In vivo treatment of HCV core-positive HepG2 cells with the transfer of recombinant caspase-3 using a 2'-5' OAS promoter

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Abstract. Hepatitis C virus (HCV) is one of the most common pathogens causing liver-related morbidity and mortality, which affect 170 million individuals worldwide. There is no vaccine available, and current therapy is only partially effective. In a previous study, we constructed a recombinant caspase-3 expression vector under the 2'-5'-oligoadenylate synthetase gene (OAS) promoter (pGL3-OAS-re-caspase-3) and demonstrated that it is an effective gene therapy for HCV core-positive liver cells *in vitro*. In the present study, the human hepatoma cell line HepG2 was transfected with the pcDNA3.1-HCV-core-EGFP plasmid and selected by G418. Expression of HCV core protein was confirmed by RT-PCR and immunocytochemistry. Both HepG2-expressing HCV core protein and parental HepG2 cells were inoculated subcutaneously into BALB/c mice, respectively. Tumor-bearing mice were treated with an intratumoral injection of pGL3-OAS-re-caspase-3. The mice were sacrificed after 48 h. The correlation between HCV core and caspase-3 expression in tumor tissues was analyzed by immunohistochemical staining and double-label immunofluorescence staining. The subcutaneous hepatoma *in vivo* mouse models stably expressing HCV core protein and co-expressing HCV core protein and pGL3-OAS-re-caspase-3 were established. Double-label immunofluorescence staining showed that the percentage of co-expression of both HCV core and caspase-3 was 76±6% in the group treated with pGL3-OAS-re-caspase-3. There was a significant increase in the number of apoptotic cells in the group treated with the pGL3-OAS-re-caspase-3 system by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling and transmission electron microscopy. The results suggest that the pGL3-OAS-re-caspase-3 construct can effectively induce apoptosis in HCV core-positive hepatocytes *in vivo*. The results presented strongly suggest that the transfer of pGL3-OAS-re-caspase-3 is an effective and promising gene therapy strategy for HCV infection.

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Introduction

Hepatitis C virus (HCV), which affects 170 million people worldwide, is one of the most common pathogens causing liver-related morbidity and mortality (1). Traditional therapy for chronic HCV infection consists of treatment with interferon-α (IFN-α) and pegylated interferon-α (PEG-IFNα) alone or in combination with ribavirin. However, a significant fraction of patients either fail to respond or relapse after cessation of therapy (2). Thus, there is an urgent need to identify and develop highly specific and potent HCV inhibitors.

It is generally believed that HCV infects only a small fraction of hepatocytes, 1-20% as judged by the detection of HCV proteins or HCV RNAs in liver biopsy samples (3,4). Therefore, specific induction of hepatic apoptosis may be a promising approach for treatment of chronic hepatitis C. Apoptosis is the process of programmed cell death that is believed to permit removal of cells from an organ without provoking inflammation. Activation of effector caspases is a central or ultimate step in many apoptosis pathways. Caspase-3 is the key executioner caspase, and it exists as an inactive zymogen that is activated by upstream signals (5). Several research groups have tried to use the human caspase-3 gene as a novel anticancer gene (6-9). However, overexpression of the wild-type caspase-3 gene in mammalian cells does not induce apoptosis, which is due to their inability to undergo autocatalytic processing without upstream caspase for activation (10). Constitutively, an active recombinant caspase-3 (re-caspase-3) has been generated by making its small subunit precede its large subunit (10). Unlike its wild-type counterpart, in which the large subunit precedes the small subunit, the re-caspase-3 is capable of autocatalytic processing and inducing apoptosis independent of the upstream initiator caspase molecules. In addition, it may be able to resist the effect of various apoptosis-restraining genes. As caspase-3 is the most effective downstream executioner of apoptosis, the re-caspase-3 could be used at a very low concentration to induce apoptosis in targeted cells. To restrict the induction of apoptosis to HCV-infected cells and to increase the safety

of this approach, establishment of an HCV-specific caspase expression system is required. Naganuma *et al* (11) found that the HCV core protein specifically activated the interferon (IFN)-inducible 2',5'-oligoadenylate synthetase (OAS) gene promoter in human hepatocytes, whereas the E1, E2, and NS5A proteins did not activate the OAS gene promoter. Moreover, the activation by the core protein is a general phenomenon, regardless of HCV genotype and strain. We also reported that the HCV core protein can specifically activate the OAS gene promoter in human hepatocytes (12). Therefore, using the OAS gene promoter that is predominantly active in HCV core-positive hepatocytes is an ideal strategy to restrict cytotoxic caspase expression.

