Inhibition of lipid peroxidation by deer antler (Rokujo) extract *in vivo* and *in vitro*

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Abstract

The effects of an ethanol extract prepared from the pilose antler of *Cervus nippon* TEMMINCK var. *mantchuricus Swinhoe* (Rokujo) on lipid peroxidation were investigated *in vivo* and *in vitro*, as measured by the content of malondialdehyde (MDA) using two methods. An oral ingestion of chloroform or ethanol to adult rodents induced unusual increase in the MDA synthesis in the liver. When the Rokujo extract had been repeatedly given to the animals, such increase in the MDA content was significantly reduced. In the microsome fraction prepared from mouse liver, the Rokujo extract suppressed *de novo* synthesis of MDA evoked by an addition of chloroform in the presence of NADPH-generating system. These results suggest that the hepatic lipid peroxidative injury induced by an increase in oxygen radicals could be protected by the treatment with Rokujo extrct *in vivo* and *in vitro*.

Key words Rokujo, *Cervus nippon* TEMMINCK var. *mantchuricus Swinhoe*, malondialdehyde, lipid peroxidation, aging.

Abbreviations MDA, malondialdehyde ; SAM, senescence-accelerated mouse ; TBA, thiobarbituric acid ; HPLC, high-performance liquid chromatography ; TCA, trichloroacetic acid ; SOD, superoxide dismutase ; Rokujo, 鹿茸.

Introduction

A consistent morphological feature of eukaryote cellular aging is the progressive accumulation of autofluorescent, lipoidal, pigmented granules called lipofuscin within the cytoplasm of non-dividing, long-lived cells such as neurones and cardiac myocytes.[1] The age-dependent increase in the amount of lipofuscin indicates that lipid peroxidation has occurred with the formation of malondialdehyde (MDA), which in turn reacts with primary amino groups in the formation of the fluorescent Schiff base.[2] The peroxidized lipids have been generally recognized to affect several cardiovascular, pulmonary, or hepatic diseases,[3] moreover, one of the end products, MDA, has mutagenic properties to be a chemical carcinogen *per se*.[4,5] Thus the peroxidation of lipids by free oxygen radicals, the formation of MDA, and the accumulation of lipofuscin are closely associated with each other, and may be one of the principal causes of aging.[2,6]

In the course of our research for anti-aging

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action of Rokujo (pilose antler of Chinese deer) extracts, we found that age-related increase in the amount of MDA in the liver and brain of senescence-accelerated mouse (SAM) was considerably restored to the normal level after a repeated administration of a water extract from Rokujo.[7] The effect of Rokujo to decrease the MDA content was highly specific to senile-prone, SAM-P/8 strain.[7] Therefore the present study was aimed to clarify the molecular basis for the protection of MDA increase by Rokujo. For this purpose, both *in vivo* experiments using normal adult animals administered with chloroform and ethanol which are known to induce MDA synthesis in the liver and *in vitro* experiment to evaluate the direct interaction of MDA with Rokujo extracts have been done. Two separate procedures were used for the determination of MDA content. One is thiobarbituric acid (TBA) assay,[11] and the other is an improved method using high-performance liquid chromatography (HPLC).[12]

Materials and Methods

Animals : Male Wistar rats (150−155 g) and male ddY mice (22−23 g) were used. They were maintained in conventional conditions at 23±1℃ in an alternating 12 hr light/dark cycle, and given food and water *ad libitum*.

Extraction from Rokujo : Unossified pilose antler of *Cervus nippon* TEMMINCK var. *mantchuricus Swinhoe* (Rokujo) was obtained from the Jilin (吉林) Province of China. Sliced Rokujo (4,240 g) was extracted with boiling water (2,000 ml) three times. Combined supernatant was condensed and added to 95% ethanol (6,000 ml). Ethanol-insoluble precipitates was removed by filtration. The filtrate was dried and the remaining yellow-brown powder (671 g) was stored at 4℃.

In vivo Rokujo treatment before a chloroform administration : Four groups of ddY mice (each consisted of 10 mice) were orally given the Rokujo extract at doses of 0 (2 groups), 200 (1 group), and 300 (1 group) mg/kg/day, respectively, for 7 successive days. Two hours after the last administration, a mixture of chloroform and mineral oil (1 : 1) was orally given to 3 out of the 4 groups (0, 200, 300 mg/kg) of mice at a dose of 50 μl/20 g body weight. The remaining control group was administered only with mineral oil. Two hours later, all mice were killed by decapitation and portions of the liver and blood were quickly sampled to measure free MDA content by the HPLC method, as described later.

