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Establishment of an Immortalized Human Hepatic Stellate Cell Line to Develop Antifibrotic Therapies

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Because human hepatic stellate cells (HSCs) perform a crucial role in the progress of hepatic fibrosis, it is of great value to establish an immortalized human cell line that exhibits HSC characteristics and grows well in tissue cultures for the development of antifibrotic therapies. Thus, we engineered an immortalized human hepatic stellate cell (HSC) line TWNT-4 by retrovirally inducing human telomerase reverse transcriptase (hTERT) into LI 90 cells established from a human liver mesenchymal tumor. Parental LI 90 entered replicative senescence, whereas TWNT-4 showed telomerase activity and proliferated for more than population doubling level (PDL) 200 without any crisis. TWNT-4 expressed platelet-derived growth factor-β receptor (PDGF-βR), α-smooth muscle actin (α-SMA), and type I collagen (α1) and was considered to be an activated form of HSCs. Treatment of TWNT-4 cells with either 100 U/ml of IFN-γ or 1 ng/ml of rapamycin (Rapa) for 14 days led to lower expression of type I collagen (α1) at RNA and protein levels. Exposure of TWNT-4 cells to both of IFN-γ (10 U/ml) and Rapa (0.1 ng/ml) for 14 days effectively decreased the expression of type I collagen (α1), PDGF-βR, and α-SMA expression and suppressed TGF-β1 secretion of TWNT-4 cells. We successfully induced apoptosis by transducing TNF-related apoptosis-inducing ligand (TRAIL) into TWNT-4 cells using adenovirus vectors Ad/GT-TRAIL and Ad/PGK-GV-17. These findings suggested that immortalized activated HSC line TWNT-4 would be a useful means to develop antifibrotic therapies.

Key words: Human hepatic stellate cells; Human telomerase reverse transcriptase; Interferon-γ; Rapamycin; Type I collagen

INTRODUCTION

Hepatic stellate cells (HSCs), one of sinusoid wall cells in the liver, play a major role in the progress of hepatic fibrosis (1,12,14,25,27). HSCs are in a resting phase in a normal liver and contain a large quantity of lipid droplet with vitamin A in the cell bodies (4). When hepatic inflammation persists, HSCs are transformed into myofibroblasts, resulting in an increase in cellular proliferation and production of extracellular matrices, including type I and III collagens and fibronectin, and disappearance of lipid droplet (9). It is known that cytokines, such as transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF), are closely involved in HSC activation (29). Therefore, inhibition of activated HSCs can be important to develop strategies for prevention and treatment of hepatic fibrosis. Because of severe donor liver shortage for human HSC isolation and difficulty in maintaining primarily isolated HSCs in culture for a long period of time, it would be extremely importance to establish an immortalized human HSC line that could provide adequate quantity and quality. Therefore, it is necessary to obtain immortalized cells by inducing an immortalizing gene into the primary culture of human HSCs.

In this study, we have endeavored to obtain immortalized HSC lines by transducing a human HSC strain LI 90, established by Murakami et al. (26), with a retrovirus vector #197 expressing human telomerase reverse transcriptase (hTERT) (41). One of the resultant cell lines, TWNT-4, exhibited characteristics compatible with those of human HSCs. TWNT-4 cells expressed PDGF-β receptor (PDGF-βR) and α-smooth muscle actin (α-SMA) and are considered to be an activated form of HSCs in vitro. We investigated the effect of interferon-γ (IFN-γ) and rapamycin (Rapa) on TWNT-4 cells. Because ex-

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pression of TRAIL was reported to induce apoptosis in activated HSCs, we also examined the efficacy of adenovirus-mediated TRAIL transduction on the apoptotic effect in TWNT-4 cells.

MATERIALS AND METHODS

Cell Culture

A human hepatic stellate cell (HSC) strain LI 90 was transduced with a retrovirus vector SSR 197, encoding hTERT and green fluorescent protein (GFP) cDNAs, as previously reported (41). One of six clones derived from SSR 197-transduced LI 90 cells, TWNT-4, was used in the present study. The cells were maintained with ASF104 medium containing 10% FBS and penicillin-G (100 U/ml) and streptomycin sulfate (100 μg/ml) (P/S).