In a previous study, we constructed the re-caspase-3 expression vector under the OAS promoter (pGL3-OAS-caspase-3) and demonstrated that it is an effective gene therapy for HCV core-positive liver cells *in vitro* (13). In the present study we explored the further possibility that pGL3-OAS-caspase-3 may effectively induce apoptosis specifically in HCV core-positive hepatocytes *in vivo*.

Materials and methods

Cell lines, transfection and establishment of stable transformants. HepG2, a human liver carcinoma cell line, was maintained in DMEM medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. The vector pcDNA3.1-HCV-core, which contains the complete coding region of HCV-core protein (1b genotype) under the control of the cytomegalovirus (CMV) immediate early promoter, was kindly provided by Professor Jun Cheng (Institute for Epidemic Disease Research, Beijing Ditan Hospital, China). The pcDNA3.1-HCV-core was transferred into HepG2 cells by electroporation using a Scientz-2C Gene Pulser (XinZhi, NingBo, China) at 250 V and a capacitance of 500 μF in a 0.4-cm electroporation cuvette. At the same time, the vector pcDNA3.1 was transferred as a control. Transfectants were selected by incubation in the presence of 800 μg/ml G418 (Merker, Germany). The HCV-core gene-transferred HepG2 cells were then cloned and identified. The resulting cell line was called HepG2/core and the control cell line was called HepG2/pcDNA3.1.

Reverse transcriptase-polymerase chain reaction (RT-PCR). Total-RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. cDNA was synthesized from 2 μg RNA using M-MLV reverse transcriptase (GeneCopoeia, Rockville, MD, USA). Subsequently, 2 μl cDNA was used as the template for the PCR analysis. The Taq PCR Mastermix kit (2X) (Tiangen, Beijing, China) was used according to the manufacturer's instructions. The HCV-core primers (Biosune Biotechnologies, Shanghai, China) were forward, 5'-atgagcaccaatcctaaac-3' and reverse, 5'-ggctgaagcgggcaca-3'. The β-actin primers (Biosune Biotechnologies) were forward, 5'-ccttcctgggcatggagtcct-3' and reverse, 5'-ggagcaatgatcttgatctt-3'. The protocol was 95˚C for 5 min, and 36 cycles of 96˚C for 1 min, 52˚C for 1 min and 72˚ for 1 min. After completion of the cycles, one 10-min extension at 72˚C was carried out. The HepG2/pcDNA3.1 and HepG2 cell lines were used as the negative and blank control, respectively. RT-PCR was performed using β-actin as an internal control.

Immunocytochemistry. We plated cells in 2 ml of medium in 6-well tissue culture plastic dishes at a concentration of 10^5 cells/ml. After 24 h, the cultured cells were rinsed with D-Hanks' solution and were fixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 20 min at room temperature. After immersion in PBS containing 3% H_2O_2 for 30 min at room temperature, followed by a 30-min incubation in normal goat serum at 37˚C, cells were incubated in PBS with monoclonal antibody against HCV-core (dilution of 1:100; Millipore, Boston, MA, USA) overnight at 4˚C. After washing with PBS, cells were incubated in poly-peroxidase-anti-mouse IgG for 30 min at 37˚C. As a negative control, the primary antibody was replaced with PBS. Cells showing light brown or yellow brown grains in the cytoplasm were classified as positively stained.

Animal models. BALB/c nude mice (5- to 6-week-old, male, 20-25 g) were provided by the Laboratory Animal Center of Central South University (Changsha, China). The mice were maintained under specific pathogen-free conditions and fed a standard laboratory diet. All of the mice were randomly assigned to two groups: HepG2/core and HepG2. Each group contained 9 mice. HepG2/core and HepG2 cells were harvested by trypsinization, respectively, and $1x10^7$ cells with viability >95% were injected subcutaneously into the dorsal region of nude mice. After 2 weeks, when the tumors reached 0.5 cm in diameter, each group was randomly assigned to 3 treatment subgroups: pcDNA3.1-re-caspase-3 (positive control), pGL3-OAS-re-caspase-3 and pcDNA3.1 (negative control). Each subgroup contained 3 tumor-bearing mice. The plasmid and lipid (20 μl; Superfect™, Qiagen) dissolved in 200 μl of sterile PBS was injected directly into the tumor. After 48 h, the mice were sacrificed, and tumors were resected for study. The tumors were stored in liquid nitrogen. Before and after treatment, tumor sizes were determined with calipers by measuring two perpendicular diameters and the differences were compared. Tumor volume was calculated according to the formula: $0.52 \times a \times b^2$, where a and b is the largest and smallest diameter, respectively.

