Total Saponins of Panax Ginseng Induces K562 Cell Differentiation by Promoting Internalization of the Erythropoietin Receptor

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Abstract: Ginseng is a commonly used herbal medicine with a wide range of therapeutic benefits. Total saponins of Panax ginseng (TSPG) is one of the main effective components of ginseng. Our previous studies have shown that TSPG could promote the production of normal blood cells and inhibition of the leukemia cell proliferation. However, whether ginseng can induce the differentiation of leukemia cells is still unclear. This study was to examine the effect of TSPG or the combination of erythropoietin (EPO) and TSPG on the erythroid differentiation of K562 cells, and their corresponding mechanisms regarding erythropoietin receptor (EPOR) expression. Under light and electron microscopes, the TSPG- or TSPG + EPO-treated K562 cells showed a tendency to undergo erythroid differentiation; early and intermediate erythroblast-like cells were observed. Hemoglobin and HIR2 expressions were significantly increased. As determined by Western blotting analysis, the EPOR protein level in the K562 cytoplasmic membrane was significantly decreased after TSPG treatment, while its cytoplasm level increased in a dose-dependent manner. However, the total cellular EPOR level was unchanged. These results indicate that TSPG-induced erythroid differentiation of K562 cells may be accompanied by the internalization of EPOR. Thus, our study suggests that treatment with a combination of TSPG and EPO may induce erythroid differentiation of K562 cells at least in part through induction of EPOR internalization.

Keywords: TSPG; K562 Cells; Erythroid Differentiation; EPOR.
Introduction

Leukemia is a leading cause of death among people under the age of 20. Present strategies for the treatment of leukemia are focused on the removal or deactivation of cancerous cells. One such strategy is chemotherapy. However, it has a number of predominant disadvantages and toxicities. Therefore, differentiation-inducing therapy seems to be a promising approach, especially in some patients who cannot tolerate intensive chemotherapy or bone marrow transplantation. Therefore, exploring potent differentiation inducers, devoid of general toxicities, and their applications in the treatment of leukemia have been the subject of universal clinical interest (Hait et al., 1993).

Inducing differentiation has been demonstrated in an increasing number of compounds extracted from traditional Chinese medicines (TCMs) (Huang et al., 2005; Tai and Cheung, 2007; Zhao et al., 2008). Ginseng is an herbal medicine derived from the roots of species from the genus Panax and is a very important TCM for “invigorating qi.” The most effective components of ginseng are the total saponins of Panax ginseng (TSPG). Our previous studies have proven that TSPG favored the development of normal blood cells and inhibited the proliferation of leukemia cells (Wang et al., 2006a; 2006b). It has been reported that ginseng induced neuronal differentiation of PC12 cells (Mizumaki et al., 2002). However, whether ginseng can induce differentiation of leukemia cells is still unclear.

The human cell line K562, which was established from a patient with chronic myelogenous leukemia (Lozzio and Lozzio, 1975), has been widely used for in vitro studies of erythropoiesis (Gambari and Fibach, 2007). These cells indeed can be triggered to undergo erythroid differentiation by a variety of chemicals, such as butyric acid, cytosine arabinoside, and hemin (He and Yuan, 2007; Moosavi et al., 2007; Rabizadahe et al., 2007; Sutherland et al., 1986; Gambari and Fibach, 2007). In the presence of these compounds, K562 cells develop phenotypic characteristics similar to those of normal red blood cells, including surface antigens and hemoglobin in synthesis. Our study was undertaken to examine whether TSPG would induce K562 cells to differentiate into erythroid cells.

Erythropoietin receptor (EPOR) expression in hematopoietic cells plays an important role in erythropoiesis. It was demonstrated that, in addition to cytostatic properties, antitumor drugs such as aclacinomycin, could modulate the expression of differentiation factor receptors (i.e., EPOR) to induce erythroid differentiation of K562 leukemia cells (Jeannesson et al., 1991). The aim of the present study was to investigate the effect of TSPG or the combination of erythropoietin (EPO) and TSPG on the erythroid differentiation of K562 cells, and their corresponding mechanisms of EPOR expression.

Materials and Methods

Reagents and Drugs

Roswell Park Memorial Institute (RPMI-1640) medium was purchased from Gibco-BRL (Gaithersburg, MD, USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), from Sigma (St. Louis, MO, USA); rabbit anti-human EPOR, from Santa Cruz
Biotechnology (Santa Cruz, CA, USA); and horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG, Histostain™ Plus immunohistochemical kits, and diaminobenzidine (DAB) reagent, from Beijing Zhongshan Goldbridge Biotechnology Co. (Beijing, China). The standard protein was from the Shanghai Institute of Biochemistry, Chinese Academy of Sciences, and the polyvinylidene difluoride (PVDF) membrane was purchased from NEN-Dupont (Boston, MA, USA). The reagents for extracting the total, cytoplasmic, and membrane proteins were from BioVision (Palo Alto, CA, USA).

