Down-regulation of Phosphoglucose Isomerase/Autocrine Motility Factor Enhances Gensenoside Rh2 Pharmacological Action on Leukemia KG1α Cells

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Abstract

Aims and Background: Ginsenoside Rh2, which exerts the potent anticancer action both in vitro and in vivo, is one of the most well characterized ginsenosides extracted from ginseng. Although its effects on cancer are significant, the underlying mechanisms remain unknown. In this study, we sought to elucidate possible links between ginsenoside Rh2 and phosphoglucose isomerase/autocrine motility factor (PGI/AMF). Methods: KG1α, a leukemia cell line highly expressing PGI/AMF was assessed by western blot analysis and reverse transcription-PCR (RT-PCR) assay after transfection of a small interfering (si)-RNA to silence PGI/AMF. The effect of PGI/AMF on proliferation was measured by trypan blue assay and antibody array. A cell counting kit (CCK)-8 and flow cytometry (FCM) were adopted to investigate the effects of Rh2 on PGI/AMF. The relationships between PGI/AMF and Rh2 associated with Akt, mTOR, Raptor, Rag were detected by western blot analysis. Results: KG1α cells expressed PGI/AMF and its down-regulation significantly inhibited proliferation. The antibody array indicated that the probable mechanism was reduced expression of PARP, State1, SAPK/JNK and Erk1/2, while those of PRAS40 and p38 were up-regulated. Silencing of PGI/AMF enhanced the sensibility of KG1α to Rh2 by suppressing the expression of mTOR, Raptor and Akt. Conclusion: These results suggested that ginsenoside Rh2 suppressed the proliferation of KG1α, the same as down-regulation of PGI/AMF. Down-regulation of PGI/AMF enhanced the pharmacological effects of ginsenoside Rh2 on KG1α by reducing Akt/mTOR signaling.

Keywords: Phosphoglucose isomerase - autocrine motility factor - ginsenoside Rh2 - KG1α-mTOR

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Introduction

Leukemia is a type of cancer of the blood or bone marrow characterized by an abnormal increase of immature white blood cells called "blasts". The major interventions of conventional medicine failed to have its effects because of its side effects. Thus, it is urgent for us to find another effective therapy. An increasing number of researches have been focused on ginseng. Ginsenosides are the main components of Ginseng extracts, includes more than 100 ginsenosides were identified so far (Christensen, 2009). The chemoprevention and anticancer mechanism of ginsenosides include mitigation of DNA damage, induction of apoptosis, inhibition of proliferation, and positive immunomodulation (Helms., 2004). Ginsenoside Rh2 is one of the most studied ginsenosides and it displays potent anticancer activity both in vitro and in vivo (Cheng et al., 2005; Wang et al., 2006; Chung et al., 2013).

Phosphoglucose isomerase/autocrine motility factor (PGI/AMF), a housekeeping gene, plays a critical role in both glycolysis and gluconeogenesis, catalyzing the interconversion of glucose-6-phosphate and fructose 6-phosphate in intracellular environment (Yakirevich and Naot., 2000), which behaves extracellularly as a cytokine. Molecular cloning and sequencing have identified PGI as an autocrine motility factor (AMF) which is associated with cancer progression and metastasis (Matsumoto et al., 1999; Cao et al., 2000; Yakirevich and Naot., 2000), and its presence in the serum and urine is of prognostic value which is associated with cancer progression (Lee et al., 2006; Thiery and Sleeman., 2006). PGI/AMF was identified as neuroleukin that promotes growth of embryonic spinal and sensory neurons, maturation factor mediating differentiation of human myeloid leukemia cells (Xu et al., 1996). Tumor cells protect themselves with auto products, such as AMF, and proliferate despite various stresses and chemical insults; AMF regulates expression of Apaf-1 and caspase-9 genes via a complex signaling