Immunohistochemistry. We performed immnunohistochemistry on formalin-fixed paraffin sections. The sections were dewaxed and dehydrated. After rehydration, antigen was retrieved in citrate buffer. Endogenous peroxidase activity was blocked for 30 min using a methanol solution containing 3% hydrogen peroxide. Sections were incubated with the primary antibody (HCV-core monoclonal antibody at a dilution of 1:100) at 4˚C for one night. The sections were then incubated in poly-peroxidase-anti-mouse IgG for 30 min at 37˚C. Slides were subsequently visualized with diaminobenzidine (DAB; ZhongShan Goldenbridge, Beijing, China) and counterstained with hematoxylin for microscopic examination. In the negative control, the sections were incubated with PBS instead of the primary antibody. Cells showing light brown or yellow brown grains in the cytoplasm were classified as positively stained. We randomly selected 10 high-power fields and counted the number of positive cells in 100 cells from each field, and

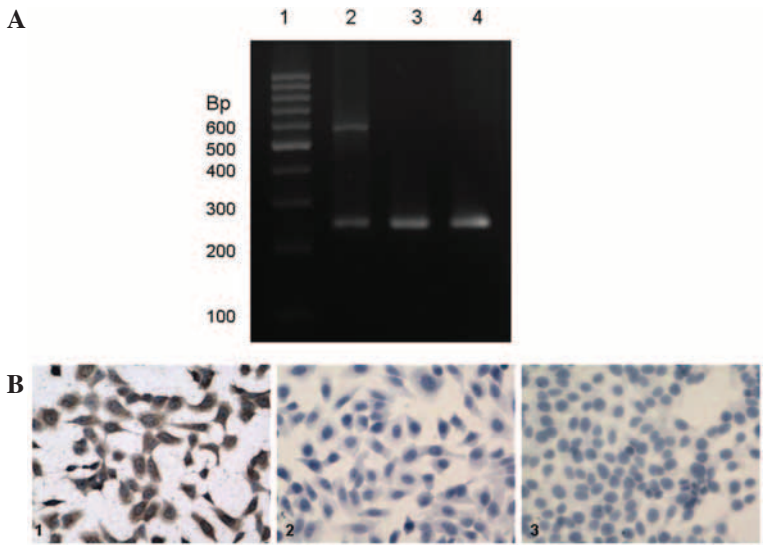

Figure 1. Establishment of the HepG2 cell line stably expressing the HCV-core gene. (A) Detection of HCV-core mRNA by RT-PCR. The visible fragments of 573 bp, consistent with the predicted size of HCV-core mRNA, were detected in the HepG2/core cell clones. Lane 1, 100-bp marker; lane 2, HepG2 cells transfected with pcDNA3.1-core (HepG2/core); lane 3, HepG2 cells transfected with pcDNA3.1 (empty vector); lane 4, untransfected HepG2 cells. (B) Detection of HCV-core protein by immunocytochemical staining: 1, HepG2/core cells; 2, HepG2 cells transfected with empty vector; 3, untransfected HepG2 cells.

regarded the mean percentage of positive cells as the positive ratio of cells expressing HCV-core protein.

Double-label immunofluorescence. Double-label immunofluorescence staining was performed on frozen-sliced tissues. Sections were thoroughly washed in PBS and incubated with 1% BSA for 30 min. Then, half of the sections were incubated with primary antibody HCV-core (1:100) overnight at 4˚C and FITC-labeled goat anti-mouse IgG (1:50; ZhongShan Goldenbridge) for 30 min at 37˚C by turns. The other sections were incubated with the secondary primary antibody caspase-3 (1:100) and TRITC-labeled goat anti-rabbit IgG (1:50) (ZhongShan Goldenbridge) which were added to the sections in order overnight at 4˚C and 30 min at 37˚C, respectively. The protein expression was observed by fluorescence microscopy after being mounted with water-soluble mounting agents. Caspase-3-positive cells and double-stained cells for caspase-3 and HCV-core were counted in 10 fields in each subgroup displayed randomly on the monitor at high-power view. For the negative control, sections were incubated with PBS instead of the primary antibodies.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Apoptotic cells were visualized using a TUNEL assay. The TUNEL procedure was performed using an *In Situ* Cell Death detection kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. TUNEL-positive apoptotic tumor cells were counted in 3 fields and each section was displayed randomly on the monitor at high-power view. The mean values and standard deviations were calculated from each subgroup. Fluorescence images were analyzed using the software package Image-pro Plus 6.0.

