

Effects of recombinant human adenovirus-p53 on the regression of hepatic fibrosis

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Abstract. Hepatic fibrosis is a scarring process that may progress to hepatic cirrhosis and even hepatic carcinoma if left untreated. Hepatic stellate cells (HSCs) play essential roles in the development of hepatic fibrosis. The tumor suppressor protein p53 is a transcription factor that is involved in cell proliferation, cell cycle regulation, apoptosis and DNA repair. Recombinant human adenovirus-p53 (Ad-p53) has been demonstrated to act as a promising antitumor gene therapy in various types of cancer. However, there is limited information regarding the therapeutic effect of Ad-p53 on the regression of hepatic fibrosis. In order to examine the underlying molecular mechanism responsible for the effects of Ad-p53 on HSCs, a rat model of hepatic fibrosis was established and HSC-T₆ cells were cultured under different conditions. The expression of p53, transforming growth factor (TGF- β 1) and α -smooth muscle actin (α -SMA), which is a marker of activated HSCs, was detected by immunohistochemical assays and RT-qPCR. *In vitro*, five different concentrations (1x10⁶, 5x10⁶, 1x10⁷, 2x10⁷ and 5x10⁷ PFU/ml) of Ad-p53 were selected for use in the MTT assay to analyze the proliferation of HSCs at 0, 24, 48 and 72 h. Flow cytometric analysis was applied to determine the effect of three different concentrations of Ad-p53 (5x10⁶, 1x10⁷ and 2x10⁷ PFU/ml) on the cell cycle and the apoptosis of HSC-T₆ cells at 24 and 48 h. The results of immunohistochemical studies and RT-qPCR showed that Ad-p53 upregulated the expression of p53, and downregulated the expression of TGF- β 1 and α -SMA. The MTT assay revealed that when treated with various doses of Ad-p53, the proliferation of HSCs was inhibited within a certain range of concentrations and time periods. Analysis of flow cytometric data showed that Ad-p53 arrested the cell cycle in G1 phase and significantly induced apoptosis. Taken together, these findings suggest that Ad-p53 promotes apoptosis and inhibits the

proliferation of HSCs in a time- and dose-dependent manner by modulating the expression of p53, TGF- β 1 and α -SMA.

Introduction

Hepatic fibrosis, defined as the deposition of excessive extracellular matrix (ECM) in liver tissue, is the major complication of chronic liver damage and is associated with significant morbidity and mortality (1). Fibrosis is considered as a wound-healing response to various chronic liver diseases (2); however, it may progress to liver cirrhosis and even hepatocellular carcinoma if left untreated (3,4). Hepatic stellate cells (HSCs) have been reported to play vital roles in liver fibrogenesis and in chronic injuries (5). Injury to the liver results in the activation of HSCs which subsequently transdifferentiate into myofibroblasts that typically express α -smooth muscle actin (α -SMA) and over-produce fibroblast-like matrix (particularly collagen I and III), leading to the massive deposition of ECM and the prevention of further liver damage (6). However, accumulating evidence has demonstrated that liver fibrosis subsequently develops due to the redundant and the extended activation of HSCs (7). The characteristics of HSCs, as well as their functions in liver development and regeneration remain crucial areas for liver research (8). The progression of hepatic fibrosis is a highly dynamic process involving various complex cellular and molecular mechanisms (9). The development of novel antifibrotic therapies targeted at the regulation of HSCs appears to be a pivotal strategy for the treatment of hepatic fibrosis.

The tumor suppressor protein p53 is widely known as the guardian of the genome and is involved in cell cycle regulation, DNA repair and anticancer responses (10). The activation of p53 by a stressful environment in the liver is associated with altered metabolic pathways or the induction of apoptosis (11). Emerging data suggest that the p53 mutation rates in patients with either chronic hepatitis B or C virus (HBV or HCV, respectively) infections were apparently higher than those in healthy controls, and these findings confirmed that genetic polymorphisms of p53 affect the prognosis of hepatic diseases (12). Furthermore, in HSCs, the activity of p53 protein provides protection against liver fibrosis (13). Animal experiments revealed that loss of p53 promoted hepatic fibrosis and tumor development, and a combination of p53 and myeloid cell leukemia 1 (Mcl-1) exerted protective effects on hepatocytes as well as effects on fibrosis and tumor control (14). Accumulating evidence has

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confirmed that antifibrotic drugs function through the p53 pathway. There is a transition from hepatic fibrosis to cirrhosis to hepatocellular carcinoma, and the induction of hepatocellular carcinoma correlates closely with both p53 mutation and deficiency (15-18). For example, tetramethylpyrazine (TMP) as a pharmacological product induced the apoptosis of HSCs through p53 activation, and exerted anti-fibrotic effects in the liver *in vitro* (19). Artesunate has also been found to inhibit HSC proliferation in a time- and dose-dependent manner *in vitro* by increasing p53 expression (20).

