Rg1 Induces Apoptosis through Inhibition of the EpoR-Mediated JAK2/STAT5 Signalling Pathway in the TF-1/Epo Human Leukemia Cell Line

Jing Li¹, Qiang Wei¹, Guo-Wei Zuo², Jing Xia¹, Zhi-Mei You¹, Chun-Li Li³, Di-Long Chen¹*

Abstract

Ginsenoside Rg1 is one effective anticancer and antioxidant constituent of total saponins of Panax ginseng (TSPG), which has been shown to have various pharmacological effects. Our previous study demonstrated that Rg1 had anti-tumor activity in K562 leukemia cells. The aim of this study was designed to investigate whether Rg1 could induce apoptosis in TF-1/Epo cells and further to explore the underlying molecular mechanisms. Here we found that Rg1 could inhibit TF-1/Epo cell proliferation and induce cell apoptosis in vitro in a concentration and time dependent manner. It also suppressed the expression of EpoR on the surface membrane and inhibited JAK2/STAT5 pathway activity. Rg1 induced up-regulation of Bax, cleaved caspase-3 and C-PAPR protein and down-regulation of Bcl-2 and AG490, a JAK2 specific inhibitor, could enhance the effects of Rg1. Our studies showed that EpoR-mediated JAK2/STAT5 signaling played a key role in Rg1-induced apoptosis in TF-1/Epo cells. These results may provide new insights of Rg1 protective roles in the prevention and treatment of leukemia.

Keywords: Ginsenoside Rg1 - apoptosis - erythropoietin receptor - JAK2 - STAT5 - leukemia

Introduction

Acute myeloid leukemia (AML) is the most common malignancy of unknown etiology in childhood. Although remissions can be achieved in most patients with interventions of hematopoietic stem cell transplantation and conventional chemical drugs. The poor overall survival rate and common relapse for most cases remain a major cause of treatment failure, as well as the unavoidable toxicities to local norm tissues such as myelosuppression, which stress the need to identify more effective strategies. Many studies have revealed that some abnormally activated signal pathways including Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway dysregulation correlated with the pathogenesis or survival of acute leukemia cells (Lee et al., 2013). Erythropoietin receptor (EpoR) is chosen as a candidate molecular target because of its key role in the regulation of cell growth, proliferation, differentiation and survival (Wu et al., 2012). Epo exerts its action through interaction with two adjacent monomeric EpoR molecules and activates phosphorylation of EpoR cytoplasmic domain-associated protein Janus kinase 2 (JAK2). Among the seven STAT proteins, STAT5 signaling is essential for cell proliferation and universally activated in leukemias. In addition, STAT5 can bind to some fusion oncoprotein that confers self-renewal capability to long-term hematopoietic stem cells and suffices to induce leukemia, leading to the phosphorylation of STAT5 and activation of the STAT5 signalling pathway (Tam et al., 2013). Monomeric STAT5 is phosphorylated by activated JAK2 and then themselves become tyrosine-phosphorylated forming homo- or heterodimeric complexes that translocate to the nucleus. Here they bind to specific gene promoters to activate transcription of a range of target genes, including caspase proteins and Bcl-2 protein family (O’Shea et al., 2002). Since JAK2/STAT5 pathway has been implicated to promote tumour growth directly, especially in hematological malignancies, inhibiting aberrantly activated JAK2, STAT5 or rewired downstream signaling pathways is considered a promising therapeutic approach (Kilpivaara and Levine, 2008; Santos and Verstovsek, 2011).

Ginsenosides are the main components identified in ginseng, which has traditionally been used as a well known herbal medicine for its wide spectrum of pharmacological effects, such as immunomodulatory, anti-ageing, and anti-cancer. Previous researches have found ginsenoside Rg1...
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exerted cytotoxic or cytostatic activities against several human cancer cell lines (Wang et al., 2008; Shi et al., 2009; Liu et al., 2012) and vascular smooth muscle cells (Ma et al., 2006; Gao et al., 2011) through reversal of gene expression by antioxidant, anti-inflammatory and apoptosis mechanisms. Our previous study has proven that Rg1 could not only to decrease proliferation of human cancer cells but also induce chronic myeloid leukemia cells line K562 senescence in vitro (Liu et al., 2012). However, the anti-carcinogenic role of Rg1 in acute myeloid leukemia cells remains to be clarified.