Expression of PDGF-βR and α-SMA and TGF-β1 Production of TWNT-4 Cells After IFN-γ or Rapa Treatment

TWNT-4 cells (2×10^5) were inoculated on the coverslips placed in six-well culture plates and IFN-γ (100 U/ml) or Rapa (1 ng/ml) was added to the culture every other day for 14 days. The cells were subjected to indirect immunofluorescent staining for α-SMA and PDGF-βR, as previously reported (41). The levels of TGF-β1 secreted by TWNT-4 cells into the culture for 24 h at the end of 14-day administration of either 100 U/ml IFN-γ or 1 ng/ml Rapa were measured using a TGF-β1 ELISA kit according to the manufacturer's protocol (Rand D Co.).

Expression of Type I Collagen (α1) of TWNT-4 Cells After IFN-γ and/or Rapa Treatment

When the TWNT-4 cells reached 50% confluence in T75 culture flasks (Falcon Co.), IFN-γ (100 U/ml) (Roche, Tokyo, Japan), Rapa (1 ng/ml), or IFN-γ (10 U/ml) plus Rapa (0.1 ng/ml) was added to each culture every other day for 14 days. After the treatment, total RNAs of three independent cultures were isolated using RNAzol (Cinna/BioTecx, Friendswood, TX) and used as templates. Reverse transcription was performed at 22°C for 10 min and then at 42°C for 20 min using 1.0 μg of RNA per reaction to ensure that the amount of amplified DNA was proportional to that of specific mRNA in the original sample. Polymerase chain reaction (PCR) was performed with specific primers in volumes of 50 μl containing 2.0 μg RT products according to the manufacturer's protocol (PCR kit; Perkin-Elmer/Cetus, Norwalk, CT). The amplification reaction involved denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and at 72°C for 1 min using a thermal cycler (Perkin-Elmer, Foster City, CA) using 30–35 cycles. The PCR products were resolved on 1% agarose gels and visualized control. Primers used were as follows: type I procollagen α1 (601 bp): sense, 5′-CTGGTCCTGATGGCAAAACT-3′, antisense, 5′-ACCAGCATCACCCTTAGCAC-3′. Total cell lysates were prepared from three independent similar TWNT-4 cell cultures using a cell lysis buffer, resolved on 12% SDS-PAGE and transferred onto nitrocellulose membranes (Amersham, Japan), as previously described (33). Samples were treated with a mouse monoclonal antibody against human type I collagen α1 (DAKO, Tokyo, Japan) followed by a horseradish-peroxidase-conjugated anti-rabbit IgG secondary antibody (1:2000) (MBL, Nagoya, Japan). Then type I collagen α1 was detected using an enhanced chemiluminescence system (ECL detection kit, Amersham). Human tubulin was used as an endogenous control.

Transduction of TWNT-4 Cells With TNF-Related Apoptosis-Inducing Ligand (TRAIL)

The recombinant adenovirus vectors Ad/GT-TRAIL expressing TRAIL cDNA downward of GT promoter and Ad/PGK-GV-16 (kindly provided by Drs. J. A. Roth and B. Fang, University of Texas M.D. Anderson Cancer Center, Houston, TX, USA) were used to transduce TWNT-4 cells with TRAIL. The TWNT-4 cells (5×10^5) were inoculated on sterilized coverslips in a well of six-well plates and were concomitantly infected with Ad/GT-TRAIL and Ad/PGK-GV-16 at multiplicity of infection (MOI) ranging from 1 to 100, for 2 h. At 72 h after infection of Ad/GT-TRAIL and Ad/PGK-GV-16, Hoechst nuclear staining for assessing fragmentation of DNAs and cell cycle analysis with FACS (Becton Deckinson Co.) was conducted to evaluate an apoptosis-inducing effect of TRAIL in TWNT-4 cells. For Hoechst nuclear staining, the cells were washed with phosphate-buffered saline (PBS) three times and treated with trypsin. Samples were centrifuged at 1000 rpm for 5 min, and added with 1 ml of 1% glutaraldehyde solution overnight at 4°C. Then samples were centrifuged at 1000 rpm at 5 min and cell pellets were diluted with 300 μl of PBS. Hoechst 33342 solution (2 μl) was added to 10 μl of the PBS-diluted cell-floating solution. One drop of the solution was placed on a glass slide, which was covered with a cover glass, to observe DNA fragmentation with a fluorescence microscope. For cell cycle analysis, the cells were washed with PBS three times, treated with trypsin, and centrifuged at 1000 rpm for 5 min. Samples were suspended in 1 ml of PBS, and 3 ml of cold 100% ethanol was added one drop at a time while agitating. The cells were immobilized at a final concentration of 75% ethanol, left still for 30 min at 4°C, and centrifuged again at 1000 rpm for 5 min. After 10 μl of RNase treatment, the cells were suspended in 1 ml of PBS to incubate for 20 min at 37°C. Propidium iodide (PI) solution (Wako, Tokyo, Japan) was sequentially added to the solution, which adjusted the final concentration to 50 μg/ml to stain DNAs for 10 min at 4°C in a dark place. The

