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Inhibitory Effects of Ginsenoside Rb1 on Apoptosis Caused by HSV-1 in Human Glioma Cells*

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Abstract: To investigate the inhibitory effects of Ginsenoside Rb1 (GRb1) on apoptosis caused by Herpes Simplex Virus-1(HSV-1) in Human Glioma Cells (U251), U251 cells were infected by HSV-1 at a multiplicity of infection of 5 and GRb1, GRb1+HSV-1, HSV-1 and control groups. MTT and cell apoptosis assays were used to detect the inhibitory effects of GRb1 on the apoptosis of U251 cells that caused by HSV-1 infection for various concentrations of drug and virus treatments by MTT assay. We found that in the 400 μg/mL GRb1 and 400 μg/mL GRb1+HSV-1 groups, MTT values were higher than control group at all times ($P<0.05$). Moreover, the apoptosis rate in the 400 μg/mL GRb1+HSV-1 group was lower than the HSV-1 group ($P<0.05$). These results confirmed that, at appropriate concentrations, GRb1 could inhibit nerve cell apoptosis in HSV-1 infections.

Key words: Ginsenoside Rb1; Herpes Simplex Virus-1; Human Glioma Cells; Apoptosis

Herpes Simplex virus type 1(HSV-1) is a neurotropic double-stranded DNA virus that belongs to the herpes virus family that establishes life-long latency infection in the human nervous system. HSV-1 infection can cause various of human diseases, such as gingivo-stomatitis, herpes labialis, meningitis, as well as eye and genital infection[6]. Among these diseases, herpes simplex encephalitis (HSE) is a rare disease with high mortality and significant morbidity. Though some patients with severe viral encephalitis after treatment can survive, neural function defect symptoms always result to differing extents. Therefore, it much research has focused on identification of drugs that may inhibit or reduce nerve damage caused by HSV-1 infection.

Ginseng, the root of Panax ginseng C.A. Meyer, has been widely used in traditional oriental medicine for over two thousand years in the prevention and treatment of aging-associated disorders in Asia[7].

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Ginsenosides, as the main bioactive components of ginseng, play an important role in the central nervous system (CNS)[11]. Ginsenoside Rb1 (GRb1)(Fig. 1), one of the saponin components of ginseng, has been widely reported to exert neurotrophic and neuroprotective activities on the CNS. It has been known that GRb1 enhances the stimulatory effect of neurite outgrowth[13]. Recent studies have also revealed that GRb1 can reduce neural cell apoptosis[24]. Later, it has been demonstrated that GRb1 can promote the proliferation and expression of neurotrophic factors in primary Schwann cell cultures[10]. Furthermore, recent studies have proved that ginseng can inhibit virus infection[14,18,22].

In spite of these many investigations into the properties of GRb1, there has been no report about the effect of GRb1 in HSV-1 infections. In this study, human glioma Cell line U251 was selected to investigate the effects of GRb1 on the apoptosis caused by HSV-1, and we illustrate the potential neuroprotective effect of GRb1 after HSV-1 infection. This study provides theoretical and experimental evidence to support the further study of ginsenoside on HSV-1 infection.

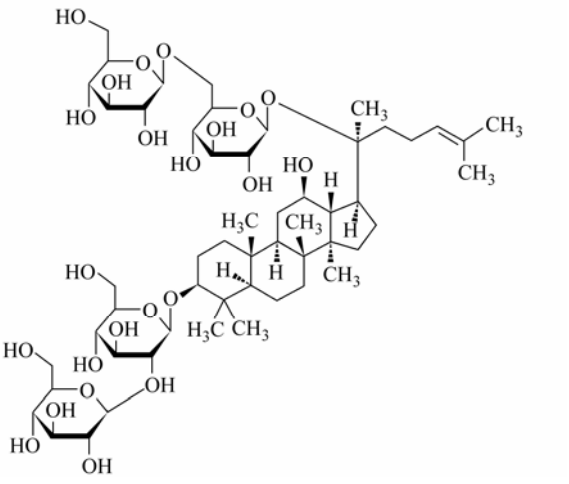

Fig. 1. Chemical structure of ginsenoside Rb1.

MATERIALS AND METHODS

Virus and cell culture

HSV-1-SM44 from Beijing union medical college was amplified in Vero cells. When more than 80% cells have pathological change after HSV-1 infection, virus was collected. The empty spot filamentous experiment was chosen to detect the degree of virus drops. Virus with 10^8 PFU/mL was stored at -86 °C.

