HSG provides antitumor efficacy on hepatocellular carcinoma both in vitro and in vivo

WEILIN WANG, FENG ZHU, SHUQIAN WANG, JIANFENG WEI, CHANGKU JIA, YUANBIAO ZHANG, LIN ZHOU, HAIYANG XIE and SHUSEN ZHENG

Key Laboratory of Combined Multi-organ Transplantation, Ministry of Public Health, Key Laboratory of Organ Transplantation, Department of Hepatobiliary and Pancreatic Surgery, First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang 310003, P.R. China

Received February 23, 2010; Accepted March 31, 2010

DOI: 10.3892/or_00000844

Abstract. Hyperplasia suppressor gene (HSG) is a novel gene that markedly suppresses the mitogenetic stimuli or injury mediated by vascular smooth muscle cell proliferation. Herein we provide experimental evidence to show that HSG can also play a key role in tumor proliferation. Down-regulation of HSG protein in hepatocellular carcinoma tissues compared to adjacent tissues. Overexpression of HSG suppressed the growth of liver cancer cell lines, resulted in cell cycle arrest in the G0/G1 phase, increased expression of the cyclin dependent kinase inhibitors (CKIs), and reduced expression of proliferating cell nuclear antigen (PCNA). It also showed that adenovirus-mediated HSG overexpression induced apoptosis. Up-regulation of HSG by adenovirus also significantly suppressed the growth of subcutaneous tumors in nude mice both ex vivo and in vivo. Collectively, our data suggest that HSG is a potential therapy for tumors and possibly other proliferative diseases as well and it has antitumor efficacy on hepatocellular carcinoma by using adenovirus vectors, which may be a new therapeutic target for liver cancer prevention.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide. Eighty-two percent of cases (and death) are in developing countries, especially in sub-Saharan Africa and southeastern Asia and the death rate is the second highest among males in China (1,2). Although there are many advances in HCC therapy, such as recent target therapies, and liver transplantation, the overall patient outcome has not been substantially improved. The five-year survival rate is limited to 25-39% after surgery and much lower elsewhere (3-5).

Materials and methods

Tissue samples, cell lines and culture conditions. Twelve pair samples were obtained from HCC and adjacent benign liver tissues (>2 cm from tumor margin) of patients who underwent surgical treatment for HCC in the First Affiliated Hospital, College of Medicine, Zhejiang University. HepG2 and Hep3B liver cancer cell lines, were preserved in our institute. HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Biosciences). Hep3B cells were cultured in minimum essential medium (Gibco) supplemented with 10% fetal bovine serum (Biosciences). All cells were cultured at 37°C in a humidified chamber with 5% CO2.

Virus construction and adenovirus infection. Replication-defective adenoviruses encoding the complete HSG open reading frame (AdHSG) and an adenoviral vector encoding green fluorescent protein (AdGFP) which was made as a
control were constructed by Vector Gene Technology Company Ltd. (Beijing, China). Cells synchronization was achieved by culturing cells in DMEM or MEM with 0.2% FBS for 24 h. Cells were incubated with adenovirus at a multiplicity of infection (MOI) of 100 pfu per cell in a small volume of serum-free medium. After adsorption for 4 h, DMEM with adenovirus was discarded and fresh complete growth medium was added and cells were cultured for additional time for the following experiments. Infection was efficient showing almost 90% GFP-positive cells only 24 h after infection.

**Reverse transcription-PCR analysis.** Detection of HSG mRNA expression was carried out by reverse transcription-PCR as described previously (10). The sequence primers were: 5'-GGAGCTGGACAGCTGGATTGAT-3' (forward), and 5'-AGCTCCAGCTGTTGTCATGA-3' (reverse) for HSG (301 bp); 5'-CTTAGTTGCGTTACACCCTTTC-3' (forward), and 5'-CACCTTCACCGTTCCAGTTT-3' (reverse) for β-actin (151 bp). The PCR reaction for HSG amplification was carried out with 1 cycle of 94˚C for 10 min and 30 cycles of 94˚C for 1 min, 58˚C for 10 sec, 72˚C for 10 sec, followed by a 1 cycle extension at 72˚C for 10 min. The PCR reaction for β-actin amplification was carried out with 1 cycle of 94˚C for 10 min and 30 cycles of 94˚C for 1 min, 55˚C for 10 sec, 72˚C for 15 sec, followed by a 1 cycle extension at 72˚C for 10 min.

