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Original Article

Inhibitory effect of propolis on the development of AA amyloidosis

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Abstract: In the several types of amyloidoses, participation of oxidative stresses in the pathogenesis and the effect of antioxidants on amyloidosis have been reported. Meanwhile, the relationship between oxidative stresses and pathogenesis of amyloid A (AA) amyloidosis is still unclear. In this study, we used an antioxidant, Brazilian propolis, to investigate the inhibitory effects on AA amyloidosis. The results showed that AA deposition was inhibited by administration of propolis. Increased expression of antioxidant markers was detected in molecular biological examinations of mice treated with propolis. Although serum amyloid A (SAA) levels were strongly correlated with the immunoreactive area of AA deposits in the control group, the correlation was weaker in the propolis-treated groups. In addition, there were no changes in SAA levels between the control group and the propolis-treated groups. The results indicate that propolis, an antioxidant, may induce inhibitory effects against AA amyloidosis. (DOI: 10.1293/tox.2017-0044; J Toxicol Pathol 2018; 31: 89–93)

Key words: AA amyloidosis, antioxidant, propolis

Introduction

Amyloid A (AA) amyloidosis is a fatal disease characterized by deposition of AA fibrils in systemic organs including the spleen, liver, and kidneys. The precursor protein of AA is serum amyloid A (SAA), which is synthesized in the liver during chronic inflammation that occurs in conditions such as rheumatoid arthritis[1]. In experimental animals, AA amyloidosis can be induced by the administration of amyloid fibrils as an amyloid enhancing factor (AEF) with concurrent inflammatory stimulation[2]. This model is known as a transmission model of AA amyloidosis, and it has been used in research on the condition[3, 4]. However, the pathogenesis of AA amyloidosis has not been elucidated, and there is no standardized treatment for the disease[5, 6].

In the current study, the effects of an antioxidant on an experimental AA amyloidosis model were investigated. In the case of amyloid β (Aβ) amyloidosis, various antioxidants have been shown to reduce oxidative stress caused by the deposition of Aβ[7], and it is known that antioxidants like vitamin E or curcumin suppress the progression of Aβ deposition in mice[8–10]. Furthermore, inhibitory effects of antioxidants on the formation of Aβ *in vitro* have been reported[11]. In a mouse model of familial amyloid polyneuropathy, administration of drugs with strong antioxidant properties decreased the deposition of transthyretin[12]. In a study on islet amyloidosis, DNA damage was induced by oxidative stress resulting from islet amyloid polypeptide deposition[13]. In the case of AA amyloidosis, detection of lipoperoxidation in the organs of disease patients has been reported[14]. However, the relationship between oxidative stress and the pathogenesis of AA amyloidosis as well as the effects of antioxidants on the deposition of AA fibrils have remained unclear.

Propolis is a resinous substance produced by honeybees from plants. It has been shown to possess various biological properties including antimicrobial activity, anti-inflammatory effects, and antioxidative effects, which have made it a popular ingredient in health foods[15]. Previous research shows that the active components of propolis include flavonoid-like compounds called phenylpropanoids, which protect against Aβ-induced toxicity and exhibit inhibitory effects against Aβ formation[16, 17]. Brazilian green propolis contains cinnamic acid derivatives and flavonoids, which possess antioxidant properties[18].

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In this study, we administered propolis to mice by mixing it in their diet and examined the inhibitory effects on AA amyloidosis.

Materials and Methods

Animals

Twenty male C57BL/6J mice, 7 weeks of age, were purchased from Charles River Laboratories (Tokyo, Japan). All mice were maintained under conventional conditions. Tap water from a bottle and powder feed (CRF-1, Charles River Laboratories) were supplied without restriction. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Approval from an animal care and use committee (27-96) was obtained for the research program at the Tokyo University of Agriculture and Technology.

Experimental design

Ethanol extract of Brazilian green propolis was obtained from API Co., Ltd. (Gifu, Japan). A 20-fold dilution of spleens from mice with AA amyloidosis in saline was prepared as AEF. For administration of propolis, 20 mice were divided into 4 groups, each consisting of 4-6 mice, and administered propolis at 0, 200, 1,000, or 5,000 ppm with powder feed, respectively, by mixing the appropriate volumes in their diet. Following 7 days of propolis administration, all mice were subcutaneously inoculated with 0.5 mL of 2.0% $AgNO_3$ solution as an inflammatory stimulus and intraperitoneally inoculated with 0.3 mL of AEF. On the 10th day following this injection, all mice were euthanized under anesthesia and necropsied, upon which their spleens and livers were collected. A portion of livers was preserved by freezing for molecular biological analysis, and the rest of the livers and whole spleens were fixed in Methacarn liquid for histological analysis. In addition, sera were collected from the mice for the measurement of SAA at the following time points: 3 days after the inflammatory stimulus and at the time of necropsy. These were stored at −20°C.

Histological and immunohistochemical examinations

Fixed organs were embedded in paraffin and cut into 2-μm sections. Subsequently, they were stained with hematoxylin and eosin (H&E). Immunohistochemistry was conducted using anti-mouse SAA goat monoclonal antibody (1:160, R&D Systems, Minneapolis, MN, USA).

Quantification of AA deposition was performed by ImageJ software (National Institutes of Health, Bethesda, MD, USA), with immunohistochemical analysis conducted in three regions of the spleen, and a low-powered image of the liver was obtained from each mouse. The immunoreactive area was measured in these regions for each mouse, and in the case of the spleen, the average of the values for the three regions examined was identified as the individual score.

The individual scores for AA deposition obtained from the mouse spleens were used to compare the treated animals, and linear regression analysis was performed using scatter diagrams.

