Inhibitory Effect of Ginseng Total Saponins on Glutamate-Induced Swelling of Cultured Astrocytes

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The effects of ginseng total saponins (GTS) on L-glutamate-induced swelling of cultured astrocytes from rat brain were studied. Following exposure to 0.5 mM glutamate for 1 h, the intracellular water space (as measured by [³H]O-methyl-D-glucose uptake) of astrocytes increased three-fold with a morphological change: the disappearance of cellular processes. Simultaneous addition of GTS with glutamate reduced the astrocytic swelling in a dose-dependent manner. GTS at 0.5 mg/ml did not affect the viability of astrocytes for up to 18 h, which was determined by a colorimetric assay for cellular growth and survival. These data suggest that GTS prevents the cell swelling of astrocytes induced by glutamate.

Key words ginseng total saponin; glutamate; astrocytic swelling

Astrocytes are involved in the regulation of the electrolyte concentration and water volume of the extracellular space in the central nervous system (CNS). The cells are known to swell in some pathological states, such as hypoxia or ischemia.¹⁾ Glutamate, the major excitatory neurotransmitter amino acid, is known to be involved in the pathophysiology of neuronal cell death in hypoxic-ischemic brain injury,^{2,3)} epileptic brain damage⁴⁾ and other neurodegenerative disorders.^{5–7)} Glutamate, at concentrations similar to those required to induce neuronal cell death, causes the swelling of brain slices and astrocytes in primary cell culture.^{8–11)} Ketamine and MK-801, noncompetitive NMDA receptor antagonists that bind to the ion channel complexes, are effective in combatting the swelling of cultured astrocytes induced by glutamate.^{12,13)}

Much attention has been focused on ginseng saponins, the main effective components of ginseng, because of their multiple pharmacological action. Their central actions include the suppression of exploratory and spontaneous movements,¹⁴⁾ prolongation of hexobarbital sleeping time¹⁵⁾ and inhibition of reverse-tolerance development to dependence-labile drugs.¹⁶⁾ There have been several reports on the *in vitro* action of some active components extracted from *Panax Ginseng* on cultured cell lines. Ginsenosides, Rb1 and Rd, saponins isolated from *Panax Ginseng*, were reported to potentiate nerve growth factor-mediated neurite extension in organ cultures of chicken embryonic dorsal root ganglia and lumbar sympathetic ganglia.¹⁷⁾ A protective effect of the lipophilic components of *Panax Ginseng* on the differentiation of an established culture cell of rat pheochromocytoma, PC12 cells, was also reported.¹⁸⁾ The present study was performed to investigate the effect of ginseng total saponins (GTS) as an active component fraction of ginseng extract on the glutamate-induced swelling of cultured astrocytes.

MATERIALS AND METHODS

Materials [³H]O-Methyl-D-glucose ([³H]OMG) was purchased from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). Eagle's minimum essential medium (MEM) and fetal bovine serum were from Nissui (Tokyo,

Japan) and Gibco (U.S.A.), respectively. Dibutyl cyclic AMP (dBcAMP) was from Yamasa Shoyu (Chiba, Japan). Phroletin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). GTS [quantitatively containing at least 11 glycosides known as ginsenosides: Rb₁ (18.26%), Rb₂ (9.07%), Rc (9.65%), Rd (8.24%), Re (9.28%), Rf (3.48%), Rg₁ (6.42%), Rg₂ (3.62%), Rg₃ (4.7%), Ro (3.82%), Ra (2.91%), and other minor ginsenosides and components (20.55%), from *Panax ginseng*, extracted and purified by the method of Namba *et al.*¹⁹⁾] was supplied from Korea Ginseng and Tobacco Research Institute. All other chemicals used were of the highest grade available.

Cell Cultures Cultured rat astrocytes were prepared from the cerebral cortices of 1- to 2-d old Sprague-Dawley rats by the method of Frangakis and Kimelberg.²⁰⁾ Briefly, the dissociated cells were suspended in the culture medium (MEM with 10% fetal bovine serum) and plated at 1 × 10⁴ cells/cm² in plastic tissue culture wells (12 wells, Corning). The cells were grown in a 5% CO₂/95% air humidified atmosphere at 37 °C with an exchange of growth medium twice a week. The cells were grown to confluence in 2 weeks and then further differentiated in a culture medium containing 1 mM dBcAMP. The astrocyte cultures were used 3–5 d after dBcAMP treatment. At this stage, more than 90% of the cells show glial fibrillary acidic protein immunoreactivity, as described previously.¹⁰⁾

Exposure to Glutamate and GTS, and Determination of [³H]OMG Space After aspiration of the growth medium, cultured astrocytes were rinsed three times with HEPES-buffered Krebs-Ringer solution (HBKR (in mM); NaCl 156, KCl 5.6, NaHCO₃ 11, D-glucose 10, MgSO₄ 1, CaCl₂ 1, NaH₂PO₄ 1, HEPES-Na 20, pH 7.4) and were preincubated at 37 °C for 30 min in HBKR. The cells were exposed to glutamate and/or GTS in the fresh HBKR at 37 °C for 60 min. The cells were observed at the end of each incubation under a phasecontrast microscope. Astrocytic swelling was quantitatively studied by measuring the intracellular water space by [³H]OMG equilibrium uptake in intact cell cultures, as described elsewhere.¹⁰⁾ The uptake of [³H]OMG (0.5 mCi/well, 1 mM) was carried

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out for the last 20 min of incubation. The uptake was terminated by aspiration of the medium and subsequent rinsing 3 times with ice-cold HBKR containing 0.1 mM phloretin. The cells were then digested by 0.1 N NaOH and the subsequent aliquot was taken for protein determination and scintillation counting. Experiments were performed in duplicate with at least 3 different batches. Protein was determined by the method of Lowry *et al.*²¹⁾ The survival cell number was measured by a colorimetric assay using MTT.²²⁾ The relationship between cell number and the amount of generated MTT formazan was found to be directly proportional to the number of cells in a range from 1×10^4 to 1.3×10^5 cells.