Transmission electron microscopy (TEM). Fresh tumor tissues were fixed with 2.5% glutaraldehyde at room temperature for 24 h, and then incubated for 2 h in 2% osmium, serially dehydrated in acetone, and embedded in Spurrs epoxy resin. Thin sections were obtained and stained with 0.5% uranyl acetate and 0.5% lead citrate. Samples were observed using a Hitachi H7500 transmission electron microscope operating at 80 kV.

Statistical analysis. Results are expressed as the mean ± SD. Statistical comparisons were carried out using an unpaired two-tailed Students' t-test. $P<0.05$ was considered to denote statistical significance.

Results

Establishment of the HepG2 cell line stably expressing HCV-core protein. RT-PCR and immunocytochemistry were performed to detect HCV-core mRNA and protein expression in the three cell lines, respectively. The HCV-core mRNA (573 bp) was amplified in the HepG2/core cells by RT-PCR, while for the two control cell lines, HepG2/pcDNA3.1 and HepG2, no signals were detected (Fig. 1A). Results of the immunocytochemical assay showed that expression of the HCV-core protein was in accordance with the RT-PCR results (Fig. 1B). Thus, we confirmed that the HepG2/core cell line stably expressing the HCV-core protein was established.

Establishment of the subcutaneous hepatoma model stably expressing the HCV-core protein. Tumors were established by subcutaneously inoculating HepG2/core cells into mice. Then the tumor-bearing mice were treated with an intratumoral injection of either pGL3-OAS-re-caspase-3, pcDNA3.1-re-caspase-3 or pcDNA3.1. There were no significant differences in tumor sizes before and after treatment.

To confirm whether the hepatoma model expressing the HCV-core protein was established, immunohistochemical staining was carried out. The HepG2/core group exhibited positive expression of the HCV-core protein and the positive ratio was 78±8% (Fig. 2A), whereas the HepG2 group did not express the core protein (Fig. 2B).

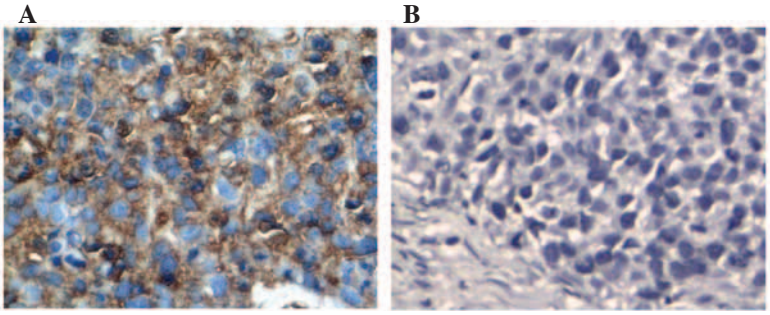

Figure 2. Establishment of a subcutaneous hepatoma model of infected HCV. (A) Immunohistochemistry of the HCV-core protein in tumors of mice subcutaneously inoculated with HepG2/core cells. Positive expression ratio of HCV-core protein was 78±8%. (B) Immunohistochemistry of the HCV-core protein in tumors of mice inoculated subcutaneously with HepG2 cells.

