNFα/IFNα (Fig. 8b). Because TNFα alone thus triggered cytochrome c release and the activation of caspase 3 and failed to induce the active apoptosis of HSC, we hypothesized that IFNα may play a critical role in finalizing DNA fragmentation. After searching for the mechanism, we found that IFNα significantly reduced the level of ICAD without affecting the amount of CAD (Fig. 8c).

Discussion

IFNα is now a first-choice therapy for chronic hepatitis C (CH–C). In combination with rivabirin, IFN therapy leads to about a 30% and 80% eradication of HCV genotypes 1 and 2, respectively. Recently, pegylated IFNα has improved the efficacy of antiviral therapy [16, 17]. IFNα as well as IFNβ binds to cell surface receptors composed of IFNAR1 and IFNAR2 subunits. IFNα binding leads to ligand-induced receptor dimerization and then to the auto- and trans-phosphorylation of Janus protein tyrosine kinases, which successively induces the phosphorylation of STAT1/STAT2. Phosphorylated STAT1/STAT2 binds to IFN regulatory factor 9 (IRF-9) to form IFN-stimulated gene factor 3 (ISGF3), which translocates into the nucleus and binds to the IFN-stimulated response element (ISRE), initiating the transcription of IFN-dependent genes such as 2′-5′-oligoadenylate synthetase. These IFN-induced intracellular signalings suppress viral replication to complete viral eradication [18, 19].

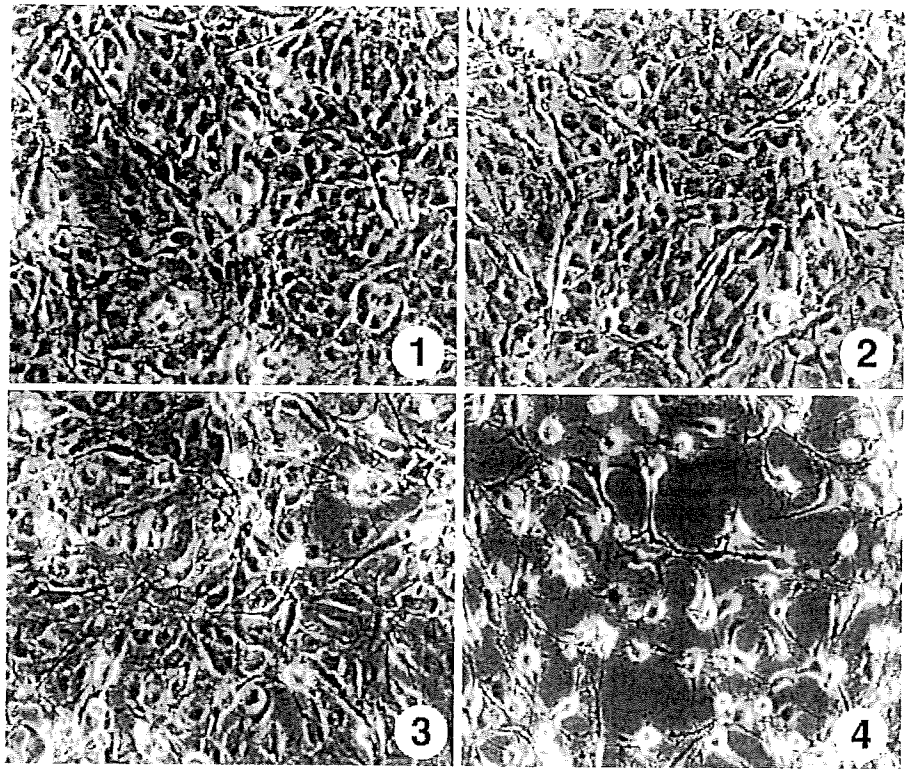

Fig. 5 Effect of IFNα and TNFα on the cell number of HSC. HSC were maintained on plastic culture plates in DMEM supplemented with 10% fetal bovine serum. Then, the medium was replaced by serum-free DMEM with IFNα and/or TNFα and the culture was continued for another 48 h. Cell appearance of HSC were determined under a microscope at a magnification of ×200. 1: Control; 2: IFNα; 3: TNFα; 4: IFNα/TNFα. Note that the cell number was markedly decreased by incubating them with IFNα plus TNFα. IFNα: 100 IU/ml. TNFα: 10 ng/ml

