J. Ginseng Res.
Vol. 22, No. 2, 126~132(1998)

Effects of Ginsenosides on pp60$^{c\text{-}src}$ Kinase, Intracellular Calcium and Cell Proliferation in NIH 3T3 Cells

Hee-Youn Hong, Gyurng-Soo Yoo and Jung-Kap Choi

College of Pharmacy, Chonnam National University, Kwangju 500-757, Korea

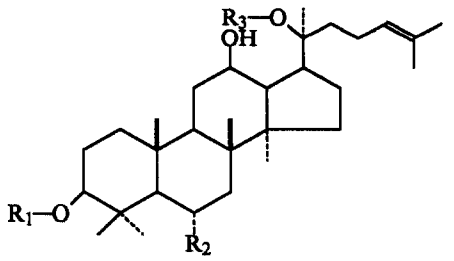
(Received April 16, 1998)

Abstract : In the present study, we examined effects of ginseng saponins (ginsenosides) on pp60$^{c\text{-}src}$ protein tyrosine kinase (PTK) activity, intracellular calcium concentration ($[Ca^{2+}]_i$), and cell proliferation in NIH3T3 cells. Eight different ginsenosides [ginsenoside-Rb_1 (G-Rb_1), -Rb_2, -Rc, -Rd, -Re, -Rf, -Rg_1, -Rg_2] and ginseng total saponin (GTS) were used for these experiments. All ginsenosides and GTS tested stimulated the activation of pp60$^{c\text{-}src}$ kinase, and especially G-Rb_1, -Rd, -Rg_1, and -Rg_2 showed a higher stimulatory effect than others at 16.7 μg/m*l* of ginsenosides with a 18 hr-incubation, increasing the activity by 4.5, 3.5, 3.5, and 3.0-fold, respectively, over that of untreated control. In addition, both G-Rd and -Rg_2 increased $[Ca^{2+}]_i$ to 202 and 334 nM, respectively, about 2-3-fold above the basal level within 7min at 250 μg/m*l* of ginsenosides. The increases of $[Ca^{2+}]_i$ were eliminated by pretreatment of EGTA, an extracellular calcium chelator, suggesting that they result from an influx of calcium ion from extracellular medium rather than an efflux from intracellular calcium store, endoplasmic reticulum (ER). All ginsenosides studied enhanced cell proliferation to 1.2-1.4-fold over that of untreated control at 5~250 μg/m*l* of concentrations. Interestingly the promotion of cell proliferation by ginsenosides corresponded with the activation of *c-src* kinase which is an early step in the mitogenic signaling cascade. Taken together, we suggest that some ginsenosides may lead to cell proliferation via the activation of cellular signal transduction pathway involving pp60$^{c\text{-}src}$ kinase.

Key words : *Panax ginseng* C. A. Meyer, ginsenosides, pp60$^{c\text{-}src}$ protein tyrosine kinase, intracellular calcium concentration, cell proliferation.

Introduction

Ginseng, the root of *Panax ginseng* C. A. Meyer, is an oriental folk medicine that is well-known to alleviate various diseases and to replenish vital functions of body. Ginseng saponin (ginsenoside) is one of the major active ingradient of ginseng. Compared to saponins found in other plants, ginseng saponin has quite different chemical structure (Fig. 1), which has triterpenoid dammarane skeleton, and effect. From Korean ginseng, approximately 30 different ginsenosides have been identified with a variety of effects. Kim and Lu reported that the saponin fraction of Korean ginseng is active to promote protein biosynthesis in erythrocytes and human lung fibroblasts, respectively.[1,2] There are many evidences implicating cyclic AMP as a secondary messenger for the biological actions of ginsenosides.[3,4] A crude ginseng saponin had a proliferative effect on neurite extension and a protective effect on distortion of neurites.[5] Recently it was reported that ginsenosides could inhibit lipid peroxidation of rat liver and brain microsomes[6] and also protect pulmonary vascular endothelium against free radical-induced injury.[7] Ginsenosides have various other effects such as anti-inflammation, anti-fatigue, anti-neoplastic effect, *etc.*. Many of the biological effects of ginsenosides imply that ginsenosides are closely related to a series of events leading to cellular activation. So we examined effects of ginsenosides on signal transduction pathway, primarily involving pp60$^{c\text{-}src}$ protein tyrosine kinase (PTK).