TSPG characterized saponin mixture quantitatively containing at least eight glycosides as known ginsenosides [Rb1 (20.48%), Rb2 (0.13%), Rc (0.52%), Rd (0.34%), Re (2.98%), Rf (6.53%), Rg1 (22.16%), Rg2 (9.61%), Rg3 (3.0%) and other minor ginsenosides and components (34.25%), according to an HPLC-method for separation and quantitative determination of ginsenosides by Chen et al. (2000) with minor modification] from roots of Panax ginseng C. A. Meyer, extracted and purified by the methods described by Chi et al. (1992). It was supplied from the Chongqing Institute of Traditional Chinese Medicine and diluted to 1 g/L with IMDM medium followed by sterile filtration.

**Cell Culture and Treatment**

K562 cells were kindly provided by the Department of Clinical Biochemistry, Chongqing Medical University, and cultured in IMDM containing 10% fetal bovine serum (FBS), and cells were passaged every 2–3 days. Cells in their logarithmic growth phase were divided into the following groups: control (without treatment); TSPG (TSPG was added to a final concentration of 100 or 200 mg/L); TSPG + EPO (100 or 200 mg/L TSPG combined with 1 × 10^3 U/L EPO).

**Morphological Observation under Light Microscope and Cytochemical Staining**

Cells were harvested 3 days after incubation with TSPG or TSPG + EPO, cytopspun onto slides and stained with Wright’s stain. The slides were then observed under a light microscope. The volumes, diameters, and nuclei/cytoplasm ratios of 300 cells were measured by using an image Pro Plus software (Media Cybernetics, MD). The cells were also subjected to benzidine or α-naphthyl acetate esterase (α-NAE) staining and visualized under a microscopy. Three visual fields (each containing 100 cells) were randomly selected, and the percentage of cells with positive stain was then calculated using the image analysis system.

**Electron Microscope Examination**

K562 cells were harvested 3 days after incubation and fixed with 0.2 M sodium cacodylate buffer containing 2.5% glutaraldehyde and 0.8% paraformaldehyde, osmificated, and embeded into epon according to standard procedures for ultrastructural examination. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined under a Hitachi H600 transmission electron microscope.
Detection of Hemoglobin by Colorimetry

K562 cells were treated with TSPG of various concentrations (0, 50, 100, 200 or 400 mg/L) for 3 days. Cells were harvested and lysed, and the content of hemoglobin was determined spectrophotometrically.

Flow Cytometry Assay

K562 cells were treated with various concentrations (0, 100, 200, 300, or 400 mg/L) of TSPG for 3 days. The cells were washed with phosphate-buffered saline (PBS), resuspended at a concentration of $1 \times 10^6$ cells/ml and incubated with fluorescein isothiocyanate (FITC)-EPOR antibody for 25 min at room temperature in dark. Flow cytometry was performed using a FACS Vantage flow cytometer (Becton Dickinson), and the percentage of positively stained cells was determined.

Immunocytochemical Staining

K562 cells were treated with TSPG (0 or 200 mg/L) for 3 days. The cells were subsequently cytospun onto slides. Immunocytochemical staining was performed by the primary anti-EPOR antibody at 1:400.

In another immunocytochemical staining experiment, K562 cells were treated with TSPG + EPO for 3 days. The cells on the slides were stained by the primary anti-HIR2 antibody (1:300).

Western Blot Analysis

K562 cells were treated with various concentrations (0, 100, 200, 300, or 400 mg/L) of TSPG for 3 days. The cells were harvested and the total, cytoplasmic, and membrane proteins were extracted by using the commercial kits according to the manufacturer’s instructions. Aliquots of cell lysates containing 50 µg proteins were separated on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to PVDF membranes. The membranes were blocked with TBST buffer containing 5% skimmed milk and incubated overnight with an EPOR antibody (1:400) at 4°C, and followed by the addition of HRP-labeled goat anti-rabbit IgG (1:500) and DAB visualization of the bands according to a previously described method (Wang et al., 2006b).

