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Full-length article

Ginsenoside Rg1 promotes bone marrow stromal cells proliferation *via* the activation of the estrogen receptor-mediated signaling pathway[1]

Xin-zheng LU[2,7], Jun-hong WANG[2], Xin WU[4], Lei ZHOU[3], Li WANG[5], Xiao-wen ZHANG[6], Ke-jiang CAO[2], Jun HUANG[2]

[2]Department of Cardiology, the First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China; [3]Department of Medicine, Baylor College of Medicine, Houston, Texas, USA; [4]Department of Clinical Laboratory Medicine, Nanjing Benq Hospital, Nanjing 210029, China; [5]Department of Internal Medicine, the Hospital Affiliated with Shanghai Meishan Group, Nanjing 210039, China; [6]Department of Pharmacology, Nanjing Medical University, Nanjing 210029, China

Key words

ginsenoside Rg1; bone marrow stromal cell; estrogen receptor; proliferation

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[7] Correspondence to Prof Xin-zheng LU.
Phn 86-25-8371-8836, ext 6325.
Fax 86-25-8367-3396.
E-mail xzlu@njmu.edu.cn

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Abstract

Aim: To investigate the possible mechanisms of ginsenoside Rg1 promoting bone marrow stromal cell (BMSC) proliferation. **Methods:** BMSC were isolated from bone marrow of Sprague–Dawley rats and maintained *in vitro*. After stimulation with 1 μmol/L ginsenoside Rg1 for the indicated time, the proliferation ability of BMSC were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and [^{3}H]-thymidine incorporation assays. The estrogen receptor (ER) binding activity of BMSC was determined by a specific ER antagonist and an ER binding assay. Furthermore, the influence of ginsenoside Rg1 on the expression of ERα was investigated by RT–PCR and Western blotting assays. **Results:** BMSC proliferation stimulated by 1 μmol/L ginsenoside Rg1 can be completely blocked by 1 μmol/L ER antagonist ICI 182, 780, or ERα-specific antagonist methylpiperidinopyrazole. Moreover, Rg1 failed to displace the specific binding of [^{3}H]17β-estradiol to BMSC cell lysates, suggesting that no direct interaction of Rg1 with the ER is needed for its estrogenic effects. In addition, 1 μmol/L Rg1 had no effects on the expression of ERα in either the mRNA or protein levels. **Conclusion:** Our results indicate that ERα is essential for mediating the effects of Rg1 on stimulating BMSC proliferation, which might involve the ligand/receptor-independent activation of ERα.

Introduction

The ginseng root, commonly used in traditional Chinese medicine, is widely studied in the West. It has been safely being used in China for more than 2000 years as a tonic against stress. Ginsenosides (steroidal glycosides), in the extracts of various kinds of ginsengs, are the major active components responsible for the pharmacological effects of ginseng on the central nervous, cardiovascular, endocrine, and immune systems[1–6]. There are 2 major classes of ginsenosides, protopanaxatriols (Rg1, Rg2, Re, and Rf) and protopanaxadiols (Rb1, Rb2, Rc, and Rd). They both possess 4 trans-ring rigid steroid skeletons with a modified side-chain at C20, which is absent in estradiol[7]. Ginsenoside Rg1, a steroidal saponin abundantly contained in ginseng, is one of the most active components in ginseng and contributes to many of its effects.

Bone marrow stromal cells (BMSC) were initially identified in the bone marrow (BM) as multipotent, non-hematopoietic progenitor cells that can differentiate into osteoblasts, adipocytes, chondrocytes, tenocytes, skeletal myocytes, and visceral mesodermal cells[8–10]. BMSC can influence hematopoietic stem cell homing and differentiation through cell–cell interaction and by the production of cytokines and chemoattractants. They can also be used to regenerate bone, cartilage, myocardial, and hepatic tissues[10,11]. Since the autologous transplantation of BMSC does not generate immune response and has a limited ten-

dency of tumorigenesis, BMSC have become ideal candidates for cell and gene therapies[12].

