TGF-β1 signaling pathway serves a role in HepG2 cell regulation by affecting the protein expression of PCNA, gankyrin, p115, XIAP and survivin

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Abstract. The transforming growth factor-β (TGF-β) signaling pathway serves a key role in the pathogenesis of liver cancer. To investigate the association between TGF-β1 and the following proteins: Proliferating cell nuclear antigen (PCNA), gankyrin, general vesicular transport factor p115 (p115), X-linked inhibitor of apoptosis protein (XIAP) and survivin, HepG2 liver cancer cells were transfected with small interfering RNA (siRNA) directed against TGF-β1, or were treated with exogenous TGF-β1. TGF-β1 protein expression levels were assessed at 72 and 96 h using western blotting, cell growth was evaluated using a Cell Counting kit-8 assay, and flow cytometry was used to examine cell cycle distribution and apoptosis. In addition, PCNA, gankyrin, p115, XIAP and survivin protein levels were evaluated using western blotting. TGF-β1 protein expression levels were decreased at 72 and 96 h following siRNA transfection, indicating that the siRNA against TGF-β1 was effective. In the TGF-β1-knockdown group, the HepG2 cells exhibited G1 or S-phase cell cycle arrest; therefore, the number of G2-phase cells was decreased, cell growth was inhibited and apoptotic peaks were observed. By contrast, no significant alteration in cell cycle distribution or apoptosis was observed in the cells treated with exogenous TGF-β1. In the exogenous TGF-β1 group, PCNA and XIAP protein expression levels were increased, whereas gankyrin, p115 and survivin protein expression was observed to be dependent on the duration of treatment. By contrast, PCNA, gankyrin, XIAP and survivin protein expression decreased following TGF-β1 knockdown; however, p115 protein expression increased. In conclusion, the TGF-β1 signaling pathway may affect cell growth, cell cycle distribution and apoptosis through the regulation of PCNA, gankyrin, p115, XIAP and survivin protein expression in liver cancer. The results of the present study may improve the current understanding of the role of the TGF-β1 signaling pathway during the pathogenesis of liver cancer.

Introduction

Liver cancer is one of the most common human malignancies. A number of signaling pathways, including the transforming growth factor β (TGF-β)/mothers against decapentaplegic homolog (1), proto-oncogene Wnt/β-catenin (2), rat sarcoma GTPase/mitogen-activated protein kinase (MAPK) (3), phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) (4), c-Jun N-terminal kinase (JNK)/signal transducer and activator of transcription (STAT) (5,6), hedgehog and tumor protein 53 transduction pathways, serve key roles in the pathogenesis of liver cancer. Among these signaling pathways, the TGF-β signaling pathway is one of the most important (1).

TGF-β1 is as a member of the TGF-β family able to produce tumor-inhibiting and promoting effects (7-9). TGF-β1 has been associated with immunosuppression, tumor angiogenesis, tumor cell migration, proliferation, differentiation, development, apoptosis and invasion, as well as other processes, in numerous types of cancer (10,11). For example, TGF-β1 expression is increased in liver cancer (12), intrahepatic cholangiocarcinoma (13), prostate cancer (14,15) and...
in head and neck squamous cell carcinoma (16), and leads to increased tumor growth. Conversely, TGF-β1 expression levels in patients with leukemia are significantly decreased compared with healthy subjects (17).