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pathway and indirectly regulates formation of the apoptosome (Haga et al., 2003). It has been reported that PGI/AMF could induce cell proliferation, migration and reduce apoptosis through PI3K/Akt pathway. PGI/AMF induced PI3K/MAPK signaling pathways in a HER family dependent manner (Kho et al., 2013). On the contrary, down-regulation of AMF/PGI sensitized fibrosarcoma cells to oxidative stress to cellular senescence (Funasaka et al., 2007a) and resulted in mesenchymal to epithelial transition (MET) with reduced malignancy and metastasis (Funasaka et al., 2009; Niinaka et al., 2010). Akt activation is generally associated with increased tumor progression, tumor cell invasion and anti-apoptosis (Nicholson and Anderson, 2002). The mammalian target of rapamycin (mTOR) is a Ser/Thr protein kinase that functions as an ATP and amino sensor to homeostasis, cell growth (Dennis et al., 2001; Gingras et al., 2001). Also, mTOR is currently under investigation as a potential target for anti-cancer therapy (Huang and Houghton., 2003).

Numerous evidences proved that Ginsenoside Rh2 displayed dramatic inhibitory effect on leukemia cells, although its specific molecular mechanism was not well understood; Meanwhile, PGI apparently related to tumor progression. Therefore, we hypothesized that there may be some correlation between Rh2 and PGI genes. We presented evidence that silencing of PGI/AMF could increase the sensitivity of KG1α cell to Rh2. Mechanisms insights into such phenomena was that Rh2 exerts its anti-cancer effects via PGI-mediated Akt/mTOR pathway.

Materials and Methods

Cell culture

Human leukemia cell line K562, KG1α and HL-60 were kept in my own laboratory, and frozen as original stocks in 2009. Human monocyte was obtained from department of Hematology and Oncology, the first affiliated hospital of Chongqing Medical University. All cells were cultured in Roswell Park Memorial Institute ( RPMI-1640) medium containing 10% heat-inactivated fetal bovine serum (FBS) (Hyclone). Transfected KG1α cells which cultured in cultures were maintained at 37°C in air-5% CO₂ incubator at constant humidity.

Antibodies and chemicals

Ginsenoside-Rh2 (purity≥98% HPLC) was purchased from Beijing century audiocodes biotechnology co., LTD. Cell Counting Kit-8 (WST®, Japan); Annexin V- FITC notation apoptosis detection kit (KeyGEN Biotech co., LTD); GPI antibody (Santa Cruz Biotechnology Inc, CA); PathScan® Intracellular Signaling Array Kit (Chemiluminescent Readout) (Cell Signaling Technology); PGI ELISA Assay Kit (CUSBIO Biotech co., LTD); The secondary antibodies: horseradish peroxidase (HRP-conjugated goat anti-rabbit IgG antibody, HRP-conjugated goat anti-mouse IgG antibody (Beiyotem Institute of Biotechnology).

Plasmid construction and transfection

To design specific small interfering RNA (siRNA) targeting PGI/AMF, several sequences from different parts of the human PGI/AMF gene were selected using the siRNA Target Finder available at the Ambion website. The GPI siRNA Lentiviral Particles were constructed by Neuron Biotechnology Co., Ltd. KG1α cells were transfected with PGI siRNA (h) Lentiviral Particles containing plasmids and pSilencer vector control containing no siRNA. Stably expressed single clones were established by puromycin selection (1 ug/ml).