Saponins	R_1	R_2	R_3
20S-Protopanaxadiol	H	H	H
Ginsenoside-Rb_1	Glc-Glc	H	Glc-Glc
Ginsenoside-Rb_2	Glc-Glc	H	Ara(*p*)-Glc
Ginsenoside-Rc	Glc-Glc	H	Ara(*f*)-Glc
Ginsenoside-Rd	Glc-Glc	H	Glc
20S-Protopanaxatriol	H	OH	H
Ginsenoside-Re	H	Rha-Glc-O	Glc
Ginsenoside-Rf	H	Glc-Glc-O	H
Ginsenoside-Rg_1	H	Glc-O	Glc
Ginsenoside-Rg_2	H	Rha-Glc-O	H

Fig. 1. Structures of ginsenosides used.

pp60$^{c\text{-}src}$ is the prototype of nonreceptor-type *src*-family PTKs. The kinase acts upstream of *ras* and *raf* and is a functionally important component of the receptor-initiated mitogenic signaling cascade.[8] Although the normal physiological function of the kinase is obscure, many reports suggest that pp60$^{c\text{-}src}$ is involved in the normal regulation of cell growth and differentiation. An activation of *c-src* kinase correlates with phosphorylations on tyrosine residues of a wide range of substrates, e.g., phosphoinositide-3-kinase, connexin 43, MAPK (mitogen-activated protein kinase), many of which are similarly phosphorylated during the mitogenesis of normal cells. Some oncogene products including *v-src* cause multiple alterations in the metabolism of phosphatidylinositol (PI) and its derivatives by both stimulating PI 4,5-bisphosphate (PIP_2) hydrolysis and activating IP_3-3-kinase and PI-3-kinase.[9] Inositol 1,4,5-triphosphate (IP_3) is an intracellular secondary messenger, which is produced by PIP_2 hydrolysis, and releases Ca^{2+} from the ER to cytosol by binding to IP_3-gated Ca^{2+}-release channels in the ER membrane. The following increase of intracellular free calcium concentration ($[Ca^{2+}]_i$) induces ultimately cell proliferation.[10] Therefore regulation of $[Ca^{2+}]_i$ has been proposed to be an important mechanism for cellular signaling, which functions as a trigger for initiation of diverse intracellular processes.[11]

Here we describe effects of ginsenosides on the activity of pp60$^{c\text{-}src}$ kinase, intracellular calcium concentration, and cell proliferation in *c-src* overexpressed NIH 3T3 mouse fibroblasts.

Materials and Methods

1. Preparation and treatment of drugs

Samples of ginsenosides obtained from Korean ginseng were kindly provided by Korea Ginseng and Tobacco Research Institute (Taejeon, Korea). An appropriate amount of each ginsenoside was dissolved in 50% (v/v) of ethanol before use. The solubility of each ginsenoside was different; soluble to 25 μg/μl of concentration for G-Re and -Rg_2 and 50 μg/μl for other ginsenosides and GTS.

2. Kinase assay of pp60$^{c\text{-}src}$

(1) Cells and cell culture

Wild-type *c-src* protein was immunoprecipitated from NIH (pMcsrc/focus)B *c-src* overexpressor cells.[12] Cells were plated at 1.0×10^6 cells per 100 mm plate 16-24 hr before the initiation of experiments and then grown at 37℃ and 5% CO_2/95% air in Dulbecco's modified Eagle's medium (DMEM; GIBCO, USA) supplemented with 10% calf serum. Cells prepared as above were treated with ginsenosides, incubated for the designated times, and then harvested for further experiments.