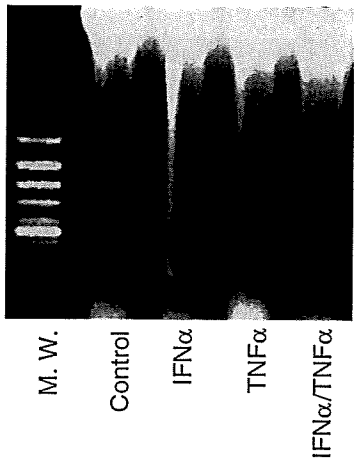

Fig. 6 DNA fragmentation of HSC treated with IFNα and TNFα. Isolated DNA was loaded onto a 1.5% agarose gel containing ethidium bromide, electrophoresed in Tris acetate/EDTA buffer for 2 h at 50 V, and photographed under ultraviolet illumination. Note that treatment of HSC with IFNα plus TNFα induced DNA flagmentation. IFNα: 100 IU/ml. TNFα: 10 ng/ml. M. W.: molecular weight

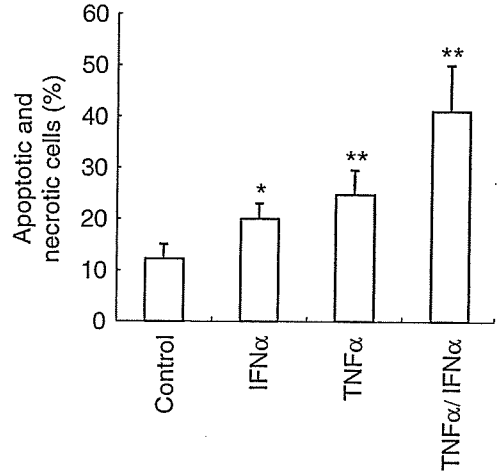

Fig. 7 Flow cytometric quantification of apoptosis in LX-2 treated with IFNα or TNFα, IFNα/TNFα. LX-2 cells were maintained on plastic culture plates in DMEM supplemented with 10% FBS. Then, the medium was replaced by serum-free DMEM with IFNα and/or TNFα and the culture was continued for another 48 h. The data show the percentile portion of apoptotic cells per total LX-2 population using flow cytometry. $*P < 0.05$; $**P < 0.01$

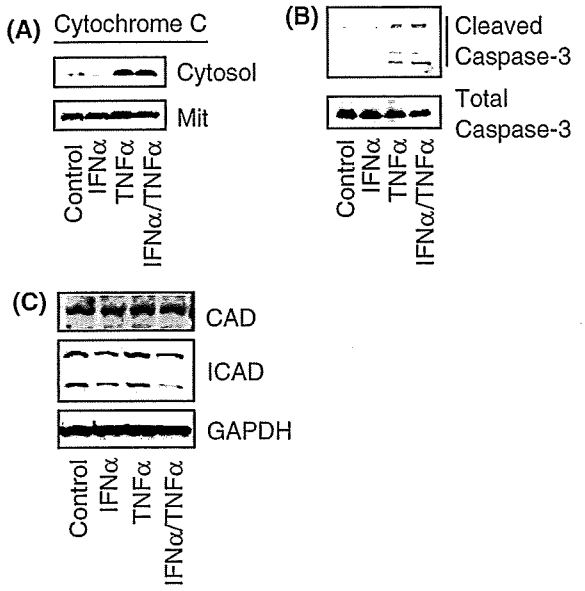

Fig. 8 Apoptosis-related protein expressions in HSC. (**a**) The cytochrome c content in the cytosol and mitochondria was determined by Western blot. (**b**) The activation of caspase-3 was studied by detecting the amount of cleaved caspase-3. (**c**) Expression of CAD and ICAD in HSC treated with IFNα and/or TNFα. IFNα: 100 IU/ml. TNFα: 10 ng/ml