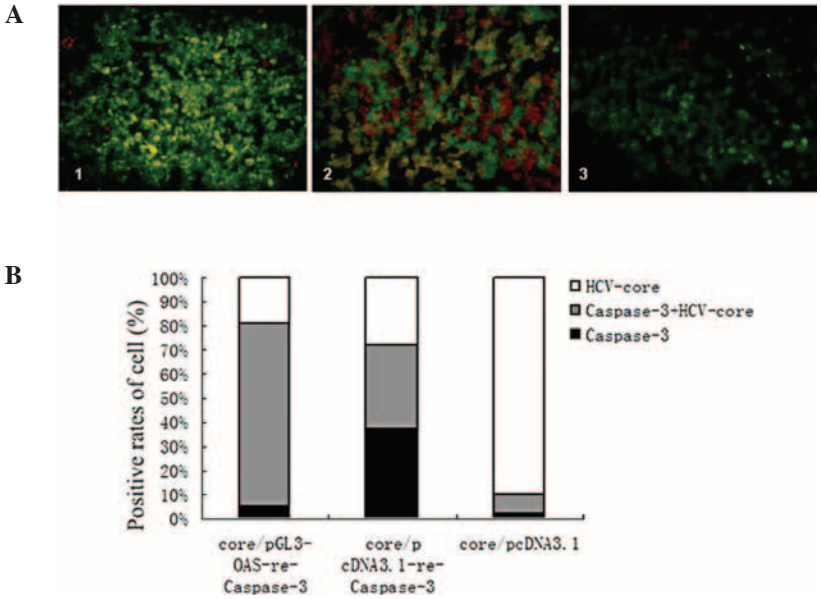

Figure 3. Correlation between HCV-core and caspase-3 expression in tumor tissues. (A) Double-label immunofluorescence for caspase-3 and HCV-core. HCV-core protein expression was localized in the cytoplasm of tumor cells (green fluorescence). Caspase-3 expression was chiefly observed in the cytoplasm of tumor cells, but also partly in nuclei (red fluorescence), and the phenomenon of co-expression was also found in tumor tissue (yellow): 1, tumor-bearing mice infected with the HCV-core protein were treated with an intratumoral injection of pGL3-OAS-re-caspase-3 (core/pGL3-OAS-re-caspase-3); 2, tumor-bearing mice infected with the HCV-core protein were treated with an intratumoral injection of pcDNA3.1-re-caspase-3 (core/pcDNA3.1-re-caspase-3); 3, tumor-bearing mice infected with the HCV-core protein were treated with an intratumoral injection of pcDNA3.1 (core/pcDNA3.1). (B) Expression of active caspase-3 was significantly increased and mainly express together with HCV-core in the core/pGL3-OAS-re-caspase-3 group. The pcDNA3.1-re-caspase-3 (positive control) and pcDNA3.1 (negative control) were used as controls.

Correlation between HCV-core and caspase-3 expression in tumor tissues. To investigate whether the correlation between HCV-core and caspase-3 expression existed *in vivo*, double-label immunofluorescence was conducted. HCV-core protein expression was found to be localized in the cytoplasm of the tumor cells (green fluorescence). Likewise, caspase-3 expression was mainly observed in the cytoplasm of the tumor cells, but also in nuclei (red fluorescence). The phenomenon of co-expression was also found in the tumor tissue (yellow) (Fig. 3A). The image analyses revealed that the percentage of HCV-core and caspase-3 co-expression was 76±6% in HepG2/core-bearing mice treated with pGL3-OAS-re-caspase-3, 35±8% in the pcDNA3.1-re-caspase-3 group, and 8±3% in the pcDNA3.1 group (Fig. 3B). The expression of active caspase-3 was significantly increased with the expression of HCV-core. These results suggest that the pGL3-OAS-re-caspase-3 system specifically induced the expression of active caspase-3 in the HCV core-positive cells *in vivo*.

Apoptosis of the tumor cells. TUNEL staining was performed to determine whether the pGL3-OAS-re-caspase-3 construct induced apoptosis only in HCV core-positive liver cells. As shown in Fig. 4A and B, in the mice inoculated with the HepG2/core, significant numbers of apoptotic cells (111±9/HP) were observed in the tumors treated with the pGL3-OAS-re-caspase-3, while tumors treated with the negative control (pcDNA3.1) showed almost no apoptotic cells ($P<0.05$). In contrast, in the mice inoculated with HepG2, tumors treated with the pGL3-OAS-re-caspase-3 did not undergo apoptosis, with no significant difference compared with the negative control ($P<0.05$). In addition, TEM analysis revealed features of early apoptosis in the HepG2/core-bearing mice treated with pGL3-OAS-re-caspase-3, including chro-