Statistical Analysis

All statistical analyses were performed by using SAS (SAS Institute, Cary, NC). One-way analysis of variance (ANOVA) and the Dunnett test were performed to analyze the mean value of each group. The critical p value was set as 0.05; a probability value of $p < 0.05$ was considered to be statistically significant.
TOTAL SAPONINS OF *PANAX GINSENG* INDUCES K562 CELL DIFFERENTIATION 751

**Results**

**Morphological Changes**

In control group, the cell volume, particularly the nuclear volume, was relatively large. The nucleoli were clearly visible and the nuclei/cytoplasm ratio was high. The cytoplasm showed strong basophilia; no specialized granule was observed (Fig. 1a). In the groups treated with TSPG at 100 or 200 mg/L, the cell volume and nuclear diameter were smaller on day 3, the nucleoli were not very distinct or invisible, the nuclear chromatin was condensed, the
cytoplasm was abundant, and the nuclei/cytoplasm ratio was decreased (Fig. 1b, Table 1). The TSPG + EPO-treated K562 cells showed a tendency to undergo erythroid differentiation; early and intermediate erythroblast-like cells could be observed (Fig. 1c).

**Ultrastructure**

In the control group, the cell volume was relatively large and the nucleus was immature. The nucleoli were clearly visible (3–4 nucleoli each cell). There was little heterochromatin, much euchromatin, and a number of mitochondria in the cytoplasm. The matrix electron density was high; ribosomes were abundant; and the nuclei/cytoplasm ratio was high. In the TSPG + EPO-treated group, the cell volume was smaller, the heterochromatin within the nuclei increased and the electron density increased, and the nucleoli were not distinct or invisible. The proportion of cytoplasm and the number of organelles increased. Some early, intermediate, and late erythroblast-like cells were observed (Figs. 1h, 1i).
Cytochemical Changes

In the control group, the percentage of K562 cells stained positively with benzidine was 5 ± 1.2%, while none of the cells were stained with α-NAE. After the cells were treated with 200 mg/L TSPG + EPO (1 × 10^3 U/L) for 3 days, their hemoglobin granules were relatively more distinct, and the percentage of cells stained with benzidine increased up to 15 ± 4.3%; the difference was significant when compared with the control group (p < 0.05). Six days after the treatment, the hemoglobin granules were more distinct and more visible, and the number of cells stained with benzidine increased up to 34 ± 1.9%; the difference was significant when compared with the control group (p < 0.01, Figs. 1d, 1e, 1g).

The Effect of TSPG + EPO on the HIR2 Expression of K562 Cells

K562 cells were treated with TSPG + EPO for 3 days, immunocytochemical staining showed that the percentage of cells stained positively with HIR2 was 48.67 ± 3.55%, which was significantly higher than that of the control group (19.91 ± 2.13%, p < 0.01). After treatment for 6 days, the number of positively stained cells had increased to 89.93 ± 3.98% (Figs. 1f, 1g).

The Effect of TSPG on the Hemoglobin Content of K562 Cells

K562 cells were treated with TSPG at various concentrations (0, 50, 100, 200, 400 mg/L) for 3 days. High concentration of TSPG in the range of 100–400 mg/L significantly increased the hemoglobin content (Fig. 2).

Effect of TSPG on EPOR Expression

K562 cells were treated with TSPG at various concentrations (100, 200, 300, 400 mg/L) for 3 days, flow cytometry assay showed that the percentages of EPOR-positive cells

![Figure 2](image_url)

Figure 2. The effect of TSPG on the hemoglobin content of K562 cells. TSPG in the range of 100–400 mg/L significantly increased the hemoglobin content (*p < 0.05, **p < 0.01, vs. control).
decreased in a dose-dependent manner (37.15 ± 1.15%, 20.65 ± 2.30%, 16.21 ± 1.46%, and 15.59 ± 2.19%, respectively). As determined by immunocytochemical staining, the cell membrane and the cytoplasm showed intense and weak staining for EPOR, respectively, and the nuclei/cytoplasm ratio was high in the control group. Three days after the treatment with 200 mg/L TSPG, the cell membrane showed weak staining, while the cytoplasm displayed intense staining, and the nuclei/cytoplasm ratio was significantly decreased. Western blot analysis showed that EPOR levels in the total protein of K562 cells after treatment with TSPG at various concentrations (0, 100, 200, 300, 400 mg/L) were 148.80 ± 6.61, 148.40 ± 6.07, 149.20 ± 5.54, 149.80 ± 7.40, and 149.40 ± 5.68 optical density (OD)/µg. There was no significant difference among these groups. The cytoplasmic EPOR levels were increased in the TSPG-treated K562 cells in a dose-dependent manner to a greater extent. However, the EPOR levels in the membranes were decreased.