Materials and Methods

Drugs and reagents

Ginsenoside Rg1 was purchased from Jinlin Hongjiu Co., LTD (Jilin, China), and the purity was more than 98.6%; It was dissolved in dimethylsulfoxide (DMSO) to create a stock solution for subsequent dilution and use. Roswell Park Memorial Institute (RPMI-1640), foetal bovine serum (FBS) and phosphate-buffered saline (PBS) were obtained from Gibco (Grand Island, NY, U.S.A.). DMSO, AG490 and Hoechst 33258 were obtained from Sigma (St. Louis, MO, U.S.A.). Trizol Reagent kit and Reverse Transcription (RT) Kit were obtained from Invitrogen (Carlsbad, CA, U.S.A.). 2X SYBR pre-mix EX Taq was obtained from Takara (Dalian, China). Cell Lysis Buffer for Western blot, propidium iodide (PI), fluorescein isothiocyanate (FITC)-conjugated Annexin-V, and Enhanced BCA Protein Assay Kit were obtained from Beyotime (Nanjing, China). Antibodies against EpoR, JAK2, STAT5, Bcl-2, Bax, Cleaved Caspase-3, C-PARP, phospho-JAK2(Tyr1007/1008), phosphor-STAT5(Tyr705), phosphor - EpoR(Tyr485) and B-actin were purchased from Epitomics (Burlingame, CA, U.S.A.). The secondary horseradish peroxidase (HRP)-linked anti-rabbit IgG antibody was obtained from Zhongshan Goldbridge Biotechnology (Beijing China). Enhanced chemiluminescence kit was obtained from Millipore (Billerica, MA, U.S.A.).

Cell line and culture condition

The leukemia cell line TF-1/Epo was obtained from the Institute of Cell Biology (Shanghai, China). TF-1/Epo cells were grown in RPMI-1640 plus10% FBS with essential growth factors: 1U/ml rHuEpo (R&D, CA, U.S.A.) in the incubator at 37°C, 5% CO2; 98% humidity. Cells were used for different assays during logarithmic growth phase.

Cell viability analysis

Cell growth and viability were determined by CCK-8 assay (Dojindo, Japan). Briefly, cells were seeded in 96-well plates (200µl/well) at a density of 1×10^4/ml. After exposure to various concentrations of Rg1 (12.5-200µmol·L-1) for 24, 48, and 72 h, 20µl CCK-8 was added to each well and incubated for an additional 4 h at 37°C. In the experiment with inhibitor, cells were pretreated with or without 75µM AG490 2 h before Rg1(50µM) treatment for an additional 48h. The absorbance of the formazan dye, which is directly proportional to the number of living cells, was measured at 450 nm by a spectrophotometric plate reader (Bio-Rad, CA, U.S.A.).The untreated control cultures received only the vehicle (DMSO<0.1%). All experiments were run in triplicate.

Flow Cytometry Assay

To quantitatively assess the rate of apoptosis, Annexin V-FITC apoptosis detection kit was used. Briefly, 1×10^6 TF-1/Epo cells were seeded in 24-well plates and treated with desired Rg1 treatments for 48 h. Then, the cells were washed twice with cold PBS and resuspended in 500 µl binding buffer containing 5 µl Annexin V-FITC and 5 µl propidium iodide (PI) for 15 min at room temperature in dark. Early apoptosis (AV+PI-) cells were used to determine the level of apoptosis.

In parallel, the percentages of EpoR positively stained cells were determined. After the indicated treatment, cells were washed with PBS, resuspended at a concentration of 1×10⁶ cells/ml and incubated with fluorescein isothiocyanate (FITC)-EpoR (Becton Dickinson, CA, U.S.A.) antibody for 25 min at room temperature in dark. Quantification of apoptotic and EpoR positive cells were analyzed by flow cytometry (Becton Dickinson, CA, U.S.A.) and obtained data were analyzed with CellQuest software.