In vivo Rokujo treatment before an acute ethanol intoxication : Rats were divided into 3 groups and one group was subjected to an oral treatment with the Rokujo extract (300 mg/kg/day) every 30 days. The other 2 groups of rats were treated with saline for the same period as the control. After the last administration, all rats were fasted for 16 hr. Then 30% ethanol was given orally to one non-treated and the Rokujo-treated groups at a dose of 5 ml/kg. Six hours later, rats were decapitated and the liver was dissected out to measure free MDA content by HPLC method.

In vitro Rokujo treatment in the liver microsome exposed to chloroform : The liver of ddY mice was homogenized with 4 volume of 50 mM Tris-HCl pH 7.4 containing 1.15% KCl in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 12,000 *g* for 15 min and the supernatant was further centrifuged at 105,000 *g* for 90 min. The pellet of the second separation was washed once with the homogenizing buffer and precipitated again by the centrifugation at 105,000 *g*. Portions of the microsomal fraction corresponding to 5 mg protein/tube were added to glass tubes containing the Rokujo extract, chloroform, and/or NADPH-generating system,[13] as indicated in the Table IV, in a total 1 ml volume supplemented with 100 mM Tris-HCl pH 7.5. The reaction mixtures were incubated at 37℃ for 20 min until 0.5 ml of 35% trichloroacetic acid (TCA) was added to them, and subjected to the measurement of MDA by TBA method.

Measurement of MDA by TBA assay : An aqueous solution of TBA (0.6%) was added to the tissue suspensions and heated for 15 min on a boiling water bath. After cooling, 4 ml of *n*-butanol was added and mixed vigorously. The butanol phase was separated by a brief centri-

fugation (1,000 *g*, 15 min) and absorbance was measured at 535 nm. Values were normalized by the protein content determined by the method of Lowry.[14]

Measurement of free MDA by HPLC method : Although the TBA method is certainly easy to use, the assay is not specific for free MDA itself since many other substances that may occur in biological material give positive reactions with TBA.[11,12] Contaminant organic solvent in the sample also affects the reaction of MDA with TBA.[11,12] The usage of HPLC equipped with an amino-phase column and an ultra-violet detector can solve such a problem. The principle of this method was described elsewhere.[12] In practice, the liver was homogenized with a mixture of acetonitrile (10%) and 30 mM Tris-HCl pH 7.4 (90 %) and centrifuged at 3,000 *g* for 5 min. The clear supernatant was directly injected into the inject loop (20 μl). The MDA peak was identified by comparison with a reference chromatogram of standard MDA solution (10 mM) freshly made of 1,1,3,3-tetramethoxypropane (Sigma). A typical chromatogram with peak identification is shown as an example in Fig. 1.

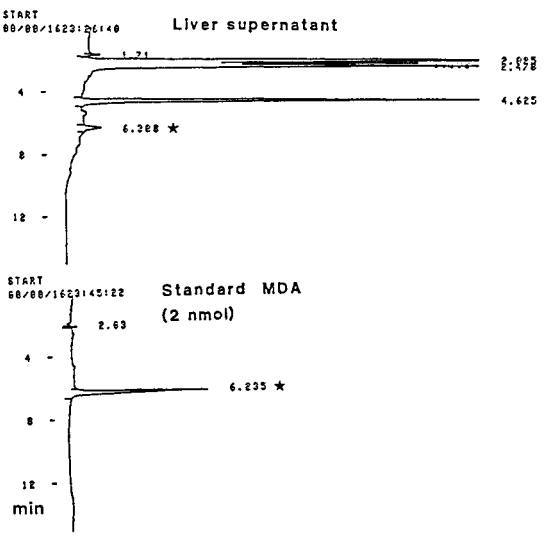

Fig. 1 HPLC separation of MDA in the mouse liver.
Standard MDA (2 nmol) or deproteinized liver supernatant was applied in a volume of 20 μl on a Cosmosil 5NH_2 aminophase column (4.6×150 mm) equilibrated with 0.03 M Tris-HCl pH 7.4 (90%) and acetonitrile (10%). Flow rate was 1 ml/min at room temperature. The effluent was monitored at 270 nm. The peak indicated with stars shows free MDA.

