Ginsenoside Rh1 Eliminates the Cytoprotective Phenotype of Human Immunodeficiency Virus Type 1-Transduced Human Macrophages by Inhibiting the Phosphorylation of Pyruvate Dehydrogenase Lipoamide Kinase Isozyme 1

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Abstract

Red ginseng (the steamed root of Panax ginseng C.A. Meyer, Araliaceae), which contains ginsenosides as its main constituents, is frequently used to treat tumor, inflammation, diabetes, stress and acquired immunodeficiency syndrome in Asian countries. Of these ginsenosides, only protopanaxadiol compound K has been reported to abolish the cytoprotective phenotype of human immunodeficiency virus type 1 (HIV-1)-transfected human macrophages. Here, we investigated the anti-cytoprotective effect of protopanaxatriol ginsenoside Rh1 on Tat-expressing cytoprotective CHME5 cells and D3-infected human primary macrophages. Treatment with ginsenoside Rh1 in the presence of lipopolysaccharide/cycloheximide (LPS/CHX) potently abolished the cytoprotective phenotype of Tat-transduced CHME5 cells as well as D3-infected human primary macrophages. Ginsenoside Rh1 significantly inhibited LPS/CHX-induced Akt phosphorylation, as well as mammalian target of rapamycin and Bcl-2-associated death promoter activation in both cell types. Furthermore, ginsenoside Rh1 inhibited pyruvate dehydrogenase lipoamide kinase isozyme 1 (PDK-1) phosphorylation. However, ginsenoside Rh1 did not inhibit phosphoinositide 3-kinase phosphorylation. Ginsenosides Rh1 in the presence of miltefosine (5µM) additively increased the anti-cytoprotective activity against HIV-1 Tat-expressing macrophages. On the basis of these findings, we propose that ginsenoside Rh1 could possibly eliminate HIV-1 infected macrophages by inhibiting the PDK1/Akt pathway.

Key words human immunodeficiency virus type 1; macrophage; ginsenoside Rh1

The phosphoinositide 3-kinase (PI3K)/Akt pathway is an important cellular process that viruses use to ensure continual and productive viral replication by extending the life-span of the infected cells, and consequently, by inducing either cell transformation (onco-viruses) or persistent viral production [human immunodeficiency virus type 1 (HIV-1)]. HIV-1 infection activates the PI3K/Akt cell survival pathway in primary human macrophages and renders these cells resistant to cytotoxic insults. Expression of the Tat protein, which is an HIV-1 antigen, elevates the cytoprotective phenotype of the human microglial cell line CHME5 as well as human primary macrophages. Tat-expressing CHME5 cells proactively activate the PI3K/Akt pathway upon exposure to cellular stresses by reducing the levels of phosphate and tensin homology, which is a negative regulator of the Akt pathway. These cellular changes ensure a strong resistance to extracellular stresses such as lipopolysaccharide (LPS) or nitric oxide. Therefore, Tat-expressing human microglia and macrophages play important roles in the establishment of long-living HIV-1 reservoirs in the central nervous system. Cytoprotective Tat-expressing microglia induce neuronal death and HIV-1-associated neurodegenerative diseases. Therefore, many studies have attempted to use chemicals to control the cytoprotective effects of HIV-1-infected macrophages.

Red ginseng (the steamed root of Panax ginseng C.A. Meyer, Araliaceae), which contains protopanaxadiol and protopanaxatriol ginsenosides as its main constituents, is frequently used to treat tumor, diabetes, inflammation, stress and acquired immunodeficiency syndrome (AIDS) in Asian countries. When ginseng is orally administered to humans or rats, protopanaxadiol and protopanaxatriol ginsenosides are metabolized to 20-O-β-D-glucopyranosyl-20(β-D)-glucopyranosyl-20(S)-protopanaxadiol (compound K) and ginsenoside Rh1 by intestinal microbiota, respectively. Previous studies have shown that these metabolites are absorbed into the blood, subsequent research has demonstrated that these transformed ginsenosides exhibit more potent biological activities than the parental ginsenosides. Recently, Cho et al. reported the beneficial effects of red ginseng in HIV-1 infected individuals. Most patients treated with red ginseng remained healthy for >20 years in the absence of highly active antiretroviral therapy (HAART). Compound K, which is a representative metabolite of protopanaxadiol ginsenosides of red ginseng, has been reported to abolish the cytoprotective phenotype of HIV-1-transfected human macrophages. However, the anti-HIV-1 effect of red ginseng constituents and their metabolites such as ginsenoside Rh1 have not been thoroughly studied.