Nuclear staining with Hoechst

Apoptosis of TF-1/Epo was observed morphologically by nuclear staining with Hoechst 33258 dye. Cells were spun onto glass slides by cytopsin centrifuge, fixed with 4% paraformaldehyde for 10 min at 37°C, and incubated with 50 µM Hoechst 33258 staining solution for 15 min in dark, then washed with PBS for three times. The cells were viewed under a fluorescence microscope (Olympus, Tokyo, Japan).

Ultra structure analyses

For the ultra-structural characteristics observation assay, cells were harvested and fixed at 1×10^6 with 2.5% glutaraldehyde for 6h at 4°C and then with 1% osmium tetroxide for 2h prior to dehydration with ethylalcohol. Ultra-thin sections (60 nm) were prepared and placed on grids, stained with 2% uranyl acetate solution and 0.2% lead citrate in 0.1 M NaOH. The cells were observed by H-600 transmission electron microscopy (Hitachi, Japan).

RNA extraction and quantitative real-time reverse transcription-PCR

The total RNA from TF-1/Epo cells was isolated with TRIZol and cDNA was generated using a High Capacity Invitrogen RT kit and an oligo (dT) primer. cDNA from with or without Rg1 treatment cell samples were amplified by quantitative Real-time PCR with specific primers for F-EpoR(5’-ATCCTGACGCTTCCCTCATC-3’) and R-EpoR (5’-GCCTTCAAACTCGCTC-TCTGG-3’). GAPDH gene was used as an endogenous reference to obtain relative expression values. The primer sequences were F-GAPDH (5’-CATCAAGAAGGTGGTGAAGCA-3’) and R-GAPDH (5’-CGTCAAAGGTGGAGGAGTGG-3’), respectively. The reaction mixture was carried out using 20
ng of template cDNA, 1X SYBR pre-mix EX Taq, and 0.5 μM forward and reverse primers in a final volume of 25 μL. Samples were amplified in the IQ SYBR Green PCR Master Mix (Bio-Rad, CA, U.S.A.) for 40 cycles under the following conditions: 95°C for 5 min, denaturing at 95°C for 20s, annealing at 55°C for 15s, and extension at 72°C for 30s. The efficiency of the target gene amplification was proven by examining the absolute value of the slope of log input amount versus ΔCT. Fold changes of EPOR transcripts were calculated after normalization to endogenous GAPDH, using comparative 2^{-ΔΔCT} method calculated for each observed value where ΔCT was the difference in the observed CT values between the gene of interest and GAPDH. The control group was set as 1. All procedures were repeated in triplicate.

Preparation of cell lysates and Western blot analysis

Cell lysates were prepared with cell lysis buffer (50 mmol/L Tris–Hcl, pH7.5, 150 mmol/L Nacl, 1 mmol/L EDTA, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 0.5 mmol/L Na3VO4, 10 mmol/L NaPpi, 1 mmol/L phenylmethylsulphonyl fluoride, 2μmol/L pepstatin A, 2μmol/L leupeptin) at 4°C. Unsolubilized material was removed by centrifugation and total protein concentration was measured with the bicinchoninic acid assay Kit. After electrophoresis, proteins were then transferred to PVDF membrane and subsequently blocked with 5% non-fat dry milk and 3% bovine serum albumin in Tris-buffered saline and Tween 20 (10 mmol/L Tris, pH7.5; 100 mmol/L Nacl; and 0.1% Tween20), incubated with primary, and then with appropriate horseradish peroxidase–conjugated secondary antibodies. After washing, the signals were visualized by enhanced chemiluminescence and exposed on X-ray film. Actin protein was used as a loading control. Density of each band was quantitated with Quantity One Image software.

Statistics analysis

Results were presented as mean±standard error of the mean for a given number of observations. Statistical significance of differences between control and treated samples were calculated by Statistics Package for Social Sciences (SPSS) software vision 17.0 to Student’s t test. p<0.05 were considered significant. All the experiments were repeated at least three times, each time with three or more independent observations.

