20(S)-Ginsenoside Rh2 Induces Apoptosis in Human Leukaemia Reh Cells through Mitochondrial Signaling Pathways

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Abstract

Acute lymphoblastic leukaemia (ALL), a malignant disorder of lymphoid progenitor cells, affects both children and adults, with peak prevalence between the ages of 2 and 5 years. The Reh cell line is one of pre-B ALL lines without gene translocation. Unfortunately, chemotherapy drugs can kill the normal cells as well as the tumour cells, leading to significant adverse effects. As a step in overcoming this limitation in chemotherapy, medicines prepared from natural traditional Chinese medicine (TCM) products are currently being considered as anticancer therapy. In the past decades, a number of studies have shown that several natural TCM products can activate cell death pathway in cancer cells including leukemia.

Ginseng, the root of Panax ginseng C.A. Meyer, is a medicinal plant used worldwide and has been reported to have various biological effects including anti-tumor activity. Ginsenosides have been suggested to be the major effective ingredients in ginseng. 20(S)-Ginsenoside Rh2 (GRh2), with a dammarane skeleton, belongs to the protopanaxadiol family and has drawn attention in chemopreventive research. Recently, researchers have found that 20(S)-GRh2 exhibits anticancer effects and could inhibit growth of several cancer cell lines and induce apoptosis in mammalian tumor cells. However, the details of the signal transduction cascade involved in 20(S)-GRh2-induced death in Reh cells remain unclear.

Apoptosis, or programmed cell death, is a highly regulated form of cell death distinguished by the activation of a family of cysteine-aspartate proteases (caspases) that cleave various proteins resulting in morphological and biochemical changes. Two main caspase activation cascades have been described. Especially, the mitochondria play a central role in apoptosis resulting from many chemotherapeutic agents. A mitochondrial pathway is triggered by cytochrome c released from mitochondria, which would then bind the caspase-activating protein Apaf-1, stimulating binding of Apaf-1 to pro-caspase 9 and then activating caspase 9. Caspase-9 can signal downstream and activate pro-caspase-3 and -7.

In the present study, the effect of 20(S)-GRh2 and ginsenoside Rg3 (GRg3) on Reh cell viability was compared. Moreover, we examined nuclear morphology, mitochondrial membrane potential, and status of mitochondria-associated apoptotic proteins analysis in order to elucidate the mechanism of 20(S)-GRh2-induced apoptosis in human acute leukaemia line-Reh cells.

Key words: acute leukaemia; 20(S)-ginsenoside Rh2; apoptosis; Reh cell; mitochondrial pathway

MATERIALS AND METHODS

Reagents 20(S)-GRh2 (purity 99.48%) and GRg3 (purity 99.50%) were purchased from Beijing North Carolina Chuanglian Biological Technology Research Institute (Beijing, China). Structures of both 20(S)-GRh2 and GRg3 are shown (Figs. 1 a, b). 20(S)-GRh2 was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mM, and GRg3 was dissolved in DMSO at a concentration of 300 mM, stored at −20°C, and diluted with fresh complete medium immediately before use. An equal volume of DMSO (final concentration <0.1%) was added to the controls.

Cell Culture Human pre-B ALL line (Reh cells) was purchased from Cell Bank of Chinese Academic of Science (Shanghai, China) and grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Grand Island, NY, U.S.A.), 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were seeded at 5×10^5 cells/mL in a 100-mm petri dish, and grown in a humidified 5% CO₂-95%
Double Staining Briefly, Reh cells were treated with DMSO (Tokyo, Japan) and were immediately analyzed by flow cytometry (FACS Calibur, BD Biosciences, San Jose, CA, U.S.A.). Flow cytometry was performed on Reh cells gated on the basis of their forward and side light scatter with any cell debris excluded from analysis. Apoptotic cells were defined as APC+/-7-AAD− cells.

