Biomedicine & Pharmacotherapy 68 (2014) 887–892

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Original article

The cell death and DNA damages caused by the Tet-On regulating HSV-tk/GCV suicide gene system in MCF-7 cells

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ARTICLE INFO

Article history:
Received 2 May 2014
Accepted 20 July 2014

Keywords:
Suicide gene system
Ganciclovir
HSV-tk
Human breast cancer

ABSTRACT

Ganciclovir (GCV) affects the molecular mechanism of cell death and DNA damage by the rAAV (recombinant adeno-associated virus)-mediated Tet-On/HSV-tk/GCV suicide gene system in human breast cancer cell line MCF-7. A rAAV/TRE/Tet-On/HSV-tk combining a Tet-On regulating system and a suicide gene HSV-tk was used to transfect human breast cancer cell line MCF-7, and therapeutic effects on this system were studied. Afterwards, we used RT–PCR, western blotting, and a modified comet-assay to explore the potential mechanism of the HSV-tk/GCV suicide gene system in breast cancer treatments. MTT assay has shown that the cell number of GCV + rAAV + Dox group was significantly decreased compared with that of other groups after treatment and flow cytometric analysis detected that there was a substantial increase of S phase cells in this group, which means the HSV-tk/GCV suicide gene system probably works on the cell cycle. RT–PCR detected the expression level of p21 increased and PCNA had an opposite trend. Western blotting detected the protein expression of p21 and p53 increased and PCNA, CDK1, cyclin B decreased in GCV + rAAV + Dox group. The modified comet-assay shown that the very small extra fragments generated by the GCV + rAAV + Dox group treatment are visible as a small cloud extending from the comet in the direction of electrophoresis. The therapeutic mechanism of the HSV-tk/GCV suicide gene system on human breast cancer cell line MCF-7 is probably by upregulating the expression of p21 through a p53-dependent DNA damage signalling pathway, leading the decrease of protein expression of PCNA, cyclin B, CDK1 in MCF-7 cells and promoting the cell cycle arrest at G1/S phase. In summary, the HSV-tk/GCV suicide gene system arouses the death of MCF-7 cells from blocking the cell cycle and DNA damage.

Ganciclovir (GCV) is also known as 9-[1,3-dihydroxy-2-(propoxy) methyl] that is a derivative of acyclovir (ACV) and also an effective drug to treat severe low immune function, such as cytomegalovirus infectious disease, CMV retinitis blindness, organ transplantation and malignant tumor [1,2]. As a kind of substrate for chemical-reaction, GCV can be used in gene therapy system, for example, the HSV-tk/GCV tumor treatment. After HSV-tk was transfected into tumor cells, its encoding product (thymidine kinase) can phosphorylate non-toxic GCV to cytotoxic GCVTP. GCVTP is involved in the DNA replication and leads an advanced replicate termination with the following death of cancer cells. This treatment has an advantage named "bystander effect", which means that tumor cells untransfected with the suicide gene HSV-tk can also be killed [3]. In our previous study, we focused on the significant anti-tumor effects of HSV-tk/GCV/Tet-On. We found that this regulation system has a better therapeutic achievement by adjusting the dosage of GCV, or inducing the adjustment of gene expression level in suicide gene therapy system in our continuous exploration [4]. However, the anti-tumor effect of the regulation system remains unknown. Therefore, it is important to determine the therapeutic effect and explore the mechanism of how the GCV in this regulatory system can affect human breast cancer cells and induced cell death.

1. Materials and methods

1.1. Materials

1.1.1. Cell culture

Human breast cancer cell line MCF-7 was derived from the pleural exudate of a Caucasian adenocarcinoma female sufferer.

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http://dx.doi.org/10.1016/j.biopha.2014.07.018
0753-3322/

This cell line is stored by the Molecular Biology Research Center of Central South University. MCF-7 cells were cultured with DMEM medium containing 10% calf serum at 37 °C in a 5% CO_2 environment.

1.1.2. Main reagents

Normal melting point agarose, GCV and doxycycline were obtained from Sigma-Aldrich Company (USA, Shanghai). Cell lysis solution was prepared according to certain formula (2.5 M NaCL, 100 mM Na_2EDTA, 1% sodium dodecyl-sarcosinate, 10 mM Tris, pH 10.0, 1% add Triton X-100 and 1% DMSO before use). DMEM medium and the calf serum were purchased from Gibco® Life Technologies Corporation (USA, Shanghai), and rTaq DNA polymerase, dNTP, DNA Hind III Marker were purchased from TaKaRa (Janpan, Dalian). Recombinant rAAV/TRE/Tet-On/HSV-tk was constructed by our laboratory.

1.1.3. Antibodies and primers

Rabbit anti-p21 monoclonal antibody, mouse anti-PCNA monoclonal antibody and mouse anti-CDK1 monoclonal antibody were purchased from Boster Biological Technology Company (China, Wuhan). Mouse anti-cyclin B monoclonal antibody, mouse anti-β-actin monoclonal antibody, rabbit anti-P53 monoclonal antibody and rabbit anti-phospho-p53 (Ser6) monoclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase-conjugated goat anti-mouse IgG (H + L) and peroxidase-conjugated goat anti-rabbit IgG (H + L) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Primer :TK3 5′-TGG AGC AGA AAA TGC CCA CG-3′; TK4 5′-TGC TGC CCA TAA GGT ATC GC-3 ′; p21 gene upstream primer sequence: 5′-CGA CTG AGA TGG GCT AAT GG-3′, downstream primer sequence: 5′-TGG TAG AAA TCT GTC ATG CTG GT-3′; p27 gene upstream primer sequence: 5′-TGG GGC AAA AAT CCG AGG T-3′ and downstream primer sequence: 5′- CCT ATT CTA CCC AAC ACA GCA T-3′; β-actin gene upstream primer sequence: 5′-AGT TGC GTT ACA CCC TTT C-3′and downstream primer sequence: 5′-GTG GCT TTT AGG A-3′; cdk2 gene upstream primer sequence: 5′-TCA GTG GTG CGA CAT-3′; downstream primer sequence: 5′-ACT TTG CCC TGT TTG ATG CCA-3′; PCNA gene upstream primer sequence: 5′-GAT GCT GTT GTA ATT TCC TG-3′ and downstream primer sequence: 5′-CAT ACT GAG TGT CAC CGT TG-3′; p53 gene upstream primer sequence: 5′-TCC CTG TTG GTC GG-3′ and downstream primer sequence: 5′-GGG AGG CAA AGG CT-3′.