Fig. 1. Effect of GTS on Glutamate-Induced Swelling in Cultured Astrocytes

Phasecontrast photomicrographs of cultured astrocytes exposed to: (A) normal HBKR (control); (B) glutamate (0.5 mM); (C) glutamate in the presence of GTS (0.5 mg/ml). The incubation was carried out at 37°C for 60 min.

RESULTS

Figure 1 shows the morphological changes of the cells observed under a phasecontrast microscope. Control cells have a stellate shape with many finely developed processes. Following incubation with 0.5 mM glutamate for 60 min, morphological changes in the astrocytes, such as swollen nuclei and the disappearance of obvious cell bodies and processes, were observed (Fig. 1B). In the previous study, we showed that these morphological changes are accompanied by a large increase in cell volume and concluded that glutamate causes astrocytic swelling.¹⁰⁾ In contrast, astrocytic morphology was not obviously changed after the simultaneous addition of GTS (0.5 mg/ml) with glutamate (Fig. 1C). Figure 2 shows the inhibitory effects of GTS on the glutamate-induced increase in astrocytic cell volume. Glutamate at 0.5 mM increased the intracellular water space from 3.17 ± 0.17 to $9.09 \pm 0.23 \mu\text{l H}_2\text{O/mg protein}$. GTS inhibited the glutamate-induced increase in the [^3H]OMG space of astrocytes in a dose-dependent manner (0.3–1.0 mg/ml). GTS alone did not affect the intracellular water space ($3.16 \pm 0.25 \mu\text{l/mg protein}$ at 0.5 mg/ml GTS).

Fig. 2. Effect of GTS on Glutamate-Induced Increase in [^3H]OMG Space in Astrocyte Cultures

Cultured astrocytes were treated with 0.5 mM glutamate (L-Glu) for 60 min in the presence or absence of GTS. [^3H]OMG space was measured in 3–5 different cultures by duplicate determination. Results are means $\pm$ S.E.M. *** $p < 0.001$ vs. control, ††† $p < 0.001$ vs. glutamate.

Table 1. Changes in Cell Numbers of Cultured Astrocytes by Treatment with GTS

Cell numbers ($\times 10^4/\text{cm}^2$)						
Pretreatment time (h)	0	1	2	4	8	18
GTS	4.25 ± 0.30	4.31 ± 0.01	4.05 ± 0.07	4.15 ± 0.18	4.47 ± 0.11	5.24 ± 0.09

Cultured astrocytes were further grown for various times in the culture medium containing 0.5 mg/ml GS. Cell numbers were measured by MTT assay. Results are means $\pm$ S.E.M. of four wells.

Since the MTT assay is a sensitive, quantitative and reliable colorimetric assay for cell viability, the assay was performed on the astrocytes incubated with 0.5 mg/ml GTS at various time intervals. Cell numbers of the astrocytes did not decrease, even up to 18 h following the incubation with GTS (Table 1). This result indicates that GTS do not injure the intact integrity of the cellular membrane or cell viability.

DISCUSSION

In agreement with the previous report,¹⁰⁾ glutamate (0.5 mM) caused the swelling of astrocytes in primary culture, as evidenced by morphological observations and by the increase in intracellular water space. This glutamate-induced astrocytic swelling was reduced by GTS in a dose-dependent manner. The mechanisms underlying the beneficial effect of GTS are not clear at present. However, the inhibitory effect of GTS on the glutamate-induced increase in [³H]OMG space was not due to a nonspecific detergent-like effect of saponins, because the level of cell numbers was not decreased after co-incubation with GTS, even up to 18 h. Cellular swelling is usually triggered by increased Na⁺ influx followed by an influx of water. Glutamate opens Na⁺ channels and increases Na⁺ uptake in astrocytes.^{22,23)} Thus, it is likely that GTS reduced the astrocytic swelling by blocking glutamate-induced Na⁺ influx. Since it is not known whether GTS contains active substances which specifically interact with Na⁺ channels, the action of GTS on astrocytic swelling may be due to a membrane stabilizing effect.

In previous reports, we indicated the central actions of GTS administered systemically (100–200 mg/kg) in mice. GTS injected intraperitoneally showed antagonism of opioid agonists-induced antinociception.²⁴⁾ Also, GTS blocked the development of cocaine- and methamphetamine-induced reverse tolerance and dopamine receptor supersensitivity in mice.^{25,26)} These findings suggest that active components of GTS can pass through the blood-brain barrier and thus interact with brain cells. We have no evidence regarding whether the concentration of GTS *in vitro* in the present study is within the range pharmacologically available *in vivo*. In view of the concentration used here, it does not seem to be high, because Namba *et al.*²⁷⁾ suggested that GTS has protective activity against the hemolytic action of a saponin, producing a 50% effect at 2.6 mg/ml concentration. Furthermore, in support of the present results, Liu *et al.*²⁸⁾ have reported that ginsenosides could significantly increase the survival rate of mice exposed to acute hypoxia.

In conclusion, the present results indicate that GTS have a protective effect on glutamate-induced astrocytic swelling *in vitro*. To assess the pharmacological aspect of this action of GTS, it is necessary to determine the active components of GTS.

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