sample was filtered with 50-μm nylon mesh to analyze the cell cycle with FACS (Becton Dickinson). Ad/GT-LacZ, which expressed *E. coli* LacZ cDNA under GT promoter, and Ad/PGK-GV-16 were used as the controls for Ad/GT-TRAIL. Normal human hepatocytes and liver endothelial cells (purchased from Cell Systems Co., Seattle, WA) and unmodified LI 90 cells population doubling level (PDL) 10 were used as controls for TWNT-4.

RESULTS

Biological Characteristics of TWNT-4 Cells

TWNT-4 cells were uniformly positive for GFP, indicating that the cells were not a spontaneously derived subpopulation of parental LI 90 cells (Fig. 1A). TWNT-4 cells were longitudinally spindle shaped and displayed long cytoplasmic extensions. When reaching confluence, they formed hills-and-valleys morphology (Fig. 1B). TWNT-4 cells grew well in tissue culture for more than population doublings 200 without any crisis, demonstrating that the cells were considered to be immortal.

Decreased Expression of PDGF-βR and α-SMA After IFN-γ or Rapa Treatment

We examined the effect of IFN-γ (100 U/ml) or Rapa (1 ng/ml) on the expression of activated HSC markers PDGF-βR and α-SMA in TWNT-4 cells. Untreated TWNT-4 cells strongly expressed both of PDGF-βR and α-SMA; in contrast, IFN-γ- or Rapa-treated TWNT-4 cells showed markedly lower expression of these markers (Fig. 2).

IFN-γ and Rapa Decreased TGF-β1 Secretion of TWNT-4 Cells

At day 14 after administration of either IFN-γ (100 U/ml) or Rapa (1 ng/ml), the average levels of TGF-β1 secreted by TWNT-4 into the culture medium were 0.42 ng/ml for IFN-γ and 0.53 ng/ml for Rapa treatment. These values were significantly ($p < 0.05$) lower compared with that of untreated TWNT-4 cells (0.67 ng/ml) using a Student *t*-test (Fig. 3).

Decreased Expression of Type I Collagen (α1) in IFN-γ- and/or Rapa-Treated TWNT-4 Cells

We evaluated the expression of type I collagen (α1) in TWNT-4 cells after 14-day treatment with IFN-γ (100 U/ml), Rapa (1 ng/ml), or IFN-γ (10 U/ml) plus Rapa (0.1 ng/ml) by RT-PCR and Western blot. In the monotherapy study, either IFN-γ (100 U/ml) or Rapa (1 ng/ml) decreased expression of type I procollagen (α1) by RT-PCR (Fig. 4A). Combined therapy of IFN-γ (10 U/ml) with Rapa (0.1 ng/ml) effectively inhibited type I procollagen (α1) expression as low as a 10th of each concentration in the monotherapy. These findings were consistent with the results of Western blot for type I collagen (α1), as shown in Figure 4B. Equal loading of the samples was confirmed by the expression of GAPDH and tubulin.

TRAIL Induced Apoptosis in TWNT-4 Cells

We examined cell morphology and cell cycle profiles of TWNT-4 cells infected with Ad/GT-TRAIL and Ad/PGK-GV-16. Marked decrease in cell density, chromatin agglutination, and fragmentation (Fig. 5, top) was observed; cell cycle was arrested in the sub-G_1 phase in TWNT-4 cells transduced with Ad/GT-TRAIL at MOI ≥10 (Fig. 5, bottom). Such observations were not detected in the control Ad/LacZ and Ad/PGK-GV-17 experiments or in the control experiments using normal human hepatocytes and liver endothelial cells. These findings indicated that TRAIL transduction efficiently induced apoptosis in TWNT-4 cells.