**Western blot analysis.** The expression level of HSG protein and PCNA, p27, p21, cleaved caspase-3 and cleaved PARP were examined by Western blotting. Cells were infected and then fresh complete growth medium was added for an additional 48 h. Tissues were homogenized in nitrogen liquid. The total protein was isolated from cells and tissues using a lysis buffer (Cell Signaling) and subjected to Western blotting as described previously (10). The primary antibody were: against HSG (Sigma, at 1:1000 dilution); PCNA (Santa Cruz, at 1:500 dilution); p27 (Santa Cruz, at 1:250 dilution); p21 (Santa Cruz, at 1:250 dilution); cleaved caspase-3 (Cell Signaling, at 1:1000 dilution); cleaved PARP (Cell Signaling, at 1:1000 dilution) and β-actin (Sigma, at 1:1000 dilution).

**Cell viability assay.** Cell viability assay was achieved by using Cell Counting kit-8 (Dojindo Laboratories). Liver cancer cells were plated on 96-well plates at a density of 6x10^3 per well. When cells were grown to subconfluence, they were infected with AdGFP or AdHSG at a MOI of 100 pfu per cell, with the uninfected cells as control. We added 10 μl of the Cell Counting kit-8 solution with 90 μl growth medium into each well after 48 h. Two hours later, we read the absorbance at 450 nm to determine the cell viability in each well. Cell viability was calculated as follows:

\[
\text{Cell viability} = \frac{A_{450 \text{ nm mean value of infected cells}}}{A_{450 \text{ nm mean value of uninfected cells}}} \times 100\%
\]

**Flow cytometry analysis for cell cycle distribution.** HepG2 cells were synchronized and infected. After that, cells were kept in low serum DMEM (0.2% FBS) for 24 h and then stimulated by 10% FBS for 24 h. Then cells were collected and flow cytometric analysis (Beckman Coulter) was used to examine cell cycle distribution as described previously (10).

**Ex vivo and in vivo treatments with adenoviruses.** Male BALB/c nude mice at 4-5 weeks obtained from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China) were
used in all of the experiments and kept in a dedicated animal facility with 6 mice per cage. The experiments were performed according to the institutional ethics guidelines. For the *ex vivo* experiment, mice were divided randomly into three groups with 6 mice each. HepG2 cells, AdGFP-infected HepG2 cells and AdHSG-infected HepG2 cells were injected subcutaneously into the armpit of mice in a total volume of 100 μl (2x10^6 cells in PBS). For the *in vivo* experiment, HepG2 cells were injected subcutaneously into the armpit of mice (2x10^6 cells in PBS/100 μl). When majority of the tumor volume reached 100 mm³ in size, mice were randomly allocated into three groups (6 mice each). These mice were treated with multiple-point intratumor injection 100 μl of AdHSG or AdGFP at 2x10^8 pfu per animal or PBS as a control every other day for 4 times. After cell inoculation, tumor volumes for each mouse were monitored with a caliper every 5 days by measuring in two directions (length and width). The volume was calculated as length x (width)^2/2. Then mice were sacrificed. Tumors were removed and weighed. HSG expression in tumors of different groups was analyzed by Western blotting as described above.

Statistical analysis. Data are displayed as mean ± SD. Densitometry analysis of protein and mRNA levels was achieved by using Image-Pro Plus 5.0.2 software (Media Cybernetics, USA). The statistical significance of difference between the three groups was analyzed by the Student-Newman-Keuls test for multiple comparisons. Also, all the statistical analysis was performed by the SPSS 16.0 program for Windows (SPSS, Chicago, IL, USA). p<0.05 was considered as statistically significant.

Results

**HSG expression is decreased in liver tumor tissue.** Compared to its adjacent normal tissues, the expression of HSG was decreased in hepatocellular carcinoma samples. The density value ratio of cancer and corresponding normatic tissue (β-actin expression as a loading control) was evaluated in all patients, as shown in Fig. 1A and B. The density value of tumor was significantly lower than corresponding normal tissue (p<0.01, compared with uninfected or AdGFP). (C) HepG2 cells were infected with AdGFP or AdHSG at a MOI of 100. After incubation for 48 h, the expression of HSG, PCNA, p27 and p21 protein was analyzed by Western blotting. β-actin was used as the protein loading control.

