Neurotrophic Actions of Ginsenoside Rb_1, Peptide Growth Factors and Cytokines

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ABSTRACT

Ginseng root has been considered to prevent neuronal degeneration associated with brain ischemia, but experimental proof in support of this speculation is limited. Moreover, few studies have compared the neuroprotective actions of ginseng ingredients with those of peptide growth factors and cytokines *in vivo*. Using a gerbil forebrain ischemia model, we demonstrated that the oral administration of red ginseng powder before an ischemic insult prevents delayed neuronal death in the hippocampal CA1 field and that a neuroprotective molecule within red ginseng powder is ginsenoside Rb_1. The neurotrophic effect of ginsenoside Rb_1, when examined in the gerbil ischemia model and in neuronal cultures, was as potent as or more potent than the effects of epidermal growth factor, ciliary neurotrophic factor, erythropoietin, prosaposin, interleukin-6 and interleukin-3. Besides the protection of hippocampal CA1 neurons against brain ischemia/reperfusion injuries, ginsenoside Rb_1 was shown to prevent place navigation disability, cortical infarction and secondary thalamic degeneration in stroke-prone spontaneous hypertensive rats with permanent occlusion of the unilateral middle cerebral artery distal to the striate branches. These findings may validate the empirical use of ginseng root for the treatment of cerebrovascular diseases.

Introduction

Ginseng root (*Panax ginseng* C.A. Meyer) has served as an important component of Chinese prescriptions (Kampou) for thousands of years. Since the introduction of ginseng root into oriental medicine, this crude drug has been thoughout to prevent a number of degenerative processes associated with brain ischemia, but experimental proof in support of this speculation is limited.

Ginseng root consists of two major ingredients; crude ginseng saponin (CGS) and crude ginseng non-saponin (CGNS) fractions. To date, more than 20 saponins have been isolated from ginseng root and identified chemically. They can be classified into three major groups according to their chemical structures; protopanaxadiol, protopanaxatriol and oleanolic acid saponins. Ginsenoside Rb_1, ginsenoside Rg_1 and ginsenoside Ro are representative substances, respectively (Shibata *et al.*, 1985).

Using the gerbil forebrain ischemia model, we first investigated whether or not red ginseng powder and ginseng ingredients prevented ischemia-induced learning disability and delayed neuronal

death in the hippocampal CA1 field. In this experimental design, ginsenoside Rb_1 but not ginsenoside Rg_1 or ginsenoside Ro was proved to exhibit a neuroprotective action *in vivo* when administered intraperitoneally prior to forebrain ischemia (Wen *et al.* 1996). To ascertain the central action of ginsenoside Rb_1, we next examined the effect of cerebroventricular infusion of ginsenoside Rb_1 in the gerbil ischemia model (Lim *et al.* 1997) and in stroke-prone spontaneously hypertensive (SH-SP) rats with permanent occlusion of the unilateral middle cerebral artery (MCA) distal to the striate branches (Zhang *et al.* 1998). Finally, the neuroprotective action of ginsenoside Rb_1 was compared with those of peptide growth factors and cytokines in the gerbil ischemia model and in neuronal cultures.

Materials and Methods

Animals

Male Mongolian gerbils weighing 70-80 g (approximately 12 weeks of age) and SH-SP rats at the age of 12 to 13 weeks, weighing 250 to 320 g, were housed in air-conditioned rooms at constant temperature (22 ± 1℃) with a 12:12 hour light-dark cycle, and given food and water ad libitum throughout the experiments. The animals were handled once a week for cage cleaning. The following experiments were conducted in accordance with the Guide for Animal Experimentation at Ehime University School of Medicine.