Therefore, we investigated the anti-cytoprotective effect of ginsenoside Rh1, a protopanaxatriol metabolite, on the Tat-expressing cytoprotective cell line CHME5 and D3-infected human primary macrophages.

MATERIALS AND METHODS

Chemicals Dulbecco’s modified Eagle’s medium (DMEM), LPS purified from Escherichia coli O26:B6, cycloheximide (CHX), propidium iodide solution (PI), and miltefosine were purchased from Sigma Co. (St. Louis, MO, U.S.A.).
Antibodies for PI3K, Akt, p-Akt, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Antibodies for phospho-pyruvate dehydrogenase lipoamide kinase isozyme 1 (pPDK-1), mammalian target of rapamycin (mTOR), p-mTOR, Bcl-2-associated death promoter (BAD) and p-BAD were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). Polyvinylidene difluoride membranes and the enhanced chemiluminescence detection kit (Luminata™ Forte Western horse-radish peroxidase substrate) were purchased from Millipore (Billerica, MA, U.S.A.).

Isolation of Ginsenoside Rh1  Ginsenoside Rh1 was isolated from dried ginseng according to the previously reported method. Briefly, the dried ginseng extract was prepared in KuAn Industry Co. (Seoul, Korea) and its main constituent ginsenoside Re was isolated from the extract according to the method described by Shin et al. The ginsenoside Re (0.2 g) was anaerobically incubated with a human fecal suspension (1 g) in an anaerobic medium (0.5 L) according to the method described by Bae et al. The reaction mixture was extracted with n-BuOH (1 L) twice, and evaporated (dried extract, 0.15 g) followed by defatting with n-hexane. The defatted n-BuOH extract was subjected to chromatography using a silica gel column (2 × 20 cm) and eluted using a stepwise gradient of CH$_2$Cl$_2$-methanol. The eluent was further separated by chromatography on a silica gel column, employing the same eluent system, thereby isolating ginsenoside Rh1 (25 mg). The isolated ginsenoside Rh1 was identified by comparing it to an

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Fig. 1. The Anti-cytoprotective Effect of Ginsenoside Rh1 against Cytoprotective Tat-Expressing CHME5 Cells

(A) (a) Trypan blue staining assay was used to determine cell death induced by ginsenoside Rh1. (b) Cytotoxic effect of ginsenoside Rh1 against CHME5 cells transduced with pcDNA3.1 hygro-Tat gene) in the absence or presence of lipopolysaccharide/cycloheximide (LPS/CHX). (B) PI-FACS assay was used to measure the anti-cytoprotective effect of ginsenoside Rh1 (Rh1). Tat-expressing CHME5 cells were treated with and without 50 µg/mL LPS and 10 µg/mL CHX in the absence or presence of ginsenoside Rh1 for 48 h. The normal control (control) was treated with the vehicle alone. Cells were trypsinized and mixed with 1 µg/mL PI (in saline buffer). The samples were incubated on ice for 15 min under a light-protected conditions and then analyzed by FACS cytometry. PI-stained samples were identified using the phycoerythrin fluorescence (FL2) detector in FACS, which detect PI fluorescence. All studies were performed in triplicate. Data are presented as the mean ± standard deviation.
authentic standard by nuclear magnetic resonance spectrometry (Bruker AM-500: 500 MHz) and fast atom bombardment mass spectrometry.