Evidence from *in vitro* and *in vivo* studies has demonstrated that recombinant human adenovirus-p53 (Ad-p53), as a novel drug for gene therapy, has therapeutic effects on various types of tumors including breast, prostate, head and neck, and ovarian cancer (21). However, there have been no studies to date, to the best of our knowledge, examining the mechanism responsible for the effects of Ad-p53 in hepatic fibrosis. To further examine the effect of Ad-p53 on the development of hepatic fibrosis, a rat model of hepatic fibrosis was established and HSC-T₆ cells were cultured under different conditions. We found that Ad-p53 promotes apoptosis and inhibits HSC proliferation in a time- and dose-dependent manner by modulating the expression of p53, transforming growth factor (TGF)- β 1 and α -SMA.

Materials and methods

Reagents. Ad-p53 (1x10¹² virus particles/ml) were obtained from Shenzhen SiBiono GeneTech Co., Ltd. (Shenzhen, China). Cell culture media, high glucose Dulbecco's modified Eagle's medium (DMEM) and supplements were purchased from HyClone (Burlington, ON, Canada). Carbon tetrachloride (CCl₄) was purchased from Xi'an Helin Biological Engineering Co., Ltd. (Xi'an, China). 3,3'-Diaminobenzidine (DAB) mix was purchased from Beyotime Institute of Biotechnology (Beijing, China). TRIzol reagent was purchased from Life Technologies (Gaithersburg, MD, USA). The primary antibodies anti-p53 (sc-13580), TGF- β 1 (sc-66904) and α -SMA (sc-324317) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and horseradish peroxidase (HRP)-conjugated secondary antibody was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

Cell culture. HSC-T₆ cells (Fuxiang Biological Co., Ltd, Shanghai, China) were maintained in high glucose DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, in a 5% CO₂ incubator at 37°C. The cells in the logarithmic growth phase were used for all experiments.

Establishment of a model of hepatic fibrosis. Forty Sprague Dawley (SD) rats (male, weighing 240-260 g) were purchased from the Experimental Animal Center of the Medical School of Xi'an Jiaotong University (Xi'an, China). The animals were housed and handled in accordance with the approved guidelines of the Animal Welfare Committee of Xi'an Jiaotong University. All rats were randomly divided into either the fibrosis model group or the normal control group. The fibrosis model group (5 out of 32 rats died during model preparation) was prepared by subcutaneously injecting the fibrosis-inducer, 40% CCl₄, diluted in salad oil (Jinlongyu, Xi'an, China) (an initial dose of

0.5 ml/100 g body weight followed by 0.3 ml/100 g body weight thereafter), twice per week for 12 weeks. This group was further divided randomly into the following three subgroups: 8 rats in the experimental subgroup (Ad-p53 group), 8 rats in the control subgroup (normal saline group) and 11 rats in the model test subgroup (hepatic fibrosis model group). Eight rats in the normal control group received routine nursing. The animal research protocol was reviewed and approved by the Animal Care and Use Committee of Xi'an Jiaotong University (Xi'an, China).

Immunohistochemical assay. The animals were euthanized and the liver tissues were dissected and fixed immediately with 4% paraformaldehyde for 2 days and subsequently embedded in paraffin and sectioned (4 μ m) onto the slides. The sections were baked at 60°C for 2 h, deparaffinized by two changes of xylene, and rehydrated in graded alcohol solutions. For antigen retrieval, the sections were heated in 25 mmol/l sodium citrate (pH 9.0) at 95°C for 15 min. The slides were treated with 3% H₂O₂ to block endogenous peroxidase activity, and then incubated overnight at 4°C with anti-p53 (1:500), TGF- β 1 (1:1,000) or α -SMA (1:1,000) antibodies, respectively. Subsequently, the sections were incubated with a secondary antibody (1:500) for 30 min at room temperature and DAB mix was applied for staining. The percentage of positive-stained liver cells was quantified under a microscope (Olympus, Tokyo, Japan) and the mean value of 5 randomly-selected fields was used for comparison.

Hematoxylin and eosin (H&E) staining. The separated livers were fixed in 4% paraformaldehyde for 2 days and subsequently embedded with paraffin and cut into sections of 4 μ m thickness, and then stained with H&E (Beyotime Institute of Biotechnology) according to the standard protocols.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the liver tissues of the experimental subgroup and the control subgroup using TRIzol reagent. cDNA was synthesized using a reverse transcription kit (Applied Biosystems, Beijing, China) according to the manufacturer's instructions. RT-qPCR was performed using SYBR-Green Master Mix (Takara Bio, Dalian, China) according to the manufacturer's instructions and the following program was used: reverse transcription at 37°C for 15 min, 85°C for 5 sec and 4°C thereafter. The primers were as follows: β -actin forward, 5'-GGAGATTACTGCCCTGGCTCCTA-3' and reverse, 5'-GACTCATCGTACTCCTGCTTGCTG-3'; p53 forward, 5'-TATGCTGAGTATCTGGACGACA-3' and reverse, 5'-CCAGCGTGATGATGGTAAGG-3'; TGF- β 1 forward, 5'-ATGGTGGACCGCAACAAC-3' and reverse, 5'-TGAGCACTGAAGCGAAAGC-3'; and α -SMA forward, 5'-CGGGCATCCACGAAACCACC-3' and reverse, 5'-GAGCCGCCGATCCAGACAGA-3'. The expected sizes of the PCR products were as follows: 150 bp for β -actin, 169 bp for p53, 329 bp for TGF- β 1 and 217 bp for α -SMA. PCR was performed for 40 cycles, consisting of denaturation (95°C, 30 sec), annealing (56, 54, 55 and 56°C, 30 sec) and extension (60°C, 30 sec). Triplicate experiments were performed for each result. The Ct values for each gene amplification were then normalized by subtracting the Ct value calculated for β -actin. Data were reported according to the 2^{- $\Delta\Delta$ Ct} method:

