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Ginsenoside-Rd attenuates oxidative damage related to aging in senescence-accelerated mice

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Abstract

Among the various theories of the aging process, the free radical theory, which proposes that deleterious actions of free radicals are responsible for the functional deterioration associated with aging, has received widespread attention. The theory suggests that enhancement of the antioxidative defence system to attenuate free-radical-induced damage will counteract the aging process. We used senescence-accelerated mice (SAM) to investigate the relationship between aging and the antioxidative defence system and evaluated the effects of ginsenoside-Rd, the saponin from ginseng, by measuring antioxidative defence system parameters, including the glutathione (GSH)/glutathione disulfide (GSSG) redox status, antioxidative enzyme activity and level of lipid peroxidation. SAM at 11 months of age (old SAM) showed a significantly lower hepatic GSH/GSSG ratio, due to decreased GSH and increased GSSG levels, than SAM at 5 weeks of age (young SAM). However, the administration of ginsenoside-Rd at a dose of 1 or 5 mg kg^{-1} daily for 30 days to 10-month-old SAM significantly increased GSH, but decreased GSSG, resulting in elevation of the GSH/GSSG ratio. In addition, ginsenoside-Rd increased the activity of glutathione peroxidase (GSH-Px) and glutathione reductase that were both significantly lower in old SAM than in young SAM. This suggests that ginsenoside-Rd could play a crucial role in enhancing the defence system through regulation of the GSH/GSSG redox status. Moreover, decreases in the superoxide dismutase (SOD) and catalase activity in old SAM compared with young SAM were also revealed, indicating that the aging process resulted in suppression of the antioxidative defence system. However, ginsenoside-Rd did not affect SOD and catalase activity. As catalase is localized in peroxisome granules and GSH-Px is present in the cytoplasm and mitochondrial matrix, the site of ginsenoside-Rd action may be the cytoplasm and mitochondrial matrix. Furthermore, the serum and liver malondialdehyde levels, indicators of lipid peroxidation, were elevated with aging, while ginsenoside-Rd inhibited lipid peroxidation. This study indicates that the aging process leads to suppression of the antioxidative defence system and accumulation of lipid peroxidation products, while ginsenoside-Rd attenuates the oxidative damage, which may be responsible for the intervention of GSH/GSSG redox status.

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Introduction

Reactive oxygen species (ROS) are deeply involved in various pathologic conditions, such as inflammation, ischaemia and carcinogenesis. It has also been pointed out that, in relation to aging, generation of ROS, which is inevitable in aerobic organisms, and accumulation of injuries caused by ROS are important factors determining the lifespans of living cells and the body. In fact, since Harman (1956) first presented his view on the relationship between ROS and aging, a number of studies to investigate this issue have been carried out. However, the living body has a defence mechanism against peroxidation-derived injuries associated with aging. If the mechanism is functioning efficiently, such injuries are not apparent, but if this defence mechanism deteriorates, injuries may become manifest.

Although hypotheses such as antioxidant intake increases lifespan, antioxidant defences decline with age or the antioxidant defense status is positively correlated with lifespan have not been proven yet, it has been widely accepted that an adequate supply of antioxidants and an effective antioxidative defence system might delay and prevent the aging process and its related degenerative pathological conditions. The antioxidative

defence system consists of a network of antioxidative enzymes, such as superoxide dismutase (SOD), catalase and glutathione peroxidase (GSH-Px), and redox cycles that are linked together with small antioxidant molecules, such as glutathione (GSH), vitamin E, vitamin C, ubiquinol, uric acid and bilirubin (Reiter 1995; Sies 1997; Evans & Halliwell 1999; Hu et al 2000). It has been demonstrated that supplementation with antioxidants attenuates the damage induced by ROS and might increase the lifespan (Miquel 2002). In addition, Tolmasoff et al (1980) analysed the activity of SOD in relation to lifespan. They plotted the ratios of the specific activity of SOD in the hearts, livers and brains of various animals and man to the specific metabolic rates on the longitudinal axis and the maximum lifespans of the respective species on the horizontal axis and found a positive linear correlation; namely, the higher the SOD activity relative to the metabolic rate, the longer the lifespan. On the basis of the positive evidence relating to antioxidative defence systems and anti-aging effects, extensive studies have been carried out on the use of antioxidants to ameliorate or attenuate aging and its related degenerative diseases (Doctrow et al 1997; Lipman et al 1998; Melov 2002).