Preparation of cell lysate and conditioned medium

After cultured for 0, 24, 48 h, Cell medium was was removed. The cells were collected and washed twice with PBS and lysed in 200 ul plate (Six orifice plate) of cell lysis buffer, 0.1 Mm PMSF for cell lystate, or incubated with 3 ml/dish of RPMI for 48 h to obtain conditioned medium. Cell supernatants were concentrated up to 100-fold using Amicon Ultra (30kDa cut-off; Millipore, Billerica, MA). Protein concentrations of each sample were determined using Enhanced BCA Protein Assay Kit.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted using TRizol Reagent (Invitrogen). The cDNA for PCR template was generated by using first-strand cDNA for PCR synthesis kit (TaKaRa Biotechnology (Dalian) Co., Ltd.) as recommended in the manufactures’ protocols. For quantitative evaluation of the amplified product, PCR encompassing 20 to 30 cycles was preliminarily done to determine the most suitable amplifications for each reaction. Each PCR consisted of the following: 1 min at 95°C, 1 min at 55°C, and 2min at 72°C for PGI/AMF and 1 min at 95°C, 1 min at 60°C, 2 min at 72°C for β-actin. PCR-amplified products were electrophoresed in 1% agarose gel and stained with ethidium bromide. The sequences of oligonucleotides primers were as follows: for PGI/AMF, forward: 5’-AATGCAGAGACCCACACTGTGCCCCAGG-3’ and reverse: 5’-AGGAAGAAGGAGGGAGTC-3; for β-actin, forward: 5’-TGACGGGGTCACCCACACTGTGCCCCAGG-3’, reverse: 5’-CTAAGACCATTTGGCGGAGCA-3’. The amount of RNA was normalized to β-actin expression.

Western blot analysis

Equal amounts of the protein were separated on SDS-PAGE gels and transferred to 0.45 μm Polyvinylidene fluoride (PVDF) membrane (Millipore) at constant current 250 mA, time was depend on protein molecular weight for 1min/KD. The membrane was blocked with 5% skim milk solution in TBST for 1.5 h at room temperature and then incubated with primary antibody and secondary antibodies conjugated with fluorophore. Blots were visualized by using ECL. Density of each band was quantitated with Quantity One Image software.

CCK-8 assay and trypan blue stain

For cell proliferation, a CCK-8 assay was done. Briefly, 1×10⁵ cells per well were plated in 96-well plates and cultured for different times indicated. At the end of time, 20ul CCK-8 was added to each well and then incubated at 37°C for 3 h. Then plates were read at 450 nm on a
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**Figure 1. Down-regulating the Expression of PGI/AMF by Small Interfering RNA (siRNA).** (A) The results of PCR and Western blot results showed the expression of PGI/AMF in KG1α at the highest level among chosen cell lines, so we chose KG1α cells as study objects. (B) KG1α cells were stably transfected with plasmid containing PGI/AMF-specific siRNA (siPGI/AMF) or controlled plasmid (mock) as described in Material and methods. PGI/AMF expression was analyzed by PCR and immunoblot analysis. (C) Extracellular PGI/AMF was analysed by ELISA kit. The results showed that PGI/AMF content was decreased by transfection. On the right, quantificational analysis of PGI/AMF expression was displayed. Representative results of three different experiments. *P < 0.05; **P < 0.01, compared with control cells

**Figure 2. Down-regulation PGI/AMF Inhibited the Growth of KG1α.** (A) The cells were seeded at low density and grew for 8 days. The in vitro trypan blue assay was designed to test the number of viable cells. (B) Intracellular Signaling Antibody Array Kit was used to detect the changes caused by down-regulation of PGI/AMF

**Cell cycle assay**

The cells were seeded at a concentration of 2×10^4 cells/ml and incubated for 24-72 h with Rh2 at various concentrations. Rh2 dissolved in dimethyl sulfoxide was added to the medium in serial dilution. The cells were collected by centrifugation at 2500 r.p.m. for 5 min, fixed in 70% ethanol then washed once with PBS and resuspended in 1 ml of PBS containing 2.5 μg/ml ribonuclease and 50 μg/ml propidium iodide, incubated in the dark for 30 min at room temperature and analyzed using flow cytometry (FCM).

**Annexin V assay**

Briefly, for the cell death assay, KG1α and transfected cells were seeded at a concentration of 3×10^4 cells per ml and incubated as usual. Cells were loaded on a hemocytometer, and viable cell number was determined based on exclusion of trypan blue dye.

**Statistics analysis**

The intensity of the immunoreactive bands was determined by a densitometer (Bio-Rad, Hercules, CA). Statistical significance of differences between control and treated samples were calculated by Student’s t-test (SSPS 17.0). *P < 0.05 were considered significant. All the experiments were repeated at least three times, each time with three or more independent observations.