Africa green monkey kidney cell lines (Vero) were preserved in our laboratory. Human Glioma Cell line U251 (American Type Culture Collection, Manassas, VA, cell line number: 99522) was purchased from the Shanghai Cell Resource Center of the Chinese Academy of Sciences. U251 cells were grown in tissue culture flasks in Roswell Park Memorial Institute (RPMI 1640) (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco). The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium was changed every day. U251 cells were grown to anastomose every three days.

Analysis of cell viability by MTT assay

Ginsenoside Rb1 was purchased from Shanghai Aladdin reagents Co.,Ltd. (Shanghai, China) and dissolved in RPMI 1640. The U251 cells were inoculated to 96-well plates when grown to anastomose and cell concentration was 5×10^4 cell/well. GRb1 group, GRb1+HSV-1 group, HSV-1 group and control group were set up. After 24 h, medium was thrown away, and the GRb1 group and GRb1+HSV-1 group were treated with 100 μL RPMI 1640 medium containing different concentrations (50, 100, 200 and 400 μg/mL) of GRb1 and 2% FBS. The HSV-1 group and control group were treated with same volume of RPMI 1640 containing 2% FBS. After 8 h, the GRb1

+ HSV-1 group and HSV-1 group were treated with HSV-1 (MOI = 5) and the GRb1 group and the control group were treated with the same volume of RPMI 1640 containing 2% FBS. Briefly, at 0, 24, 36 and 48 hpi (hour post infection), 20 μL of 5 mg/mL 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Ameresco, USA) was added to the culture medium. The plates were cultured for 4 h at 37°C, followed by the addition of 150 μL of dimethyl sulfoxide. The 96-well plates were shaken for 10 min and the OD values were detected by a DG3022 type A microplate reader using a detection wavelength of 492 nm, with a reference wavelength of 620 nm. In the experiments, zero setting cells were set up (not containing cells) for a baseline references. The above experiments were repeated 3 times and the average value was taken for statistical analysis.

Analysis of cell apoptosis by flow cytometric assay

At 12, 24 and 36hpi (n=3), the cells of GRb1 (400 μg/mL) group, GRb1 +HSV-1 (400 μg/mL) group, HSV-1 group and the control group were collected with the old culture medium and the adherent U251 cells, centrifuged, and the old culture medium was discarded. Afterwards, 100 μL RPMI 1640 containing 2% FBS was added in order to resuspend the cells, followed by labeling by addition of 100 μL of Nexin Reagent for 20 minutes in room temperature and avoiding light conditions. The Guava EasyCyte™ CellGrowth Kit (0500-1430, Guava technologies Inc, Millipore, USA) was used to detect red and orange fluorescent signals of 5000 cells. The experimental results were shown with a four quadrant scatterplot chart.

Statistical analysis

All data was presented as "mean±standard error mean" and the paired t-test was performed to detect the difference between mean values for the same time points among these groups. Statistical significance was set at $P < 0.05$.

RESULTS

Effects of GRb1 on proliferation of U251 cells by MTT assay

To investigate the effects of GRb1 on proliferation of normal U251 cells and U251 cells infected by HSV-1, cell proliferation was detected for various concentrations of drug and virus treatments by MTT assay (Fig. 2). Compared with control cells (without GRb1 and HSV-1), both 200 μg/mL GRb1 and 400 μg/mL GRb1 significantly promoted proliferation of U251 cells (n=3; $P < 0.05$). The OD values of both the 200 μg/mL GRb1 group at 24 hpi and 400 μg/mL GRb1 group at 12 hpi were higher than the HSV-1 group (n=3; $P < 0.05$). Therefore, 400 μg/mL GRb1 was chosen for further study.