Cell growth, the cell cycle and apoptosis are closely associated with the genesis and development of liver cancer, and multiple factors are involved in their regulation, including proliferating cell nuclear antigen (PCNA), gankyrin, general vesicular transport factor p115 (p115), X-linked inhibitor of apoptosis protein (XIAP), survivin and caspase-3 (18-21). PCNA is a highly conserved protein; in addition to DNA replication, the functions of PCNA are associated with other vital cellular processes, including chromatin remodeling, DNA repair, sister-chromatid cohesion and cell cycle control (22). Recent studies have reported that tumor cells express increased levels of PCNA, identifying it as a potential target for cancer therapy (23,24). Gankyrin is a chaperone of the ubiquitin-protein, and a novel oncogene, and has been demonstrated to be overexpressed in numerous types of cancer (25-27), including liver cancer (28-30), breast cancer (31,32), colorectal cancer (33), estrogen-driven endometrial carcinoma (26) and oral cancer (34). Gankyrin serves an essential role in tumor occurrence and development (25-34). p115 is a tether protein that has an important role in a number of signaling pathways required for cell proliferation, and has been extensively studied (35,36). A previous study demonstrated that p115 is a potential tumor biomarker and therapeutic target that is overexpressed in human gastric cancer cells (36). The inhibitor of apoptosis protein (IAP) family comprises internal apoptosis suppressors, and its members are able to bind to caspase and inhibit cell apoptosis. XIAP, an important member of the IAP family, possesses inhibitory activity and serves an important role in cell apoptosis (19,20). Survivin, a novel member of the IAP family with the lowest relative molecular weight, is the most potent suppressor of apoptosis that has been identified so far (21). However, there are currently few studies concerning the association between TGF-β1 and PCNA, gankyrin, p115, XIAP and survivin in liver cancer.

Following previous observations, TGF-β1 expression was knocked down in the present study using small interfering RNA (siRNA). Subsequently, cell growth, cell cycle distribution and apoptosis, as well as PCNA, gankyrin, p115, XIAP and survivin protein expression, was observed in HepG2 liver cancer cells. The present study aimed to further elucidate the association between TGF-β1 and the aforementioned factors, and the role of TGF-β1 during the genesis and development of liver cancer.

Materials and methods

Cell culture. HepG2 liver cancer cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and plated in culture flasks. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% v/v fresh fetal calf serum (FCS; TBD Biotechnology Corporation, Tianjin, China), at 37°C in a humidified atmosphere containing 5% CO₂. To maintain the cell line, cells were replated following digestion for 1 min in 0.25% trypsin when they reached confluence.

Transient transfection. siRNA directed against TGF-β1 was designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The TGF-β1 siRNA sequences were as follows: Sense, 5'-GAC ACC AAC UAU UGC UUC ATT-3' and antisense, 5'-UGA AGC AAU AGU UGG UGU CTT-3'. The scrambled negative control siRNA sequences were as follows: Sense 5'-UUC GCC GAU GUC AGC UTT-3' and antisense, 5'-ACG UGA CAC GGU CGG AGA ATT-3'. A total of 3x10⁵ cells were plated in 6-well plates in triplicate and grown to 30-50% confluency. For the transfection, 10 μl X-tremeGENE siRNA Transfection Reagent (Roche Diagnostics, Basel, Switzerland) and 2 μg siRNA were mixed in 200 μl DMEM for 15-20 min. The 210-μl mixture was added to the cells alongside 2 ml DMEM supplemented with 10% v/v FCS, and the plates were subsequently agitated. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂. Cells were harvested at 72 and 96 h, and the proteins were isolated for further analysis. In addition, a negative control was created using the control siRNA and subsequently analyzed. Untreated cells were also analyzed. All experiments were performed in triplicate.

Detection of TGF-β1 protein expression using western blotting. Transfected cells were lysed in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) supplemented with phenylmethylsulfonyl fluoride (1:200) and phosphatase inhibitors (1:200) on ice for 15 min, at 72 and 96 h following transfection, prior to protein isolation. The total protein concentration was determined using a bicinchoninic acid kit (Beyotime Institute of Biotechnology). Protein samples (60 μg) were separated using 12% SDS-PAGE and then transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked by incubation in PBS with 5% skimmed milk at room temperature for 1 h. The membranes were subsequently incubated with primary antibodies against TGF-β1 (1:200; cat. no. sc-146; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and β-actin (1:200; cat. no. sc-47778; Santa Cruz Biotechnology, Inc.) at 4°C overnight. The membranes were washed three times using PBS, followed by incubation with a horseradish peroxidase-conjugated secondary antibody (1:5,000; cat. no. sc-2005; Santa Cruz Biotechnology, Inc.) at 37°C for 1 h. The membranes were washed three times using PBS and visualized using a BeyoECL Plus kit (cat. no. BYT-P0018; Beyotime Institute of Biotechnology). Images were captured using a fluorescence imager (Champgel 5500; Beijing Sage Creation Science and Technology Ltd., Beijing, China) and analyzed using Quantity One® software (version 4.5.2; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The quantitative results of grayscale analysis were used for the statistical analysis.