$$\Delta\Delta Ct = \Delta Ct_{\text{sample}} - \Delta Ct_{\text{reference}}$$

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay. The effect of Ad-p53 on HSC-T₆ cell proliferation were determined using an MTT assay. The cells (3×10^3 cells/well) were plated in 96-well culture plates and cultured for 24 h. The cells were then treated with various concentrations of Ad-p53 (1×10^6 , 5×10^6 , 1×10^7 , 2×10^7 and 5×10^7 PFU/ml) for 0, 24, 48 and 72 h. The control group (treated with normal saline only) and each concentration group were set up with six duplicate wells, and the absorbance was measured using a microplate reader (Bio-Rad, Hercules, CA, USA; $\gamma 570$ nm). The inhibition rate (IR) was calculated using the following formula: $IR = [(A \text{ value of control group} - A \text{ value of experimental group}) / A \text{ value of control group}] \times 100\%$.

Cell cycle analysis. To analyze HSC-T₆ cell cycle distribution, DNA content was determined by flow cytometry using propidium iodide (PI) staining. Briefly, the cells were pre-treated with Ad-p53 (5×10^6 , 1×10^7 and 2×10^7 PFU/ml, respectively) for 24 or 48 h, harvested, washed twice with phosphate-buffered saline (PBS) and fixed in 75% ethanol (in 0.01 mol/l PBS) at -20°C overnight. Following centrifugation, the cells were incubated in PBS containing 100 $\mu\text{g/ml}$ DNase-free RNase and stained with 50 $\mu\text{g/ml}$ PI at room temperature in the dark for 30 min. Flow cytometry was performed using a BD LSR II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and analysis was performed using FlowJo Software (Tree Star, Ashland, OR, USA). Dead cells were excluded on the basis of forward angle and 90° light scatter.

Analysis of cell apoptosis. The HSC-T₆ cells were collected following treatment with Ad-p53 (5×10^6 , 1×10^7 , and 2×10^7 PFU/ml, respectively) for 24 or 48 h, and then washed with cold PBS twice, centrifuged (1,000 rpm/min, 5 min) and suspended for 20 min at room temperature without light after being added to 500 μl binding buffer, 5 μl Annexin V-FITC and 5 μl PI. Flow cytometry was performed using a BD LSR II flow cytometer (BD Biosciences) and analysis was performed using FlowJo software (Tree Star). Dead cells were excluded on the basis of forward angle and 90° light scatter.

Statistical analysis. Unless otherwise stated, all experiments were performed with triplicate samples and repeated at least three times. The results are presented as the means \pm SD. Statistical comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by the Student's t-test. A P-value < 0.05 was considered to indicate a statistically significant difference.

Results

Effect of Ad-p53 on the positive expression of TGF- β 1, α -SMA and p53. HSC survival is a hallmark of liver fibrosis (6). The major event in hepatic fibrogenesis is the transdifferentiation of quiescent HSCs to a myofibroblastic cell type (22). This process is driven by a number of compounds, among which TGF- β 1 is the most potent fibrogenic cytokine, and α -SMA is recognised as a typical marker of activated HSCs during this process (23). In order to evaluate the role of Ad-p53 in the regression of hepatic fibrosis, the expression of TGF- β 1 and α -SMA as well as p53 was determined.

We confirmed that a model of hepatic fibrosis was established successfully due to the obvious ECM deposition in the rat liver sections (Fig. 1A). Our data showed that p53 was mainly expressed in the cell nuclei rather than in the cytoplasm. When treated with Ad-p53, p53 expression was more distinct in the nuclei compared with that in the normal saline group, and the particle depositions in the nuclei ranged from buff to dark brown (Fig. 1B). However, TGF- β 1 was preferentially expressed in the cytoplasm. When treated with Ad-p53, the expression of TGF- β 1 was significantly decreased compared with that in the normal saline group, particularly in the regions of hyperplastic fibrous septa and the cytoplasm of non-parenchymal cells in the portal area, HSCs and inflammatory cells (Fig. 1C). In the normal saline group, overexpression of α -SMA was mainly found in the portal areas, fibrous septa, and in areas of inflammatory cell infiltration, which was reversed by treatment with Ad-p53 (Fig. 1D).