Previously, we found that ginsenoside Rg1 could significantly promote the proliferation of cultured porcine BMSC in a dose-dependent manner[13]. This may be particularly ideal for accelerating the recovery of hematopoietic cells and protecting the bone micro-environment after chemotherapy or radiotherapy, facilitating the reconstruction of hematopoiesis after transplantation and promoting the *ex vivo* expansion of BMSC for cell and gene therapies. However, the underlying mechanisms of ginsenoside Rg1 promoting BMSC proliferation remain largely unknown. Since ginsenoside Rg1 is a kind of potent phytoestrogen[7] which shares many targets with estrogens, we postulate that ginsenoside Rg1 might promote BMSC proliferation via the estrogen receptor (ER). Therefore, the ability of ginsenoside Rg1 to activate the ER in BMSC was assessed by using non-specific ERα or ERβ subunit antagonists ICI 182, 780, and specific ERα antagonist methylpiperidinopyrazole (MPP). In addition, the effects of ginsenoside Rg1 on BMSC ERα receptor expression were studied. Our findings indicated that ginsenoside Rg1 promoted BMSC proliferation and required ERα. However, ERα activation might be ligand/receptor independent.

Materials and methods

Isolation of BMSC The protocol was approved by the Committee of Animal Research at Nanjing Medical University (Nanjing, China). Eight-week-old male Sprague–Dawley (SD) rats were purchased from Shanghai SLAC Laboratory Animal Co, Ltd (Shanghai, China). BM was flushed from SD rat tibias and femurs using a 23-gauge needle. BM mononuclear cells were separated by gradient density centrifugation (Lymphoprep; Nycomed Pharma, Oslo, Norway) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (GibcoBRL Life Technologies, USA) in 25-cm^2 flasks at 37 °C in a 5% CO_2 atmosphere. Seventy-two hours later, non-adherent cells were removed. Adherent cells were split at 70%–80% confluence (0.05% trypsin at 37 °C for 5 min; GibcoBRL Life Technologies, USA) and expanded *in vitro*. A homogenous cell population was obtained after 2–3 weeks of continuous culturing[13].

BMSC proliferation assays To assess BMSC proliferation after the treatment of 1 μmol/L ginsenoside Rg1 (Sigma, St Louis, MO, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and [^{3}H]-thymidine incorporation assays were applied as previously described elsewhere[7,13]. Furthermore, in order to determine if ginsenoside Rg1 promotes BMSC proliferation via the ER, ER inhibitor ICI 182, 780, or MPP was added to the culture medium alone or 1 h before the addition of ginsenoside Rg1.

MTT assay BMSC were seeded onto 96-well plates (3×10^3 cells/well) in phenol-red free DMEM supplemented with 10% FBS 24 h before treatment with or without 1 μmol/L Rg1 and 1×10^{-7}, 1×10^{-6}, and 1×10^{-5} mol/L ICI 182, 780, or MPP. Forty-eight hours later, 100 μL of 5 mg/mL MTT solution was added into each well. The plates were then incubated at 37 °C for 4 h. In total, 100 μL DMSO was added into each well and mixed thoroughly before reading on an ultra microplate reader (Bio-Tek Instruments, USA; wavelengths: test, 570 nm; reference, 630 nm).

[^{3}H]-thymidine incorporation assay We previously described the procedures for the [^{3}H]-thymidine incorporation assay. Briefly, BMSC were seeded onto 96-well plates at a density of 3×10^3 cells per well and grown in DMEM supplemented with 15% FBS for 7 d. On the third day, half the medium was replaced with fresh growing medium, and the cells were treated with or without ginsenoside Rg1 (1 μmol/L) and 1×10^{-7}, 1×10^{-6}, and 1×10^{-5} mol/L ICI 182, 780 or MPP. Then, on the sixth day, 185 GBq/L of [^{3}H] thymidine was added to each well and incubated for 24 h. The cells were then rinsed with ice-cold phosphate-buffered saline (PBS) for 3–5 min and 10% trichloroacetic acid for 30 min. Finally, the cells were lysed in 200 μL of 200 mmol/L NaOH and left overnight at 4 °C. Radioactivity was determined by scintillation counting.

ER binding assay The ER binding assay used in the present study was described elsewhere with slight modification[7]. Briefly, the BMSC were cultured in phenol red-free DMEM supplemented with 5% charcoal-stripped FBS for 72 h before the cellular proteins were prepared. The cells were washed twice with ice-cold PBS and scraped off the flasks. The cells were suspended in PBS with 1 nmol/L phenylmethylsulfonyl fluoride and 50 μmol/L protease inhibitor cocktail P8340 (Sigma, USA). The cells were then passed 20 times through a 26-gauge needle for complete breakage. The cell lysate was centrifuged, and the supernatant was used for the receptor binding assay. The final protein concentration in the binding assay was 1 mg/mL in a total volume of 500 μL containing different concentrations of [2,4,6,7-^{3}H] estradiol (GE Healthcare, USA) with or without a 500-fold excess of either non-labeled estradiol or Rg1. Binding reactions were carried

out at 4 °C for 24 h before the lysate–ligand mixture was mixed thoroughly with dextran-coated charcoal. The protein was then separated from the dextran-coated charcoal by centrifugation and transferred to scintillation vials for radioactivity measurement. Specific ligand binding was determined at each concentration of 17β-estradiol by subtracting the radioactive count in the vials containing both radiolabeled and cold ligand [displacement count (DC)] from the vials containing only the radiolabeled ligand [total binding (TB)]. The percentage of specific ligand binding was calculated as: 100%×(TB–DC)/TB.