**Determination of the Mitochondrial Membrane Potential (MMP) by JC-1 Fluorescence** JC-1 mitochondrial membrane potential detection assay kit (Sigma-Aldrich) is for detection of mitochondrial depolarization during the early stages of apoptosis. Reh cells cultured in 12-well plates (5×10^5/well) and were treated with DMSO or with different concentrations (20, 40, 50 μM) of 20(S)-GRh2. After 24h incubation, cells were washed twice with PBS and incubated in complete medium containing 5 μg/mL JC-1 in the dark for 30min at 37°C in a humidified incubator with 5% CO₂. Then, cells were washed twice with PBS and immediately observed under a fluorescence plate reader GENios plus (Tecan) with 550nm excitation/600nm emission for red fluorescence, and 485nm excitation/535nm emission for green fluorescence. For each sample, the results were calculated as the ratio (red/green) of fluorescence of sample, averaged after the fluorescence values have been corrected for the background and protein content.

**Western Blot Analysis** Reh cells cultured in 6-well plates were treated with DMSO or with different concentrations (20, 40, 50 μM) of 20(S)-GRh2. Cells were collected after 24h of incubation, and lysates were analyzed by Western blot. Briefly, the harvested cells were lysed for 30 min in RIPA buffer (Sigma-Aldrich). The sample was heated to 100°C for 5 min and placed briefly on ice. A total of 20 μL of the supernatant was loaded onto a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. After electrophoresis, the protein was electrotransferred to a Hybond-ECL polyvinylidene fluoride membrane. The membrane was blocked with 5% milk for 1h. The membrane was then incubated overnight with primary antibodies such as rabbit anti-caspase-9 antibody, rabbit anti-cytochrome c (Abcam Company, Cambridge, MA, U.S.A.) and rabbit anti-active-caspase-3 p17 antibody (Bioworld Technology, NanJing, China), at a 1:1000 dilution in Tris-buffered saline (TBS) with 5% milk at 4°C. The blot was washed three times for 10min in TBST at room temperature. The membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell signaling Tech., Danvers, MA, U.S.A.) for 1h at room temperature. The immunoreactive bands were visualized by enhanced chemiluminescence method. Each membrane was stripped and re-probed with anti-actin antibody or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody to ensure equal protein loading.

**Statistical Analysis** Results were presented as means±standard error of the mean (S.E.M.). Statistical significance between groups was analyzed by one-way ANOVA followed by the Bonferroni post hoc test, as appropriate, using Prism 5.03 (GraphPad Software Inc., San Diego, CA, U.S.A.). A p-value of <0.05 was considered significant.

### RESULTS

**Effect of 20(S)-GRh2 and GRg3 on Cell Viability**
The CCK-8 assay was done to examine if GRh2 could affect Reh cell growth in culture. Reh cells were treated with different concentration (20, 30, 40, and 50 µM) of 20(S)-GRh2 and GRg3 for 24 h. When Reh cells were treated with the compounds for 24 h, 20(S)-GRh2 exhibited a significantly potent toxic effect with IC_{50} values of around 40 µM (Fig. 2a), and GRg3 with IC_{50} values of around 100 µM (Fig. 2b). After a 24 h exposure to 20(S)-GRh2 and GRg3, survival of Reh cells was decreased in a concentration-dependent manner, and analysis of cell viability revealed that 20(S)-GRh2 is significantly more potent at inhibiting proliferation than GRg3.

**Effect of 20(S)-GRh2 on Nuclear Morphology of Reh Cells** To characterize the cell death induced by 20(S)-GRh2, we examined the nuclear morphology of dying cells with a fluorescent DNA-binding dye, Hoechst 33342. After 24 h of treatment with various concentrations of 20(S)-GRh2, cells clearly exhibited condensed and fragmented nuclei, indicative of apoptotic cell death (Fig. 3, indicated by white arrows). When Reh cells were treated with increasing concentrations of 20(S)-GRh2, the number of nuclei exhibiting chromatin condensation and apoptotic bodies increased. Collectively, these findings suggest that 20(S)-GRh2 inhibits cellular proliferation and is cytotoxic to Reh cells.