The inhibitory effects of GRb1 on the apoptosis caused by HSV-1 in U251 Cells

GRb1 inhibited morphological changes of U251

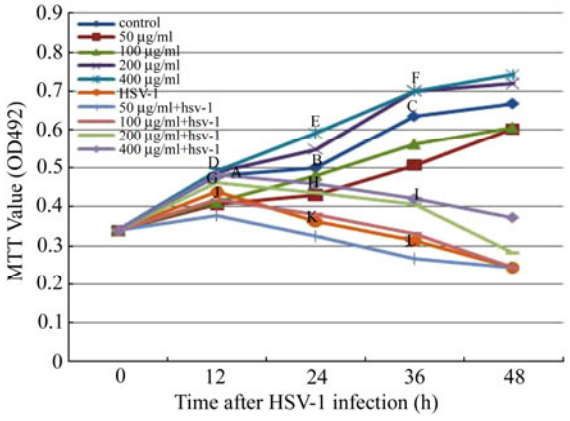

Fig. 2. Effects of GRb1, GRb1+HSV-1, HSV-1 and control groups at different concentations on the proliferation of U251.

cells caused by HSV-1 as observed by inverted microscope observation. Compared with control cells (without GRb1 and HSV-1), in the HSV-1 group, cells gradually appeared to fuse after HSV-1 infection, cell gaps became larger, granularity increased in the cytoplasm and typical cytopathic effects (CPE) occurred at 36 hpi (Fig. 3 A, B, C, J, K and L). However, in 400 μg/mL GRb1+HSV-1 group, cells did not appear to exhibit obvious lesions, such as the clear outline of the body and good refractive index at 12hpi (Fig. 3 G). Moreover, in the 400 μg/mL GRb1+HSV-1 group, very few cells appeared to fuse at 24 hpi but by 36 hpi fusion was increasing, but most of the cells remained in the normal form (Fig. 3 H, I). The results suggests that GRb1 can inhibit the change of U251 cells infected by HSV-1. Compared with control cells, the cells of the GRb1 group had no obvious differences (Fig. 3 A, B, C, D, E, F).

GRb1 inhibited the apoptosis caused by HSV-1 in U251 Cells by flow cytometric assay

The apoptosis rate of the GRb1 group at each time point was lower than that of the control group at the corresponding time ($P < 0.05$) (Fig. 4, Fig. 5). It is notable that the apoptosis rate of the GRb1+HSV-1 group at each time point was lower than that of the HSV-1 group at the corresponding time ($P < 0.05$) (Fig. 4, Fig. 5). Therefore, GRb1 itself does not cause cell death, but it can promote cell proliferation and inhibit the apoptosis of U251 cells caused by HSV-1 infection.

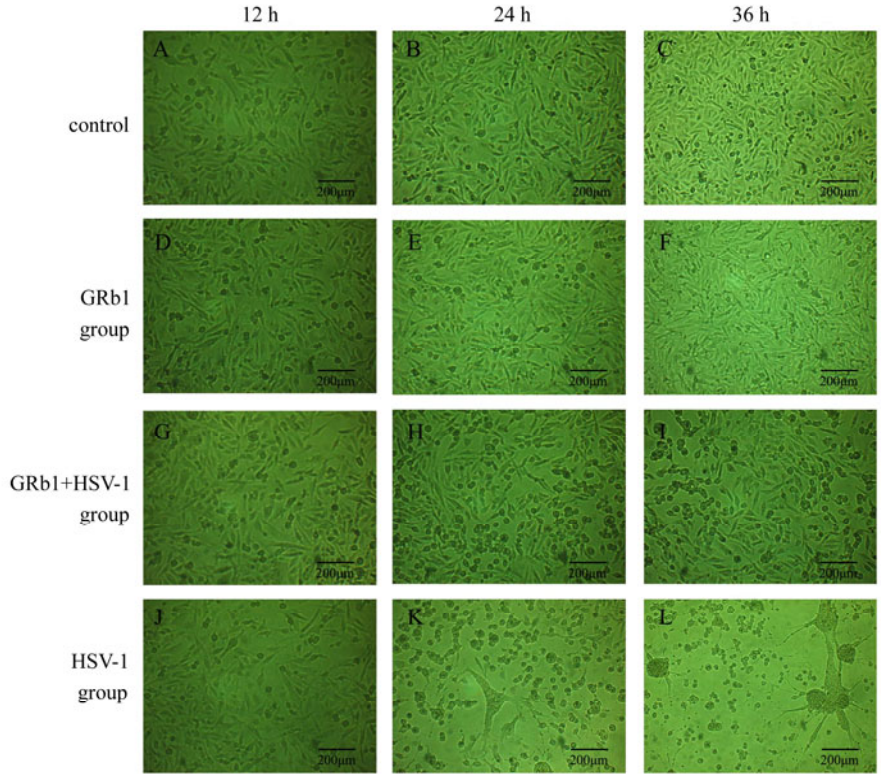

Fig. 3. Morphological changes of U251 cells in each group. U251 cells were seeded in tissue culture flasks and treated with RPMI 1640 (A, B, C, J, K, L) and 400 μg/mL GRb1 (D, E, F, G, H, I). Eight hours after treatment, cells were infected with HSV-1(G, H, I, J, K, L). After 12h (A, D, G, J), 24h (B, E, H, K), 36 h (C, F, I, L) post-infection, cells were observed by inverted microscope. *Bar = 200 μm*.