RT–PCR and Western blotting for ERα expression The cells were incubated with ginsenoside Rg1 for 30 min, 1, 4, and 8 h for the analysis of the expression of ERα in BMSC, After incubation, the BMSC were washed with cold PBS and harvested for RT–PCR and the Western blot analysis. For RT–PCR, total RNA was isolated from the cells by using TRIzol reagent. Total RNA was used to generate cDNA in each sample using SuperScript II reverse transcriptase with oligo(dT) 12–18 primers (Invitrogen, CA, USA). The expression of ERα was performed by semiquantitative RT–PCR. The primer sets for ERα and the housekeeping gene GAPDH were 5′-TGAAGCCTCAATGATGGG-3′ (forward) and 5′-CAGGACTCGGTGGATGTG-3′ (reverse) for ERα, and 5′-TATCGGACGCCTGGTTA-3′ (forward) and 5′-CATTTGATGTTAGCGGGAT-3′ (reverse) for GAPDH to yield products of 424 and 222 bp, respectively. PCR amplification was performed on a GeneAmp 9600 PCR system (Perkin–Elmer, USA). The PCR products were analyzed using agarose gel electrophoresis. Optical densities of ethidium bromide-bound DNA bands were quantified using Quantity-One software (Bio-Rad, USA), and the mRNA expression levels were normalized to the expression of GAPDH. For Western blotting, the cells were lysed in 100 μL RIPA sample buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, and 2 mmol/L orthovanadate]. Lysates were clarified, and protein contents were quantified by the Bradford method. Thirty micrograms of the clarified cell lysate was applied to 12% SDS–PAGE followed by transferring to a polyvinylidene difluoride membrane. The membrane was incubated with a monoclonal antibody against ER (Abcam, USA) and then with horseradish peroxidase-conjugated appropriate secondary antibodies followed by enhanced chemiluminescence reaction (Pierce, USA).

Statistical analysis Data were reported as mean±SEM. Significance of difference between group means was determined by one-way ANOVA. The independent Student's *t*-test was used to calculate statistical significance between the control group and each treatment group in the MTT and [^{3}H]-thymidine incorporation assays. A P-value <0.05 was considered statistically significant.

Results

With the exception of 1×10^{-5}mol/L MPP which presented cytotoxicity, in the absence of ginsenoside Rg1, the other compounds used did not affect the cell cultures. In general, the compounds used in this study did not have a dose-dependent effect on the proliferative action of ginsenoside Rg1. The results presented here therefore only refer to the concentration of each drug that elicited the most effective response: 1×10^{-6}mol/L ICI 182 780, and 1×10^{-6}mol/L MPP.

Ginsenoside Rg1 stimulates rat BMSC proliferation The MTT and [^{3}H]thymidine incorporation assays were used to evaluate the effects of ginsenoside Rg1 on the proliferative ability of BMSC *in vitro*. After incubation with 1 μmol/L Rg1, the absorbency value of the cells increased significantly (0.38±0.05 *vs* 0.68±0.07; P<0.01). The thymidine incorporation value was enhanced from 3569.80±809.75 to 8321.00±2408.42 (P<0.01; Table 1).

Role of ER in mediating ginsenoside Rg1 action The binding of [^{3}H] 17β-estradiol to BMSC lysates in the presence and absence of a 500-fold excess of cold Rg1 and 17β-estradiol was determined. In the case of 17β-estradiol, a 500-fold excess of cold ligand was sufficient to displace the specific binding of [^{3}H] 17β-estradiol with its receptor in the BMSC cell lysates. Figure 1 demonstrates the percentage of specific binding of 17β-estradiol at different concentrations of 17β-estradiol. In contrast, 0.1 μmol/L non-labeled Rg1 failed to displace the specific binding of

Table 1. Effects of Rg1, ICI 182, 780, and MPP on BMSC proliferation in rats. n=10. mean±SEM (Student's *t*-test). [b]P<0.05, [c]P<0.01 *vs* control.