**Detection of Apoptosis by APC-Annexin V/7-AAD Staining** In the early stages of apoptosis, phosphatidylserine (PS) is translocated from the inner side of the plasma membrane to the outer layer. Annexin V, a calcium dependent phospholipid-binding protein with a high affinity for PS, can therefore be used as a sensitive probe for the exposure of PS on the cell membrane and hence as a marker of apoptosis. Annexin V+ and 7-AAD− cells were designated as early apoptotic and Annexin V+ and 7-AAD− cells were designated as necrotic. More apoptotic cells were observed in 20(S)-GRh2 treated group (20, 40, 50 µM) than in control group (Fig. 4a–d). Analysis of the cell population revealed distinct sets of population (Fig. 5). With increasing concentration of 20(S)-GRh2 for 24 h, the population of Annexin V+ and 7-AAD− apoptotic cells increased gradually from 2.76 to 17.51% while that of FITC+/7-AAD+ necrotic cells increased from 0.78 to 11.42%. These results demonstrate that 20(S)-GRh2 induced apoptotic cell-death in Reh cells.

**Effect of 20(S)-GRh2 on MMP of Reh Cells** In order to determine the cause for reduced mitochondrial activity in the presence of 20(S)-GRh2, Reh cells were stained with the MMP sensing dye JC-1. The uptake of JC-1 by Reh cells treated with increasing concentration of compounds was measured by the increase in monomer (green) and aggregate (orange-red) fluorescence. The fluorescence of the cationic dye JC-1 was detected by a fluorescence plate reader GENios plus. As shown in Fig. 6, 20(S)-GRh2 induced a dramatic decrease in the ratio of JC-1 (red/green ratio). Treatment with 20µM 20(S)-GRh2 yielded a ratio that was 59.2±6.6% of the control (p<0.01) while treatment with 40µM and 50µM 20(S)-GRh2 induced a ratio that was 28.0±3.2% and 13.1±2.9% of the control, respectively (p<0.005). These data suggest that 20(S)-GRh2 induced MMP depolarization in the apoptotic cells.

**Effect of 20(S)-GRh2 on Expression of Mitochondria-associated Pro-apoptotic Proteins** To explore the possible role of a mitochondrial-related pathway in 20(S)-GRh2-induced apoptosis, we examined the effects of 20(S)-GRh2 on the expression of caspase-9, -3 and cytochrome c by Western blot analysis. In the mitochondrial apoptosis pathway, cytochrome c is released from the intermembrane space to the mitochondrion and is cytotoxic to Reh cells.
cytoplasm. 20(S)-GRh2 treatment caused an accumulation of cytochrome c in the cytosol, most probably due to the release of mitochondrial cytochrome c (Fig. 7a). These results suggest an involvement of caspases in the apoptotic process downstream of mitochondria. Next we investigated the roles of specific caspases, namely caspase-9, and the downstream effector caspases, caspase-3. As shown in Fig. 7, Treatment of Reh cells with 20(S)-GRh2 caused a marked increase in the cleavage of caspase-9 and caspase-3 proteins compared to the control. These results suggest an involvement of caspases in the intrinsic apoptotic process downstream of mitochondria. A mitochondrial pathway plays an important role in 20(S)-GRh2-induced apoptosis.

**DISCUSSION**

GRh2 and GRg3 are the main bioactive components in Asian ginseng and North American ginseng extracts, which exhibits potent ability in killing cancer cells. However the anticancer mechanisms of GRh2 and GRg3 to ALL cell lines remain unknown. In the present study, we observed that 20(S)-GRh2 is significantly more potent at inhibiting proliferation of human acute leukaemia line-Reh cells than GRg3, and that 20(S)-GRh2-induced apoptosis is mediated partially...
by caspase-dependent mitochondrial signaling pathways.

Previous reports have demonstrated that GRh2 and GRg3 are able to induce apoptosis in different strains of human cancer cells.15–28 Moreover, GRh2 can kill colorectal cancer cells and its cytotoxic effect is significantly more potent than GRg3.29 Our findings are consistent with these previous reports: As shown in Fig. 2, 20(S)-GRh2 had a more potent toxic effect on Reh cells with an IC_{50} value of around 40 \mu M than GRg3 with an IC_{50} value of around 100 \mu M. We show that 20(S)-GRh2 and GRg3 can inhibit Reh cellular proliferation in a concentration-dependent manner, while 20(S)-GRh2 is significantly more potent at inhibiting the proliferation than GRg3.