In addition to the antiviral effect, IFNα is also known as a negative growth factor and shows anti-oncogenic activity [14]. Several lines of evidence have revealed that IFN therapy for CH–C lowers the occurrence of hepatocellular carcinoma (HCC), although the precise molecular mechanism has not yet been elucidated [20–22]. In addition, treatment using IFN in combination with 5′-fluorouracil (5-FU) was reported to be effective for advanced HCC with portal vein thrombosis [23, 24]. It has recently been reported that IFNα/β induces p53 gene transcription and increases the p53 protein level in HepG2 and HLE cells, two human hepatic cancer cell lines, thereby contributing to tumor suppression. However, the effect of IFN on the proliferation and cell survival of hepatic non-parenchymal collagen-producing HSCs has yet to be fully elucidated.

Several lines of evidence show that IFNα treatment for chronic hepatitis C leads to the regression of liver fibrosis even if HCV is not successfully eradicated [11, 12]. This fact indicates that IFNα has a direct anti-fibrotic action, most likely through its action against HSC. Initially, IFNγ was reported to successfully inhibit HSC activation, as revealed by its inhibitory effect on collagen synthesis and smooth muscle α-actin expression [25, 26]. Its in vivo anti-fibrotic action has also been elucidated [27, 28]. Successively, IFNα was proven to inhibit DNA synthesis of human HSC although a relatively weak inhibitory effect was seen at a concentration above 10^4 U/ml [29]. In the present study, we observed an almost 100-times stronger anti-proliferative effect using OIF, a natural IFNα. This discrepancy may be derived from the difference in IFN used and from the condition and culture system of HSC. Furthermore, in that report, Mallat et al. showed that IFNα suppressed [^{3}H]thymidine incorporation of human HSC stimulated with 5% fetal calf serum, PDGF-BB (20 ng/ml), PDGF-AA (20 ng/ml), or TGFβ1 (0.5 ng/ml), and that IFNα reduced the number of HSC stimulated with 5% fetal calf serum. However, the authors did not refer to the mechanism, especially to the induction of apoptosis of human HSC exposed to IFNα. Although we failed to clarify the mechanism of IFNα-dependent inhibition of cell cycle progression in human HSC-line, we found that natural IFNα contributes to the induction of apoptosis of human HSC by activating the caspase cascade, which provides a novel insight into the fibrosis regression in patients treated with IFNα derivatives.

Mallat et al. [29] additionally showed that IFNα as well as IFNγ reduced the secretion of prolines and the mRNA expressions of collagens α1(I) and α1(III). With respect to this mechanism, Inagaki et al. [30] recently reported that IFNα blocked promoter activation and prevented the progression of liver fibrosis induced by CCl_4 injection when administered to transgenic mice harboring the α2(I) collagen gene (COL1A2) promoter sequence, and that, in transient transfection assays, IFNα decreased the steady-state levels of COL1A2 mRNA and inhibited TGFβ/Smad3-stimulated COL1A2 transcription.

In addition to these previous observations, we herein found that IFNα in combination with a small amount of TNFα shows a pro-apoptotic effect in HSC. This effect is considered to be related to cytochrome c release into the cytosol from mitochondria and caspase-3 activation and be regulated by ICAD (Fig. 7). Although human HSC have been proven to be resistant to apoptosis [31], the data shown here clearly indicate that they undergo apoptosis at inflammatory liver sites in the presence of IFNα, which may at least partially account for the regression of liver fibrosis during IFN therapy due to chronic hepatitis C.

In conclusion, the present study demonstrates the anti-proliferative and pro-apoptotic actions of natural human IFNα against human HSC-line, and provides useful information regarding the mechanism of IFN-dependent regression of human liver fibrosis caused by viral infection.