Results

Rg1 Inhibits TF-1/Epo Cell Viability

Our results indicated the proliferation of cells was inhibited in a time and concentration-dependent manner after Rg1 treatment for 48h and 72h (Figure1A). However, no obvious toxic effects were observed at 24h, except when the concentration of Rg1 reached over 100μM (Figure 1B ). The IC50 determined after 48h and 72h Rg1 incubation, were 62 and 40 μmol/L respectively. Moreover, addition of AG490, a JAK2 specific inhibitor, could inhibit TF-1/Epo cells growth and make the cells more sensitive to Rg1-induced cell injury (Figure1C). These findings indicated that JAK2 signalling might play an important role in the decreased viability of TF-1/Epo cells.

Figure 1. The Effects of Rg1 on Cell Viability of TF-1/Epo Cells. Cultured cells were treated with various concentrations of Rg1 (12.5, 25, 50, 100, 200 μmol/L) for 24, 48 and 72 h, then examined cell viability were showed over 48 and 72 h; A) and absorbance of 450 nm; B) using CCK-8 assay; C) TF-1/Epo cells under treatment with or without AG490 (75 μmol/L) 2 h before Rg1(50μM) treatment for an additional 48h and then cell viability was analyzed by CCK-8 assay. The results were expressed as the mean±SD (n=3; *p<0.05)
TF-1/Epo cells. As shown in (Figure 3A,B), TF-1/Epo cells treated with Rg1 at various concentrations (12.5, 25, 50μM) for 48h, flow cytometry assay showed that the percentages of EpoR-positive cells decreased in a dose-dependent manner (Rg1-induced cells: 63.8±2.7%, 34.9±3.2% and 23.6±2.9% respectively, control group: 71.4±3.4%). Moreover, low-level EPOR mRNA and protein expression were also found among Rg1-treated TF-1/Epo cells compared with control group by real-time PCR and Western blot (Figure 3C). These results suggested that Rg1 reduced the expression of EpoR and might modulate the EpoR-mediated signalling pathways in TF-1/Epo cells.

**Rg1 induces reduction of EpoR signaling intensity**

Epo is an important cytokine, which stimulates signaling pathways that induce cell growth and survival in the microenvironment surrounding the tumour. To further study whether Rg1 reduces the activation of EpoR that relates to the anti-apoptotic effect of Epo, the effect of Rg1-pretreatment on Epo-induced tyrosine phosphorylation of EpoR was analyzed by Western blot using p-EpoR
Rg1 inactivates the JAK2/STAT5 signalling pathway and regulates the expression of JAK2/STAT5 downstream genes related to cell apoptosis

JAK2/STAT5 constitutively activated in cancer cells plays key role in EpoR-mediated signaling, and regulates the expression of genes that control cell proliferation and cell survival. In order to confirm the effects of Rg1 on JAK2/STAT5 survival pathway, which has an effect on Bcl-2 family and the caspase cascades, Western blot was performed with available antibodies (Anti-JAK2, anti-P-JAK2, anti-STAT5, anti-P-STAT5, anti-Bcl-2, anti-Bax and Cleaved Caspase-3) to determine the specific targets of Rg1(Figure 4C, D). When cells treated with 0, 12.5, 25, and 50µM Rg1 for 48h, there was a concentration-dependent decrease in levels of anti-apoptotic Bcl-2 and dephosphorylation of JAK2 and STAT5, but it had no obvious effects on the expression of JAK2 and STAT5. In addition, the protein levels of Bax and Cleaved Caspase-3 were up-regulated by Rg1 in a concentration-dependent manner. These results demonstrated that Rg1 could regulate the expression of genes involved in cell apoptosis in TF-1/Epo cells, which was at least in part through EpoR-mediated JAK2/STAT5 signal pathway inhibition.