The mRNA levels of p53, TGF- β 1 and α -SMA were evaluated by RT-qPCR. Data analysis showed that Ad-p53 upregulated the mRNA expression of p53 and downregulated the mRNA expression of TGF- β 1 and α -SMA, compared with the normal saline group, and the differences were statistically significant ($P < 0.05$) (Fig. 1E). The results were consistent with the immunohistochemistry results described above.

Effect of Ad-p53 on HSC-T₆ proliferation. The HSC-T₆ cell line has been widely used in fibrosis research as it retains all the features of activated HSCs (24). Thus, in the present study we used HSC-T₆ cells to determine the effect of Ad-p53 on HSCs. The results of the MTT colorimetric assay showed that exposure to different doses of Ad-p53 for different time periods had different inhibitory effects on the proliferation of HSC-T₆ cells. The inhibitory effect was gradually enhanced as the Ad-p53 dose increased at the same time-point (Fig. 2). Furthermore, when given the same Ad-p53 dose, the inhibitory effects on the HSC-T₆ cells increased with prolonged exposure time. At a concentration of 1×10^7 PFU/ml, the inhibition rates were $35.42 \pm 2.02\%$ and $64.03 \pm 2.45\%$ after 24 and 48 h, respectively, which suggested that Ad-p53 significantly arrested HSC-T₆ cell proliferation ($P < 0.05$). However, these effects were not observed at a dose of 5×10^7 PFU/ml. These findings demonstrated that the inhibitory action of Ad-p53 occurred in a time- and dose-dependent manner within a certain range of doses and times.

Effect of Ad-p53 on HSC-T₆ cell cycle. Following treatment with increasing concentrations of Ad-p53 for 24 h, the percentage of the HSC-T₆ cells in G1 and G2/M phase decreased significantly ($P < 0.05$), whereas the percentage of cells in S phase increased significantly ($P < 0.05$), which suggested that Ad-p53 blocked the cell cycle in S phase after being cultured with the cells for 24 h. Following treatment with increasing concentrations of Ad-p53 for 48 h, the percentage of the HSC-T₆ cells in S phase and G2/M phase decreased significantly ($P < 0.05$), whereas the percentage of cells in G1 phase increased significantly ($P < 0.05$), which suggested that Ad-p53 induced cell cycle arrest at the G1 checkpoint after being cultured with the cells for > 24 h (Table I and Fig. 3).

Effect of Ad-p53 on HSC-T₆ cell apoptosis. Several studies have focused on the apoptosis of activated HSCs, indicating that apop-