Treatment of gerbils with ginseng before ischemia

Red ginseng powder (RGP) dissolved in distilled water (0.6 to 1.5 g/kg/day) was orally administered and CGS (50 or 100 mg/kg/day), CGNS(50 or 100 mg/kg/day), ginsenoside Rb_1 (10 or 20 mg/kg/day), ginsenoside Rg_1 (10 or 20 mg/kg/day) or ginsenoside Ro (10 or 20 mg/ka/day) in saline was injected into the intraperitoneal cavity once a day for 7 days before 5-min forebrain ischemia in gerbils (n=8 per group). Ischemic controls and sham-operated animals were given the same volume of water or saline (n=8 per group). Twenty-four hours after the 7th treatment with ginseng, the gerbils were subjected to 5-min forebrain ischemia as described elsewhere (Wen *et al.* 1996). Then they were examined with a step-down passive avoidance task and processed for histopathological analyses of the hippocampal CA1 field (Wen *et al.* 1996).

Cerebroventricular infusion of ginsenoside Rb_1

Immediately after 3.5-min or 3-min forebrain ischemia, ginsenoside Rb_1 in a dose of 60 or 600 ng/day was continuously infused for 7 days into the cerebral ventricles of gerbils through an osmotic minipump (n=8 per group) (Lim *et al.* 1997). Ischemic controls and sham-operated animals received saline infusion. Subsequently the gerbils were examined as described above. Besides ginsenoside

Rb_1, basic fibroblast growth factor, ciliary neurotrophic factor, prosaposin, interleukin-6, erythropoietin, epidermal growth factor and interleukin-3 were infused in 3-min ischemic gerbils and their effects were investigated in the same way (Sano *et al.*, 1994; Wen *et al.*, 1995a, 1995b, 1998; Kotani *et al.*, 1996; Matsuda *et al.*, 1996; Sakanaka *et al.*, 1998; Peng *et al.*, 1998).

Two hours before or just after permanent occlusion of the unilateral MCA in SH-SP rats, the cerebroventricular infusion of ginsenoside Rb_1 (0.006-60 μg/day) was started (n=8 per group), and it lasted 4 weeks. At the 2nd and 4th weeks after MCA occlusion, the rats were subjected to repeated Morris water-maze tests (Okada et al., 1995; Zhang *et al.*, 1998; Igase *et al.*, 1998). Each test included three trials per day for 4 consecutive days. After the last water maze test, the rats were processed for the morphological analyses of cerebrocortical infarcts and secondary thalamic degeneration (Zhang *et al.*, 1998).

Neuronal cultures

Cortical neurons and hippocampal neurons in cultures were prepared as described elsewhere (Lim *et al.*, 1997, Zhang *et al.*, 1998; Peng *et al.*, 1998; Wen *et al.*, 1998). We investigated whether ginsenoside Rb_1, epidermal growth factor and interleukin-3 protected neurons against lethal damage caused by the free radical-promoting agent $FeSO_4$, according to the method of Zhang *et al.*, (1993).

Results and Discussion

The possible neuroprotective action of ginseng in 5-min ischemic gerbils was investigated by using a step-down passive avoidance task and subsequent neuron counts in the hippocampal CA1 region. Oral administration of red ginseng powder (RGP) significantly prevented the ischemia-induced decrease in response latency, as determined by the passive avoidance test, and rescued a significant number of ischemic hippocampal CA1 pyramidal neurons in a dose-dependent manner (Fig. 1). Intraperitoneal injections of crude ginseng saponin (CGS) exhibited a similar neuroprotective effect (Fig. 1). Crude ginseng non-saponin (CGNS) had a significant but less potent protective effect against impaired passive avoidance task and degeneration of hippocampal CA1 neurons. Ginsenoside Rb_1 significantly prolonged the response latency of ischemic gerbils and rescued a significant number of ischemic CA1 pyramidal neurons, whereas ginsenoside Rg_1 and Ro were ineffective (Fig. 1). These findings suggest that RGP and CGS are effective in the prevention of delayed neuronal death, and that ginsenoside Rb_1 is one of the neuroprotective molecules within ginseng (Wen *et al.*, 1996).

