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Stimulatory Effects of Ginsenoside-Rg_1 on $p56^{lck}$ Kinase and Cell Proliferation in Jurkat T Cells

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Abstract□We studied the effects of ginsenoside-Rg_1 (G-Rg_1) extracted from *Panax ginseng* C.A. Meyer on $p56^{lck}$ kinase and cell proliferation in Jurkat T cells. $p56^{lck}$ was maximally activated within 5 min after the treatment of 16.7 μg/m*l* of G-Rg_1 increasing the activity by 1.2~2 times relative to untreated control, thereafter its activity was gradually decreased to the level of untreated control. The action of EGTA on the kinase was altered by the addition of G-Rg_1, accompanying the band shift of $p56^{lck}$ to $p60^{lck}$. In addition, G-Rg_1 promoted cell proliferation in a concentration-dependent manner. These results suggest that G-Rg_1 may be involved in T cell receptor-CD3 (TCR) signaling via the activation of $p56^{lck}$ and the change of cellular calcium concentration.

Key words □*Panax ginseng*, ginsenoside-Rg_1, $p56^{lck}$, TCR signaling, cell proliferation.

Introduction

The root of *Panax ginseng* C.A. Meyer (Araliaceae) has been widely used as a drug to replenish vital function and cure a variety of diseases in Asian countries for thousands of years. It is general opinion that the major pharmacological activity of ginseng may be due to saponin. To the present, 25 different ginsenosides have been identified and it is reported that they have a variety effects such as the augmentation of central nervous system,[1] the promotion of protein and cAMP synthesis,[2,3] insulin-like action,[4] anti-inflammatory effect,[5] anti-neoplastic effect,[6] and hepatic regeneration effect,[7] *etc*. Recently ginseng has been taken a growing interest in its synergistic effects on theraphy against cancer and AIDS.[8] In 1958, Brekhman reported that ginseng extract has adaptogenic actions, which increase the non-specific resistance to stress and diseases and thus allow to keep up homeostasis.[9] It is thought that the effects of ginseng mentioned above may result from the potentiation of immune system.[8] Meanwhile, we previously reported that G-Rg_1 among 4 ginsenosides (G-Rb_2, Rc, Re, and Rg_1) had the most stimulatory effect on $pp60^{c\text{-}src}$, a member of src-family, in NIH3T3 cells.[10] G-Rg_1 is a dammarane-type protopanaxatriol saponin present most abundantly in ginseng. It has been shown to possess immunomodulating activity in mice[11] as well as DNA, RNA, and protein anabolic effect,[12,13] anti-thrombotic effect,[14] and the promotion of the survival of rat neuronal cells.[15] Based on these reports, we were stimulated to examine the effect of G-Rg_1 on the pathway of T cell activation, particularly on the lck kinase ($p56^{lck}$), since $p56^{lck}$ shares extensive structural and functional homology with $pp60^{c\text{-}src}$.[16]

$p56^{lck}$ is a nonreceptor-type src-family protein tyrosine kinase (PTK) and is expressed exclusively in lymphoid cells.[17] $p56^{lck}$ binds noncovalently to the cytoplasmic tails of the cell surface glycoproteins CD4 and CD8.[16] It has also been shown to interact with the subunit of the interleukin-2 (IL-2) receptor in a manner distinct from its interaction with CD4 and CD8.[18] Recent studies have revealed that the kinase plays a vital role in both T cell

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activation through the TCR and in lymphocyte development.[19]

In the present study, we describe the effect of G-Rg_1 on the activation of $p56^{lck}$ and cell proliferation in Jurkat T cells.

Materials and Methods

1. Cells and cell culture

Jurkat cells (human T lymphoma) were passed in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (100 U/m*l*) and streptomycin (50 U/m*l*) (GIBCO BRL, USA). Cells were plated to be 5×10^6 cells per 10 cm plate and maintained in a rapid growth phase for use in experiments.

2. Cell activation

Cells prepared as above were treated with the specified drugs. The treatments consist of the addition of G-Rg_1 (16.7 μg/m*l*) (Korea Ginseng and Tobacco Research Institute) and/or each specified drugs. After these drugs were added, cells were incubated for 5 min and then collected for *in vitro* kinase assay.

3. Immunoprecipitation of $p56^{lck}$

Cells (5×10^6) were washed three times in phosphate-buffered saline and lysed for 40 min in 0.5 m*l* of cold RIPA buffer (10 mM sodium phosphate, pH 7.0, 0.15 M sodium chloride, 0.1% SDS, 1% nonidet P-40, 0.75% sodium deoxycholate, 2 mM EDTA, 1 mM EGTA, 50 mM sodium fluoride, 1 mM sodium vanadate, 1%(v/v) aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol, 1 μg/m*l* antipain, 1 μg/m*l* pepstatin A) (Sigma Chemical Co., USA). Lysates were pelleted at 15,000 rpm for 30 min and the amount of total cell protein (TCP) in supernatant was determined by Bradford's method.[20] $p56^{lck}$ was immunoprecipitated by incubating the lysate containing 200 μg of TCP at 4℃ for 1 hr with 10 μ*l* (1/50) of anti-$p56^{lck}$ monoclonal antibody against the C-terminal fragment (amino acids 145-509) of human $p56^{lck}$ (Boehringer Mannheim GmbH, Germany). It is followed by a 30-min incubation at 4℃ with 10 μ*l* of a 10% *S. aureus* suspension that had been precoated with anti-mouse IgG by 20 min of incubation at 4℃. Immune complexes were collected by centrifugation at 4℃ and subsequently washed with RIPA buffer lacking SDS, sodium deoxycholate, EDTA, EGTA and sodium fluoride, high-salt buffer (1 M sodium chloride, 0.5% Triton X-100, 10 mM Tris-HCl, pH 7.2), and kinase buffer (100 mM Pipes, pH 7.0, 10 mM manganese chloride, 1% aprotinin, and 1 mM PMSF).