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ORIGINAL ARTICLE

Induction of tropomyosin during hepatic stellate cell activation and the progression of liver fibrosis

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Abstract The activation of hepatic stellate cells (HSCs) is a cue to initiate liver fibrosis. Activated stellate cells acquire contractile activity similar to pericytes and myofibroblasts in other organs by inducing the contractile machinery of cytoskeletons such as smooth muscle α-actin (α-SMA), a well-known marker of activated stellate cells, and actin-binding proteins. We further show herein the expression of tropomyosin in rat HSCs in the course of their activation during primary culture and liver tissue damaged by thioacetamide intoxication. In immunoblot analysis, tropomyosin became detectable in an early stage of the primary culture of rat stellate cells in a manner similar to the expression of α-SMA and platelet-derived growth factor receptor-β. Tropomyosin was found to be colocalized with α-SMA on fluorescent immunocytochemistry. At the liver tissue level, an increased expression of tropomyosin was observed by immunoblot analysis and immunohistochemistry along the septum of fibrosis, where α-SMA was enriched. These results strongly suggest that tropomyosin is a new marker of activated stellate cells and may serve as a useful diagnostic marker of liver fibrosis.

Keywords Actin · Cell contraction · Vitamin A · Liver injury · Liver sinusoid

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Abbreviations

DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular matrix
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HSC	Hepatic stellate cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGFR-β	Platelet-derived growth factor receptor-β
TAA	Thioacetamide
α-SMA	Smooth muscle-α actin

Introduction

Hepatic stellate cells (HSCs) play a key role in liver fibrogenesis regardless of the pathogenesis [1–4]. In response to local tissue damage and hepatocyte necrosis, HSCs undergo activation characterized by the proliferation, migration, contraction, secretion of several profibrogenic mediators such as cytokines, growth factors, chemokines, and tissue inhibitors of matrix metalloproteinases, and generation of extracellular matrix (ECM) materials such as type I collagen. HSC activation thus contributes to scar formation in chronically injured liver tissue.

One of the indicators of activated HSCs is smooth muscle α-actin (α-SMA), the actin isoform typical of smooth muscle cell differentiation [5–10]. Similar to the expression of γ-actin, α-SMA was first demonstrated in primary-cultured rat HSCs in the course of the culture-dependent HSC activation process [5]. α-SMA-positive HSCs are also seen along the fibrotic septum of chronically damaged livers of rodent models, in which ECM proteins such as type I collagen and fibronectin are dominantly produced and deposited [11, 12]. Furthermore, in the human liver, the augmented expression of α-SMA has been

documented in and around the area of hepatocyte damage [13, 14].

Tropomyosin is one of the actin-associated proteins, present in virtually all eukaryotic cells, and modulates the interaction between actin and myosin to stabilize the actin-filament structure. Tropomyosin assembles into an α-helical coiled heterodimer composed of an α-chain and a β-chain, with each molecule interacting with six or seven monomers of actin [15, 16]. Tropomyosin regulates the contractility of striated muscle by blocking myosin-binding sites on actin in the relaxed state. On activation, tropomyosin moves away from these sites [17]. X-ray studies have suggested that the initiation of smooth muscle contraction leads to the movement of tropomyosin in a manner similar to that in striated muscle [18]. Although one recent study has shown the expression and organization of actin filaments and tropomyosin in the cloned hepatic GRX cell line [19], tropomyosin expression patterns in primary cultured HSCs and the fibrotic liver remain to be studied.

This study aimed to demonstrate the presence of tropomyosin in HSCs and clarify the dynamics of its expression pattern during HSC activation. Herein, we report in detail the expression pattern of tropomyosin in cultured rat HSCs and fibrotic liver tissue induced in rats by thioacetamide (TAA) administration, and further demonstrate the network of α-SMA filaments and tropomyosin in primary HSC cultures.