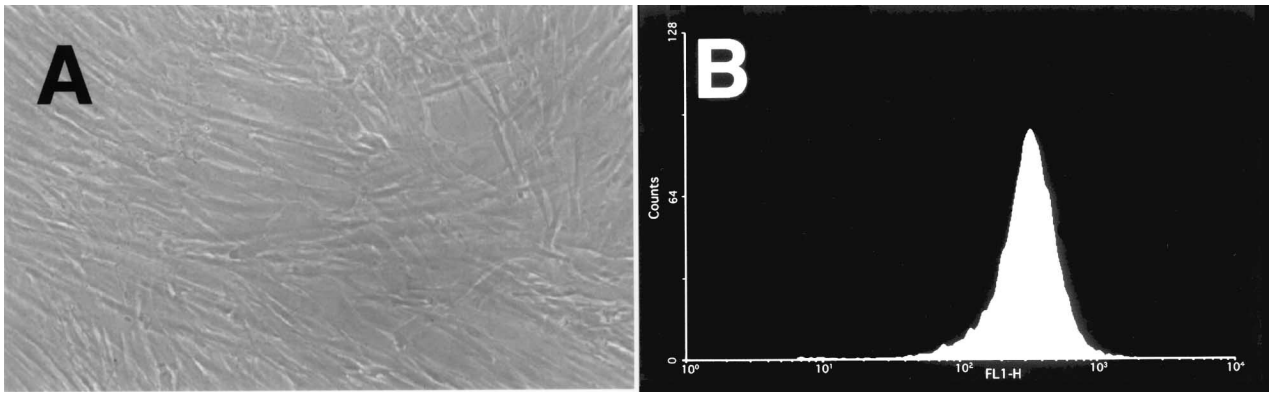

Figure 1. Morphology and GFP expression of TWNT-4 cells. (A) TWNT-4 cells showed spindle shape and typical hills-and-valleys morphology at confluence (a phase contrast micrograph, original magnification ×100). (B) Flow cytometric analysis demonstrated homogeneous GFP expression of TWNT-4 cells.