In the above study, however, the intraperitoneal injections of CGS or ginsenoside Rb_1 starting immediately after ischemic insult did not rescue ischemic CA1 neurons (Wen *et al.*, 1996). A possible explanation for this is that peripherally administered ginsenoside Rb_1, even though having neuro-

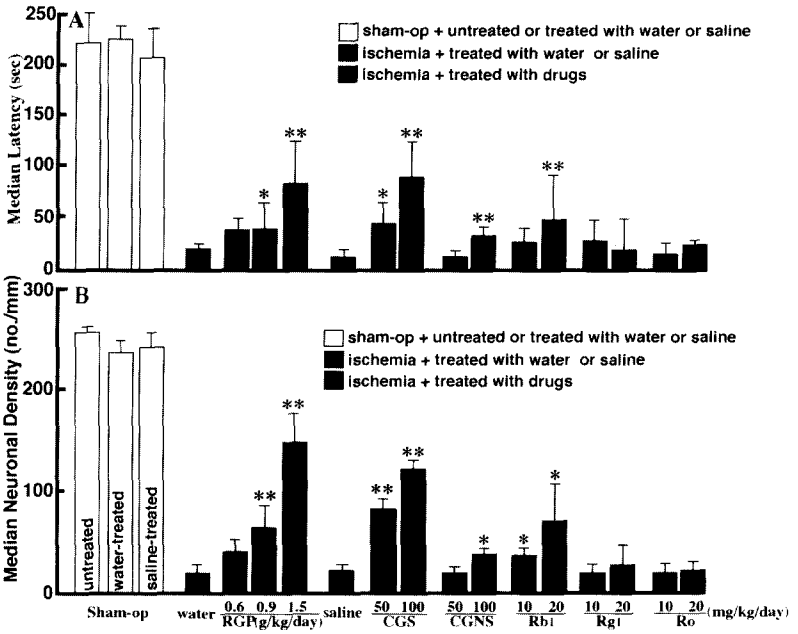

Fig. 1Effects of red ginseng powder (RGP), crude ginseng saponin (CGS), crude ginseng non-saponin (CGNS), ginsenoside Rb_1, ginsenoside Rg_1 and ginsenoside Ro on response latency in a passive avoidance task (A) and on number of hippocampal CA1 neurons (B) in 5-min ischemic gerbils (closed columns). When RGP, CGS or ginsenoside Rb_1 was administered for 7 days before ischemia, it caused a dose-dependent increase in response latency in the passive avoidance task (A) and in the number of viable neurons within the hippocampal CA1 region (B), in comparison with the response latency and CA1 neuronal density of ischemic gerbils treated with distilled water or saline (shaded columns). Note that CGNS pre-treatment was less effective and that ginsenoside Rg_1 or Ro pre-treatment was ineffective. The open columns indicate the median response latency and neuronal density of sham-operated (sham-op) animals. Each value represents median ± interquartile range (n=6-10). *P<0.05,**P<0.01; significantly different from the corresponding distilled water-treated or saline-treated ischemic group.

protective action, could not reach the ischemic brain by the time when neuronal death or survival was determined. If this speculation is the case, the central administration of ginsenoside Rb_1 after an ischemic insult should rescue hippocampal CA1 neurons. We infused ginsenoside Rb_1 into the cerebroventricles of gerbils immediately after 3.5-min or 3-min forebrain ischemia, and its effects were examined. The intracerebroventricular infusion of ginsenoside Rb1 after 3.5-min or 3-min forebrain ischemia precluded significantly the ischemia-induced reduction in response latency in the step-down passive avoidance task and rescued a significant number of hippocampal CA1 neurons from lethal ischemic damage (Figs. 2, 3), although the intraperitoneal injections of ginsenoside Rb_1 after 3.5-min or 3-min ischemia were ineffective (data not shown). The intracerebroventricular infusion of ginsenoside Rb1 did not affect hippocampal blood flow or hippocampal temperature except that it caused a slight but significant increase in hippocampal blood flow at 5 min after transient forebrain ischemia (data not shown). The neurotrophic action of centrally infused ginsenoside Rb_1 was as