(2) Immunoprecipitation of pp60$^{c\text{-}src}$

Cells were washed twice in STE buffer [0.15M NaCl, 1 mM EDTA, 20 mM Tris-HCl (pH 7.2)] and lysed in 0.5 m*l* of RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 20 mM NaH_2PO_4) supplemented freshly with 1mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 50 mM NaF, 0.2 mM Na_3VO_4, and 100 KIU of aprotinin (Sigma Chemical Co., USA). Lysates were clarified at 25,000×g for 30 min. The amount of total cell protein (TCP) was det-

ermined by Bradford's method[13] and normalized to equal amount of proteins. $pp60^{c\text{-}src}$ was immunoprecipitated from a volume of lysate containing 100 μg of TCP with 1 μl of monoclonal antibody 327[14] for 45 min at 0℃. Immune complexes were collected on 30 μl of 10% *S. aureus* suspension that had been precoated with 1 μg of anti-mouse IgG (heavy plus light chains) by a 20 min-incubation at 0℃. It was washed once with high-salt buffer [1M NaCl, 0.5% Triton X-100, 10mM Tris-HCl (pH 7.2)] and twice with RIPA buffer.

(3) *In vitro* kinase assay of $pp60^{c\text{-}src}$

For kinase reactions, $pp60^{c\text{-}src}$ immunoprecipitates were resuspended in phosphorylation buffer [5 mM $MnCl_2$, 20 mM HEPES (pH 7.0), 2 mM β-mercaptoethanol]. Aliquots equivalent to 6 μg of TCP for each sample were used for the kinase reactions at room temperature for 5 min in 40 μl of reaction mixture consisting of phosphorylation buffer, acid-denatured rabbit muscle enolase (4 μg/sample), and 1 μM [γ-^{32}P] ATP (400 Ci/mmol, Amersham, England). At the end of 5 min, the reactions were stopped by adding sample buffer for SDS-PAGE [210 mM Tris-HCl (pH 6.8), 34.2% glycerol, 9% SDS, 0.03% bromphenol blue, 15% β-mercaptoethanol] and analysed on 10% SDS-PAGE followed by autoradiography. Band intensity was quantified by densitometry of autoradiograms using a Model CS-9000 densitometer (Shimadzu Co., Japan).

3. Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was determined as originally described for quin-2 by Tsien *et al.*.[15] Briefly, cells were loaded with a fluorescent Ca^{2+} indicator, fura-2/AM, to the final concentration of 2 μM in Hanks' balanced salts (HBSS) buffer and then incubated for 20 min at 37℃. Subsequently the cells were diluted one-fifth in HBSS containing 0.25% of BSA and incubated for 30 min at 37℃. After loading the dye, the cells were pelleted and washed three times with HBSS to remove the extracellular dye. Sulfinpyrazone was added to both loaded medium and washing solution to a final concentration of 250 μM to prevent dye leakage.[16] For fluorometric measurement, the cells (1.0×10^6) were placed in a quartz cuvette in the thermostatically controlled cell holder at 37℃ and suspended thoroughly. Fluorescence of fura-2-labelled cells was measured at 335 nm (excitation) and 510 nm (emission) in arbitrary fluorescence unit. Absolute $[Ca^{2+}]_i$ was calculated using the equation.[15]

$$[Ca^{2+}]_i = K_d * \frac{[F - Fmin]}{[F_{max} - F]}$$

K_d represents the dissociation constant for Ca^{2+}-bound fura-2 and is 224 nM. F_{max} and F_{min} are obtained when fura-2 is saturated with Ca^{2+} and when EGTA is used to remove Ca^{2+}, respectively. The values were measured at final concentrations of 4 mM EGTA, 30 mM Tris base, 0.1% Triton X-100, and 4 mM $CaCl_2$.

4. Cell proliferation assay

NIH (pMcsrc/focus)B cells were divided to be 1×10^3 cells/well in flat-bottomed 96-well plates in a final volume of 100 μl per well and grown at 37℃ and 6.5% CO_2 in humidified atmosphere. After treatmemt of ginsenosides at various concentrations, cells were incubated for 72 hr followed by adding to each well 50 μl 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, absorbance was measured at 492 nm using ELISA reader, Labsystems Uniskan II (Labsystems, Finland).