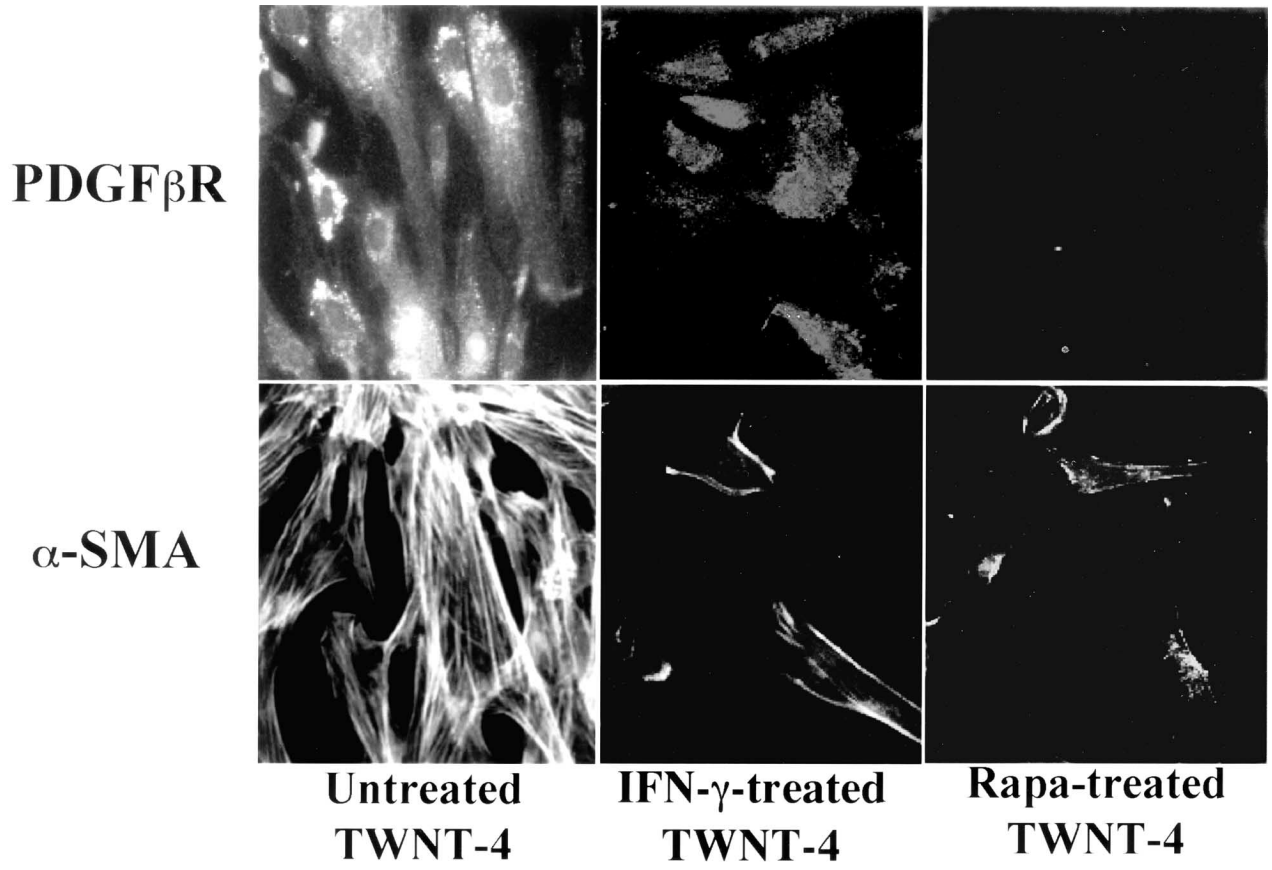

Figure 2. Expression of PDGF-βR and α-SMA after IFN-γ and Rapa treatment. When either 100 U/ml IFN-γ or 1 ng/ml of Rapa was administered for 14 days, expression of PDGF-βR and α-SMA of TWNT-4 cells was dramatically decreased (original magnification ×400 for each micrograph). The data are representative of three independent experiments.

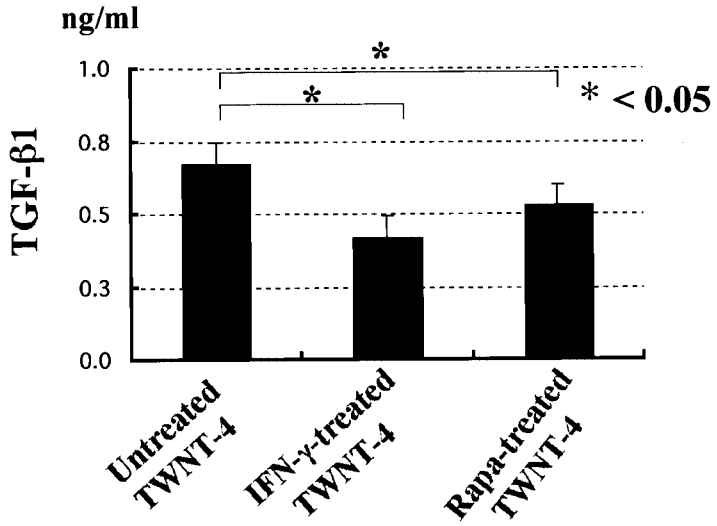

Figure 3. TGF-β1 secretion of TWNT-4 cells after IFN-γ and Rapa treatment. The average levels of TGF-β1 secreted were 0.67 ng/ml for untreated TWNT-4 cells, 0.42 ng/ml for IFN-γ-treated cells, and 0.53 ng/ml for Rapa-treated cells. TGF-β1 secretion of TWNT-4 cells was significantly ($p < 0.05$) decreased by IFN-γ or Rapa treatment. The data are shown as mean ± SD.