4. *In vitro* kinase assay

Immune complexes prepared in previous section were resuspended in kinase buffer and used for the kinase reaction of each sample at 25℃ for 10 min in 30 μ*l* reaction mixture, containing kinase buffer, acid-denatured rabbit muscle enolase (4 μg /sample) and 1 μM[γ-^{32}P] ATP (400 Ci/mmol, Amersham, England). Reactions were stopped by the addition of 15 μ*l* of sample buffer for SDS-PAGE (210 mM Tris-HCl, pH 6.8, 34.2% glycerol, 9% SDS, 0.03% bromphenol blue, 15% β-mercaptoethanol). Proteins were released for 10 min and resolved on 10% SDS-PAGE followed by autoradiography.

5. Cell proliferation

Jurkat T cells were divided to be 3×10^3 cells/well in flat-bottomed 96-well plates in a final volume of 100 μ*l* cell suspension per well and grown at 37℃, 6.5% CO_2 in humidified atmosphere. After treated at different concentrations of G-Rg_1, cells were incubated for 72 hr followed by adding to each well 50 μ*l* of XTT (sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) labeling mixture (Boehringer Mannheim GmbH, Germany). After a 6 hr-incubation, the spectrophotometrical absorbance of the samples was measured using ELISA reader at 492 nm.

Results and Discussion

Jurkat T cells highly expressing $p56^{lck}$ were treated with G-Rg_1 at various concentrations in the presence of PMA (phorbol-12-myristate-13-acetate) and collected for *in vitro* kinase assay. G-Rg_1 showed the most stimulatory effect at 16.7 μg/m*l* of concentration, increasing the activity of lck kinase by two times compared with untreated control or PMA alone (Fig. 1). When cells were treated with 16.7 μg/m*l* of G-Rg_1 for different times, $p56^{lck}$ PTK was maximally activated within 5 min (Fig. 2).

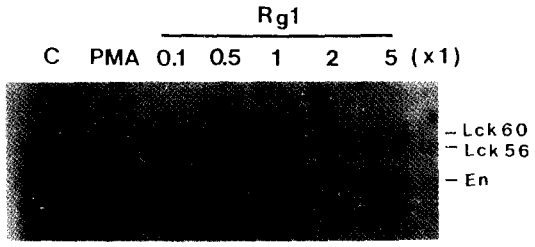

Fig. 1. Concentration-dependent relative protein tyrosine kinase activity of $p56^{lck}$. Jurkat T cells were treated with a different concentration of ginsenoside Rg_1 (G-Rg_1) for 5 min and collected. $p56^{lck}$ was immunoprecipitated with monoclonal antibody of $p56^{lck}$ from cell lysate, incubated with [γ-^{32}P] ATP and rabbit muscle enolase (En) as a substrate, and analyzed by 10% SDS-PAGE and autoradiography. Drug concentrations and times treated were: PMA (phorbol-12-myristate-13-acetate; 30 ng/ml, 20 min), G-Rg_1 (0.1; 5, 0.5; 25, 1; 50, 2; 100, 5; 250 μg/3 ml, 5 min). En indicates the band of phosphorylated enolase.

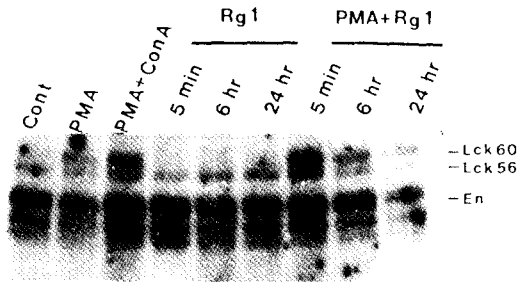

Fig. 2. Band shift of $p56^{lck}$ to $p60^{lck}$ after G-Rg_1 treatment with time-dependency. Cells were treated with specified drugs for the incubated times and in vitro kinase assay was performed as in Fig. 1. Drug concentrations and times treated were: PMA (30 ng/ml, 20 min), ConA (concanavalin A; 12.5 μg/ml, 20 min), and G-Rg_1 (16.7 μg/ml, 5 min, 6 hr, 24 hr). En indicates the band of phosphorylated enolase.