**Increased expression of HSG mediated by adenovirus vector in liver cancer cell lines and its effect on cancer cell proliferation.** Fig. 1 shows that down-regulated expression or loss-of-function of HSG in HCC might play a role in the development of HCC. We hypothesized that overexpression of HSG mediated by adenovirus may inhibit the growth of HCC. The cells infected with AdHSG expressed much decreased HSG protein than those infected with AdGFP (Fig. 2A), which indicated that AdHSG infection resulted in...
an efficient overexpression of HSG. In order to examine the cytotoxicity induced by overexpression of HSG, HepG2 and Hep3B cells were infected with AdHSG or AdGFP and cell viability was analyzed at 48 h after infection. As shown in Fig. 2B, AdHSG infection caused a significant inhibition on proliferation of liver cancer cell lines compared with AdGFP infection (p<0.05).

The role of HSG in regulating the cell cycle. By using flow cytometry, the alteration of cell cycle distribution in response to increased expression of HSG was observed (Fig. 3A). After 10% FBS stimulation for 24 h, a significant increase of G0/G1 arrest in AdHSG infected HepG2 cells was found, compared with the uninfected and AdGFP groups. Overexpression of HSG also caused alterations in key components of the cell-proliferation regulatory machinery. An increased expression of the cell proliferation inhibitors p27 and p21 and a significant reduction of PCNA were observed in the AdHSG group (Fig. 3C).

Overexpression of HSG triggers apoptosis in liver cancer cells. To explore the potential role of HSG in regulating liver cancer cell apoptosis, HepG2 and Hep3B cells were infected with AdHSG and AdGFP. After 48 h, AdHSG induced liver cancer cell apoptosis, as manifested by activation of caspase-3 (Fig. 4) by Western blotting. Also PARP, as a representative substrate of caspase-3, was cleaved in AdHSG infected cells (Fig. 4).

AdHSG mediates inhibition of tumorigenicity ex vivo and in vivo. We next sought to determine whether exogenous HSG affected tumorigenicity both ex vivo and in vivo. Cells were treated with PBS or with 100 MOI of AdGFP and AdHSG and injected into nude mice 12 h later. AdHSG-treated tumor cells suppressed tumor growth ex vivo, whereas AdGFP-treated and PBS-treated cells resulted in tumor growth (p<0.001; Fig. 5A). At the end of our experiments, the average weight of tumors in the AdHSG-treated group was significantly lighter compared with AdGFP-treated group or in the PBS-treated group (p<0.001, Fig. 5B), and there was no difference in the AdGFP-treated group and PBS-treated group (p>0.05). It indicated that HSG protein may have therapeutic efficacy. The HSG expression of the three groups is showed in Fig. 5C. The therapeutic potential of AdHSG was further examined using an in vivo tumor model. PBS, AdGFP, or AdHSG was injected into HepG2 tumors. Also intratumoral injection of AdHSG resulted in significant tumor growth delay compared with injection of AdGFP or PBS (p<0.001, Fig. 6A), whereas the AdGFP treated group has a similar tumor volume to the...
PBS treated group (p>0.05). The tumor weight of the three groups is shown in Fig. 6B. The tumor image and HSG expression of the three groups are shown in Fig. 6C and D.

Discussion

Liver cancer is the third most common cause of death and China alone accounts for 55% of all liver cancer cases worldwide (1). Novel treatments for liver cancers are urgently needed. In recent years gene therapy has shown great potential for the treatment of liver cancer (8,9). HSG is a novel hyperplasia suppressor gene. Overexpression of HSG markedly prevented cell proliferation through the Ras-Raf-MEK-ERK1/2 signaling cascade and resulted in cell cycle arrest in the G0/G1 phases. It also prevented balloon injury induced re-stenosis and reduced atherosclerosis (10). HSG was also found to be involved in the mitochondrial fusion reaction. Furthermore, it was found that the HSG has apoptotic effect and this effect is mediated by the mitochondria apoptosis pathway. The level of mitochondria anti-apoptotic protein Bcl-2 was decreased, while Bax mitochondrial accumulation was increased by overexpression of HSG, which resulted in mitochondrial cytochrome c releasing and activation of caspase-9 and caspase-3 (12-14). The anti-proliferative effect of HSG may be more effective than p53, a well known tumor suppressor (10,11). However, whether the HSG contributes to cancer development remains unclear. Moreover, its potential effect for cancer therapy is completely unknown. In this study, we first confirmed the antitumor activity of hyperplasia suppressor gene (HSG) in hepatocellular carcinoma.