Results and Discussion

When cells overexpressing wild-type chicken $pp60^{c\text{-}src}$ were treated with 16.7 μg/ml (100 μg/6 ml) of ginsenosides for 18 hr, all ginsenosides used for this experiment (G-Rb_1, -Rb_2, -Rc, -Rd, -Re, -Rf, -Rg_1, -Rg_2, and GTS) activated $pp60^{c\text{-}src}$ PTK with different levels of activation (Fig. 2A, B). Among these, G-Rb_1, -Rd, -Rg_1, and -Rg_2 showed a higher stimulatory effect than others, increasing the activity by 4.5, 3.5, 3.5 and 3.0-

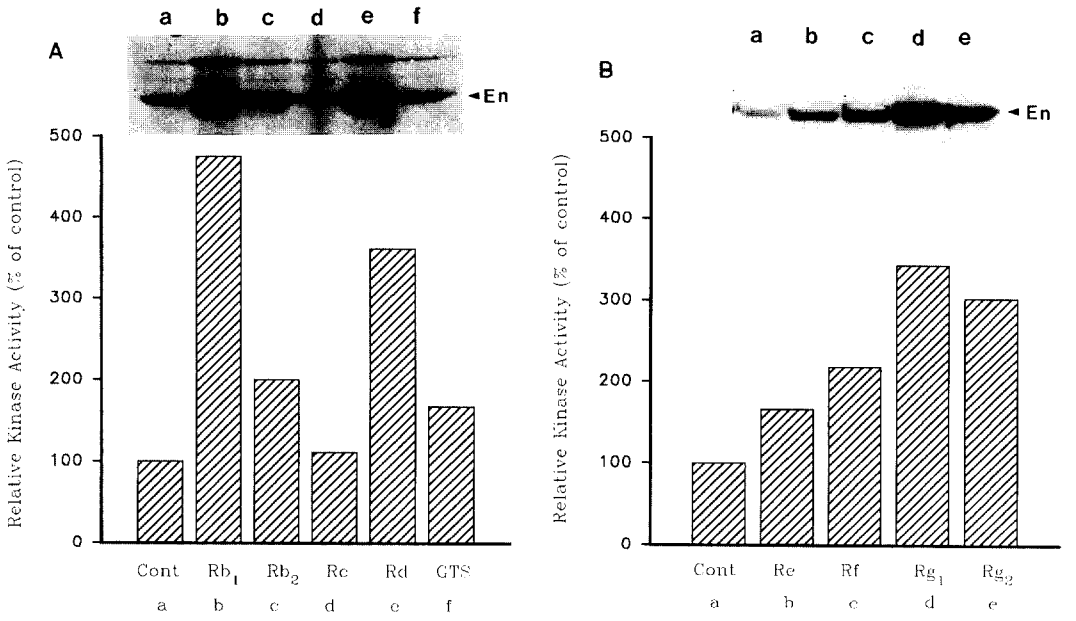


Fig. 2. Protein tyrosine kinase activity of pp60$^{c\text{-}src}$ in ginsenosides-treated cells. NIH (pMcsrc/focus) B cells were treated with 16.7 μg/m*l* (100 μg/6 m*l*) of ginsenosides for 18 hr and collected. pp60$^{c\text{-}src}$ was immunoprecipitated with MAb 327 from cell lysate, incubated with [γ-^{32}p] ATP and rabbit muscle enolase (En) as a substrate, and analysed by 10% SDS-PAGE and autoradiography. Band intensity was quantified by densitometry as described in Materials and Methods.

fold over that of untreated control, respectively. Time and concentration of ginsenosides for maximal *c-src* kinase activation were within the range of 12~18 hr and 16.7~33.4 μg/m*l* (100~200 μg/6 m*l*), respectively (data not shown). The ginsenosides-induced activation of *c-src* kinase was gradually up to optimal concentration and thereafter the effect rather reduced. The activation of the kinase by most of ginsenosides was dependent on incubation times in the same manner as concentration. However, G-Rd sustainedly activated the kinase from 6 to 24 hr with similar degrees of activation. The group difference between protopanaxadiol and protopanaxatriol saponins was not found in the stimulatory effect on *c-src* kinase. According to Cooper,[8] the *src* -family kinases are associated with a variety of receptors that lack intrinsic protein kinase activity, are activated by ligand binding, and couple non-tyrosine kinase receptors to their targets such as PLCγ and PI 3-kinase. These indicate that the *src* kinases are functionally important components of the receptor-initiated mitogenic signaling cascade. Based on these, our results suggest that the ginsenosides-induced activation of *c-src* kinase leads to other downstream events in cellular signaling.