Statistics : Values were employed to Student's *t* test to evaluate significant differences.

Results

A comparison of the two methods to measure MDA

To evaluate the validity of equating the TBA reaction with the content of free MDA itself in the sample, we examined the parallelism between these methods. For this purpose, MDA content in the liver tissues of SAM which were stored at −80℃ since our previous work[7] was measured using TBA and HPLC methods (Table I).

Table I Comparison of the two methods measuring free MDA and MDA-like substances in the liver of SAM after administration with water extract from Rokujo for 8 days.

Dose (mg/kg/day, p.o.)	Free MDA by HPLC assay (nmol/100 mg tissue)	MDA-like substance by TBA assay (OD_{535})
0	9.4±2.2	0.29±0.05
100	4.9±0.5*	0.10±0.03***
200	3.1±0.9**	0.12±0.05***

Groups of 5 male SAM-P/8 mice (11 or 12 months of age) per treatment were administered orally with a water extract from Rokujo for 8 successive days.[7] One hour after the last administration, the animals were decapitated and the liver were quickly dissected out and stored for about 1 month at −80 ℃ until this measurement was carried out. Values are means±S.E.M. of 5 determinations. Significant differences are indicated as *(5%), **(1%) and ***(0.1%).

Table II Changes in the chloroform-induced MDA formation in the liver and plasma of ddY mice by a preceding 7 day-administration with ethanol extract from Rokujo.

Dose (mg/kg/day, p.o.)	Free MDA (nmol/100 mg weight) Liver	Plasma
Control	17.8±1.0[a)]	21.4±1.5
Chloroform[b)]	24.5±1.9**	41.9±1.6***
Extract 200 + chloroform	17.1±0.8#	33.4±3.8**
Extract 300 + chloroform	13.8±0.9*,##	27.8±2.2*,##

a) Values are means±S.E.M. of 4 determinations.
b) A mixture of chloroform and mineral oil (1 : 1) was administered orally at a dose of 0.05 ml/20 g body weight at 2 hr prior to decapitation.
*(5%), **(1%), ***(0.1%), vs. control. #(5%), ##(1%), vs. chloroform alone.

There was a good agreement between the TBA value and free MDA content in each preparation. The effect of a subchronic Rokujo treatment to decrease MDA which had been significant in the TBA assay,[7] was also confirmed by the HPLC method.

Effect of Rokujo treatment on chloroform-induced MDA formation in vivo

The acute toxic action of chloroform is known to involve the formation of free radical products in the liver.[9,13] The effect of a preceding 7 day-administration of the Rokujo extract to mice on the chloroform-induced MDA accumulation in the liver and plasma was examined (Table II). Two hours after a single oral administration of chloroform to control (not-treated with Rokujo) mice, an obvious increase in the free MDA level in the liver and plasma was produced. In the mice treated orally with the Rokujo ethanol extract for 7 days at doses of 200 and 300 mg/kg/day, such increase was significantly reduced in a dose-dependent manner.

Effect of Rokujo treatment on ethanol-induced MDA formation in vivo

In experimental animals, acute or chronic ethanol administration produce a depletion of reduced glutathione concomitantly with an enhancement in lipid peroxidation in the liver.[10,15] The effect of a preceding 30 day-treatment with the Rokujo extract to rats on the hepatic MDA accumulation elicited by an acute ethanol intoxication was examined (Table III). The free MDA content was doubled by the oral ethanol administration after 6 hr in control rats. In the rats chronically administered with Rokujo extract at a dose of 300 mg/kg/day, a significant reduction in the ethanol-induced MDA formation was observed.

Table III A change in the ethanol-induced MDA formation in the liver of rats by a preceding 30 day-administration with ethanol extract from Rokujo.

Dose (mg/kg/day, p.o.)	Free MDA (nmol/100 mg tissue)
Control	19.8±0.90[a)]
Ethanol[b)]	38.0±1.61***
Extract 300 + ethanol	30.1±2.60**,##

a) Values are means ± of 5 determinations.
b) 30% ethanol was administered orally at a dose of 5 ml/kg body weight at 16 hr prior to decapitation.
(1%), *(0.1%), vs. control.
##(1%), vs. ethanol alone.