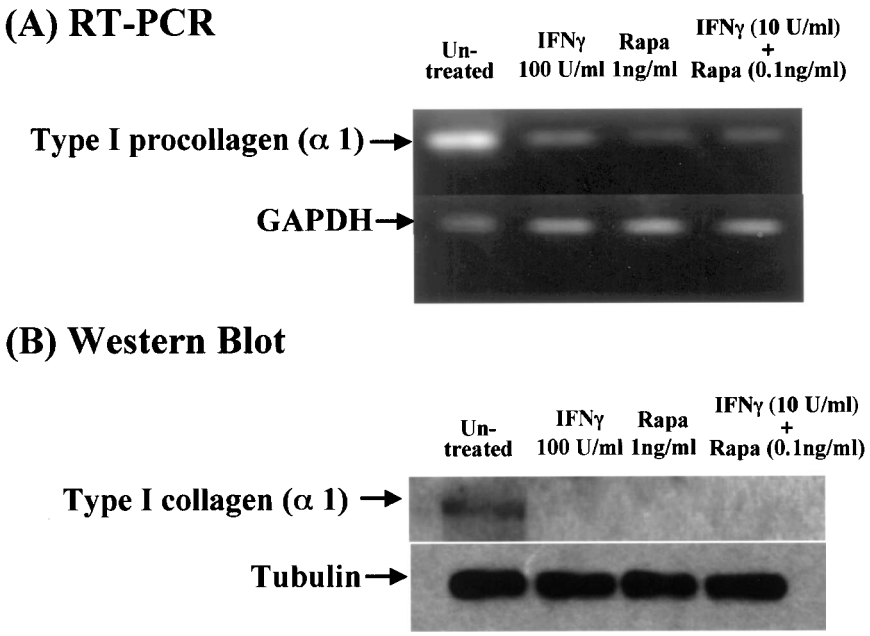

Figure 4. Expression of type I collagen (α1) in IFN-γ- and/or Rapa-treated TWNT-4 cells. In the monotherapy, either of IFN-γ (100 U/ml) or Rapa (1 ng/ml) decreased expression of type I procollagen (α1) by RT-PCR (A). Combined therapy of IFN-γ (10 U/ml) with Rapa (0.1 ng/ml) effectively inhibited type I procollagen (α1) expression, which was confirmed by Western blot for type I collagen (α1) (B). GAPDH and tubulin expression demonstrated an equal loading of the samples.

DISCUSSION

In hepatic fibrosis process, the hepatic stellate cells (HSCs) play a central role in production of extracellular matrices (1,12–14,25,27). Production of excess matrices by HSCs is a key process for the progress to cirrhosis. Therefore, to inhibit activation of HSCs is of extreme importance to develop antifibrotic therapies in cirrhosis (3,10,15,24,28,31,37,38,44,45). The following approaches should be considered to control production of excess matrices by HSCs: 1) inhibit activation of HSCs by eliminating causes of hepatitis and by suppressing inflammations; 2) directly suppress activation of HSCs; 3) decrease extracellular matrix production from HSCs; and 4) decrease the number of activated HSCs by inducing them into apoptosis. The most common approach in such current clinical practices is the use of IFN-α or IFN-β, which targets eliminating the cause of hepatitis B and/or hepatitis C virus-mediated hepatitis, but the effect of IFN-α or INF-β is not adequate. Thus, an efficient strategy for hepatic fibrosis should be explored. However, the primary culture of human HSCs has some drawbacks: 1) fewer cell divisions, 2) frequent contamination by other cells, and 3) morphological and biodynamic changes caused by serial cell passages in vitro. Alternate HSCs derived from other animals, including rodents, which can be maintained in vitro for a longer term, has been widely used in basic research. However, it often raises the issue whether such cells are compatible with human diseases. Unfortunately, a reliable human HSC line, not strain, to determine the effectiveness of such a strategy is not available. Establishment of an immortalized human HSC line that exhibits the characteristics of human HSCs would be of great value to facilitate the research of hepatic fibrosis. Regarding the mechanism of cell division, it has been thought that cell crisis occurs when telomere contraction reaches the second critical point at which chromosomes become unstable (35,40). Because of such phenomenon, it may be highly unlikely that the primary culture of human cells may continue to proliferate without limitation over crisis (7,16).

Introduction and expression of specific genes to primary human cells have enabled construction of immortalized cells by bypassing the first senescence crisis and expanding population doubling level (PDL). Spontaneous immortalization of cells with simian virus 40 large T antigen (SV40T) has been reported to be approximately 3.3×10^{-7}, which is thought to be due to activation of endogenous telomerase activity. Previous reports indicated that telomerase activation in SV40T-introduced HEK cells or human pancreatic cells and fibroblast with SV40T or Ras gene could eliminate crisis (8,13). Thus, expression of human telomerase reverse transcriptase (hTERT) in human cells may allow dramatic improve-