Apoptosis is an evolutionarily conserved form of cell suicide and accomplished by distinctive morphological changes.30 GRh2 and GRg3 have been shown to have potent ability in inhibiting growth of different strains of tumor cells and to cause distinctive morphological changes.31–33. It was reported that after 24 h of treatment with GRg3 (10 \mu M) and GRh2 (10 \mu M), hepatoma cells clearly exhibited condensed and fragmented nuclei, indicative of apoptotic cell death.15 In this report, we observed that increasing concentration of 20(S)-GRh2 induced a marked increase in condensed apoptotic nuclei (Fig. 3), as evidenced by chromosomal condensation and formation of apoptotic bodies. In addition to morphological changes, apoptosis are accompanied by the phosphatidylserine translocation from the inner leaflet of the plasma membrane to the outer leaflet. 20(S)-GRh2 increased the percentage of annexin V-positive/7-AAD-negative Reh cells that were apoptotic but not necrotic in a concentration-dependent manner based on results from flow cytometric analysis (Fig. 4). Our findings reveal that 20(S)-GRh2 has cytotoxic ability and can induce apoptosis in human acute leukaemia line-Reh cells.

It is well known that apoptosis is a type of cell death accomplished by a specialized cellular mechanism and distinct biochemical pathways. Two pathways lead to the activation of caspases: the extrinsic pathway associated with membrane receptors and their ligands, and the intrinsic pathway dependent on mitochondria.24 The mechanism underlying 20(S)-GRh2 induced Reh cells apoptosis was unclear until now. It has been reported that reduction of MMP is among the very first intracellular events preceding the execution phase of apoptosis via the mitochondria-mediated death pathway.25 Our experimental results showed that treatment of 20(S)-GRh2 with Reh caused a reduction of MMP in a concentration-dependent manner (Fig. 6). The most important enzymes involved in apoptosis are caspases, which hydrolyze structural and functional proteins, ultimately leading to cell death.36,37 The mitochondria-mediated apoptosis pathway is activated by various cellular stress stimuli and is dependent on the release of cytochrome c from the mitochondria.36,38 Following the efflux of mitochondrial cytochrome c to cytosol, pro-caspase-9 is cleaved and activated by the formation of apoptosome and then causes caspase-3 activation, finally resulting in apoptosis.39,40 There was a report that 20(S)-GRh2 induced apoptosis stimulated the release of mitochondrial cytochrome c, activation of caspase-3 and production of intracellular reactive oxygen species by direct activation of the mitochondrial pathway in human hepatoma cells.15 Our results showed that 20(S)-GRh2 activated...
the mitochondria-mediated apoptosis pathway by stimulating the release of mitochondrial cytochrome c and activation of caspase-9 and caspases-3 proteins in Reh cells. We revealed that intrinsic apoptotic death played a critical role in 20(S)-GRh2-treated Reh cells because we observed an increase in apoptotic cells, mitochondrial membrane degradation, and release of cytochrome c and cleavage of caspase-9, -3.

In conclusion, 20(S)-GRh2-induced apoptosis in Reh cells is associated with the mitochondria-mediated apoptosis pathway via a caspase-dependent mechanism. 20(S)-GRh2 could stimulate the activation of caspase-9 through the mitochondrial pathway, as demonstrated by the early loss of MMP and cytochrome c release from mitochondria. These results, together with previous findings, demonstrate that 20(S)-GRh2 induced apoptosis through mitochondrial signaling pathways (cytochrome c and caspase-3) in Reh cells. Moreover, the activation of caspase-9 plays an important role in 20(S)-GRh2-induced cell apoptosis in vitro. Therefore, 20(S)-GRh2 could be a potential and powerful chemopreventive agent to treat human leukemia, although further research must be carried out to fully investigate these mechanisms.

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