The band shift of $p56^{lck}$ to $p60^{lck}$ is well known as a indication to T cell activation either by cross-linking the surface TCR or by the treatment with PMA. It follows the initial burst in PTK activity in the processes of cell activation.[21,22] Fig. 2 shows that the band shift of $p56^{lck}$ to $p60^{lck}$ was significant when cells were treated with PMA and G-Rg_1 for 5 min, although it was not by the treatment of G-

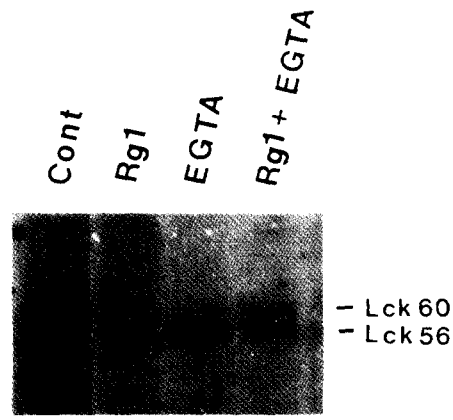

Fig. 3. Effect of G-Rg_1 on lck kinase activity in EGTA-treated cells. Cells were treated with specified drugs for the incubated times and in vitro kinase assay was performed as in Fig. 1. Drug concentrations and times treated were: G-Rg_1 (16.7 μg/ml, 5 min), EGTA (2.5 mM, 30 min).

Rg_1 alone. $p60^{lck}$ band by the treatments with PMA plus G-Rg_1 was as intense as by that of PMA plus ConA (concanavalin A), positive control. And also the addition of G-Rg_1 much intensified band shift to 60 kD by PMA alone. Thereafter 5 min, the activity of lck kinase was gradually decreased. From these results, we assume that when cells are stimulated with G-Rg_1, lck kinase is maximally activated in a short time followed by inactivation to basal level and the activated signal is transduced to the downstreams of TCR signaling pathway.

Straus and Weiss reported that lck kinase activity is important to the increase of calcium as well as the tyrosine phosphorylations of cellular proteins during T cell activation.[23] To investigate the involvement of the activation of lck kinase by G-Rg_1 with the change of cellular calcium concentration, extracellular Ca^{2+} was removed by the treatment with EGTA, a Ca^{2+} chelator and an inhibitor of T cell activation (Fig. 3). Although the band of $p56^{lck}$ was somewhat intensified by EGTA, the band shift to 60 KD did not show clearly. By the addition of G-Rg_1 to EGTA-treated cells, however, $p60^{lck}$ band was strengthened while $p56^{lck}$ band became weak. The increase of $p56^{lck}$ autophosphorylation by EGTA is thought to be only the temporary response to the sudden reduction of extracellular Ca^{2+},

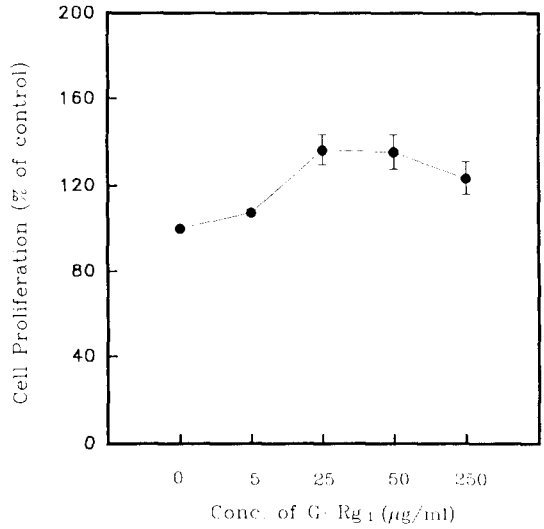

Fig. 4. Concentration-dependent cell proliferation in G-Rg_1-treated cells. Jurkat T cells were plated to be 3×10^4 cells/well in 96-well plate. Cells were treated with a different concentration of G-Rg_1 and incubated at 37℃, 6.5% CO_2. After 72 h, XTT (sodium 3'-[1-(phenylaminocarbonyl) -3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) solution was added and cell proliferation was measured by ELISA reader at 492 nm. Data were given as the mean ± SE (n=4).

which leaves not to transduce the signal into next pathway in T cell signaling. The appearance of $p60^{lck}$ intensified by G-Rg_1 suggests that G-Rg_1 may serve the compensatory mechanism(s) for the drastic change of cellular calcium by EGTA through the increase in lck kinase activity and/or the changes in its specific interactions with other cellular proteins.

Fig. 4 demonstrates that G-Rg_1 promoted the proliferation of Jurkat T cells in a concentration-dependent manner. 25~50 μg/m*l* of G-Rg_1 was required for maximal effect on cell proliferation increasing by 1.3 times of untreated control. But higher than 50 μg/m*l* of G-Rg_1 resulted in the decrease of the effect, which may be due to the nonspecific increase of membrane permeability by detergent-like action of G-Rg_1.

In conclusion, we suggest that the activation of $p56^{lck}$ by G-Rg_1 may be involved with the changes of cellular calcium concentration and finally lead to cell proliferation of Jurkat T cells. It presents that G-Rg_1 has potentialities as an immunostimulant and further it may be available to develop an immunopotentiator by introducing G-Rg_1 as a leading compound.

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