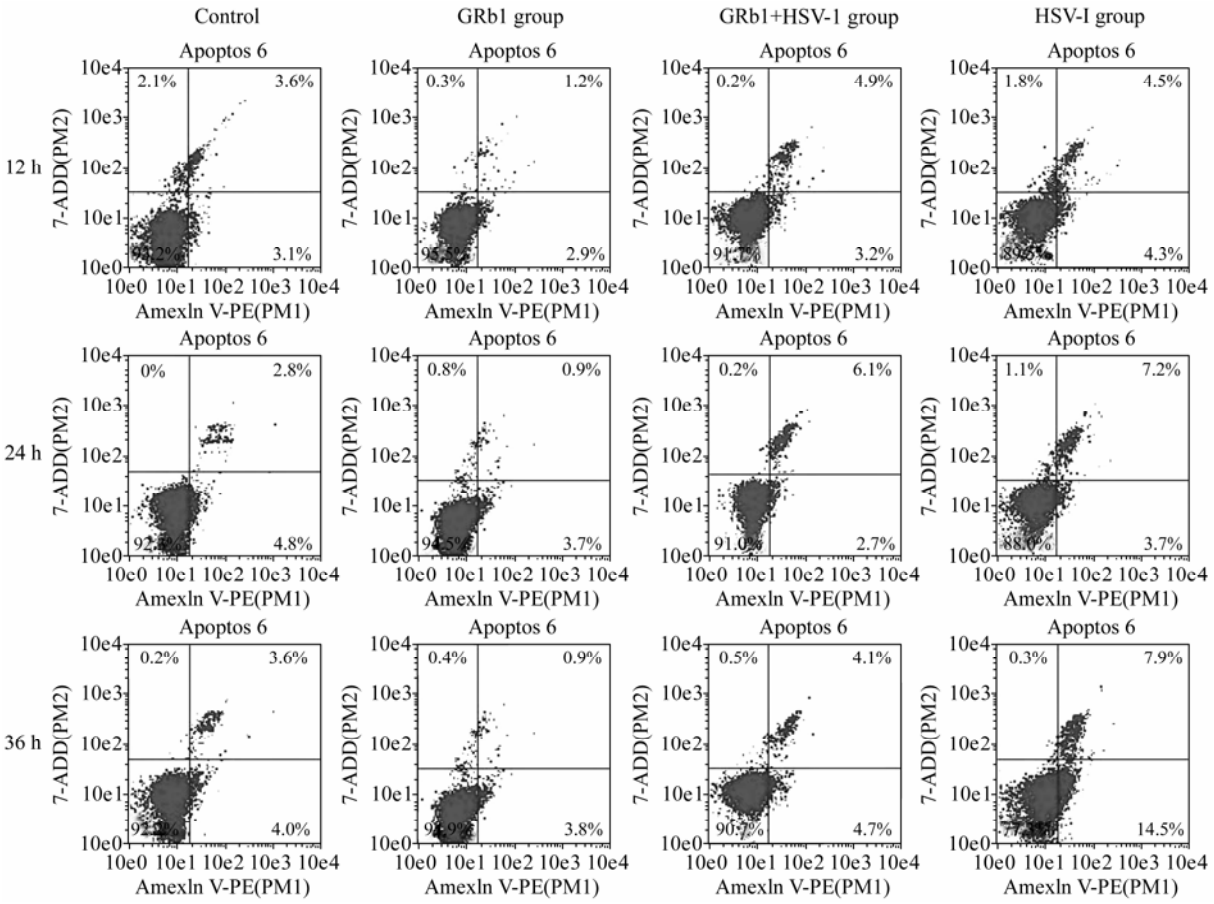

Fig. 4. Apoptosis of U251 cells of each group analyzed by flow cytometry technology.