In many cell types intracellular free calcium plays a central role in the transduction of external stimuli by changing its concentration.[17] To investigate whether ginsenosides bring about an increase of $[Ca^{2+}]_i$ and further, if it would happen, which is the source of the increased $[Ca^{2+}]_i$; either an influx from extracellular medium or an efflux from intracellular calcium store, ER, we determined $[Ca^{2+}]_i$ by spectrofluorimetry using a fluorescent Ca^{2+} indicator, fura-2. When ginsenosides (250 μg/m*l*) were treated to fura-2-loaded cells, $[Ca^{2+}]_i$ did not change except in the G-Rd or -Rg_2 treated cells. At a glance, it appeared that all ginsenosides increased significantly intracellular calcium concentration, but ginsenosides themselves gave background signals varied with a kind of ginsenosides (data not shown). G-

Rd and -Rg_2 increased $[Ca^{2+}]_i$ to 202 and 334 nM, respectively, within 7 min about 2-3-fold above the basal level (Fig. 3; A, C). After treatment of the ginsenosides, $[Ca^{2+}]_i$ gradually increased with time. However, the increases of $[Ca^{2+}]_i$ were eliminated by pretreatment of EGTA (4 mM), an extracellular calcium chelator (Fig. 3; B, D). This findings strongly suggest that the increase of $[Ca^{2+}]_i$ by G-Rd or -Rg_2 results from the influx of calcium ion from extracellular medium rather than the efflux from intracellular calcium store. The slow increase in $[Ca^{2+}]_i$ after adding G-Rd or -Rg_2 also supports that the increase does not come from IP_3-mediated calcium efflux from ER,

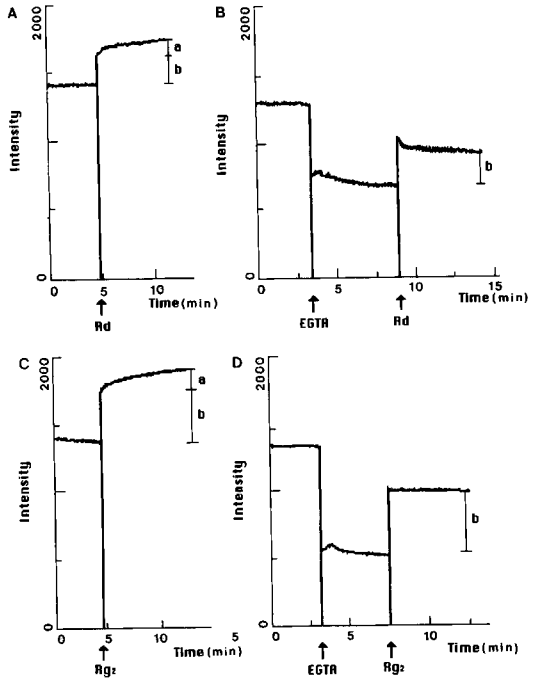


Fig. 3. Changes of the concentration of intracellular calcium ion ($[Ca^{2+}]_i$) after stimulation with G-Rd or -Rg_2, in the presence or absence of EGTA. Cells were labelled with fura-2 and treated with specified drugs. Fluorescent intensity was measured by spectrofluorimetry at 335 nm (excitation) and 510 nm (emission). Drugs added were: A, G-Rd (250 μg/m*l*); B, EGTA (4 mM) prior to G-Rd; C, G-Rg_2 (250 μg/m*l*); D, EGTA (4 mM) prior to G-Rg_2. a and b in spectra indicate $[Ca^{2+}]_i$ increase and background signal, respectively, by the ginsenosides.

which is regarded to bring about a large initial increase of $[Ca^{2+}]_i$ that is rapid in onset and often transient.[18] This influx of calcium ion to cytosol may primarily result from an increase of membrane permeability by a detergent effect, saponification, of ginsenosides common to all saponins, although it is not clear that the ginsenosides-induced activation of *c-src* kinase drives into the increase of $[Ca^{2+}]_i$. We also do not exclude that detection of a small, rapid change of $[Ca^{2+}]_i$ could be failed for technical reasons in our experiments.