Protection against chloroform-induced MDA formation by Rokujo extract in liver microsome in vitro

The general features of the reaction by which chloroform stimulates lipid peroxidation in the liver have been previously elucidated,[8] *i.e.* it requires a source of reduced $NADP^+$ (NADPH) in liver microsome fraction. In fact, as shown in Table IV, when chloroform was exposed to the mouse liver microsome in the presence of NADPH-generating system, a drastic MDA synthesis was evoked in comparison with the treatments of microsomes with chloroform alone, or

Table IV Effect of ethanol extract from Rokujo on the chloroform-induced MDA formation in mouse liver microsome *in vitro*.

Additions to microsome preparation	MDA by TBA assay[a] (μg/mg/protein)
No addition	2.55[b]
Extract (100 μg/ml)	3.30
Extract (200 μg/ml)	3.39
NADPH-generating system[c]	3.10
Chloroform (20 μl)	5.03
NADPH + chloroform	22.10
NADPH + chloroform + extract (100 μg/ml)	4.10
NADPH + chloroform + extract (200 μg/ml)	3.93

a) In this experiment, TBA assay was used because NADPH-generating reagents disturbed the HPLC identfication of MDA.
b) Values are the means of duplicate tubes.
c) NADPH-generating system contained 56 mM glucose-6-phosphate, 3 mM NADP and 0.5 unit glucose-6-phosphate dehydrogenase (Sigma).

NADPH-generating system. Rokujo ethanol extract in itself did not affect endogenous MDA synthesis, but when combined with chloroform and NADPH-generating system, strong inhibition of the chloroform-induced MDA formation was observed at concentrations of 100 and 200 μg/ml microsomal suspension.

Discussion

Previously, we demonstrated a novel decrease in the liver MDA content by a subchronic Rokujo treatment, using a specific murine model of self-promoted aging,[16] SAM. In this study, the anti-lipoperoxydative action of Rokujo extract in the liver was further characterized using normal mice and rats *in vivo* as the protection against acute chloroform-or ethanol-induced MDA increase. As we described before,[7] Rokujo treatment in itself does not affect the physiological MDA level in the liver of normal animals. Similar oral treatments with ethanol extract from Rokujo produced a marked reduction in the hepatic peroxidative damage, that is, the content of MDA, elicited by the ingestion of toxic radical generators. The anti-lipoperoxidative of Rokujo extract was also evident *in vitro*. Chloroform-induced MDA induction in the microsome fraction from mouse liver was dependent on NADPH-generating system, and almost suppressed in the presence of the Rokujo extract. In our preliminary experiment, direct conjugation of a Rokujo ingredient (diethylether-soluble fraction) with free MDA molecule *in vitro* was found (data not shown). Some substance (s) included in Rokujo may remove excessive free MDA from the cytoplasm of liver cells. It was previously shown[17] that net protein synthesis in the liver of senile mice was increased after the Rokujo treatment because of an increase in RNA polymerase II activity to produce messenger RNA molecules. The increased protein synthesis resulted in the recovery of diminished superoxide dismutase (SOD) activity in aged mice liver.[7] It is possible that the Rokujo extract may stimulate liver SOD which scavenge oxygen radicals causing lipid peroxidation and MDA formation.

和文抄録

マンシュウジカ (*Cervus nippon* TEMMINCK var. *mantchuricus Swinhoe*) の幼角 (鹿茸) から調製したエタノールエキスが, 脂質酸化に対して生体内で, あるいは試験管内でおよぼす影響について, マロンジアルデヒド (MDA) を2種類の方法を用いて定量して調べた。成熟した齧歯類へのクロロホルムやエタノールの1回経口投与は, 肝臓において非正常的なMDA合成の増加を誘導した。鹿茸エキスを予めこれらの動物に半慢性的に与えておくと, そのようなMDA量の上昇は有意に減弱した。マ

ウス肝臓のミクロソーム画分において，鹿茸エキスはNADPH生成系の存在下で添加したクロロホルムで誘発される新たなMDAの合成を抑制した。これらの結果は，酸素ラジカルの増加で誘発される肝臓の脂質の過酸化的障害が，鹿茸エキスの処置によってまるごとの動物の体内でも，試験管内での反応でも，保護されることを示唆している。

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