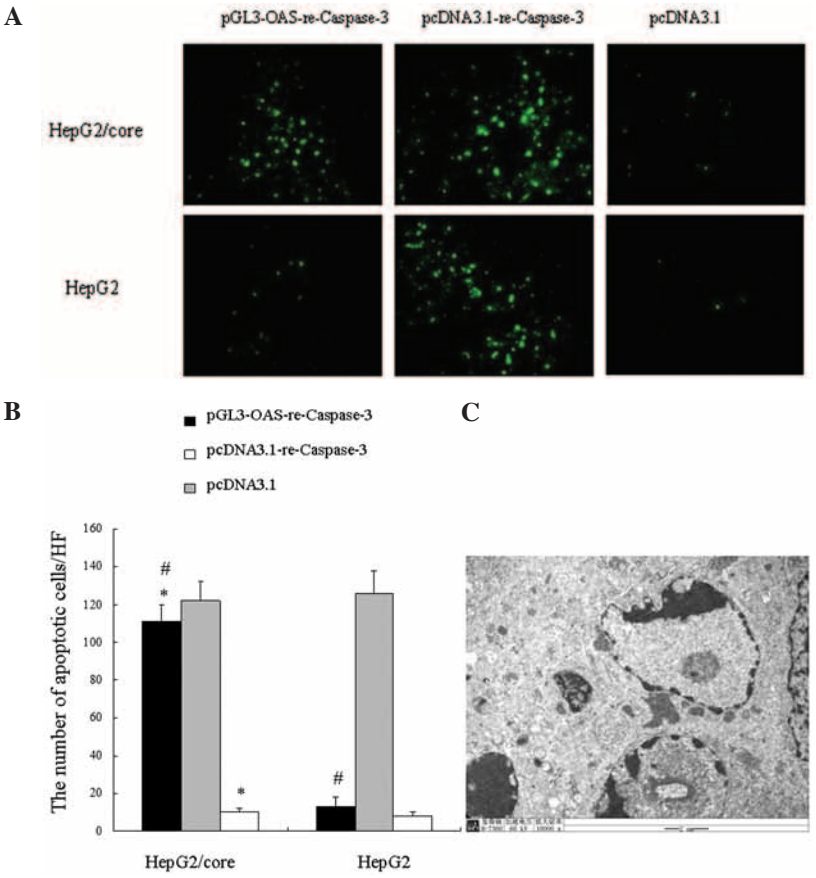

Figure 4. Apoptosis of the tumor cells. (A) TUNEL assay of the tumor tissues. (B) The number of TUNEL-positive cells in the tumor tissues with infected and non-infected HCV-core protein after intratumoral injection of pcDNA3.1-re- caspase-3, pGL3-OAS-re-caspase-3 or pcDNA3.1. The results illustrate that the pGL3-OAS-re-caspase-3 system specifically induced the expression of active caspase-3 in the HCV core-positive cells *in vivo*. $^{\#}$P<0.05 compared with same group; *P<0.05 compared with other group. Data are shown as the mean ± SD from three independent experiments. (C) TEM of the cells undergoing apoptosis in the HepG2/core/pGL3-OAS-re-caspase-3 group. The majority of cells show features of early apoptosis, including chromatin aggregation, nuclear and cytoplasmic condensation.

matin aggregation, and nuclear and cytoplasmic condensation (Fig. 4C).

The results above illustrated that pGL3-OAS-caspase-3 effectively induced apoptosis, specifically in HCV core-positive hepatocytes *in vivo*.

Discussion

Since the discovery of HCV in 1989 (14), many significant advances have been achieved in the development of HCV therapy. However, no vaccine is available, and current therapy is only partially effective. In a previous study, we constructed the re-caspase-3 expression vector under the OAS promoter (pGL3-OAS-caspase-3) and demonstrated that it is an effective gene therapy for HCV core-positive liver cells *in vitro*.

As the previous study was based on *in vitro* cell cultures stably expressing HCV core protein, further experiments using animal models with HCV infection are necessary to determine whether this approach may be applied for the treatment of patients with hepatitis C. Since the discovery of HCV, the development of preventive and therapeutic drugs has been severely hampered by the lack of suitable animal models, a deficit resulting from the limited species tropism of HCV. Chimpanzees are the only available immunocompetent *in vivo* experimental system, but their use is limited by ethical concerns, restricted availability and prohibitively high costs (15). An amenable small-animal model with exogenously introduced HCV susceptibility traits could significantly accelerate the preclinical testing of vaccine and drug candidates, as well as facilitate *in vivo* studies of HCV pathogenesis. In this study, we first established a hepatoma animal model infected with the HCV-core gene through subcutaneous injection of HepG2 cells stably expressing the HCV-core protein into nude mice. We then used this model to evaluate the role of a pGL3-OAS-re-caspase-3 treatment system in promoting apoptosis. Although the mouse model was unable to simulate the physical state of human infection with HCV, it can illustrate the efficacy of anti-HCV drugs on the whole, and this model achieved our experimental purposes.

In conclusion, pGL3-OAS-re-caspase-3 effectively induced apoptosis specifically in HCV core-positive hepatocytes *in vivo*. The present results strongly suggest that the transfer of pGL3-OAS-re-caspase-3 is an effective and promising gene therapy for HCV infection.

Acknowledgements

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