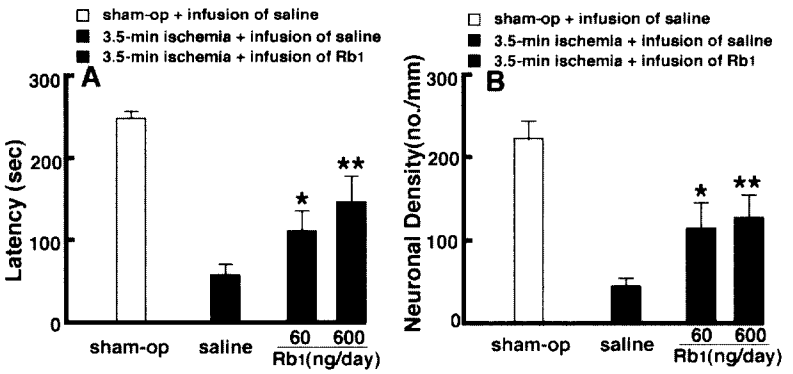

Fig. 2 **The effects of intracerebroventricular ginsenoside Rb_1 infusion on response latency in a passive avoidance task and on number of hippocampal CA1 neurons in 3.5 min ischemic gerbils (closed columns). When ginsenoside Rb_1 infusion into the lateral vehicle started just after 3.5-min forebrain ischemia and continued for 7 days, it caused a significant dose-dependent increase in response latency (A) and in the number of viable neurons within the hippocampal CA1 region (B), in comparison with the response latency and CA1 neuronal density of 3.5-min ischemic gerbils infused with saline (shaded columns). The open columns indicate the mean response latency and neuronal density of sham-operated (sham-op) animals. Each value represents mean ± S.D. (n=8). *P<0.05, **P<0.01, significantly different from the corresponding saline-infused ischemic group.**

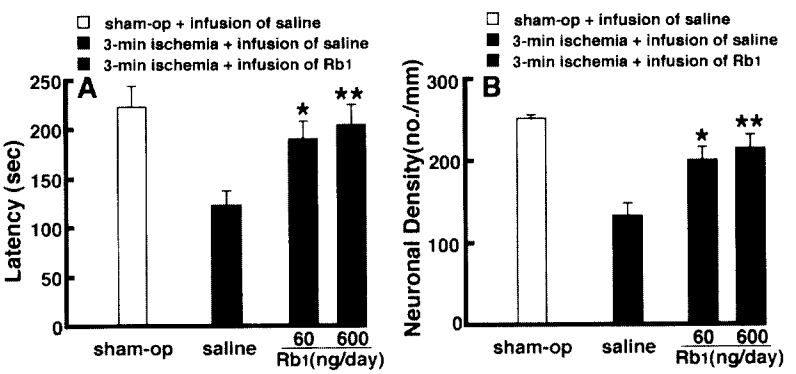

Fig. 3 Effects of intracerebroventricular ginsenoside Rb_1 infusion on the response latency (A) and CA1 neuronal density (b) of 3-min ischemic gerbils (closed columns). The infusion of ginsenoside Rb_1 after 3-min forebrain ischemia resulted in significant increases in response latency and CA1 neuronal density, in comparison with the response latency and CA1 neuronal density of saline-infused ischemic animals (shaded columns). The open columns indicate the mean response latency and neuronal density of sham-operated (sham-op) animals. Each value represents mean ± S.D. (n=8). *P<0.05, **P<0.01, significantly different from the corresponding saline-infused ischemic group.

potent as or more potent than those of basic fibroblast growth factor, ciliary neurotrophic factor, prosaposin, interleukin-6, etythropoietin, epidermal growth factor and interleukin-3 (Sano *et al.*, 1994; Wen *et al.*, 1995a, 1995b, 1998; Kotani *et al.*, 1996; Matsuda *et al.*, 1996; Sakanaka *et al.*, 1998; Peng *et al.*, 1998).