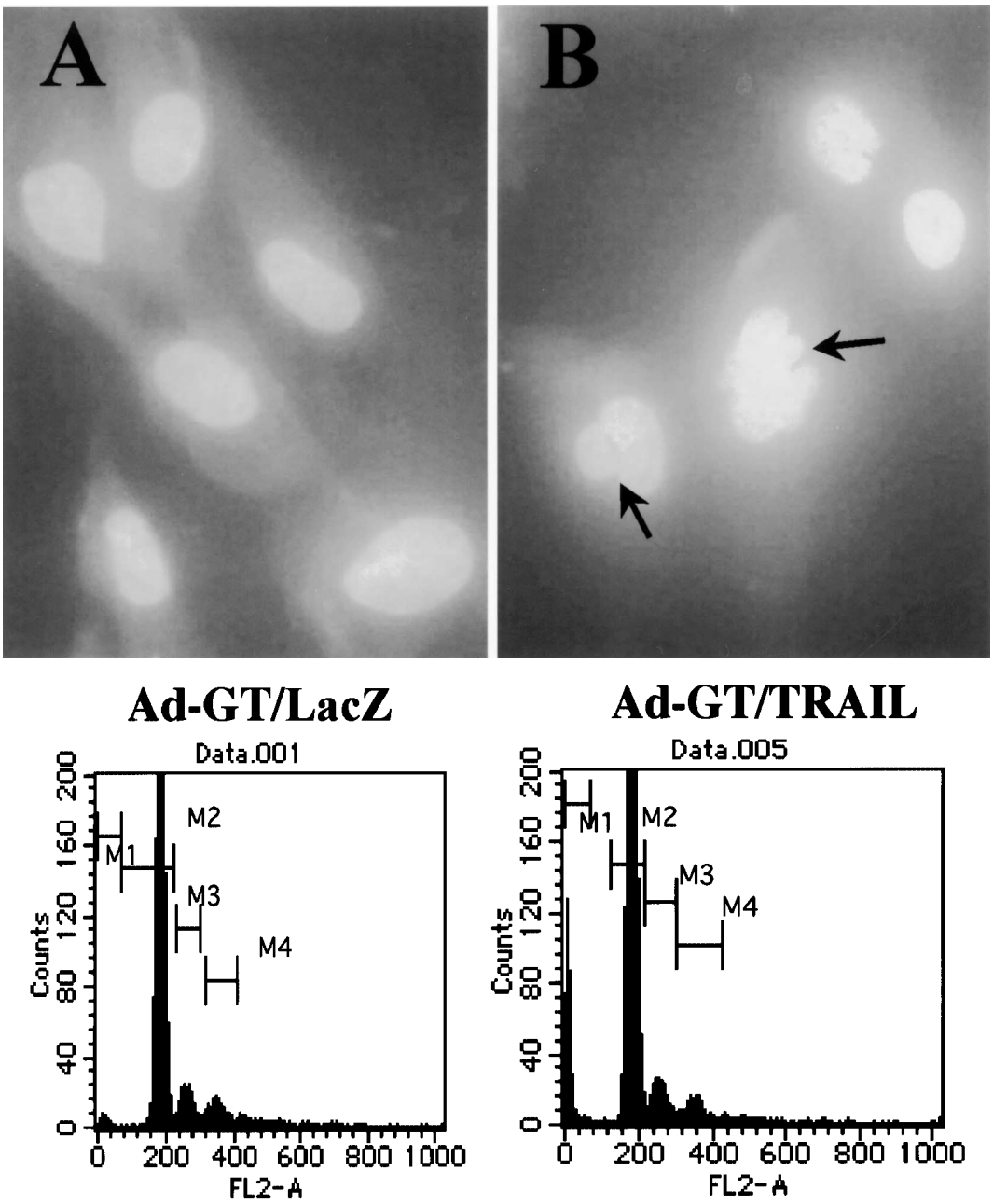

Figure 5. TRAIL induced apoptosis in TWNT-4 cells. At 72 h after the transduction of TWNT-4 cells with Ad/GT-TRAIL and Ad/PGK-GV-16 (MOI = 1, 5, 10, 25, 50, and 100), agglutination and fragmentation of chromatins were observed in the cells, as indicated by arrows (top). The apoptosis of TRAIL-transduced TWNT-4 cells was increased by 10% compared with the control in a cell cycle analysis (bottom). (A) Ad/GT-LacZ- and Ad/PGK-GV-16-infected TWNT-4 cells at MOI 50. (B) Ad/GT-TRAIL- and Ad/PGK-GV-infected cells at MOI 50.