We examined the antioxidant activity of various medicinal and edible plants and found that ginseng saponin has antioxidant activity (Yokozawa et al 1998; Yokozawa & Owada 1999; Yokozawa & Liu 2000; Yokozawa & Dong 2001). In view of the statement (in a classic book) that long-term use of ginseng lightens the load on the body and prolongs life, we examined the effects of ginseng extract on the survival rate and survival function (lifespan) of rats and reported some findings suggestive of a life-prolonging effect of ginseng extract (Yokozawa & Oura 1988). In this study, we investigated the effects of ginseng on antioxidant activity in senescence-accelerated mice (SAM) using ginsenoside-Rd of the diol-type (a saponin belonging to the damaran group), which has been demonstrated to have oxidative-stress-reducing activity in young rats.

Materials and Methods

Animals and treatment

SAM (SAM-P/1) were originally obtained from Professor Masanori Hosokawa (Kyoto University). They were bred under conventional conditions, housed at $23 \pm 1\,^{\circ}C$ with a 12-h light–dark cycle and allowed free access to food and water. At 10 months of age, male mice were used for the experiments. One group was given water while the other two groups were given ginsenoside-Rd at a dose of 1 or 5 mg kg^{-1} daily, via a feeding tube, for 30 consecutive days, and they were subjected to the experiments 330 days after birth (11 months old). Young (5-week-old) SAM were used as controls. After induction of anaesthesia by intraperitoneal administration of sodium pentobarbital, 50 mg kg^{-1}, blood samples were obtained by cardiac puncture and the serum was separated immediately by centrifugation. The liver was subsequently extirpated from each mouse, immediately frozen in liquid nitrogen and kept at $-80\,^{\circ}C$ until analysis. Seven mice were used for each experimental group. All experimental studies using mice were conducted in accordance with Recommendation on the Establishment of Animal Experimental Guidelines approved by Toyama Medical and Pharmaceutical University.

Isolation of ginsenoside-Rd

Ginseng Radix (*Panax ginseng* C. A. Meyer) was produced in Korea and 100 g was boiled gently in 1 L water for 60 min. The extract was then concentrated under reduced pressure to leave a residue. The yield of the extract was 32% (w/w). As described previously (Oura et al 1975), a saponin mixture was prepared by dissolving the ginseng extract in water, adding ammonium sulfate to 70% saturation, dissolving the resulting precipitate in de-ionized water, dialysing it against de-ionized water until salt free and then lyophilizing the internal solution to yield flakes. These flakes were extracted with 99% methanol in a water bath under reflux and the methanolic solution was concentrated under reduced pressure. Then, 15 volumes of cold ether were added to this residual solution and the slightly yellowish precipitate was dried overnight under vacuum. This fraction was dissolved in distilled water and dialysed against distilled water for 1 week in a cold room to remove traces of brown resinoid substance. A white precipitate was observed in the dialysis bag and this was recrystallized from distilled water to afford the saponin mixture. Ginsenoside-Rd was isolated from the mixture by preparative thin-layer chromatography on silica gel, which was developed with a mixture of *n*-butanol–ethyl acetate–water (4:1:5 v/v/v, upper phase) and chloroform–methanol–water (65:35:10 v/v/v, lower phase), by the procedure of Shibata et al (1966). Identification and purity were determined by ^{1}H-NMR, ^{13}C-NMR, HPLC and melting point data. The chemical structure is shown in Figure 1.

GSH and glutathione disulfide (GSSG) assays

According to the method of Floreani et al (1997), the tissue (about 250 mg) was homogenized in 1 mL 25% metaphosphoric acid plus 3.75 mL 100 mM sodium phosphate–5 mM EDTA buffer (pH 8.0) and then centrifuged at 105 000 *g* for 30 min at 4 °C. Determination of the GSH and GSSG concentrations in the supernatant was performed by the method of Hissin & Hilf (1976), using *o*-phthaldialdehyde as the fluorescent reagent.