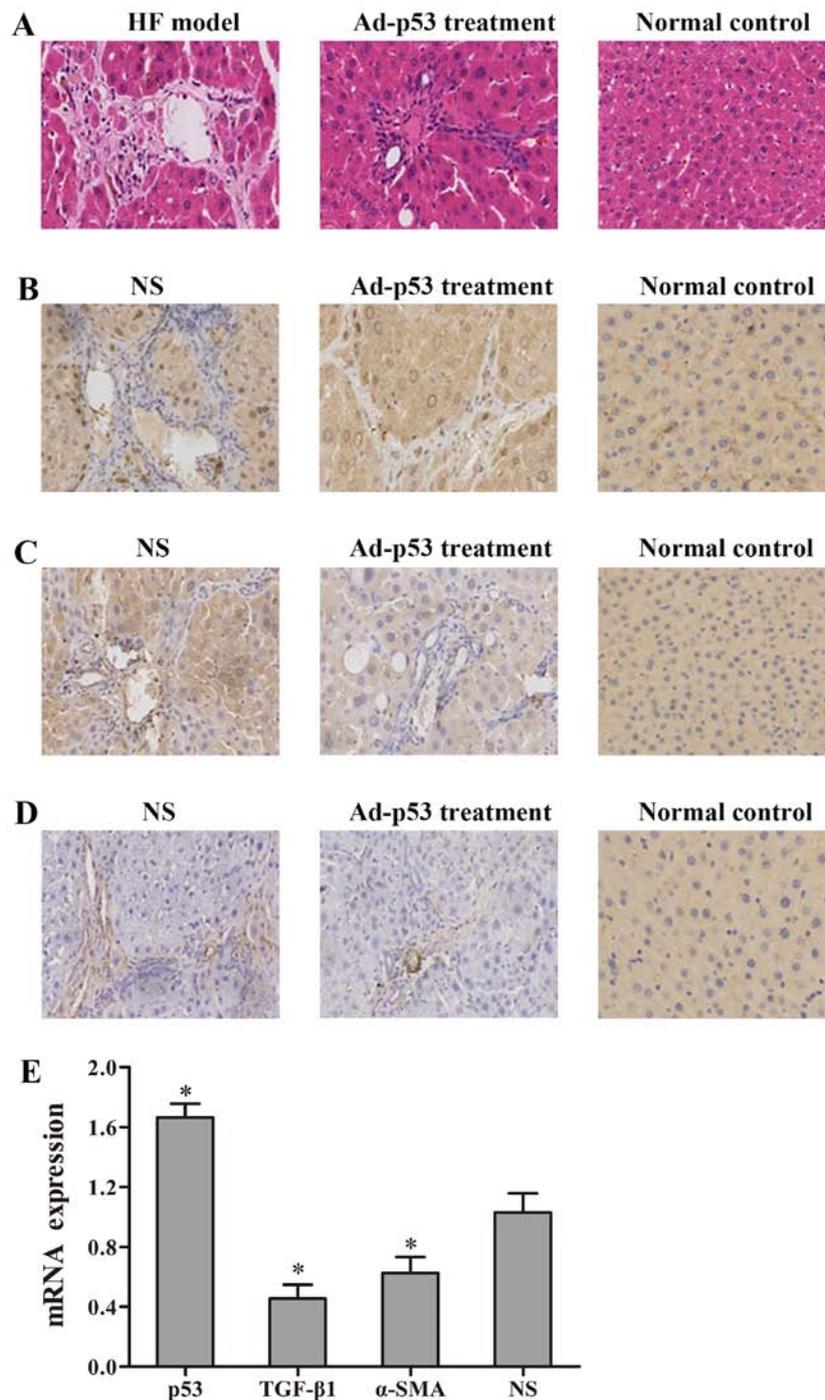


Figure 1. Effect of recombinant human adenovirus-p53 (Ad-p53) on the positive expression of transforming growth factor (TGF-β1), α-smooth muscle actin (α-SMA) and p53 in rat liver tissue. (A) H&E staining of liver tissue from the hepatic fibrosis (HF) model group, Ad-p53 treatment group and normal control group. (B) Immunohistochemical detection of p53. The expression of p53 was upregulated in the normal saline (NS) and Ad-p53 treatment group compared with the control. (C) Immunohistochemical detection of TGF-β1. TGF-β1-positive cells were decreased in the NS and Ad-p53 treatment group compared with the control. (D) Immunohistochemical detection of α-SMA. The decreased expression of α-SMA-positive cells was mainly found in the NS and Ad-p53 treatment group compared with the control. The data indicated that Ad-p53 upregulated the expression of p53 and downregulated the expression of TGF-β1 and α-SMA (magnification, x200). (E) The mRNA levels of p53, TGF-β1 and α-SMA were determined by RT-qPCR in the Ad-p53 group. The control group is referred to as the NS group. The data analysis showed that Ad-p53 significantly upregulated the mRNA expression of p53 ($P < 0.05$) and downregulated the mRNA expression of TGF-β1 and α-SMA ($P < 0.05$). * $P < 0.05$ when compared with the control.

tosis mainly contributes to the reversal of hepatic fibrosis (25,26). Hence, the effect of Ad-p53 on the apoptosis of HSC-T₆ cells was examined in the present study. Apoptosis was determined by FITC-labeled Annexin V/PI double staining and flow cytometric analysis. The dual parametric dot plots were used to calculate the percentage of non-apoptotic viable cells (Annexin V-negative/

PI-negative) in the lower left quadrant, early apoptotic cells (Annexin V-positive/PI-negative) in the lower right quadrant, late apoptotic or necrotic cells (Annexin V-positive/PI-positive) in the upper right quadrant, and mechanically injured cells (Annexin V-negative/PI-positive) in the upper left quadrant (Table II and Fig. 4). In the Ad-p53 treatment groups, at 24 h,

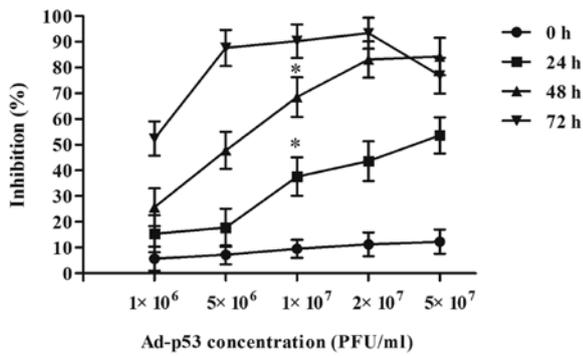


Figure 2. Effect of recombinant human adenovirus-p53 (Ad-p53) on HSC-T₆ proliferation. The results of the MTT colorimetric assay showed that different concentrations of Ad-p53 (1x10⁶, 5x10⁶, 1x10⁷, 2x10⁷ and 5x10⁷ PFU/ml) had different inhibitory effects on HSC-T₆ cell proliferation at 0, 24, 48 and 72 h, which suggested that the inhibitory action of Ad-p53 occurred in a time- and dose-dependent manner within a certain range of doses and times. At 1x10⁷ PFU/ml, Ad-p53 exerted significant inhibitory effects on HSC-T₆ cell proliferation at 24 and 48 h (compared to 0 and 24 h, respectively) (P<0.05). *P<0.05 when compared with the previous time point.

the early apoptotic rate was significantly different (P<0.05) when compared with the control group, whereas the late apoptotic rate and total apoptotic rate were not significantly different; at 48 h, the all three apoptotic rates were significantly different (P<0.05), which also illustrated that Ad-p53 promotes the apoptosis of HSC-T₆ cells in a time- and dose-dependent manner.