Assay	Control	Rg1	Rg1+ICI 182 780	Rg1+MPP	ICI 182 780	MPP
MTT (OD_{570} value)	0.38±0.05	0.68±0. 07[c]	0.37±0.08	0.36±0.07	0.33±0.06	0.31±0.07
[^{3}H]-thymidine incorporation (cpm)	3569.80±809.75	8321.00±2408.42[c]	3690.70±921.90	3759.30±826.00	3367.60±783.71	3403.03±854.66[b]

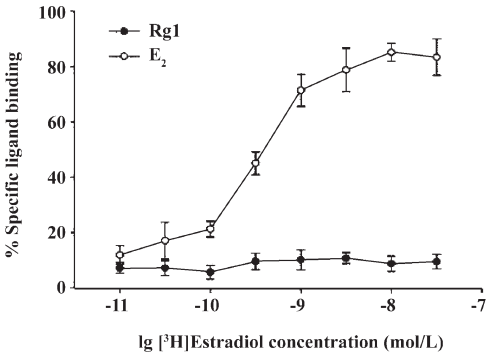

Figure 1. ER binding assay. Total [^{3}H] estradiol binding to the BMSC cellular protein was determined at different concentrations of radiolabeled ligand in the absence of non-labeled competitor. Radioactive counts were also measured in the presence of a 500-fold molar excess of either non-labeled estradiol or Rg1 attempting displacement of [^{3}H] 17β-estradiol (E_2) from ER. Percentage of specific ligand binding was calculated based on the following equation: 100%×(TB–DC)/TB. Data are the mean of duplicate determinations, and the experiment was repeated twice. Specific binding of 17β-estradiol increases with increasing doses of 17β-estradiol. No specific binding of Rg1 to ER was found at any concentration of 17β-estradiol tested.

[^{3}H] 17β-estradiol with its receptor, which indicated that no direct interaction existed between ERα and Rg1 at concentrations as high as 1 μmol/L. However, when pretreated with 1×10^{-6} mol/L ICI 182, 780, or MPP, the MTT and [^{3}H]-thymidine incorporation assays demonstrated that both ICI 182, 780, and MPP could completely repress the proliferative ability of BMSC stimulated by ginsenoside Rg1, as shown in Table 1.

Role of ginsenoside Rg1 on the expression of ERα in BMSC As ICI 182, 780, and MPP showed similar action on ginsenoside Rg1-induced BMSC proliferation, we therefore supposed that ERα, not ERβ, was an essential mediator of Rg1 action. We then examined if Rg1 exerted E_2-like activity by regulating the expression level of ERα in BMSC. As ERα was the major isomer expressed in BMSC, we studied its expression in response to Rg1 in the present study. As shown in Figure 2, the time-course response effects of Rg1 on the ERα protein and mRNA expressions were determined. Our results showed that 1 μmol/L Rg1 did not significantly alter the ERα protein expression. Similarly, its mRNA expression level in BMSC was unaltered in response to treatment with 1 μmol/L Rg1. Thus it appears that Rg1 behaves differently from E_2 and does not change the expression of ERα in BMSC.

Discussion

Ginsenoside Rg1 is a steroid saponin that shares similar structural features and targets organs with steroid hormones[1]. Ginsenosides are amphiphilic in nature and have the ability to intercalate into the plasma membrane. There is evidence suggesting that ginsenosides interact directly with specific membrane proteins[14,15]. Moreover, like steroid hormones, Rg1 has previously been shown to interact with the glucocorticoid receptor and initiate genomic effects[16,17].