**Results**

**Suppression of PGI/AMF in KG1α cell by siRNA**

Previous studies showed that the expression of PGI in KG1α cells was the highest among KG1α, K562, HL-6 and human monocytes (Figure 1A). Therefore, KG1α was chose for transfection. As one of the myeloid leukemia cell lines, KG1α cells was transfected with the constructed plasmid encoding siRNA targeting to PGI/AMF and with control plasmid, respectively. The stable transfectants with PGI/AMF siRNA plasmid (siPGI/AMF) and control plasmid (mock) were established by puromycin screening. To examine the efficiency for silencing PGI/AMF, we detected the expression level of mRNA and protein in the transfectants. The results of RT-PCR and western blotting showed that the level of PGI/AMF was apparently decreased compared with parental group, and there was no difference between parental and mock group (Figure 1B). SiPGI/AMF exhibited decreased expression of cellular and extracellular PGI/AMF at protein level, 75% and 78% decreased respectively by Western blot analysis and ELISA kit (Figure 1B and C).
Down-regulation of PGI/AMF inhibited cell growth

To investigate the effect of knockdown of PGI/AMF on cell proliferation, cell proliferation assay was done. The cells were seeded at low density and grown for 8 days. The trypan blue assay was designed to test the number of viable cells. Silencing of PGI/AMF resulted in the stagnation of viable cells, whereas control group cells continued proliferation normally. The cell proliferation was slowly decreased from the third day to the fifth day and decrease obviously from the sixth to the end in 8 days (Figure 2A).

It demonstrated PGI/AMF involves in the proliferation of leukemia KG1α. The lysates of KG1α and transfected cells were analyzed by Intracellular Signaling Array Kit, results showed the expression of PARP, State1, SAPK/JNK, Erk1/2 protein were decreased and PRAS40, p38 protein were increased in transfected cells (Figure 2B). These results demonstrated that the silencing of PGI/AMF modulated cell growth might through extensive ways.

Silencing of PGI enhanced sensibility of KG1α cells to Rh2

Previous studies demonstrated that ginsenoside Rh2 has potent anticancer capability. To assess the inhibitory effect of ginsenoside Rh2 on KG1α cells, we first determined the cytotoxicities of Rh2 using CCK-8 assay. As shown in Figure 3A, the ginsenoside Rh2 exerts obvious effect on human leukemia KG1α cells. The results showed Rh2 effect on KG1α cell in a dose-dependent manner. Accordingly, low concentration 30μM was chosen to test cell cycle inhibition (Figure 3B) and IC_{50} concentration 75μM was chosen to induce apoptosis and other studies. To examine whether silencing of PGI affected Rh2 pharmacological action, we explored the distinctions of Rh2 in control group and transfected group. Cells were seeded in 6-well plates. The results of FACScan flow cytometry showed that silencing of PGI/AMF could strengthen pharmacological effect on apoptosis of KG1α. Compared with those in control group, the KG1α cells after treated with 75μmol/L ginsenoside Rh2 showed up-regulated apoptosis rates from (1.44±0.14) % to (8.22±0.22) % at 24h and to (37.85±0.94) % at 48h. And, the transfected KG1α cells showed up-regulated apoptosis rates from (1.44±0.148) % to (50.0±0.94) % at 24h and to (37.85±0.94) % at 48h. The results showed that Rh2 could cause KG1α cells arrest in G0/G1 phase and reduced in S phase in the mean time. (C) KG1α and transfected cell line were induced by Rh2 in IC_{50} concentration of 75μmol/L in 24h and 48h respectively, and then the apoptosis analyzed. *P<0.05, **P<0.01, compared to control group. NS, non-significant. Data represent the mean±SEM of three independent experiments.

Figure 3. Silencing of PGI/AMF Enhanced Sensibility of KG1α to Rh2. (A) Leukemia cell line KG1α cells were treated with ginsenoside Rh2 in different concentrations within 24, 48, 72h respectively. The inhibition of Rh2 on KG1α was detected by CCK-8 assay kit in fixed time. (B) KG1α cells were induced by Rh2 in concentration of 30μmol/L in 24 h and 48 h, and then collected cells to test cell cycle in flow cytometry (FCM) till the indicated time. The results showed that Rh2 could lead to KG1α cells arrest in G0/G1 phase and reduced in S phase in the mean time. (C) KG1α and transfected cell line were induced by Rh2 in IC_{50} concentration of 75μmol/L in 24h and 48h respectively, and then the apoptosis analyzed. *P<0.05, **P<0.01, compared to control group. NS, non-significant. Data represent the mean±SEM of three independent experiments.