Discussion

Induced differentiation and the mechanism underlying the reversal of leukemia cells have become popular topics in biomedical fields (Yu et al., 2008; Cailleteau et al., 2008; Jakubowska et al., 2007). A possible approach to establish a new treatment is to identify agents in TCM that are capable of inhibiting the malignant proliferation of leukemia cells and inducing them to mature differentiation from treasure medical library. Our current work showed that, notably, TSPG (100–200 mg/L) induced K562 cells to differentiate into erythrocytes as assessed by the morphology, the Benzidine staining for hemoglobin synthesis, direct hemoglobin detection, and immunological marker. However, we also noted that K562 cells did not continuously differentiate into more mature cells, and that nuclear and cytoplasmic development was disrupted. The reason may be that the differentiation of K562 cells requires hematopoietic growth factors to further induce differentiation after TSPG treatment.

Hematopoietic cells in different stages of maturation and various cell lines express different hematopoietic growth factor (HGF) receptors and rely on stimulation of various HGFs (Testa et al., 1993; Ziegler and Kanz, 1998; Cluitmans et al., 1997). We observed the synergistic effect of TSPG and EPO on erythroid differentiation of K562 cells, the results showed that the level of reduction in cell diameter and volume induced by TSPG-EPO treatment was higher than that induced by TSPG alone, which suggested that the combination of TSPG and cytokine could induce K562 cells to differentiate into more mature cells. This effect was also reflected by morphological changes and higher HIR2 expression.

It has been well established that EPOR is mainly expressed in the colony-forming unit-erythroid (CFU-E) stage, with a lower level on the early erythroid progenitor cells, mature erythroid cells, and on the surface of reticulocytes and erythrocytes lacking EPOR (Wu et al., 1995; Lin et al., 1996; Kieran, 1996; Krantz, 1991). K562 cells differentiated into mature erythroid cells by treatment with TSPG for 72 hours. Therefore, the decrease of EPOR-positive cells after TSPG treatment was attributed in part to TSPG-induced differentiation of K562 cells.
TOTAL SAPONINS OF PANAX GINSENG INDUCES K562 CELL DIFFERENTIATION 755

EPO is a factor that helps with erythroid progenitor cell survival and exerts a strong anti-apoptotic effect (Koury and Bondurant, 1990; Kelley et al., 1993). The main function of EPOR, as in the case of EPO, is conducing anti-apoptotic signal transduction. Our previous studies have shown that TSPG could not only inhibit the proliferation of K562 cells but also induce the apoptosis of K562 cells; it is suggested that TSPG may induce apoptosis of K562 cells by inhibiting the expression of EPOR protein and subsequently inhibiting the proliferation of K562 cells.

EPOR is deficient on the surfaces of normal and transformed erythroid cells. Approximately 1,000 molecules per cell are expressed, and most of them are intracellular (Neumann et al., 1993). Following treatment with a combination of EPO and EPOR, internalized EPO-EPOR could be detected in cells (Yen et al., 2000; Supino-Rosin et al., 1999). Approximately 60% EPOR was internalized and the remaining 40% degraded slowly and eventually reached a final level of 25%. The EPO within cells may be degraded in the lysosome, while the EPOR may either re-enter the cycle without degradation or be degraded along with EPO. Therefore, receptors on cell surface were added and maintained in combining with EPO through re-synthesis and relocation of EPOR from cytoplasm to membrane.

As determined by the immunocytochemical staining experiment, EPOR expression increased in the cytoplasm but decreased in K562 cell membrane following treatment with TSPG. Therefore, we hypothesize that TSPG induce internalization of EPOR in K562 cells during the process of K562 cells being caused to differentiate into erythroid lineage cells. In other words, TSPG promote endocytosis of EPOR from the cell membrane to the cytoplasm, leading to a decreased EPOR expression on the membrane and an increased expression in the cytoplasm.

In order to elucidate the molecular mechanism of the effect of TSPG on EPOR on the surface of the K562 cell, we evaluated the expression of EPOR in the whole-cell, plasma, and membrane proteins of K562 cells by Western blotting analysis. The results showed that: (1) the expression levels of EPOR in a whole-cell protein did not differ significantly between the drug groups and control group; (2) EPOR protein expression in the cytoplasm of cells induced by TSPG was enhanced in a dose-dependent manner; (3) EPOR protein expression on the cell membrane was reduced. Based on these results, we concluded that TSPG could induce EPOR internalization in K562 cells.

In summary, the current study shows that TSPG may induce erythroid differentiation of K562 cells at least in part through induction of EPOR internalization. Our future investigation will determine whether TSPG affects EPOR-mediated signal transduction pathway.

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TOTAL SAPONINS OF PANAX GINSENG INDUCES K562 CELL DIFFERENTIATION


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