Enzyme assays

The tissue was homogenized with a 9-fold volume of ice-cold physiological saline and the activity of the enzymes in the homogenate was determined. The activity of SOD was measured according to the nitrous acid method described by Elstner & Heupel (1976) and Oyanagui (1984), which is based on inhibition of nitrite formation from hydroxylamine in the presence of superoxide (O_2^-) generators.

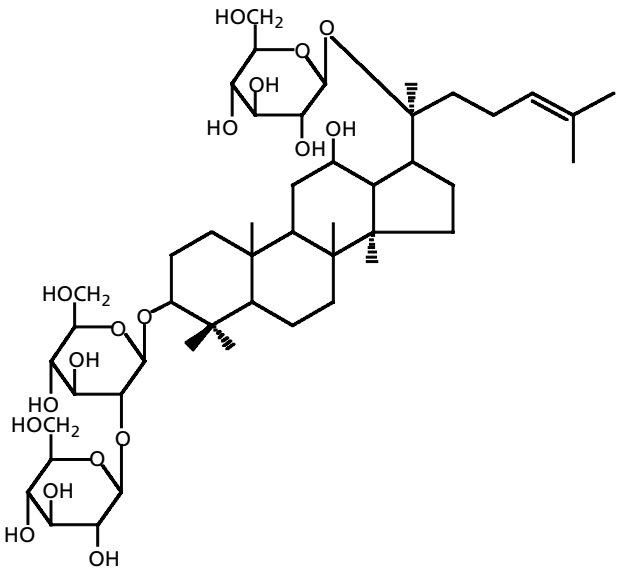

Figure 1 Chemical structure of ginsenoside-Rd.

Catalase activity was measured by following the decomposition of hydrogen peroxide (H_2O_2) directly by recording the decrease in extinction at 240 nm with time. The change in extinction per unit time was used as a measure of catalase activity (Aebi 1974). GSH-Px activity was obtained colorimetrically with 2-nitro-5-thiobenzoic acid, a compound produced by the reaction between GSH and 5,5′-dithiobis(2-nitrobenzoic acid), as described by Ellman (1959). For the glutathione reductase assay, tissue was homogenized with a 10-fold volume of ice-cold 1.5% KCl, the homogenate was centrifuged at 15 000 *g* for 40 min at 4 °C and the glutathione reductase activity in the supernatant was determined by the method of Tor-Agbidye et al (1999).

Determination of malondialdehyde (MDA) levels

The MDA level of serum was measured using the method of Naito & Yamanaka (1978) and that of liver tissue was assayed according to the method of Uchiyama & Mihara (1978).

Determination of protein levels

Protein levels were determined by the method of Itzhaki & Gill (1964) with bovine serum albumin as the standard.

Statistics

The values are presented as means ± s.e. The effect of ginsenoside-Rd on each parameter was examined using the one-way analysis of variance. Individual differences between groups were evaluated using Dunnett's test and those at $P < 0.05$ were considered to be statistically significant.

Results

As shown in Table 1, the GSH level and GSH/GSSG ratio were significantly lower in SAM at 11 months of age (old SAM) than in 5-week-old SAM. The values were about 88% and 67% of the respective 5-week-old SAM values and the hepatic GSSG level increased from 0.90 μmol g^{-1} to 1.19 μmol g^{-1}. Administration of ginsenoside-Rd efficiently suppressed the oxidation of GSH in old SAM. The content of the reduced form, GSH, increased, and that of the oxidized form, GSSG, decreased, the difference being significant for both parameters. The GSH/GSSG ratios of old SAM given ginsenoside-Rd orally at doses of 1 and 5 mg kg^{-1} daily increased significantly compared with water-treated old SAM.

The activity of enzymes participating in the glutathione redox cycle showed significant decreases in SAM at 11 months of age: the SOD, catalase, GSH-Px and glutathione reductase activity values were 16%, 13%, 16% and 16% lower, respectively, than the corresponding 5-week-old SAM values (Table 2). The administration of ginsenoside-Rd significantly increased the reduced GSH-Px and glutathione reductase activity. There was no significant variation in the SOD or catalase activity after administration of ginsenoside-Rd.