Materials and methods

Materials

Collagenase was purchased from Wako Pure Chemical Co. (Osaka, Japan). Pronase E was obtained from Merck (Damstadt, Germany). Mouse monoclonal IgG2a antibody against α-SMA, mouse monoclonal IgG1 antibody against tropomyosin, Dulbecco's modified Eagle's medium (DMEM), TAA, and fetal bovine serum (FBS) were purchased from Sigma Chemical Co. (Saint Louis, MO, USA). Rabbit polyclonal IgG antibodies against human platelet-derived growth factor receptor-β (PDGFR-β) that reacts with rat PDGFR-β were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated secondary antibodies against mouse and rabbit immunoglobulins were obtained from Dako. Alexa Fluor 488 goat antimouse IgG2a antibodies and Alexa Fluor 594 goat antimouse IgG1 antibodies were from Molecular Probes (Eugene, OR, USA). ECL immunoblotting detection reagent was purchased from Amersham Pharmacia Biotech (Buckinghamshire, England). Immobilon P membranes were from Millipore Corp. (Bedford, MA, USA). Cell culture inserts were from Falcon (Beckton Dickinson, Franklin Lakes, NJ, USA). All the other reagents were purchased from Sigma Chemical Co. or Wako Pure Chemical Co.

Animals

Pathogen-free male Wistar rats (12-week-old, body weight 200–220 g) were obtained from SLC (Shizuoka, Japan). Animals were housed at a constant temperature and supplied with laboratory chow and water ad libitum. The protocol of the experiments was approved by the Animal Research Committee of Osaka City University (Guide for Animal Experiments, Osaka City University).

Induction of liver fibrosis

Liver fibrosis was induced in rats ($n = 3$) by the intraperitoneal injection of TAA (40 mg/body weight) dissolved in 2 ml of saline twice a week for up to 10 weeks. Control rats ($n = 3$) were given 2 ml of saline during the same period.

Preparation of HSCs

HSCs were isolated from male Wistar rats, as previously described in detail [20]. Isolated HSCs were suspended in DMEM supplemented with 10% FBS at a cell density of 5×10^5 cells/ml, and 1.5 ml of the cell suspension was introduced into a 35-mm cell tissue culture plate (Falon, 3003). After the culture had continued for the indicated number of days, the cells were fixed in 4% paraformaldehyde solution for immunocytochemistry or lysed for immunoblotting.

Immunoblotting

Protein samples (10 μg) were subjected to SDS-PAGE and then transferred onto Immobilon P membranes. After blocking, the membranes were treated with primary antibodies and then with peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized using the ECL system (Amersham Pharmacia Biotech) and documented by LAS 1000 (Fuji Photo Film, Kanagawa, Japan). The density of bands was analyzed using a BIO-RAD GS-700 densitometer. Experiments were repeated thrice using samples obtained from HSCs isolated from different rats.

Immunohistochemistry and collagen staining

Immunohistochemistry was performed using the methods described in detail elsewhere [21]. After the development of fibrosis, rats were anesthetized and laparotomized. The

liver was perfused with phosphate-buffered saline (PBS) and then perfusion-fixed with 4% formaldehyde, dehydrated, and embedded in Polybed. Sections were cut at a thickness of 5 μm and stained for 1 h in 0.1% (w/v) Sirius red (Direct Red 80, Aldrich, Milwaukee, WI, USA) [22]. Double immunostaining analysis was carried out using methods described previously [22]. After blocking with 5% bovine serum albumin/PBS, they were incubated overnight with primary antibodies in the medium. They were then incubated with both 20 μg/ml Alexa Fluor 488 goat anti-mouse IgG2a antibody and 20 μg/ml Alexa Fluor 594 goat anti-mouse IgG1 antibody for 2 h. Specimens were counterstained for nuclei with DAPI. The sections were observed under an LSM510 confocal laser scanning microscope (Carl Zeiss, Germany).

Cultured HSCs on glass microscope slides were fixed with 3.7% formaldehyde for 30 min at room temperature. After washing thrice with PBS containing 0.1% Triton X-100, the fixed cells were incubated with anti-α-SMA antibody and anti-tropomyosin antibody for 1 h at room temperature and successively with FITC-labeled goat antimouse IgG1 (Alexa Fluor 488) and rhodamine-labeled goat antimouse IgG2a (Alexa Fluor 594) for 1 h at room temperature. After washing, the specimens were observed under an LSM510 confocal laser scanning microscope (Carl Zeiss, Germany). Experiments were repeated thrice using samples obtained from HSCs isolated from different rats.