Discussion

Recent evidence indicates that the halting of liver fibrogenic processes may allow the reversal of severe fibrosis and even cirrhosis (27). Although the activation of HSCs

stimulates various fibrogenic and inflammatory pathways, they may revert to quiescent HSCs if the causative agents are removed (28). Furthermore, it has been confirmed that inflammation induces liver fibrosis through some specific mechanisms and cytokines. TGF-β1 is considered to be the major fibrogenic growth factor, since in HSCs, it promotes collagen I expression, favors the transition to myofibroblast-like cells, and inhibits ECM degradation through the expression of tissue inhibitors of matrix metalloproteinases (TIMPs) (13,29). Bleser *et al* (30) found that insulin-like growth factor-II/mannose 6-phosphate (IGF-II/M6P) receptor expression in hepatic fat storing cells in a model of CCl₄-induced hepatic fibrosis was indispensable for TGF-β activation, therefore, the TGF-β pathway may be blocked by the arrest of IGF-II/M6P receptor expression in HSCs; *in vivo* experiments have shown that blocking TGF-β binding to HSC membrane receptors or inactivating the receptors of the molecular signaling pathway (Smad3 and Smad7) contributed to the anti-fibrotic effects (31,32). In addition, α-SMA, which is involved in cell motility and contractility, has been identified as the specific marker of activated HSCs (33). The increased expression of α-SMA may enhance cell migration and adhesion, increase proliferation as well as the acquisition of fibrogenic capacity (34,35)

The regulatory function of p53 is associated with cellular growth arrest and apoptosis through DNA damage or cellular stress events (36). Previous findings have demonstrated that Ad-p53 treatment mediates tumor growth arrest and regression in human cancers (37). In the present study, an immunohistochemical assay revealed that in a model of CCl₄-induced hepatic fibrosis, Ad-p53 significantly decreased the positive expression of TGF-β1 and α-SMA, and increased the positive expression of p53 compared with the normal

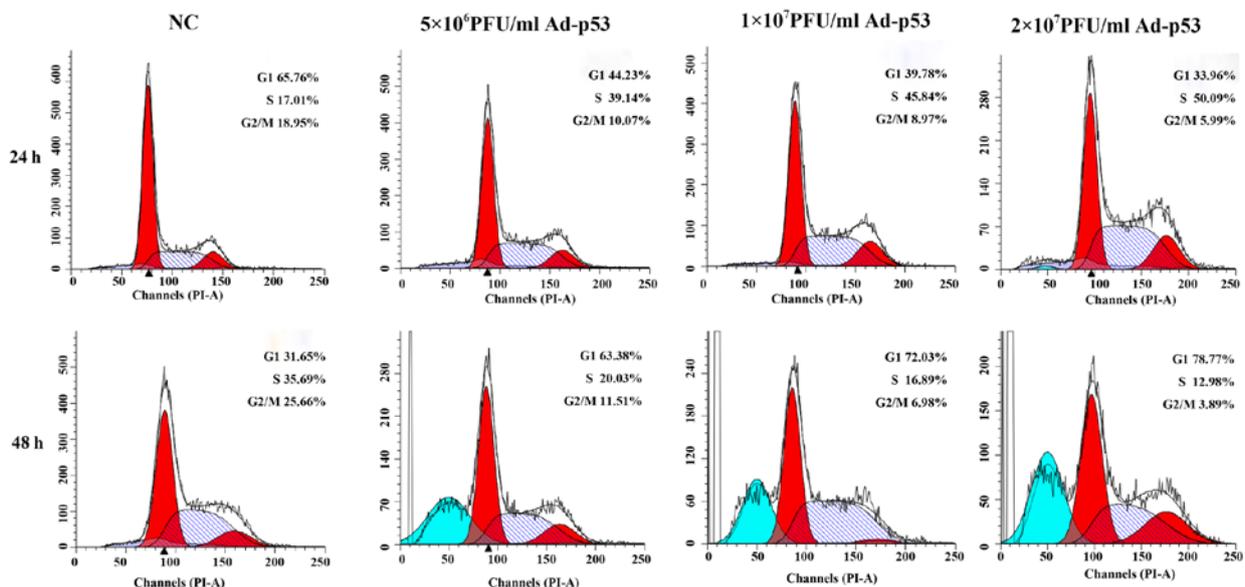


Figure 3. Effect of recombinant human adenovirus-p53 (Ad-p53) on the HSC-T₆ cell cycle. To analyze HSC-T₆ cell cycle distribution, DNA content was determined by flow cytometry using PI staining following cell treatment with Ad-p53 (5x10⁶, 1x10⁷ and 2x10⁷, respectively) for 24 or 48 h. At 24 h, the percentage of HSC-T₆ cells in G1 phase and G2/M phase decreased significantly, whereas the percentage of cells in S phase increased significantly when compared with the control, which indicated that Ad-p53 mainly acted on cells in S phase by arresting DNA replication and protein synthesis; at 48 h, the percentage of HSC-T₆ cells in S phase and G2/M phase decreased significantly, whereas the percentage of cells in G1 phase increased significantly when compared with the control, which indicated that treatment with Ad-p53 for more than 24 h, led to the arrest of most cells at the S checkpoint (which undergo apoptosis) and most remaining cells were in G1 phase. NC, normal control.