We examined the mRNA and protein expression of HSG in hepatocellular carcinoma samples and corresponding normal tissues. Compared with paired normal tissues, the decreased expression of HSG was found in hepatocellular cancer samples. HSG is located in human chromosome 1p36.3. Many types of human malignancies, from solid tumors to leukemias and myeloproliferative disorders, have shown nonrandom abnormalities on chromosome 1p36 (16,17). In the past few decades, many tumor suppressor genes have been found in this region (18,19), showing that down-regulated expression or loss-of-function of HSG in HCC might have a role in the development of HCC.

Consequently, we hypothesized that overexpression of HSG mediated by adenovirus may inhibit tumorigenic growth of HCC. Our results show that AdHSG can up-regulate expression effectively in liver cancer cells, indicating that the adenovirus-based HSG may be an effective strategy for cancer therapy. Additionally, the prevention of proliferation, the promotion of apoptosis in liver cancer cells and the tumor suppression effect in nude mice support the efficiency of the treatment. Also HSG mediates inhibition of tumorigenicity both ex vivo and in vivo, which shows the antitumor activity of AdHSG is independent of the state of endogenous HSG, making it even more effective than P53.

To date, the mechanism of the hepatogenesis is not very clear, but it has been shown that disruption of the G1/S and G2/M check points leads to uncontrolled cell growth, resulting
in the development and progression of cancers. Many studies have reported that genetic and epigenetic alterations of cell cycle regulators in hepatocellular carcinoma are involved in the carcinogenesis of many types of human cancer. In hepatocellular carcinoma, cyclin dependent kinase inhibitors (CKIs) have an important role in the early stage of carcinogenesis and in disease progression (20,21).

The outcome of CKI induction in most cells is the cessation of cell proliferation, differentiation or even cell death. In tumor cells, the regulation of the CKIs is altered leading to either lack of function or expression. Hence, if the CKIs, such as p27 and p21, could be induced consistently in tumor cells and their induction leads to G1 arrest, the goal of controlling the proliferation of cancer cells could be achieved. Particularly p27, reduced p27 expression relates with poor prognosis in most tumor types, making it a novel and powerful prognostic marker (22). High p27 expression, foreshowing long survival, is a favorable independent prognostic index for HCC (23,24).

In this study, after HepG2 cells were treated with AdHSG for 48 h, a significant decrease of S-phase population in HepG2 cells was observed, compared with the uninfected group and AdGFP group. The effect of HSG on cell cycle arrest was also proved by HSG-mediated alterations in key components of the cell cycle regulatory machinery. An increased expression of the CKIs p21 and p27 was caused by overexpression of HSG. These results showed the great antitumor efficacy of HSG on hepatocellular carcinoma. On the other hand, we also found overexpression of HSG caused a significant reduction of proliferating cell nuclear antigen (PCNA), which plays a key role in DNA repair, cell proliferation and cell cycle control (25). These results validated our hypothesis that overexpression of HSG mediated by adenovirus may inhibit tumorigenic growth in HCC.

In conclusion, we provide experimental evidence to confirm HSG as a novel tumor suppressor gene at least in heaptocellular carcinoma. Overexpression of HSG mediated by adenovirus may inhibit tumorigenic growth in HCC. HSG provides antitumor efficacy on hepatocellular carcinoma.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (No. 30671991), Natural Sciences Foundation of Zhejiang Province (No. 03020505), Scientific Research Foundation of Ministry of Public Health (No. WKJ2006-2-005), National S&T Major Project (No. 2008ZX10002-026) and Program for Changjiang Scholars and Innovative Research Team in University (No. IRT0753).

References