In comparison with SAM at 5 weeks of age, both the serum and hepatic levels of MDA were significantly higher in SAM at 11 months of age (Table 3). However, administration of ginsenoside-Rd reduced the serum MDA level. Compared with the control (11-month-old SAM given water) values, it decreased from 3.03 nmol mL^{-1} to 2.80

Table 1 Effect of ginsenoside-Rd on glutathione in mouse liver.

Group	Dose (mg kg^{-1}daily)	GSH (μmol (g liver)$^{-1}$)	GSSG (μmol (g liver)$^{-1}$)	GSH/GSSG
SAM (11 months)				
Control	—	5.10 ± 0.10^{b}	1.19 ± 0.06^{b}	4.36 ± 0.19^{b}
Ginsenoside-Rd	1	$5.45 \pm 0.13^{b,c}$	$1.09 \pm 0.04^{b,c}$	$5.06 \pm 0.32^{b,d}$
Ginsenoside-Rd	5	5.70 ± 0.12^{e}	$1.00 \pm 0.03^{a,e}$	$5.65 \pm 0.24^{b,e}$
SAM (5 weeks)	—	5.81 ± 0.09	0.90 ± 0.05	6.55 ± 0.20

[a] $P < 0.05$, [b] $P < 0.001$ vs SAM (5 weeks) values; [c] $P < 0.05$, [d] $P < 0.01$, [e] $P < 0.001$ vs SAM (11 months) control values.

Table 2 Effect of ginsenoside-Rd on the activity of mouse hepatic enzymes involved in the glutathione redox cycle.

Group	Dose ($mg\,kg^{-1}$daily)	SOD (U (mg protein)$^{-1}$)	Catalase (U (mg protein)$^{-1}$)	GSH-Px (U (mg protein)$^{-1}$)	Glutathione reductase ($nmol\,min^{-1}$ (mg protein)$^{-1}$)
SAM (11 months)					
Control	—	24.35 ± 1.96^{a}	256.6 ± 10.2^{c}	158.8 ± 6.6^{c}	15.96 ± 0.74^{c}
Ginsenoside-Rd	1	25.99 ± 2.47	260.0 ± 8.2^{c}	165.5 ± 3.9^{c}	$16.83 \pm 0.44^{c,d}$
Ginsenoside-Rd	5	27.74 ± 1.88	261.1 ± 4.6^{a}	$172.2 \pm 3.3^{b,e}$	$18.01 \pm 0.25^{a,f}$
SAM (5 weeks)	—	29.01 ± 2.67	295.5 ± 4.2	188.1 ± 6.6	19.11 ± 0.35

$^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ vs SAM (5 weeks) values; $^{d}P < 0.05$, $^{e}P < 0.01$, $^{f}P < 0.001$ vs SAM (11 months) control values.

Table 3 Effect of ginsenoside-Rd on malondialdehyde in mouse serum and liver.

Group	Dose ($mg\,kg^{-1}$ daily)	Serum MDA ($nmol\,mL^{-1}$)	Liver MDA (nmol (mg protein)$^{-1}$)
SAM (11 months)			
Control	—	3.03 ± 0.15^{a}	0.96 ± 0.08^{a}
Ginsenoside-Rd	1	$2.80 \pm 0.11^{a,b}$	0.85 ± 0.06^{a}
Ginsenoside-Rd	5	$2.54 \pm 0.09^{a,c}$	$0.74 \pm 0.08^{a,c}$
SAM (5 weeks)	—	1.98 ± 0.09	0.48 ± 0.05

$^{a}P < 0.001$ vs SAM (5 weeks) values; $^{b}P < 0.05$, $^{c}P < 0.001$ vs SAM (11 months) control values.

(8% decrease) and 2.54 $nmol\,mL^{-1}$ (16% decrease) after the administration of ginsenoside-Rd at doses of 1 and 5 $mg\,kg^{-1}$ daily, respectively. The MDA level of hepatic tissue showed larger decreases of 11% and 23%, respectively.