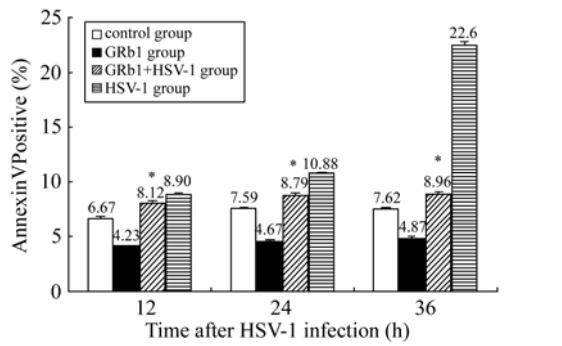

Fig. 5. Changes of apoptosis proportion of U251 cells for GRb1, GRb1+HSV-1, HSV-1 and control groups, *P<0.05.

DISCUSSION

HSV-1, a neurotropic double-stranded DNA virus, is a nerve damage pathogen that targets cells in the CNS. The exact mechanism of nervous system damage caused by HSV-1 infection is unknown. However, some studies have proved that astrocytes are most sensitive for HSV-1 infection in the central nervous system and so these cells represent an[1,3] important target in central nervous system for HSV-1 infections.

Nevertheless, there has been limited success in achieving culture astrocytes in human cells and there has been few report about human astrocytes infected by HSV-1 in previous research. Therefore, in this work, the glioma cells (U251) were selected as the target cells to investigate if GRb1 can inhibit HSV-1 infection in nervous system.

In this study, effects of GRb1 on the proliferation of U251 cells were detected by MTT assay. The results showed that 50 μg/mL and 100 μg/mL GRb1 didn't have significant effects on the cell proliferation and infection of U251 by HSV-1. However, 200 μg/mL

and 400 μg/mL GRb1 significantly inhibited HSV-1 infection in U251 cells ($P<0.05$). Also, at 200 μg/mL and 400 μg/mL GRb1 could promote proliferation of U251 cells. However, we found a much higher concentration of GRb1 could produce toxic effect on normal cells. Therefore, 400 μg/mL GRb1 was selected for further study.

To further understand the possible cause of our observations, flow cytometry was used to detect apoptosis of U251 cells with different treatments. Results clearly indicated that 400 μg/mL GRb1 itself didn't cause cell apoptosis, but inhibited apoptosis of normal U251 cells and apoptosis of U251 cells infected by HSV-1. At 36hpi, 400 μg/mL GRb1 significantly inhibited apoptosis of U251 cells. This research confirmed that GRb1 could significantly inhibit cell apoptosis induced by HSV-1.

A large number of datas show that chin traditional herb drugs inhibit virus infection in following ways: (1) protecting cells by directly restraining or kill virus; (2) exhibit antivirus effects by strengthening the resistance of cells; (3) Enhancing immunity of the organism to restrain virus infection[12,20,21].

Ginseng is a popular herbal medicine used worldwide. GRb1, a constitute of ginseng, plays a key role in exerting various beneficial effects on the central nervous system[2], such as modulation of neurotransmitters[23], stimulation of PC12 cell proliferation[9], learning, and memory[4,8]. GRb1 has been reported to improve neurite outgrowth in SK-N-SH cells and regulate the expressions of choline acetyltransferase (ChAT), nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), caspase-3 and induce neurogenesis in rats[5,17]. Combined with our data measured in this experiment, we assume that GRb1 may inhibit HSV-1 infection by enhancing the resistance of cells.

It has been reported that ginseng plays an important role in antiretroviral treatment. Synergistical action of Ginseng stem-leaf saponins (GSLS) and mineral oil can enhance the immune responses to vaccination against foot-and-mouth disease in mice[19]. Previous studies carried out in rats, guinea pigs and pigs[14,15] showed that GRb1, as a adjuvant, significantly favours the production of IgG2 over IgG1 antibodies and elicited a balanced Th1 and Th2 immune response against porcine parvovirus (PPV)[16]. It is assumed that GRb1 might inhibit virus infection by enhancing immunity of the organism. However, the exact mechanism of GRb1 inhibiting HSV-1 infection in nerve cells still is not clear and requires further research.

Above all, this study successfully establishes a model of HSV-1 infection by using human U251 cells and illustrates that GRb1 has good potential for antivirus therapy for HSV-1. Furthermore, this study also provides new ideas to explore high-efficiency and low-toxic antiviral drugs.

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