In neuronal cultures, ginsenoside Rb_1 at concentrations of 0.1-100 fg/ml (0.09-90 fM) rescued hippocampal and cortical neurons from lethal damage caused by the hydroxyl radical-producing agent $FeSO_4$ *in vitro* (data not shown) (Lim *et al.*, 1997; Zhang *et al.*, 1998). The effective concentrations of ginsenoside Rb_1 were much lower than those of basic fibroblast growth factor, nerve growth factor, insulin-like growth factor, epidermal growth factor and interleukin-3 (Zhang *et al.*, 1993; Peng *et al.*, 1998; Wen *et al.*, 1998). These *in vitro* findings, together with the *in vivo* effects of centrally infused ginsenoside Rb_1 on ischemic gerbils, suggest that post ischemic treatment with ginsenoside Rb_1 protects hippocampal CA1 neurons against lethal ischemic damage possibly by scavenging oxygen free radicals which are overproduced *in situ* after brain ischemia and reperfusion (Flamm *et al.*, 1978; Siesjo, 1981; Kogure *et al.*, 1982; Chan *et al.*, 1984).

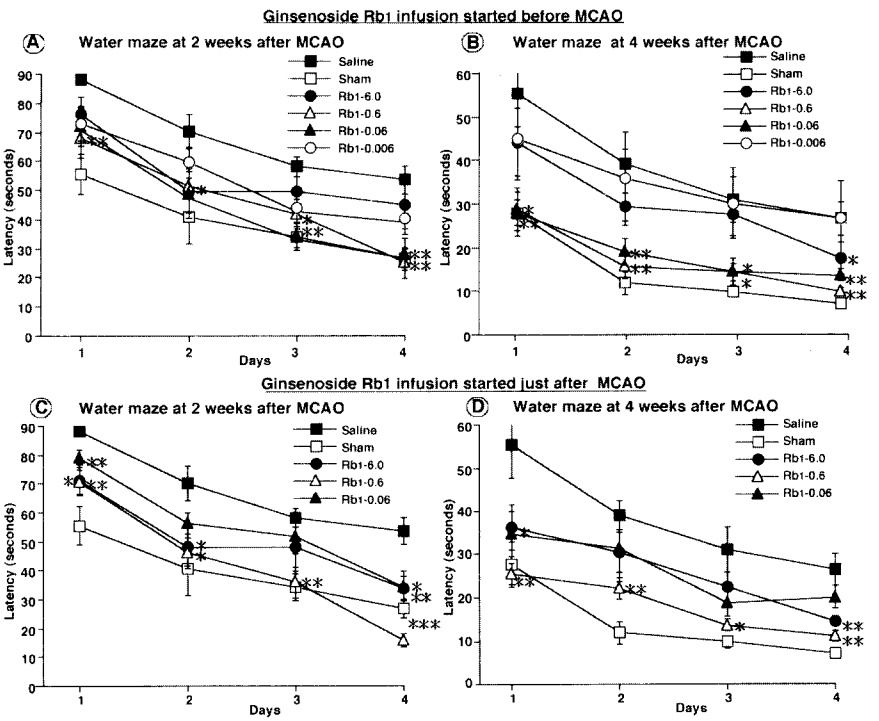

Fig. 4 A, B: Morris water maze tests carried out at 2 weeks (A) and 4 weeks (b) after MCA occlusion (MCAO) in stroke-prone spontaneously hypertensive rats that received either saline or ginsenoside Rb_1 (0.006 to 6 μg/day) infusion starting 2 hours before focal cerebral ischemia (n=8 per group). The escape latency of 0.6 μg/day ginsenoside Rb_1-treated group was significantly shorter than that of the saline-infused ischemic group on the first to fourth trial days, at the 2nd and 4th weeks after MCA occlusion. Note that treatment with 0.06 μg/day of ginsenoside Rb_1 also ameliorated the place navigation disability after MCA occlusion. *P<0.05, **P<0.01 significantly different from the escape latency of the saline-infused rats with MCA occlusion. C, D: Morris water maze tests carried out at the 2nd (C) and 4th (D) weeks after MCA occlusion (n=8 per group). Treatment with 0.6 μg/day ginsenoside Rb_1 was significantly effective in shortening escape latency. *P<0.05, **P<0.01, ***P<0.001 significantly different from the escape latency of the saline-treated ischemic rats.