Discussion

Control of aging has long been a dream and many people have tried to find the elixir of life. Among the several theories of aging that have been suggested, the free radical theory, which proposes that normal aging results from random deleterious damage to tissue by oxidative stress caused by ROS (Harman 1956, 1992), has attracted much attention. Aged animals produce higher levels of ROS than their young counterparts, resulting in the accumulation of oxidative damage to DNA, proteins and lipids. Oxidative stress increases with age, as shown by many parameters, including increases in oxidative DNA damage, levels of lipofuscin, lipid peroxidation products and oxidatively damaged proteins and the oxidation of thiol/disulfide redox state (Mecocci et al 2000; Delori et al 2001; Tahara et al 2001; Jones et al 2002). Furthermore, the decline in antioxidant defences, as well as the progressive increase in oxidative stress, is commonly associated with aging and age-related degenerative diseases. Therefore, antioxidant supplementation has been expected to counteract the aging process. The antioxidative defence in biological systems includes non-enzymatic compounds, such as GSH and vitamin E, and enzymes, such as SOD, catalase and GSH-Px.

GSH is a tripeptide consisting of glutamic acid, cysteine and glycine that is present in various parts of the body. Tateishi et al (1988) reported that the supply of cysteine is the rate-limiting factor for biosynthesis of GSH. This substance has long been known for its role in the redox system in the body and, recently, attention has been focused on its extensive physiological activity. Since GSH is one of the most effective antioxidants in biological systems, the glutathione redox status changes with aging and oxidative stress. De la Asuncion et al (1996) have shown that mitochondrial GSH oxidation correlates with age-associated oxidative damage. In addition, GSH plays an important role in the regulation of apoptosis and inhibits the oxidation of proteins and lipoproteins by oxygen free radicals (Deneke & Fanburg 1989). It is involved in metabolism of H_2O_2 and lipid peroxides in the presence of the catalyst GSH-Px through its redox and nucleophilic reactions, the major reactions of the SH group (Knight 1998). Reduced GSH acts directly as a free radical scavenger by neutralizing ·OH and serves as a coenzyme of GSH-Px that reduces the substrates H_2O_2 and LOOH to water and alcohol, respectively, while being converted to oxidized GSSG. The oxidized GSSG is then converted to GSH by glutathione reductase to complete the glutathione redox cycle. The GSH/GSSG redox value is an indicator of the antioxidative status that protects against oxidative stress.

We studied the effects of GSH on redox control and focused particular attention on the liver, because the concentration of GSH is higher in the liver than other tissues. The GSH level was found to have decreased, but the GSSG level increased, in the livers of old SAM (11 months after birth) in comparison with those of young SAM (5 weeks after birth; Table 1). Other studies showed that the GSH levels in the brain, liver and lung decreased with aging (Farooqui et al 1987; Reed 1990; Christon et al 1995; Jung & Henke 1996; Palomero et al 2001). Lang et al (1992) reported that the blood GSH level decreased in about 50% of persons aged 60 years or older in comparison with healthy young individuals, showing a correlation with progress of senility. The age-related decrease in GSH level may make the elderly more vulnerable to oxidative damage. However, the administration of ginsenoside-Rd led to an increase in the GSH level and a decrease in the GSSG level. Although anti-aging treatment involving GSH administration has hardly been attempted, the increase in the level of endogenous reduced GSH noted in mice given ginsenoside-Rd suggests that ginsenoside-Rd would enhance the defence mechanism by regulating the GSH/GSSG redox status. Furthermore, the glutathione redox ratio ($GSSG/(GSH + GSSG) \times 100$), which represents the degree of H_2O_2 generation (Soejima 1992), decreased significantly in mice given ginsenoside-Rd (values not shown in results), which provides evidence of significantly increased glutathione reductase activity. Therefore, we speculate that ginsenoside-Rd inhibits the generation of H_2O_2 and converts oxidized glutathione to reduced glutathione, thereby maintaining the antioxidant status of the body. However, the mechanism of action of ginsenoside-Rd for the intervention of GSH/GSSG redox status is still known. The concentration and redox state of GSH are related to several factors, including nutritional state, such as intake of protein, biosynthesis, utilization, degradation, oxidation/reduction, such as NAD/NADH, and transport. The administration of ginsenoside-Rd did not affect the body weight in this study (data not shown), thus we propose that ginsenoside-Rd has an effect on other factors than nutritional condition in relation to the GSH/GSSG redox state. On the basis of the reports that saiko-saponines inhibited ·OH generation by the protein kinase C activation and O_2^- generation by NADH-dependent oxidase that exists in the cell membrane (Aoyagi et al 1987, 2000), we also propose that ginsenoside-Rd that resembles saiko-saponines in its structure may function in similar ways to saiko-saponines.