Figure 4. Ginsenoside Rh2 Effected on Akt/mTOR Pathway Through Down-regulating the Expression of PGI/AMF. KG1α and Transfected Cells were Treated with or without Rh2 in IC_{50} Concentration of 75μmol/L. A. Western blot analysis for the expression of mTORC1 composition Raptor, Rag, mTOR. Suppression of PGI/AMF decrease Raptor, mTOR, Rag content obviously, especially under the effect of ginsenoside Rh2 in 48h. B. 48h after treated with Rh2, Akt and PGI expression of transfected cells dropt to 37.5%, 50% respectively vs. KG1α group, *P<0.05 and **P<0.01 vs. KG1α group, NS, non-significant.

Silencing of PGI enhanced sensibility of KG1α cells to Rh2 through Akt/mTOR pathway

Down-regulation of PGI/AMF enhanced sensibility of KG1α cells to Rh2 obviously, the relevant mechanism is not clear. Ectopic expression of PGI/AMF induced activation of the PI3K/Akt signaling pathway (Tsutsumi et al., 2003a). So we explored whether ginsenoside Rh2 could modulate the expression of PGI/AMF and Akt. KG1α and transfected cells were treated with or without Rh2 in IC_{50} concentration of 75μmol/L, we tested PGI/AMF and Akt through western blotting. Ginsenoside Rh2 restrained the expression of PGI/AMF, down-regulation of PGI/AMF made the expression of Akt decreased severely (Figure 4A). PRAS40 is found to be a second Akt substrate involved in mTORC1 regulation (Kovacina et al., 2003), it apparently gone up after silencing of PGI/AMF (Figure 2B). We explored the relationship between ginsenoside

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**Discussion**

Panax ginseng has been applied worldwide for thousands of years as a traditional herbal medicine; it was also used for adjuvant therapy in various kinds of cancer in recent years. Ginseng extracts and its chemical components have been demonstrated their cytotoxicity against a variety of cancers, including inhibition of carcinogenesis in oral cavity, stomach, lung, liver, pancreas, ovaries, and colon (Wang et al., 2008). The ingredients of ginseng were complex, more than one hundreds of ginsenosides were found (Christensen., 2009). Ginsenoside Rh2, a panaxadiol saponin, possesses various antitumor properties (Cheng et al., 2005; Park et al., 2010; Li et al., 2011; Chung et al., 2013). This study was undertaken to gain insights into the molecular mechanisms of ginsenoside Rh2 induced cell death in leukemia KG1α cell lines. It is known that Rh2 has a significant effect on inducing the apoptosis of pancreatic cancer cells, hepatoma cells and lung cancer A549 cells (Zhang et al., 2011; Park et al., 2012; Tang et al., 2013). Here, our findings revealed that Rh2 also caused the apoptosis of KG1α cells.

