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

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Received: 30 July 2008 / Accepted: 1 April 2009 / Published online: 21 April 2009
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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.

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 (PDGFR β) 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 PDGFR β .

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

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with ribavirin, provides an effective therapy for chronic hepatitis C. Although the primary action of IFN α 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 IFN α -treated patients with chronic hepatitis C [11, 12]. These reports strongly indicate that IFN α 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 IFN α against human HSC [13].

Materials and methods

Materials

Recombinant PDGF-BB and tumor necrosis factor α (TNF α) 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 IFN α 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. Immuno-reactive 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 μ Ci/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 μ l 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 trypsinization of human HSC treated with IFN α or TNF α , IFN α /TNF α 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 TNF α and/or IFN α for 24 h. Then, the cells were collected using a cell scraper, and the collected cells were pelleted by centrifugation at 600 $\times$ *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 $\pm$ 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 IFN α on DNA synthesis of human HSC-line

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

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

Because IFN α suppressed DNA synthesis of human HSC, we hypothesized that IFN α 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 IFN α . Although a previous report using hepatoma cell lines indicated that p53 is involved in growth

Fig. 1 Effect of IFN α 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 IFN α for 24 h, and then pulse-labeled with 1.0 μ Ci/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 IFN α 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 IFN α for 24 h, and then were pulse-labeled with 1.0 μ Ci/ml of [3 H]thymidine during the last 24 h. The incorporated radioactivity was counted by liquid scintillation. **P* < 0.01

suppression by IFN α [15], we failed to observe the suppression of p53 in HSC treated with IFN α . The PDGF-BB-activated MEK-MAPK cascade and Akt were also unaffected (Fig. 3b).

Fig. 3 Effects of IFN α 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 PDGFR β , 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

Induction of apoptosis of HSC by IFN α

Although the mechanism of IFN α -dependent DNA synthesis inhibition was not clear, we found that IFN α triggered the apoptosis of human HSC-line in the presence of a low dose of TNF α . As shown in Fig. 4, IFN α or TNF α alone failed to affect the number of HSC, as determined by the Alamar blue assay. However, the simultaneous addition of IFN α and TNF α 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 IFN α and TNF α for 48 h (Fig. 5). This finding indicates that cell death is actively stimulated by the combination of IFN α and TNF α . This observation was further supported by the fact that cell treatment with IFN α plus TNF α -induced DNA ladder formation (Fig. 6) and analysis using flow cytometric quantification of apoptosis of human HSC line (Fig. 7).

Role of IFN α on caspase cascade

Finally, we tested whether IFN α regulates the caspase cascade in human HSC-line. As shown in Fig. 8a, cytochrome c release from mitochondria into the cytosol was augmented by TNF α alone and TNF α /IFN α treatment. In accordance with this result, cleaved caspase 3 was enriched in cells treated with TNF α alone and TNF α /IFN α (Fig. 8b). Because TNF α alone thus triggered cytochrome c release and the activation of caspase 3 and failed to induce the active apoptosis of HSC, we hypothesized that IFN α may

Fig. 4 Effect of IFN α and TNF α 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 IFN α and/or TNF α 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. * $P < 0.01$

play a critical role in finalizing DNA fragmentation. After searching for the mechanism, we found that IFN α significantly reduced the level of ICAD without affecting the amount of CAD (Fig. 8c).

Discussion

IFN α is now a first-choice therapy for chronic hepatitis C (CH-C). In combination with ribavirin, IFN therapy leads to about a 30% and 80% eradication of HCV genotypes 1 and 2, respectively. Recently, pegylated IFN α has improved the efficacy of antiviral therapy [16, 17]. IFN α as well as IFN β binds to cell surface receptors composed of IFNAR1 and IFNAR2 subunits. IFN α 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 IFN α and TNF α 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 IFN α and/or TNF α and the culture was continued for another 48 h. Cell appearance of HSC were determined

under a microscope at a magnification of $\times 200$. 1: Control; 2: IFN α ; 3: TNF α ; 4: IFN α /TNF α . Note that the cell number was markedly decreased by incubating them with IFN α plus TNF α . IFN α : 100 IU/ml. TNF α : 10 ng/ml

Fig. 6 DNA fragmentation of HSC treated with IFN α and TNF α . 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 IFN α plus TNF α induced DNA fragmentation. IFN α : 100 IU/ml. TNF α : 10 ng/ml. M. W.: molecular weight

Fig. 7 Flow cytometric quantification of apoptosis in LX-2 treated with IFN α or TNF α , IFN α /TNF α . 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 IFN α and/or TNF α 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 IFN α and/or TNF α . IFN α : 100 IU/ml. TNF α : 10 ng/ml

In addition to the antiviral effect, IFN α 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 IFN α/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 IFN α 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 IFN α has a direct anti-fibrotic action, most likely through its action against HSC. Initially, IFN γ 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, IFN α 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 IFN α . 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 IFN α 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 TGF β 1 (0.5 ng/ml), and that IFN α 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 IFN α . Although we failed to clarify the mechanism of IFN α -dependent inhibition of cell cycle progression in human HSC-line, we found that natural IFN α 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 IFN α derivatives.

Mallat et al. [29] additionally showed that IFN α as well as IFN γ reduced the secretion of prolines and the mRNA expressions of collagens α 1(I) and α 1(III). With respect to this mechanism, Inagaki et al. [30] recently reported that IFN α blocked promoter activation and prevented the progression of liver fibrosis induced by CCl $_4$ injection when administered to transgenic mice harboring the α 2(I) collagen gene (COL1A2) promoter sequence, and that, in transient transfection assays, IFN α decreased the steady-state levels of COL1A2 mRNA and inhibited TGF β /Smad3-stimulated COL1A2 transcription.

In addition to these previous observations, we herein found that IFN α in combination with a small amount of TNF α 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 IFN α , 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 IFN α against human HSC-line, and provides useful information regarding the mechanism of IFN-dependent regression of human liver fibrosis caused by viral infection.

Acknowledgments We thank Drs. Kazuki Nakatani, Naoto Maeda, Yukihiro Imanishi, and Koji Kinoshita for their critical comments on this work. This work was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS). A part of this study was presented at the 12th International Symposium on Cells of the Hepatic Sinusoid (12th ISCHS).

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