Detection of cell growth using the Cell Counting Kit-8 (CCK-8) assay. The HepG2 groups analyzed were as follows: i) the control group; ii) the negative siRNA control group; iii) the TGF-β1 siRNA transfected group. The following doses of exogenous TGF-β1 were added to HepG2 cells (the exogenous TGF-β1 group), 0, 5, 10, 20, 30, 40 and 50 μg/l. A total of 1.5x10⁴ transfected cells were plated in 96-well plates in triplicate and grown to 30-50% confluency, prior to
transfection. A total of 0.8 µl X-tremeGENE siRNA Transfection Reagent and 0.15 µg siRNA were mixed in 30 µl DMEM for 15-20 min. The 30 µl mixture, or increasing doses of exogenous TGF-β1, were administered to the cells, in addition to 150 µl DMEM supplemented with 10% v/v FCS, and the plates were subsequently agitated. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO2. A total of 20 µl CCK-8 reagent (Beyotime Institute of Biotechnology) was added to the cell medium 24, 48, 72 and 96 h following transfection or treatment with exogenous TGF-β1. Following a 2-h incubation at 37°C, the absorbance was measured using a microplate reader (MK3; Thermo Labsystems, Inc., Beverly, MA, USA) at 450 nm in order to determine the number of viable cells. The control and negative control groups were also analyzed. All experiments were performed in triplicate. The data were normalized to their respective controls. The cell growth inhibition rates were calculated as follows: (the absorbance ratio of the control group/the absorbance ratio of the transfection group)/the absorbance ratio of the control group x100. Following analysis, the optimum duration and dose of treatment was used in subsequent experiments.

Detection of cell cycle distribution using flow cytometry. The following groups were assessed: i) the control group; ii) the 24 h exogenous TGF-β1 group; iii) the 48 h exogenous TGF-β1 group; iv) the 72 h exogenous TGF-β1 group; v) the 72 h TGF-β1-knockdown group; vi) the 96 h TGF-β1-knockdown group. Cells in the exogenous TGF-β1 groups were treated with 25 µg/l TGF-β1.

At the respective time points the culture medium was removed, and the cells were washed in PBS, trypsinized, harvested, washed in PBS, centrifuged three times at 4°C and 1,000 x g for 5 min, added to 2 ml ice-cold 70% ethanol and preserved at 4°C. The cells were washed three times, and RNases and proteins were removed using a Cell Cycle Detection kit (BD Biosciences, Franklin Lakes, NJ, USA). The cells were subsequently incubated in 10 g/ml propidium iodide (PI) at 4°C for 10 min in the dark and the cell cycle distribution was analyzed using a FACSscan flow cytometer (Sysmex Partec GmbH, Görlitz, Germany) and CyViewTM software version 6.0 (Sysmex Partec GmbH) within 2 h. All experiments were performed in triplicate.

Detection of apoptosis using flow cytometry. The cell groups described in the cell cycle distribution section were used. Cell apoptosis was detected using an Annexin V-fluorescein isothiocyanate (FITC)/PI Apoptosis Detection kit (BD Biosciences). At the respective time points, the cells were collected, centrifuged three times at 4°C and 1,000 x g for 5 min, and resuspended in 500 µl 1X binding buffer. The cell density was adjusted to 1x10⁶ cells/ml. A total of 100 µl cells were incubated with 5 µl Annexin V-FITC and 5 µl PI for 15 min in the dark at room temperature. Cell apoptosis was evaluated using a FACSScan flow cytometer. For each determination, a minimum of 50,000 cells was analyzed. All experiments were performed in triplicate.