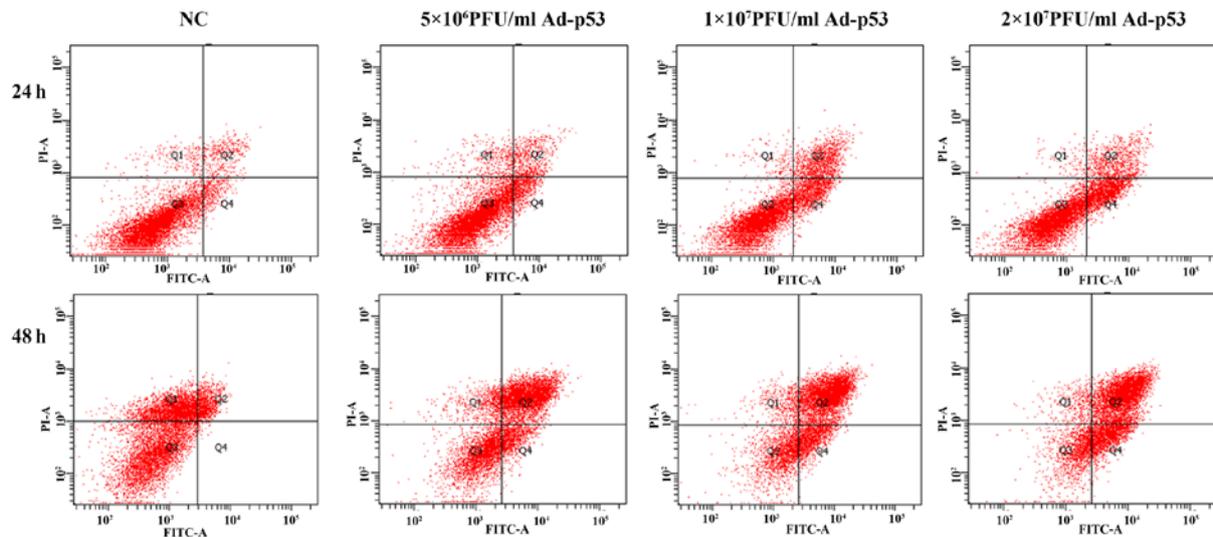


Figure 4. Effect of recombinant human adenovirus-p53 (Ad-p53) on HSC-T₆ cell apoptosis determined by FITC-labeled Annexin V/PI double staining and flow cytometric analysis. The cells were treated with Ad-p53 (5×10^6 , 1×10^7 and 2×10^7 , respectively) for 24 or 48 h. The dual parametric dot plots were used to calculate the percentage of non-apoptotic viable cells in the lower left quadrant; early apoptotic cells in the lower right quadrant; late apoptotic or necrotic cells in the upper right quadrant; and mechanically injured cells in the upper left quadrant. In the Ad-p53 treatment groups, at 24 h, the early apoptotic rate was significantly different ($P < 0.05$) when compared with the control group, whereas the late apoptotic rate and total apoptotic rate were not significantly different; at 48 h, all three rates were significantly different ($P < 0.05$) compared with the control, which illustrated that Ad-p53 promotes the apoptosis of HSC-T₆ cells in a time- and dose-dependent manner. NC, normal control.

Table I. Effect of Ad-p53 on the HSC-T₆ cell cycle (means \pm SD).

Group	Time (h)	G1	S	G2/M
NC	24	65.76 \pm 2.17	17.01 \pm 0.92	18.95 \pm 1.95
5×10^6 PFU/ml		44.23 \pm 1.83 ^a	39.14 \pm 2.41 ^a	10.07 \pm 2.01 ^a
1×10^7 PFU/ml		39.78 \pm 0.87 ^a	45.84 \pm 1.93 ^a	8.97 \pm 0.87 ^a
2×10^7 PFU/ml		33.96 \pm 1.50 ^a	50.09 \pm 1.36 ^a	5.99 \pm 3.166 ^a
NC	48	31.65 \pm 2.02	35.69 \pm 3.22	25.66 \pm 1.26
5×10^6 PFU/ml		63.38 \pm 1.25 ^a	20.03 \pm 0.89 ^a	11.51 \pm 0.78 ^a
1×10^7 PFU/ml		72.03 \pm 1.57 ^a	16.89 \pm 2.01 ^a	6.98 \pm 2.02 ^a
2×10^7 PFU/ml		78.77 \pm 3.14 ^a	12.98 \pm 1.23 ^a	3.89 \pm 1.27 ^a

n=4, ^a $P < 0.05$ was considered significant, when compared with the control. Ad-p53, recombinant human adenovirus-p53; NC, normal control.

saline group. Thus, we hypothesized that Ad-p53 effectively reduces the positive expression of TGF- β 1 and α -SMA during fibrosis in the liver, and the mechanism responsible for these anti-fibrotic effects may be associated with the TGF- β /Smad signaling pathway. In order to detect the transcriptional level of the related genes, RT-qPCR was used to evaluate the mRNA expression of p53, TGF- β 1 and α -SMA. The results showed that Ad-p53 downregulated the mRNA levels of TGF- β 1 and α -SMA, and upregulated the mRNA expression of p53. The mechanism responsible for these effects of Ad-p53 may involve the arrest of HSC activation and proliferation, which leads to ECM reduction as well as the inhibition of cytokines, in order to protect the liver from fibrosis.