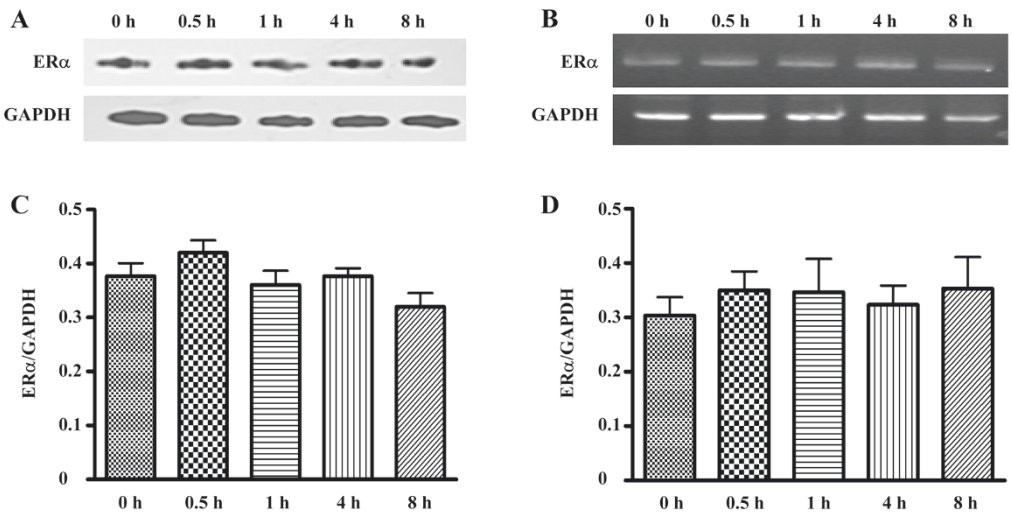

Figure 2. Effect of Rg1 on the ERα protein and ERα mRNA expressions. BMSC were treated with 1 μmol/L Rg1 with increasing time (0, 0.5, 1, 4, and 8 h). Representative protein and total RNA were isolated and subjected to Western blotting (A) and RT–PCR (B) analyses. ERα protein (C) and mRNA (D) expression levels are presented, which are expressed as a ratio to the expression of GAPDH, respectively. Results were obtained from 3 independent experiments and expressed as mean±SEM. *n*=3.

BMSC are the precursors of different mesenchymal tissues that play a crucial role in the construction of normal and pathological microenvironments. Previously, we observed that ginsenoside Rg1 could greatly enhance *ex vivo* BMSC proliferation in a dose-dependent manner, and 1 μmol/L Rg1 was sufficient to enhance the maximum proliferation of BMSC[13]. So in the present study, we chose 1 μmol/L as the appropriate concentration for Rg1 to stimulate the proliferation of BMSC. Because BMSC play important roles in hemopoiesis and in regulating the body's immunoreactivity, the ability of ginsenoside Rg1 to enhance BMSC proliferation is of potential value for clinical application. Previous studies have shown that Rg1 has estrogen-like properties and exerts its action via the activation of ERα in human breast cancer MCF-7 cells[7]. As considerable evidence has indicated that of the 2 ER (α and β), ERα is most likely the dominant ER regulating bone metabolism, at least in mice[18]. In the present study, ERα was abundantly expressed in BMSC[19], so we postulated that ginsenoside Rg1 might also exhibit an estrogen-like effect on the proliferation of BMSC via ERα. After ginsenoside Rg1 treatment, the BMSC demonstrated significant proliferative ability, which was consistent with our previous study. In total, 1 μmol/L ginsenoside Rg1 treatment led to more protein synthesis, which was proven by the MTT and [^{3}H]-thymidine incorporation assays. As anticipated, BMSC proliferation stimulated by ginsenoside Rg1 could be completely abolished by a pretreatment with a pure ER antagonist, ICI 182, 780, or ERα-specific antagonist MPP, which indicates that ERα may be the key subunit for Rg1 to exert its action on BMSC. However, unlike E_2 and other phytoestrogen, such as genistein[20], Rg1 did not directly bind to ER and change the expression of ERα in BMSC, which was confirmed by RT–PCR and Western blotting. Thus the increase in E_2-like activity by Rg1 in BMSC appears to activate ERα through its downstream signaling molecular activation. Lau found that Rg1 could activate ERα via the phosphorylation of the AF-1 domain of ERα at serine 118 residue and exert estrogen-like activities[21]. Moreover, Sims *et al* demonstrated the involvement of the crosstalk between ER–(insulin-like growth factor)IGF–insulin resistance (IR)-dependent signaling pathways in mediating the actions of Rg1 in MCF-7 cells[22], while other researchers found that genistein, the soybean phytoestrogen, promoted mouse BMSC proliferation and osteoblastic maturation via the nitric oxide (NO)/GC-mediated pathway[23]. Therefore, the involvement of other signaling pathways, such as the IGF–IR-dependent pathway or NO/GC pathway in BMSC requires further study.

In summary, the present study provides a new paradigm for characterizing the molecular actions of ginsenoside Rg1 on the proliferative ability of BMSC. Similar to the action of MCF-7 cells, the E_2-like activities of ginsenoside Rg1 are not mediated by direct binding interaction with ERα. Most importantly, our results show that ER is essential for mediating the actions of Rg1 in the stimulation of BMSC proliferation. Future studies will be needed to delineate the detailed mechanism of action of ginsenosides in BMSC, as well as to provide insights for understanding the complex actions of ginseng extract in the human body.

Author contribution

Xin-zheng LU designed research; Jun-hong WANG, Xin WU, and Li WANG performed research; Li WANG, contrbuted new analytical tools and reagents; Xiao-Wen ZHANG analyzed data; Jun-hong WANG and Lei ZHOU wrote the paper.

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