Viable cells stained negative for PI and annexin V-FITC, early apoptotic cells stained positive for annexin V-FITC and negative for PI, and late apoptotic cells stained positive for annexin V-FITC and PI. Nonviable cells, which underwent necrosis, stained positive for PI but negative for annexin V-FITC.

Evaluation of PCNA, gankyrin, p115, XIAP and survivin expression using western blotting. The cell groups described in the cell cycle distribution section were used. At the respective time points, total protein was extracted. The protein levels were evaluated using western blotting, following the aforementioned protocol used for the TGF-β1 protein. The following primary antibodies were used at 4°C overnight: Gankyrin (1:500; cat. no. GTX48519; GeneTex, Inc., Irvine, CA, USA), p115 (1:1,000; cat. no. GTX115115; GeneTex, Inc.), PCNA, XIAP (each 1:500; cat. no. BS1289 and BS1609, respectively; Bioworld Technology, Inc., St. Louis Park, MN, USA), and survivin (1:200; cat. no. sc-10811; Santa Cruz Biotechnology, Inc.). The horseradish peroxidase-conjugated secondary antibody was obtained from Santa Cruz Biotechnology, Inc. (1:5,000; cat. no. sc-2004) and incubated with the membrane for 1 h at 37°C.

Statistical analysis. Values are expressed as the mean ± standard deviation of triplicate data, and were compared using the Student's t-test and a one-way analysis of variance. Statistical analyses were conducted using GraphPad Prism (version 6; GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

TGF-β1 protein expression. Following siRNA transfection, the TGF-β1 protein expression levels at 72 and 96 h were evaluated using western blotting, and were observed to be significantly decreased (P=0.0016 and P=0.0055, respectively; Fig. 1). No significant difference was observed in the TGF-β1 protein levels of the negative siRNA control group, as compared with the control group. These results indicate that the siRNA against TGF-β1 was effective.

Effect of TGF-β1 on HepG2 cell growth

Treatment with exogenous TGF-β1 inhibits HepG2 cell growth. The results of the CCK-8 assays are presented in Fig. 2. Treatment with 0-20 µg/l TGF-β1 inhibited HepG2 cell growth in a dose-dependent manner; however, at doses >30 µg/l, the inhibitory effect of treatment with TGF-β1 on HepG2 cell growth was decreased. Therefore, the optimum dose of TGF-β1 to inhibit HepG2 cell growth was between 20 and 30 µg/l (Fig. 2). Therefore, 25 µg/l TGF-β1 was used in subsequent experiments.

The effect of silencing TGF-β1 on HepG2 cell growth. The results of the CCK-8 assays are presented in Fig. 3. No significant difference in cell viability was observed in the control group compared with the negative siRNA control group, whereas a significant decrease in the number of viable cells was observed in the TGF-β1 siRNA-transfected group (P=0.042; Fig. 3). The cell growth inhibition rates induced by the TGF-β1 siRNA were 12.9% at 24 h, 21.0% at 48 h, 34.3% at 72 h and 35.0% at 96 h. These results indicated that HepG2 cell growth was inhibited, or that cell death was increased, between 24 and 96 h following transfection, and that the optimum inhibition times were 72 and 96 h (Fig. 3). These results suggest that
short-term inhibition of cell growth or promotion of cell death occurs following transient siRNA transfection.