Some experts have shown that ovarian cancer cells are inhibited by transfection with Ad-p53 regardless of endogenous p53 status (38). Clayman *et al* also demonstrated the excellent effect of using Ad-p53 gene transfer as a surgical adjuvant

Table II. Effect of Ad-p53 on the apoptosis of HSC-T₆ cells (means \pm SD).

Group	Time (h)	Early apoptosis	Late apoptosis	Total apoptosis
NC	24	3.50 \pm 1.18	3.32 \pm 1.09	6.81 \pm 3.25
5×10^6 PFU/ml		10.89 \pm 12.14 ^a	7.55 \pm 2.13	18.44 \pm 2.17
1×10^7 PFU/ml		12.82 \pm 3.22 ^a	14.10 \pm 1.88	26.92 \pm 5.52
2×10^7 PFU/ml		9.98 \pm 1.41 ^a	20.52 \pm 1.56	30.49 \pm 1.35
NC	48	8.18 \pm 3.43	0.74 \pm 0.29	8.92 \pm 2.26
5×10^6 PFU/ml		42.39 \pm 6.31 ^a	14.43 \pm 2.25 ^a	56.81 \pm 3.15 ^a
1×10^7 PFU/ml		53.74 \pm 4.03 ^a	17.84 \pm 1.73 ^a	71.36 \pm 2.03 ^a
2×10^7 PFU/ml		50.85 \pm 3.15 ^a	21.29 \pm 3.51 ^a	74.63 \pm 5.18 ^a

n=4, ^a $P < 0.05$ was considered significant, when compared with the control. Ad-p53, recombinant human adenovirus-p53; NC, normal control.

in patients with advanced squamous cell carcinoma of the head and neck (39). He *et al* illustrated that Ad-p53 inhibits multidrug resistance gene expression and thus, enhances the chemosensitivity of human breast cancer cells (40). In the present study, the MTT assay was applied to determine the effective dose of Ad-p53 in HSCs. We found that Ad-p53 significantly inhibited the proliferation of activated HSCs *in vitro*, in a dose- and time-dependent manner particularly at a dose of 2×10^7 PFU/ml and after 48 h. Thus, we identified a dose of 2×10^7 PFU/ml and 48 h as the threshold for further experiments.

Ad-p53 has been found to strongly inhibit cell proliferation, to induce apoptosis and to arrest the cell cycle in stage G1 in cells (41,42). Thus, p53 negatively regulates cell growth by monitoring the calibration point of the G1 and G2/M phases (43). Following treatment with Ad-p53 for 24 and 48 h,

respectively, flow cytometric analysis showed that at 24 h, the percentage of HSC-T₆ cells in G1 and G2/M phase decreased, whereas the percentage of cells in S phase increased, which suggested that Ad-p53 mainly acted on the cells in S phase by arresting DNA replication, protein synthesis and the transition from S phase to G2/M phase in order to block the cell cycle in S phase. At 48 h, the percentage of HSC-T₆ cells in S phase and G2/M phase decreased significantly, whereas the percentage of cells in G1 phase increased significantly, indicating that Ad-p53 induced cell cycle arrest at the G1 checkpoint. By treating the cells with Ad-p53 for more than 24 h, the majority of the cells arrested at the S checkpoint underwent apoptosis and most remaining cells were in G1 phase.

Apoptosis is a physiological regulatory mechanism of the inner environment which is involved in the removal of unnecessary or threatened cells and in controlling the number of cells in tissues and organs together with cell division and proliferation (44). Changes in the apoptotic rate were determined by FITC-labeled Annexin V/PI double staining. The results revealed that following treatment with different doses of Ad-p53 for 24 h, the early apoptotic rate was significantly increased with increasing concentrations of Ad-p53, compared with those in the control group ($P < 0.05$). At 48 h, all three rates were significantly different ($P < 0.05$). The results showed that Ad-p53 promotes the apoptosis of HSC-T₆ cells in a time- and dose-dependent manner within a certain range of doses and times. It suggests that the anti-fibrotic effect of Ad-p53 is induced by the apoptosis of HSCs, as apoptosis may activate collagens and eliminate the source of ECM, which has a beneficial impact on liver fibrosis (20,43).

In conclusion, the present study showed that Ad-p53 directly arrests the proliferation of HSCs by modulating the expression of p53, TGF- β 1 and α -SMA. Ad-p53 plays a crucial role in the regression of liver fibrosis by inhibiting cell proliferation, regulating cell cycle progression and promoting the apoptosis of HSCs in order to decrease collagen. The findings of the present study highlight that Ad-p53 may be an effective therapeutic agent for the treatment of hepatic fibrosis.

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