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

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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 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...
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 - Epo-R(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).

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 plus 10% FBS with essential growth factors: 1U/mL rHuEpo (R&D, CA, U.S.A.) in the incubator at 37°C, 5% CO2; 98% humidity. 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'-GCCCTAAAATCGCT-CTCTG-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'-CGTCGGAAATGAGGAGTGG-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 NaP Pi, 1 mmol/L phenylmethylsulphonyl fluoride, 2 μmol/L pepstatin A, 2 μmol/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 5.5; 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 (Figure 1A). 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 (Figure 1C). These findings indicated that JAK2 signalling might play an important role in the decreased viability of TF-1/Epo cells.

Rg1 triggers apoptosis in TF-1/Epo cells

The percentages of apoptotic cells after treatment by Rg1 at a concentration of 12.5-50μM for 48 h in TF-1/Epo cells were analyzed by flow cytometry. As shown in (Figure 2A, B), an increase in early apoptotic cells (AV+PI-) was presented. The percentages of early apoptosis in TF-1/Epo cells induced by Rg1 were 4.75±0.45%, 11.43±0.57% and 17.91±1.69% respectively. The proportions of early apoptotic cells treated by Rg1 increased in a dose-dependent manner. The apoptosis of TF-1/Epo cells induced by Rg1 was also observed by chromatin condensation and nuclear fragmentation in cells stained with Hoechst 33258 fluorescent dye (Figure 2C) and the formation of apoptotic bodies with transmission electron microscope (Figure 2D). Western blot demonstrated that the expression of Cleaved PARP was increased by Rg1 after a 48-h treatment. Furthermore, when the cells were treated with Rg1 and AG490 in combination, there was a synergistic effect on Cleaved PARP which facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis (Figure 2E). These findings indicated that the anticancer effects of Rg1 might be caspase dependent.

Rg1 reduces the expression of EpoR in TF-1/Epo cells

Previous studies have demonstrated that EpoR is a key point of multiple oncogenic signalling pathways. In the present study, we investigated whether Rg1 could induce the decrease of the level of EpoR expression in

![Figure 1. The Effects of Rg1 on Cell Viability of TF-1/Epo Cells.](image-url) 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)
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TF-1/Epo cells. As shown in Figure 3A,B, TF-1/Epo cells treated with Rg1 at various concentrations (12.5, 25, 50 μM) 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 EPRO 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.
antibody. TF-1/Epo cells were incubated with or without 50µM Rg1 for 6 h, then, the cells were further stimulated with Epo for 5 min, and tyrosine phosphorylation of EpoR in response to Epo was measured. Our results revealed that, phosphorylations of EpoR were at high levels even in the absence of exogenous Epo and Rg1 attenuated the activation of EpoR by exogenous Epo in TF-1/Epo cells. Rg1 treatment for 6 h could not influence the expression of EpoR. These results demonstrated that even total EpoR levels remained unchanged, Rg1 markedly decreased EpoR signaling intensity in TF-1/Epo cells (Figure 4A,B).

Rg1 inactivates the JAK2/STAT5 signaling 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 48 h, 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 of EpoR production and reduction of 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 48 h, 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 microscopy (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 48 h (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 3C). Similar activities were observed in our previous study that TSPG could inhibit the proliferation and induce EpoR internalization in K562 cells (Zuo et al., 2009).

The antiapoptotic and anti-inflammatory effects of Epo must act through either the homodimeric (EpoR/EpoR) or the heterodimeric (EpoR/fcR) receptors. The activation of EpoR and subsequent interactions of these receptors with intracellular proteins are more important than their increased expression (Verdirer et al., 2000). Therefore, we
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Further determined whether Rg1 could inhibit the activity of EpoR in TF-1/Epo cells. Using Western blot, we found that before the expression of EpoR significantly differed between the drug group and control group in a whole-cell protein, following Rg1 treatment for 6 h, p-EpoR decreased (Figure 4A, B), which might contribute to Rg1-induced apoptosis for down-regulation of EpoR-mediated signal transduction pathway. The first step of intracellular signaling through EpoR is dependent on the activation of JAK2 tyrosine kinase, which transduces downstream signaling through STAT5, PI3K, and MAPK signaling pathways (Pelletier et al., 2006). In melanoma cells, down-regulation the level of EpoR resulted in diminished p-Erk in response to Epo stimulation (Kumar et al., 2011). There is an increasing evidence that JAK2 is the part of signaling network downstream of various forms of leukemia. JAK2 has also been implicated in the formation of tyrosine kinase fusion genes which impact on proliferation and apoptosis in a variety of hematologic malignancies, mainly acute leukemias (Walz et al., 2008). JAK2/STAT5 signaling may play crucial role as a molecular target for cancer prevention and therapy. Further investigations are needed to disclose other functions of Rg1 on EpoR and determine its potential use in oncology. As expected, immunoblot analysis showed that Rg1 attenuated JAK2 and STAT5 phosphorylation (Figure 4C, D). Similar findings were found in human renal cell carcinomas with down-regulated EpoR (Wu et al., 2012). However, the expression of JAK2 and STAT5 remained unchanged, suggesting that Rg1 might induce fusion protein like BCR-ABL1 degradation to suppress the constitutive activation of JAK2 and STAT5 (Warsch et al., 2013).

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 subdivcategorized 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 (Salomens 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). Moreover, our results also revealed that AG490 showed partial effects of inducing apoptosis (Figure 2 E). Our results also revealed that AG490 showed partial effects of inducing apoptosis (Figure 2 E). Our results also revealed that AG490 showed partial effects of inducing apoptosis (Figure 2 E). Our results also revealed that AG490 showed partial effects of inducing apoptosis (Figure 2 E). Our results also revealed that AG490 showed partial effects of inducing apoptosis (Figure 2 E). Our results also revealed that AG490 showed partial effects of inducing apoptosis (Figure 2 E). Our results also revealed that AG490 showed partial effects of inducing apoptosis (Figure 2 E). Our results also revealed that AG490 showed partial effects of inducing apoptosis (Figure 2 E). Our results also revealed that AG490 showed partial effects of inducing apoptosis (Figure 2 E). Our results also revealed that AG490 showed partial effects of inducing apoptosis (Figure 2 E). Our results also revealed that AG490 showed partial effects of inducing apoptosis (Figure 2 E). Our results also revealed that AG490 showed partial effects of inducing apoptosis (Figure 2 E). Our results also revealed that AG490 showed partial effects of inducing apoptosis (Figure 2 E). Our results also revealed that AG490 showed partial effects of inducing apoptosis (Figure 2 E). Our results also revealed that AG490 showed partial effects of inducing apoptosis (Figure 2 E). Our results also revealed that AG490 showed partial effects of inducing apoptosis (Figure 2 E). Our results also revealed that AG490 showed partial effects of inducing apoptosis (Figure 2 E). Our results also revealed that AG490 showed partial effects of inducing apoptosis (Figure 2 E). Our results also revealed that AG490 showed partial effects of inducing apoptosis (Figure 2 E). Our results also revealed that AG490 showed partial effects of inducing apoptosis (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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