Besides the long-established gerbil ischemia model showing so-called delayed neuronal death in the hippocampal CA1 region, several ischemic models have been developed using rats (Pulsinelli and Brierley, 1979: Coyle 1982; Coyle and Jakelainen, 1983; Okuyama *et al.*, 1991; Tamura *et al.*, 1991; Okada *et al.*, 1995; Kumon *et al.*, 1996; Watanabe *et al.*, 1998; Igase *et al.*, 1998). Among these, stroke-prone spontaneously hypertensive (SH-SP) rats with permanent occlusion of the MCA above the rhinal fissure and distal to the striate branches show a reproducible cortical infarct, place-navigation disability, and secondary thalamic degeneration centered on the ventroposterior nucleus (Fujii *et al.*, 1990; Iizuka *et al.*, 1990; Kumon *et al.*, 1996; Igase *et al.*, 1998). We chose this as an appropriate aminal model for the study of the effects of centrally infused ginsenoside Rb_1 on the ischemia- induced place-navigation disability, the primary ischemic lesion and on the secondary thalamic degeneration (Zhang *et al.*, 1998). We performed intracerebroventricular infusion of 0.6 μg/day ginsenoside Rb_1 before or after permanent occlusion of the left MCA in SH-SP rats. Ginsenoside Rb_1 significantly decreased escape latency on repeated trials of the Morris water maze test, throughout the first to fourth trial days at the 2nd and 4th weeks after MCA occlusion (Fig. 4). The ratio of the infarcted area to the left hemispheric area in the groups treated with 0.6 μg/day of ginsenoside Rb_1 was significantly smaller than that in the saline-treated ischemic group (Fig. 5).

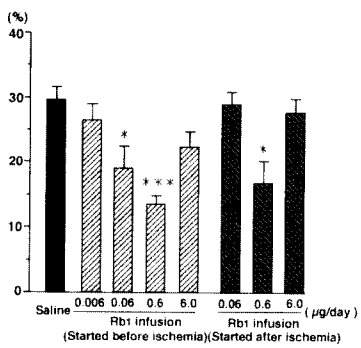

Fig. 5 Effects of different doses of ginsenoside Rb_1 on the infarcted area after MCA occlusion. Note that 0.6 μg/day of ginsenoside Rb_1 infusion, starting either before or after MCA occlusion, is effective in reducing the cortical ischemic lesion (*P<0.05, ***P<0.001). Ginsenoside Rb_1 at a dose of 0.06 μg/day ameliorates cortical infarction only when the administration is started before MCA occlusion (*P<0.05).

The continuous infusion of ginsenoside Rb_1 (0.06 μg/day) was less effective and the other doses examined were ineffective in ameliorating ischemia-induced place navigation disability and reducing cortical infarct size. There were significant differences in neuron numbers in the ventroposterior thalamic nucleus and in the left-to-right ratio of thalamic area between the saline-infused ischemic group and the ginsenoside Rb_1-treated ischemic group (data not shown). These findings suggest that ginsenoside Rb_1 exhibits a potent neuroprotective action in the MCA-occluded rats mimicking human patients with cerebrocortical infarction.

In conclusion, the present study may validate the empirical use of ginseng for the treatment of cerebrovascular diseases.

Acknowledgements

This project was supported in part by grants from the Ministry of Education, Science, Sports and Culture of Japan, the Medical Society for Red Ginseng Research and from the Ministry of Health and Welfare of Japan. The authors are grateful to Korea Tobacco and Ginseng Institute for the generous provision of ginseng. The secretarial assistance of Miss M. Fujimoto is acknowledged.

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