It is well recognized that an activation of cellular signaling pathway induces ultimately the transcription of certain genes involved in cell proliferation and differentiation. We examined that effects of ginsenosides on $pp60^{c\text{-}src}$ kinase and $[Ca^{2+}]_i$ get connected to cell proliferation in NIH (pMcsrc/focus)B cells. When cells were incubated for 3 days with different concentrations of ginsenosides (5~250 μg/m*l*), all ginsenosides enhanced cell proliferation to 1.2~1.4-fold over that of untreated control. Table 1 summarizes

Table 1. Optimum concentrations of ginsenosides and corresponded cell proliferation in 10% calf serum culture media[a]

Ginsenoside	Concentration (μg/m*l*)	Cell proliferation (%)
G-Rb_1	25	123
Rb_2	5	119
Rc	25	117
Rd	25	134[b]
Re	25	117
Rf	5	126
Rg_1	25	141
Rg_2	5	131[b]
GTS	25	128

[a] Cells were plated to be 1×10^3 cells/well in 96-well plates. Cells were treated with different concentrations of ginsenosides, 0, 5, 25, 50 and 250 μg/m*l* in culture media, and incubated at 37℃, 6.5% CO_2 in humidified atmosphere. After a 72 hr-incubation, 50 μ*l* of XTT labeling mixture was added to each well, and 6 hr later spectrophotometrical absorbance of the samples was measured using ELISA reader at 492 nm. Data were obtained from the average of four experiments.

[b] Among ginsenosides used, two ginsenosides increased intracellular calcium concentration to 2-3-fold above basal level.

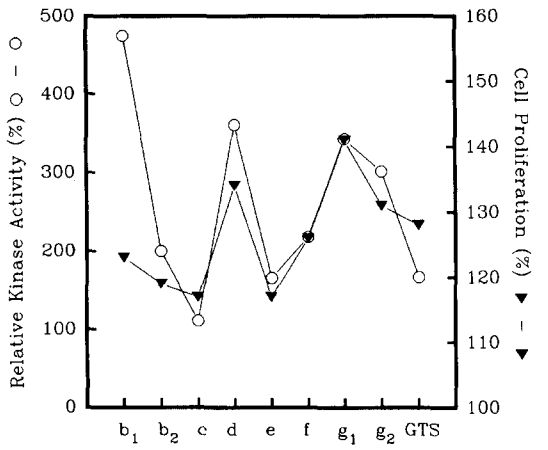


Fig. 4. Correspondence of cell proliferation with activity of *c-src* kinase after treatment of ginsenosides. Data were taken from Fig. 2 and Table 1.

the optimal concentrations of ginsenosides and the corresponded cell proliferation in 10% calf serum culture media. At higher concentrations than 50 μg/m*l* of G-Rd and 25 μg/m*l* of G-Rg_2, they showed cytotoxicity at 250 μg/m*l* of the ginsenosides, inhibiting cell proliferation to 85% and 40% of untreated contol, respectively (Data not shown). Considering that G-Rd and -Rg_2 increase significantly Ca^{2+} influx from extracellular media, we infer that the cytotoxic effects of G-Rd and -Rg_2 at high concentrations result from the destruction of calcium homeostasis in the cells coming from an excessive increase of calcium influx. In addition, the increases in cell proliferation by ginsenosides correspond with those in the activity of *c-src* kinase, although not doing exactly in case of G-Rb_1 (Fig. 4). The higher proliferation of the G-Rd or -Rg_2-treated cells indicates that increase of $[Ca^{2+}]_i$ by the ginsenosides may function in cell proliferation as a secondary messenger. Conclusively, our results imply that the ginsenosides-induced activation of *c-src* kinase that is upstream of cell signaling cascade is involved in proliferation of NIH 3T3 cells.