Immunohistochemical analysis of human liver samples

One specimen obtained by resection during surgery from subjects with normal liver function was used as a control. Informed written consent was obtained from all patients at the time of their liver biopsy, and the study was conducted in conformance with the Helsinki Declaration. The diagnosis of liver cirrhosis was established on the basis of the clinical and histopathological features. Immunohistochemistry was performed according to the methods described above.

Results

Tropomyosin expression in primary cultured HSCs

We investigated the expression of tropomyosin in primary-cultured HSCs as a model because HSC culture precisely resembles the in vivo cellular phenotypic change of HSCs from a vitamin A-storing quiescent phenotype to an activated and myofibroblastic phenotype in response to inflammatory stimuli [23]. Freshly isolated and plated HSCs resembled lipocytes, extended branching cytoplasmic processes, and enclosed multiple droplets that contained retinol (Fig. 1Aa). After culturing for 3 days, the cells expanded their cell body with enlarged processes and nuclei, and the size of fat droplets decreased (Fig. 1Ab). By

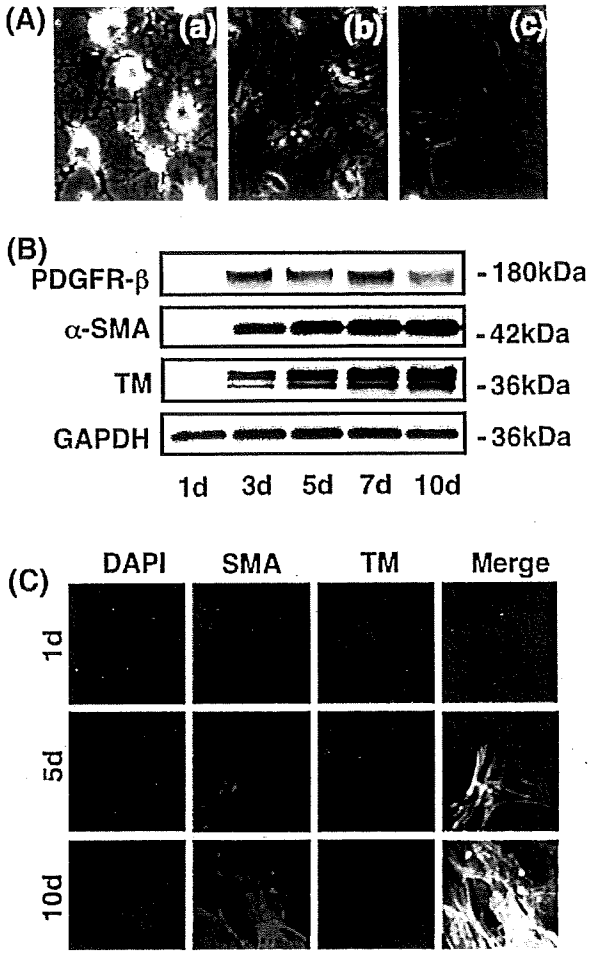

Fig. 1 Tropomyosin expression in primary cultured HSCs. **A** The cell morphology of isolated and cultured HSCs was observed under phase-contrast microscopy everyday, monitored, and digitally recorded. **a** Day 1. HSCs attached to the plate enclose droplets that contain vitamin A. **b** Day 3. HSCs start extending their processes. **c** Day 7. HSCs showed enlarged cell body and lose their droplets (magnification, ×200). **B** The expression of tropomyosin in cultured HSCs was determined by immunoblot and immunocytochemistry. Whole-cell homogenates were subjected to SDS-PAGE, transferred onto the membrane, and successively immunoreacted with PDGFR-β, α-SMA, or tropomyosin. Note that tropomyosin is induced in HSCs time dependently after starting the culture in a manner similar to the expression of PDGFR-β and α-SMA. Representative data from three independent preparations are presented here. **C** Immunocytochemistry of tropomyosin and α-SMA. Cultured HSCs on days 1, 5, and 10 were fixed in 4% paraformaldehyde and subjected to immunocytochemistry, as described in section "Materials and methods". Note that tropomyosin appears on day 5 and becomes prominent on day 10. Tropomyosin colocalizes and generates stress fibers with α-SMA (magnification ×200)