Phosphoglucose isomerase (PGI) is a multifunctional enzyme that functions in glucose metabolism as a glycolytic enzyme catalyzing an interconversion between glucose and fructose inside the cell, while, PGI acts as cytokine outside the cell, with properties that include autocrine motility factor (AMF) regulating tumor cell motility (Niinaka et al., 2010; Fu et al., 2011; Shih et al., 2012). Also, PGI/AMF has been reported to regulate proliferation and survival of cells and prevent stress-induced apoptosis in tumor cells (Haga et al., 2003; Tsutsumi et al., 2003b). Here, we reported that silencing of PGI/AMF by transfect made Rh2 more effective to KG1α. PGI/AMF is up-regulated in a variety of cancer cells (Funasaka et al., 2007b; Guarino, 2007). We examined the proliferation and cell cycle-associated change related to PGI/AMF knockdown in leukemia cells. The results in Figure 2A presented that PGI/AMF affected cell proliferation and modulated tumor growth. Over-expression of this housekeeping gene induces resistance to apoptosis in NIH-3T3 fibroblasts (Tsutsumi et al., 2003a). Down-regulation of PGI expression made KG1α much more likely to apoptosis as described in Figure 3C. AMF over-expression which suppresses the expression of Apaf-1 and caspase-9 are important for apoptosis initiation, activating PI3K and MAPK and causing mitomycin-induced apoptosis resistance (Yanagawa et al., 2004). Ectopic expression of PGI/AMF induced activation of the PI3K/Akt signaling pathway (Tsutsumi et al., 2003a). Akt activation is generally associated with increased tumor progression, tumor cell invasiveness and anti-apoptosis (Nicholson and Anderson., 2002) and is an indicator of aggressive tumor behavior. Down-regulation of PGI decreased the expression of Akt. Akt regulates cell growth through its effects on the mTOR and p70 S6 kinase pathways, as well as cell cycle and cell proliferation through its direct action on the CDK inhibitors p21 and p27, and its indirect effect on the levels of cyclinD1 and p53. Silencing of PGI/AMF inactivate Rheb-mTORC1 through decrease the expression of Erk1/2 and increase the expression of PRAS40 in downstream of PI3K/Akt pathway. Through this way, knockdown PGI/AMF could down-regulate cell growth.

The mammalian target of rapamycin (mTOR) is a Ser/Thr protein kinase that functions as an ATP and amino sensor to homeostasis, cell growth (Dennis et al., 2001; Gingras et al., 2001) and may be abnormally regulated in tumors. Hence, mTOR is currently under investigation as a potential target for anti-cancer therapy (Huang and Houghton., 2003). MTORC1 is a critical regulator of translation initiation and ribosome biogenesis and plays an evolutionarily conserved role in cell growth control (Wullschleger et al., 2006). PRAS40 is found to be a second Akt substrate involved in mTORC1 regulation (Kovacina et al., 2003), and it has been found to be negatively regulated mTORC1 signaling (Sancak et al., 2007; Vander Haar et al., 2007). The regulatory associated protein of mTOR (Raptor) is identified as an mTOR binding partner that mediates mTOR signaling. Raptor binds to mTOR substrates, including 4E-BP1 and p70 S60 kinase, through their TOR signaling motifs and is required for mTOR-mediated phosphorylation of these substrates. Raptor has been identified as a direct substrate of the AMP-activated protein kinase (AMPK). AMPK regulates fatty acid metabolism, as well as modulates protein synthesis and cell growth. AMPK phosphorylates raptor on Ser722/ Ser792. This phosphorylation is essential for inhibition of the raptor-containing mTOR complex 1 (mTORC1) and induce cell cycle arrest when cells are stressed for energy (Gwinn et al., 2008). These findings suggest that raptor is a critical switch that correlates cell cycle progression with energy status. As a glycometabolism enzyme, PGI plays a key role in cell growth and silencing of PGI decreases the cellular activity. In this study, down-regulation of PGI could coordinate with Rh2 to modulate mTOR pathway as showed in Figure 4. Alternatively, low ATP levels lead to the AMPK-dependent activation of TSC2 to reduce mTORC1 signaling. Thus, knocked-down PGI could decrease ATP levels. All of these changes reduce the mTORC1 signaling via inhibiting downstream targets and finally suppress cell growth and proliferation.

In conclusion, Ginsenoside Rh2 had obvious restraining effects on the proliferation of KG1α, it down-regulated the expression of PGI/AMF to display effects. Down-regulation of AMP/PGI constitutively inactivated mTOR by decreasing the expression of Erk1/2-Rheb and increasing PRAS40 respectively. Such effects inhibited cell growth, proliferation and induce cell apoptosis through the suppression of the PI3K/Akt signaling pathway. Down-regulation of PGI/AMF enhanced the pharmacological effects of ginsenoside Rh2 on KG1α by reducing Akt/mTOR signaling.
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