1.2. MTT detected the proliferation of MCF-7 cells with the Tet-On regulating HSV-tk/GCV suicide gene system

After the logarithmic growth phase, MCF-7 cells were digested with 0.25% trypsase, and plated on 96-well cell culture plates (2×10^3 cells/well) with DMEM medium containing 10% calf serum. This experiment was designed within five groups including GCV + rAAV + Dox group, rAAV + GCV group, rAAV + Dox group, rAAV group and negative control group. Four parallel wells were done in this test for each group. After cells attached to cell culture plates, the rAAV virus (titre 1.7×10^5 MOI) were used to infect MCF-7 cells of GCV + rAAV + Dox group, rAAV + GCV group, rAAV + Dox group and rAAV group. An amount of 1 μg/mL of Dox was added to cell culture both GCV + rAAV + Dox group and rAAV + Dox group after infected 12 h later and 4 μg/mL of GCV was added to GCV + rAAV + Dox group, rAAV + GCV group after another 12 h. Each group of cells were cultured with DMEM medium containing 10% calf serum at 37 °C in a 5% CO_2 environment for 0 h, 24 h, 48 h, 72 h and 96 h. Then 100 μL new medium containing no serum was added into every well with 20 μL of 5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to replace the former medium. Those cells were cultured for 4 h at 37 °C in a 5% CO_2 environment before culture medium was changed again. 150 μL of DMSO was added to every well and incubated for 10 min. Using the automatic microplate reader (wave length is 490 nm, calibration wavelength is 630 nm) to detect the optical density value, we calculated the survival rate of MCF-7 cells in different groups. Survival rate was calculated as the formula: (the optical density value of experimental groups/the optical density value of the negative control) × 100%.

1.3. Detecting the influence of Tet-On regulating HSV-tk/GCV suicide gene system on cell cycle by flow cytometric analysis

Cell culture and treatment were described above. Each group of cells were cultured with DMEM medium containing 10% calf serum at 37 °C in a 5% CO_2 environment for 48 h. One millimetre of pre-chilled PBS (0.01 mol/L, pH 7.2 to 7.3) was added to each well after former medium was discarded, the culture plates were gently washed three times. Finally, 1 mL of pre-chilled PBS (0.01 mol/L, pH 7.2) was added into each well again and MCF-7 cells were scraped with cell scraper. The cell suspensions were collected into different eppendorf tubes and then were centrifuged at 4 °C, 1000 *g* for 5 min. The supernatant was discarded, and pre-chilled 70% ethanol was added to each well at the same time. MCF-7 cells were fixed at 4 °C no less than 18 h or preserved for a long time at −20 °C. One millilitre of MCF cell suspension (1×10^6cells/mL) was washed three times with PBS (0.01 mol/L, pH 7.2) before resuspend them in 1 mL PI dye (50 μg/mL PI, 20 μg/mL RNase A). Flow cytometric analysis was used to detect the influence on cell cycle and the results were analyzed by software Cell Quest (BD Biosciences, Shanghai, China).

1.4. Single-cell microgel electrophoresis

To determine the DNA damage effect caused by suicide gene therapy system under the regulation of Tet-On in MCF-7 cells, a single-cell microgel electrophoresis assay (“comet-assay”), was performed under alkaline conditions. Isolated MCF-7 cells treated by rAAV + Dox + GCV or other control groups (rAAV + Dox group, rAAV + GCV group, rAAV group and normal saline group) were suspended in agarose gel and the cells were spread onto glass microscope slides pre-coated with agarose gel to set at 4 °C for 5 min. Slides were incubated in ice-cold lysis solution (2.5 mol/L NaCl, 100 mmol/L EDTA, 10 mmol/L Tris, pH 10.0, and 1% Triton X-100 with 10% DMSO) to remove cell proteins, leaving DNA as “nucleoids”. After the lysis procedure, slides were placed on a horizontal electrophoresis unit, covered with a fresh solution (300 mmol/L NaOH and 1 mmol/L EDTA, pH > 13) for 20 min at 48 °C to allow for DNA unwinding. Electrophoresis was conducted in an electrophoretic solution containing 30 mmol/L NaOH and 1 mmol/L EDTA(pH > 13), at 4 °C (temperature of the running buffer did not exceed 12 °C) for 20 min at an electrical field strength of 0.73 V/cm (28 mA). The slides were then washed in water, drained, stained with ethidium bromide, and covered with cover slips. To prevent additional DNA damage, all the steps were conducted under dimmed light or in the dark. The comets were viewed within 24 h at 200-fold magnification using a fluorescence microscope (Olympus: Beckman Coulter, Fullerton, CA, U S A) with excitation by green light (546 nm) and a barrier filter of 590 nm; photomicrographs were taken. For each sample, 3 slides were scored, and at least 100 cells were counted randomly on each slide. The cells with no DNA damage retained a circular appearance. During electrophoresis, DNA with strand breaks migrated toward the anode, giving the cell a “comet” appearance [5].