Effect of altered TGF-β1 expression on HepG2 cell cycle distribution. The flow cytometric results demonstrated that the percentage of G1-phase cells increased and the percentage of S-phase and G2-phase cells decreased, 24, 48 and 72 h following treatment with exogenous TGF-β1, as compared with the control group (Fig. 4). A total of 72 h following TGF-β1 knockdown, the percentage of G1-phase cells increased, the percentage of S-phase cells exhibited no significant alteration and the percentage of G2-phase cells decreased, as compared with the control group (P=0.0425). A total of 96 h following TGF-β1 knockdown, the percentage of G1-phase cells decreased, the percentage of S-phase cells increased and the percentage of G2-phase cells decreased, as compared with the control group (P=0.0326). In the exogenous TGF-β1 group, cells were arrested in the G1 phase, and the number of cells in the S and G2 phases decreased. In addition, cells in the TGF-β1-knockdown group were also arrested in the G1 phase, and the number of cells in the S phase remained unchanged and decreased in the G2 phase 72 h following transfection. By contrast, 96 h following knockdown, the cells were arrested in the S phase, the number of cells in the G1 phase decreased and the apoptosis peak was visible. The effect of TGF-β1 knockdown on cell cycle distribution was considered to be statistically significant. These results indicate that TGF-β1 knockdown inhibits cell cycle progression, therefore inhibiting cell growth.

Effect of altered TGF-β1 expression on PCNA, gankyrin, p115, XIAP and survivin protein expression. HepG2 cells were treated with exogenous TGF-β1 or TGF-β1 siRNA, and protein expression was evaluated using western blotting. In the exogenous TGF-β1 group, PCNA protein expression was significantly decreased at 24, 48 and 72 h following treatment with exogenous TGF-β1 compared with the control group (P=0.0016, P=0.0051 and P=0.0109, respectively); however, the most significant decrease was observed at 24 h (Fig. 6). Gankyrin expression was significantly decreased at 24 h (P=0.039), significantly increased at 48 h (P=0.0382), but significantly decreased at 48 h (P=0.0289) and 72 h (P=0.0026) compared with the control group (Fig. 6). XIAP protein expression was significantly decreased compared with the control group (P<0.05, vs. the control group). TGF-β1 knockdown decreases HepG2 cell growth. *P<0.05, vs. the control group. TGF-β1, transforming growth factor-β-1; siRNA, small interfering RNA.
Gankyrin expression was significantly increased at 96 h compared with the control group (P = 0.0292; Fig. 6). PCNA, XIAP and survivin protein expression increased following TGF-β1 knockdown in a time-dependent manner compared with 72 h and 96 h following transfection, whereas gankyrin exhibited the opposite pattern (Figs. 6 and 7).
beta-actin

Figure 6. Protein expression levels of PCNA, gankyrin, p115 and beta-actin following treatment with exogenous TGF-beta1 or TGF-beta1 siRNA in HepG2 cells. (A) Representative protein bands from the western blotting. (B) Protein band quantification. 1, control group; 2, 24 h exogenous TGF-beta1 group; 3, 48 h exogenous TGF-beta1 group; 4, 72 h exogenous TGF-beta1 group; 5, 72 h TGF-beta1-knockdown group; 6, 96 h TGF-beta1-knockdown group. *P<0.05 vs. the control group. PCNA, proliferating cell nuclear antigen; p115, general vesicular transport factor p115; TGF-beta, transforming growth factor-beta-1; siRNA, small interfering RNA.

Figure 7. Protein levels of XIAP, survivin and beta-actin following treatment with exogenous TGF-beta1 or TGF-beta1 siRNA in HepG2 cells. (A) Representative protein bands from the western blotting. (B) Protein band quantification. 1, control group; 2, 24 h exogenous TGF-beta1 group; 3, 48 h exogenous TGF-beta1 group; 4, 72 h exogenous TGF-beta1 group; 5, 72 h TGF-beta1-knockdown group; 6, 96 h TGF-beta1-knockdown group. *P<0.05 vs. the control group. XIAP, X-linked inhibitor of apoptosis protein; TGF-beta, transforming growth factor-beta-1; siRNA, small interfering RNA.

Discussion

RNA interference has been successfully used to study gene function, and has assisted in determining associations between upstream and downstream factors in various signaling pathways (37). In the present study, TGF-beta1 protein expression was observed to be decreased 72 and 96 h following siRNA transfection, indicating that the siRNA against TGF-beta1 was effective.