ment in life span or, preferably, immortalization. Therefore, in this study, we endeavored to transduce HSC strain LI 90 cells using a retrovirus vector expressing SSR 197 and GFP cDNAs. LI 90 cells proliferated slowly with replicative senescence at about PDL 20–25. TWNT-4, one of the hTERT-transduced LI 90 cells, did not exhibit crisis even after passages of more than PDL 200 and the cells expressed hTERT by RT-PCR analysis and telomerase activity in PCR-based Trap assay. TWNT-4 cells were uniformly positive for GFP. These findings indicated that TWNT-4 cells were not a spontaneous subpopulation derived from parental LI 90 cells in vitro.

TWNT-4 cells exhibited characteristics of activated HSCs. Such characteristics included fibroblastic morphology, expression of PDGF-βR and α-SMA, and maximum collagen type I expression. The observations encouraged us to study antifibrotic strategies using TWNT-4 cells. First, we examined whether activating factors of HSCs, such as PDGF and TGF-β1, increase the expression of type I procollagen (α1) by RT-PCR (8,22). No change in type I collagen expression was detected between nontreated TWNT-4 cells and PDGF- or TGF-β1-treated cells, suggesting that TWNT-4 cells were fully activated in an in vitro culture environment (data not shown). Next, we investigated whether IFN-γ, one of the most potent activation inhibitors, decreases the expression of type I procollagen (α1) (2,34). However, 24-h exposure to IFN-γ slightly decreased collagen type I expression even at the high concentration of 1000 and 10,000 U/ml (data not shown). These results suggested that 24-h administration of IFN-γ did not exert an adequate inhibition effect of type I procollagen (α1) expression of TWNT-4 cells. Based on these observations, the exposure period was extended to 14 days. The amount of IFN-γ administered was reduced to 100 U/ml, a 10th of the usual dosage, in a 14-day exposure experiment and type I procollagen (α1) expression was effectively inhibited, which was confirmed by both RT-PCR and Western blotting studies.

Then we focused on rapamycin (Rapa), a macroride antibiotic, which has been clinically applied as an effective immunosuppressant that would be replaced with FK506 or cyclosporine. Currently, Rapa has been reported to inhibit cellular proliferation through a posttranscription regulatory mechanism (45). In addition, Rapa and its derivatives have been investigated as new anticancer agents in clinical aspects (5,17,18,30,32,35). Zhu et al. demonstrated that Rapa had inhibition effect of hepatic fibrosis in a rat model of carbon tetrachloride (45). Thus, we investigated whether Rapa administration reduced activated phenotypes of TWNT-4 cells. Rapa decreased expression of type I collagen (α1), PDGF-βR, and α-SMA, and TGF-β1 secretion of TWNT-4 cells when 1 ng/ml of Rapa was administered for 14 days. Similar findings were observed in 14-day administration of 100 U/ml of IFN-γ. Additionally, combined therapy of IFN-γ (10 U/ml) with Rapa (0.1 ng/ml), which were a 10th the dose of each monotherapy, exerted similar effects. These results suggest the possibility of combination therapy of IFN-γ and Rapa at low doses to treat hepatic fibrosis through inactivation of fully activated HSCs without worries of adverse effects.

Apart from such strategy, which is designated to inhibit or suppress activated HSCs, researchers have endeavored to treat hepatic fibrosis by introducing HSCs to apoptosis actively and reducing the number of HSCs (11,19,20,39,43). We evaluated the apoptosis effect of TWNT-4 cells by transducing cDNA of TNF-related apoptosis-inducing ligand (TRAIL) cloned as a TNF family molecule using a recombinant adenovirus vector Ad/GT-TRAIL (21). Hoechst nuclear stain showed MOI-dependent nuclear chromatin agglutination and fragmentation. Flow cytometric analysis also demonstrated increase in apoptosis of TWNT-4 cells after Ad/GT-TRAIL infection. Such findings were not observed in normal human hepatocytes or liver endothelial cells. These findings suggest that activated HSCs in the liver can be selectively subjected to apoptosis with TRAIL transduction.

In conclusion, we have established an immortalized activated human stellate cell line, TWNT-4, with retrovirus-mediated hTERT transduction. TWNT-4 cells could be a useful tool to develop and establish antifibrotic therapies.

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