The enzymatic antioxidative defence mechanism also plays a crucial role in defence against free-radical-mediated oxidative damage. However, the relationship between aging and antioxidant enzyme systems is still a controversial and unresolved subject (Ji et al 1990; Sohal & Orr 1992; Mori et al 1998; Kasapoglu & Ozben 2001). This study showed that in old mice, the activity of GSH-Px and glutathione reductase, the enzymes constituting the glutathione redox cycle, declined, showing that the mice were in a state of oxidative stress (Table 2). In addition, reductions in SOD and catalase activity were also revealed in old compared with young SAM. Our results indicate that the aging process resulted in the suppression of antioxidative enzyme activity. In contrast, in old SAM given ginsenoside-Rd for 30 days, the reduced level of GSH increased and significantly higher levels of GSH-Px and glutathione reductase activity were observed compared with old SAM given water. However, ginsenoside-Rd had no effects on the activity of SOD and catalase. As catalase is localized in peroxisome granules and GSH-Px is present in the cytoplasm and mitochondrial matrix, serving as a scavenger of both LOOH and H_2O_2 (Sies 1991), the site of ginsenoside-Rd action may be the cytoplasm and mitochondrial matrix.

Change in the GSH/GSSG redox status and decline in the antioxidative enzyme activity with aging lead to the age-dependent increase in lipid peroxidation. These results indicate that the serum and liver MDA levels, indicators of lipid peroxidation, were elevated with aging (Table 3). Several reports support an increase in lipid peroxidation with aging (Cand & Verdetti 1989; Liu & Mori 1993; Christon et al 1995; Sanz et al 1997; Fukui et al 2001; Kasapoglu & Ozben 2001; Sverko et al 2002). These findings suggest that old mice may be more susceptible than young mice to oxidative stress. However, ginsenoside-Rd inhibited lipid peroxidation, determined by estimating serum and liver MDA levels. Taken together with our results, we conclude that ginsenoside-Rd attenuates the oxidative stress related to the aging process in the SAM model through the increase in GSH/GSSG and antioxidative enzyme activity, and reduction in the lipid peroxidation.

We examined various Oriental medicines and medical prescriptions that have free-radical-eliminating activity and found that ginseng has antioxidant activity (Yokozawa et al 1994, 1996; Dong et al 1996). Subsequently, we studied the active components of ginseng in an ischaemia–reperfusion model and observed the antioxidant activity of ginsenoside-Rd (Yokozawa et al 1998). It was suggested that the mechanism of action of ginsenoside-Rd could be inhibition of lipid peroxidation mediated by free radicals and, therefore, this compound might exert a beneficial effect on renal function. The results of this study suggest that ginsenoside-Rd is useful for inhibition of the senescence acceleration process under conditions of oxidative stress. However, if hypofunction in SAM is derived from a decrease in the antioxidative defence system and if ginsenoside-Rd can prevent such deterioration of the defence system, the balance of free radicals, antioxidants and the ability to repair ROS-induced injury should be determined in future investigations. When ginsenoside-Rd is incubated in rat gastric juice or 0.1 M HCl, it is converted to 20(*S*)-ginsenoside-Rg_3 and 20(*R*)-ginsenoside-Rg_3, as reported by Karikura et al (1991). There is evidence that ginsenoside-Rg_3 has a strong inhibitory effect on tumour growth and metastasis in cellular and in-vivo systems (Mochizuki et al 1995; Liu et al 2000; Keum et al 2003). On the other hand, the effect of ginsenoside-Rg_3 against oxidative damage induced by free radicals is controversial (Kim et al 1998; Liu et al 2002). Therefore, the influence of ginsenoside-Rg_3 on the aging process attracts considerable attention and further study has to be carried out to clarify the effect on aging of biometabolites of ginsenoside-Rd.

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