Ginsenoside Rh1 white amphorus powder (MeOH). (+)-FAB-MS m/z: 639 [M+H]+.

Cells We used the human fetal microglia cell line CHME5 that stably expressed full-length Tat (pTat101) or a control gene (pCDnA3.1-Hygro, without Tat). The cells were maintained in 10% fetal bovine serum in DMEM. The primary human monocyte-derived macrophages were isolated from the blood of volunteers and differentiated as previously described.17

Cell Survival Assay Tat-expressing CHME5 cells and human primary macrophages that had been transduced with the HIV-1 vector expressing eGFP were treated with 50 µg/mL LPS and 10 µg/mL CHX stress in the presence or absence of Rh1 (5, 10, 20 µM) for 48 h. Subsequently, the cells were trypsinized and mixed with Trypan blue solution and the ratio of dead cells to total cells (150–250 cells) was calculated. Alternatively, the cells were trypsinized, stained with PI (1 µg/mL), and measured by using a fluorescence activated cell sorter (FACS; C6 Flow Cytometer System). All experiments were repeated 3 times.

Isolation and Culture of Monocyte-Derived Macrophages Human macrophages were isolated from the blood of volunteer donors (men in their 20s). Peripheral blood mononuclear cells were harvested using Ficoll density gradients (Lymphoprep; Axis-Shield PoC AS, Oslo, Norway), and the monocytes were purified using immunomagnetic selection involving anti-CD14 antibody-conjugated magnetic beads according to the manufacturer’s protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). The purified human monocytes were incubated in culture plates in RPMI 1640 medium (Sigma) containing 20% human AB serum (Sigma, St. Louis, MO, U.S.A.) for 4 d in the presence of 5 ng/mL human recombinant granulocyte-macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN, U.S.A.) and then incubated for 3 additional days in the absence of the granulocyte-macrophage colony-stimulating factor to allow differentiation into macrophages. These cell preparations were used for the cytotoxicity and immunoblot assays.

Immunoblot Analysis CHME5 cells (5×10⁴ cells/well) and monocyte-derived macrophages (1×10⁶ cells/well) were stimulated with LPS/CHX in the presence or absence of test agents for 90 or 120 min and then lysed. The cell supernatant extracts were separated by 10% sodium dodecyl sulfate-poly-
acrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat dried-milk proteins in 10mM phosphate-buffered saline that contained 0.05% Tween 20 (PBST, pH 7.4) and then probed for PI3K, p-PDK1 (ser241), p-Akt (ser473), Akt, GSK (glycogen synthase kinase) 3β, p-GSK3β, p-mTOR (ser248), p-BAD, or β-actin. After washing with PBST, the proteins were probed using horse radish peroxidase-conjugated secondary antibodies for 50min. The bands were visualized using an enhanced chemiluminescence detection kit. The bands were quantified using the image analysis program Las-4000 (FUJIFILM, Tokyo, Japan).

**Statistical Analysis** The results are presented as the mean±standard deviation of ≥3 replicates. One-way ANOVA and Student’s t-tests were used. p<0.05 was regarded as statistically significant.

**RESULTS AND DISCUSSION**

We tested the anti-cytoprotective effect of ginsenoside Rh1 in the presence and absence of LPS/CHX stress against Tat-expressing CHME5 cells (Fig. 1). Trypan blue staining and PI-FACS assays revealed that LPS/CHX stress in the presence of ginsenoside Rh1 induced an anti-cytoprotective effect in a dose-dependent manner. However, treatment with either LPS/CHX or ginsenoside Rh1 alone did not induce significant amounts of cell death in Tat-expressing CHME5 cells.