Discussion

In present study, we demonstrated that Rg1 could play as a potential anti-cancer medicine via inhibition EpoR production and reduction JAK2/STAT5 signal pathway activity with a serial of assays. The capacity of various concentrations of Rg1 inhibited cell proliferation of TF-1/Epo acute myelogenous leukemia cell lines in a dose and time-dependent manner (Figure 1A, B). These antiproliferative activities were agreement with previous study that Rg1 has been suggested to inhibit human cancer cell growth (Wang et al., 2007; Li et al., 2008). Apoptosis is the most common way that anti-tumor medicine induces cell death. Apoptosis analysis by flow cytometry showed after exposure to various concentrations of Rg1 for 48h, the percentages of early apoptosis ( Annexin V-positive and PI-negative) of TF-1/Epo cells were gradually increased (Figure 2A,B). Moreover, our results indicated morphological signs of apoptosis after treatment with Rg1 by fluorescent microscope and transmission electron microscope (Figure 2C, D).

Activation of Epo–EpoR pathway is the principal regulator known to be required for growth factor-induced cellular proliferation and helps with erythroid progenitor cell survival and exerts a strong antiapoptotic effect. TF-1/Epo an Epo-dependent erythroleukemia line was considered to express physiologically relevant amount of EpoR similar to those of normal hematopoietic cells (Kitamura et al., 1989). Panax ginseng was demonstrated to exert beneficial effects on the hematopoietic system, in which Total saponins of Panax ginseng (TSPG) was reported to enhance Epo/EpoR-mediated signals in the effect of TSPG on erythropoiesis (Chen et al., 2009). Previous research has shown that biological activities were believed to be the result of the main active ingredients ginsenosides (Raghavendran et al., 2012). These results suggest that ginsenosides Rg1 may act anti-tumor effects in the TF-1/Epo cells via EpoR-mediated pathway. We have shown that the expression of EpoR was markedly decreased and Rg1 might induce TF-1/Epo cells apoptosis via JAK2/STAT5 pathway. As determined by flow cytometry, the expression of EpoR on the surface membrane of TF-1/Epo cells decreased after treatment with Rg1 for 48h (Figure 3A, B). The results of Western blot and Quantitative real-time PCR also displayed that Rg1 decreased the protein and EPOR mRNA level in TF-1/Epo cells (Figure 3A, B).
To investigate the role of JAK2/STAT5 signaling in Rg1 mediated apoptosis, we pre-incubated cells with or without pharmacological inhibitor of JAK2 (AG490) for 2h, followed by incubation with or without Rg1 treatment for 48 h, as CCK-8 results shown in (Figure 1C), Rg1 alone used significantly decreased the cell viability, the addition of AG490 evidently increased antiproliferative effect of Rg1 on TF-1/Epo cells. The JAK2/STAT5 signaling downstream genes, the proteins of Bcl-2 family, are important regulators of apoptosis and can be subcategorized into two types as either proapoptotic (Bax, Bad, Bim, Bak) or anti-apoptotic (Bcl-2, Bcl-xl, Mcl-1) proteins (Lord et al., 2000). In particular, Bcl-2 has been reported to directly inhibit the members of caspase family proteins (Zamzami et al., 1998). The ratio of anti-apoptotic members and pro-apoptotic members of this family determines whether a cell responds to an apoptotic signal (Salomons et al., 1997). Caspase-3 is the ultimate executioner of caspase family and activation of caspase-3 will induce degradation of poly (ADP-ribose) polymerase (PARP) which is substrate protein of caspase-3. Cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis. In this study, Western blot showed Rg1 commonly repressed the expression of Bcl-2, but up-regulated Bax, Cleaved caspase-3 and C-PARP (Figure 4C, D; Figure 2 E). Our results also revealed that AG490 showed partial effects of inducing apoptosis, and could enhance Rg1-induced cell apoptosis more effectively (Figure 2 E).

In conclusion, our results may reflect the mechanisms that Rg1 induces acute leukemia TF-1/Epo cells apoptosis partially via inhibition of EpoR-mediated JAK2/STAT5 signal pathway, down-regulation the ratio of Bcl-2/Bax, triggering the activation of caspase cascades. Further studies remain to be done to certify its effects on other growth factor independent and non-growth factor independent leukemia cells.

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