요 약

질병의 치료와 자양강장을 위해 보약으로 널리 사용되어 온 인삼은 그 효과가 주로 주성분인 인삼사포닌(ginsenoside; G-)에 기인한 것으로 보고되어 왔으며, 인삼의 약리학적 효과는 세포 활성화와 밀접한 관계가 있으므로 본 실험에서는 인삼사포닌이 *c-src* kinase 의 활성, 세포내 칼슘이온 농도, 나아가 세포증식에 미치는 영향을 알아보았다. 그 결과 NIH 3T3 세포에서 실험에 사용된 모든 사포닌(G-Rb_1, -Rb_2, -Rc, -Rd, -Re, -Rf, -Rg_1, -Rg_2, 인삼총사포닌) 이 *c-src* kinase 를 활성화시켰으며, 특히 G-Rb_1, -Rd, -Rg_1, -Rg_2가 16.7 μg/m*l*에서 18시간 처리로 현저한 활성화 효과를 나타냈다. 한편, 이들 중 G-Rd와 -Rg_2는 250 μg/m*l* 농도에서 세포내 칼슘이온 농도를 2~3배(각각 202, 334 nM)까지 증가시켰으며 이들 사포닌에 의한 칼슘농도의 증가는 세포내 Ca^{2+} 저장고인 ER 에서의 유리라기 보다는 세포외 Ca^{2+}의 유입에 의한 것임을 EGTA 처리로 확인하였다. 각종 kinase 활성화와 세포내 칼슘이온의 증가가 특정유전자의 발현을 증가시켜 세포증식을 유도하는 세포내 신호전달체계에 근거하여 인삼사포닌이 세포증식에 미치는 영향을 알아본 결과 9가지 사포닌 (5~250 μg/m*l*)에서 1.2~1.4배 정도의 증식 촉진효과를 관찰하였다. 특히 인삼사포닌에 의한 세포증식 촉진은 *c-src* kinase의 활성화에 대한 효과가 컸던 4가지 사포닌에서 크게 나타났으며, 위 결과들은 세포내 신호전달 체계의 초기단계인 *c-src* kinase-관련 경로의 활성화가 궁극적으로 세포증식을 유도하였을 가능성을 시사해준다.

Acknowledgement

We thank Korea Ginseng and Tobacco Research Institute for providing ginsenosides. We also thank Dr. D. Shalloway at Cornell University for providing Mab 327 antibody.

References

1. Kim, K. Y., Lee, H. B. and Joo, C. N. : *Kor. Biochem. J.* **17**, 312 (1984).
2. Lu, Z. Q. and Dice, J. F. : *Biochem. Biophys. Res. Commun.* **126**, 636 (1985).
3. Jin, R., Wan, L. L., Mitsuishi, T., Kodama, K. and Kuraashige, S. : *Yakugaku Zasshi* **114**, 533 (1994).
4. Jin, S. H., Kim, S. C. and Jung, N. P. : *Korean J. Ginseng Sci.* **10**, 151 (1986).
5. Sugaya, A., Yuzurihara, M., Tsuda, T., Yasuda,

K., Kajiwara, K. and Sugaya, E. : *J. Ethnopharmacol.* **22**, 173 (1988).
6. Deng, J. L. and Zhang, J. T. : *Chin. Med. J. Engl.* **104**, 395 (1991).
7. Kim, H., Chen, X. and Gillis, C. N. : *Biochem. Biophys. Res. Commun.* **189**, 670 (1992).
8. Cooper, G. M. : Pathways of mitogenic signal transduction. *In* Oncogenes, ed. Burns, J. E., Jones and Bartlett Publishers, Boston, p.279 (1995).
9. Fukui, Y., Saltiel, A. R. and Hanafusa, H. : *Oncogene* **6**, 407 (1991).
10. Sugimoto, Y., Whitman, M., Cantley, L. C. : *Proc. Natl. Acad. Sci. USA* **81**, 2117 (1984).
11. Lichtman, A. H., Segel, G. B. and Lichtman, M. A. : *Blood* **61**, 413 (1983).
12. Choi, J. K., Shenoy, S. and Shalloway, D. : *Cell* (USA) **57**, 763 (1989).
13. Bollag, D. M. and Edelstein, S. J. : Bradford assay. *In* Protein Methods, Wiley-Liss, Inc., New York, p.50 (1991).
14. Lipsich, L. A., Lewis, A. J. and Brugge, J. S. : *J. Virol.* **48**, 352 (1983).
15. Tsien, R. Y., Pozzan, T and Rink, T. J. : *J. Cell Biol.* **94**, 325 (1982).
16. DiVirgilio, F., Steinberg, T. H., Swanson, J. A. and Silverstein, S. C. : *J. Immunol.* **140**, 915 (1988).
17. Rasmussen, H. : *New Engl. J. Med.* **314**, 1094 (1986).
18. Bygrave, F. L. : Calcium. *In* Communication within Animal Cells, ed. Barritt, G. J., Oxford Univ. Press, New York, p.193 (1992).