In the present study, treatment with exogenous TGF-beta1 inhibited HepG2 cell growth; the degree of inhibition following treatment with concentrations between 20 and 30 micro g/l was the most significant. The effect of treatment with exogenous TGF-beta1 on cell growth may be due to TGF-beta1 having a role as a tumor suppressor in the early stage of tumor development, and is involved in two-way regulation during the genesis and development of liver cancer (7-9). In addition, an siRNA directed against TGF-beta1 inhibited HepG2 cell growth, potentially as TGF-beta1 is overexpressed in liver cancer (12). However, it remains unclear why treatment with exogenous TGF-beta1 and siRNA against TGF-beta1 inhibited HepG2 cell growth, and the underlying molecular mechanisms require further study.

Previously, TGF-beta1 has been revealed to induce G1-phase cell cycle arrest or prolong the time of the G1-S phase transition in mesothelioma and breast cancer (38), which is consistent with the results of the present study. In the exogenous TGF-beta1 group, cells were arrested in the G1 phase, and the percentage of cells in the S and G2 phases decreased. The TGF-beta1-knockdown cells were also arrested in the G1 and S phases 72 and 96 h following transfection, respectively. These results are consistent with the results of cell growth.

In the present study, treatment with exogenous TGF-beta1 or siRNA against TGF-beta1 inhibited HepG2 cell growth, cell cycle progression and apoptosis; however, the effect of TGF-beta1 knockdown was more significant. This is potentially because exogenous TGF-beta1 has inconsistent effects on the expression of related factors, including PCNA, gankyrin, p115, XIAP and survivin. As the effect was more significant in the TGF-beta1-knockdown group, the changes due to TGF-beta1 knockdown will be discussed. The effect of treatment with exogenous TGF-beta1 requires further study.

PCNA serves an important role in the priming of cell proliferation and is therefore an indicator of cell proliferation. (18) For example, antisense TGF-beta1 oligonucleotides may lead to significantly decreased expression levels of PCNA and inhibit cell growth in oral squamous cell carcinoma (39). Gankyrin, a novel oncogene, regulates the cell cycle and apoptosis (28). LBH589 inhibits the proliferation and metastasis of hepatocellular carcinoma through the inhibition of the gankyrin/STAT3/Akt signaling pathway (28). However, there are few studies on gankyrin, and the association between TGF-beta1 and gankyrin remains unclear. In the present study, treatment with exogenous TGF-beta1 resulted in decreased protein expression levels of PCNA and gankyrin compared with the control group; therefore, treatment with exogenous TGF-beta1 may inhibit cell growth and enhance apoptosis. p115 is a potential tumor biomarker and therapeutic target in human gastric cancer (36). However, in the present study, TGF-beta1 knockdown resulted in increased p115 protein expression levels as compared with the control group. The underlying molecular mechanism remains to be completely elucidated.

XIAP and survivin are considered to be IAPs and their decreased expression causes caspase-3 to become
phosphorylated, therefore increasing cellular apoptosis. TGF-β may upregulate certain anti-apoptotic genes, including B-cell lymphoma-2 like 2 and XIAP (10), and XIAP knockdown abolishes the TGF-β-induced proliferation of malignant meningioma cells (11). TGF-β signaling pathway antagonists similarly activate the survivin promoter, rendering cells refractory to further promoter activation by insulin-like growth factor-I (40). Similarly, in the present study, TGF-β1 knockdown resulted in decreased XIAP and survivin protein expression levels as compared with the control group, therefore enhancing cellular apoptosis.

In conclusion, the TGF-β signaling pathway affects cell growth, the cell cycle and apoptosis by regulating the protein expression of PCNA, gankyrin, p115, XIAP and survivin. The results of the present study may improve current understanding of liver cancerogenesis and the respective role of the TGF-β signaling pathway. By understanding these processes in detail, it may be possible to treat tumors by modulating TGF-β signal transduction cascades within cells, and to specifically control cell growth, differentiation and apoptosis.

References