To understand the anti-cytoprotective mechanism of ginsenoside Rh1 in Tat-expressing CHME5 cells, we tested its effect on the cell survival PI3K/Akt pathway (Fig. 2). In Tat-expressing CHME5 cells, treatment with LPS/CHX induced PI3K/Akt phosphorylation. However, treatment with LPS/CHX in the presence of ginsenoside Rh1 inhibited Akt phosphorylation. Furthermore, ginsenoside Rh1 also inhibited GSK3β, m-TOR, and BAD activation and PDK1 phosphorylation. However, ginsenoside Rh1 did not inhibit PI3K phosphorylation.

To confirm the anti-cytoprotective effect of ginsenoside Rh1 against HIV-1-infected macrophages, we infected human primary macrophages with the D3 vector and investigated the anti-cytoprotective effect of ginsenoside Rh1 against cytoprotective macrophages by Trypan blue staining and PI-FACS assays (Fig. 3). The ginsenoside Rh1 in the presence of LPS/CHX induced anti-cytoprotective activity in a dose-dependent manner. However, LPS/CHX in the absence of the ginsenoside Rh1 did not induce cell death (<5%) at the tested concentrations.

PI3K/Akt phosphorylation was induced in the D3-infected human primary macrophages following treatment with LPS/
However, treatment with LPS/CHX in the presence of ginsenoside Rh1 suppressed PDK1, Akt, m-TOR, and BAD phosphorylation, but did not inhibit PI3K phosphorylation (Fig. 4).

Miltefosine abolishes the cytoprotective effect of HIV-1 infected macrophages by inhibiting the PI3K/Akt pathway, but it exhibits severe side effects, such as nausea, vomiting and teratotoxicity. To reduce the required dose of miltefosine, we investigated the combined effect of miltefosine and ginsenoside Rh1 against Tat-transduced CHME5 cells. When TAT-induced CHME5 cells were administered different concentrations of miltefosine (5 or 10 µM), the anti-cytoprotective effect of ginsenoside Rh1 against the cytoprotective cells increased in a dose-dependent manner (Fig. 4). Moreover, even when the cells were subjected to various concentrations of miltefosine with a fixed concentration of ginsenoside Rh1 (5 or 10 µM), the anti-cytoprotective effect of miltefosine against the cytoprotective cells increased in a dose-dependent manner.

The PI3K/Akt pathway, which controls various downstream effectors such as GSK3β, m-TOR, and BAD, is commonly activated in many cancer cells and promotes their survival and outgrowth. Recently, it has been reported that HIV-1 infection activates the PI3K/Akt pathway. Therefore, PI3K/Akt phosphorylation inhibitors could serve not only as effective anticancer drugs but also as anti-viral agents. In this study, we found that ginsenoside Rh1 inhibits the Tat-induced PI3K/Akt cell survival pathway, thereby leading to the death of HIV-1-expressing macrophages and CHME5 cells, similar to previously reported findings using compound K. However, ginsenoside Rh1 inhibited PDK1 phosphorylation in the PI3K/Akt signaling pathway, whereas compound K did not. Furthermore, ginsenoside Rh1 also abolished the cytoprotective D3-infected human primary macrophages, as well as the PDK1/Akt pathway. These results suggest that ginsenoside Rh1 eliminates HIV-1-infected macrophages in vivo by inhibiting the PDK1/Akt pathway, unlike compound K. In addition, ginsenoside Rh1 and miltefosine, which is an effective treatment for HIV-1 dementia and Leishmania infection, synergistically increased the cytotoxicity against HIV-1 Tat-expressing macrophages. Thus, combined treatment with miltefosine and ginsenoside Rh1 or ginseng extract against HIV-1 dementia or Leishmania infection is more beneficial than miltefosine alone. These results support the finding of Cho et al. that most patients treated with red ginseng remain healthy for >20 years in the absence of HAART. Taken together, these findings...
suggested that ginseng could be a good alternative medicine for AIDS patients.

Finally, we demonstrated that ginsenoside Rh1 inhibits PDK1 phosphorylation; this finding suggests that this metabolite delivers anti-HIV effects by both shortening the lifespan of the HIV-1 infected macrophages and protecting neurons in the HIV-1 infected brains.

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