Measurement of serum SAA concentration

The SAA concentrations in mouse sera were measured using an enzyme-linked immunosorbent assay (ELISA) kit for murine SAA (Tridelta Development Limited, Maynooth, County Kildare, Ireland). ELISA was performed according to the manufacturer's instructions. Data for SAA concentration at 3 days following inflammatory stimulation and at the time of necropsy and for AA deposition in livers and spleens were used to construct scatter diagrams for linear regression analysis. Because 1 serum sample at 3 days following inflammatory stimulation and 2 serum samples at the time of necropsy could not be analyzed due to hemolysis, the remaining 19 serum samples at 3 days following inflammatory stimulation and 18 serum samples at the time of necropsy were used for ELISA.

mRNA transcript expression of inflammatory markers and antioxidant markers in the liver

Total RNA was extracted from the liver of each mouse using an RNeasy Mini Kit (Qiagen, Venlo, Netherlands). The concentrations of total RNA samples were measured using Gen5 2.0 (BioTek Instruments, Inc., Winooski, VT, USA). Subsequently, cDNA samples were prepared from 500 ng of total RNA using PrimeScript RT Master Mix (Takara Bio Inc., Kusatsu, Shiga, Japan) in a Life ECO Thermal Cycler (Hangzhou Bioer Co., Ltd, Hangzhou, China) (37°C for 15 min and 85°C for 5 s). Real-time PCR was performed using SYBR Premix Ex Taq II (Takara Bio Inc.) in a Thermal Cycler Dice Real Time System II (Takara Bio Inc.) (95°C for 30 s followed by 40 cycles of 95°C for 5 s, 60°C for 30 s, 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s). For marker analysis, three targets for inflammatory cytokines [interleukin (IL)-6, IL-1β, monocyte chemotactic protein (MCP)-1] and six targets for antioxidant-related factors [catalase (CAT), glutathione peroxidase (GPx) 1, peroxiredoxin (Prdx) 1, superoxide dismutase (SOD) 1, SOD 2, glutathione reductase (GSR)] were investigated. Primer sequences are listed in Supplementary Table S1: online only. The relative values of gene expression as compared with a control were calculated using standard curve values normalized against those of the endogenous control gene ß-actin in the same sample.

Statistical analysis

Significant differences among the experimental groups were determined by calculating P values using Student's *t*-test or Dunnett's test. Correlations were determined by calculating Spearman's rank correlation coefficient.

Results

Histological and immunohistochemical examinations

Histologically, amyloid deposits were observed around the white pulp of spleens and Disse's spaces of livers in mice of all experimental groups. Amyloid deposits were

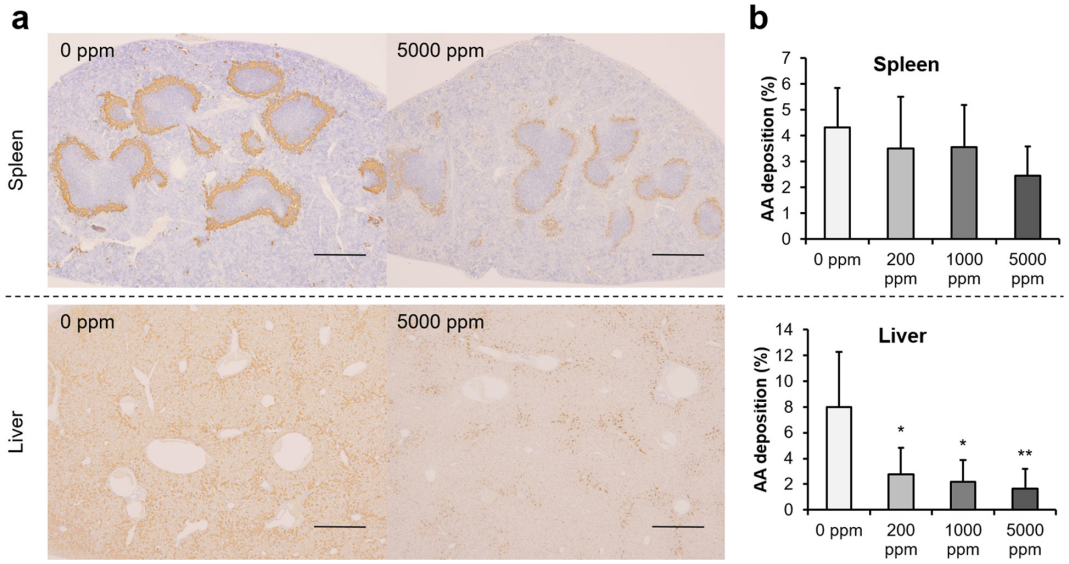

Fig. 1. Immunohistochemical analysis of amyloid deposits and immunoreactive area data. (a) Images of immunohistochemistry with anti-mouse SAA antibody in spleens and livers; bars = 500 µm. (b) ImageJ analysis revealed a downward trend for splenic AA deposition in the propolis treatment group and significant decline in hepatic AA deposition in the 200, 1,000, and 5,000 ppm propolis treatment groups. *p<0.05 and **p<0.01 when compared with the untreated control using Dunnett's test.

immunopositive for mouse SAA (Fig. 1a). ImageJ analysis revealed a downward trend in the immunoreactive area of splenic AA deposits in mice treated with propolis and a significant decline in hepatic AA deposits in mice treated with 200, 1,000, and 5,000 ppm propolis (Fig. 1b), when compared with control values.

Measurement of serum SAA concentration

There were no significant differences in serum SAA concentrations between mouse groups (Fig. 2). In the control group, moderate to strong positive correlation was observed between the serum SAA concentration on the third day following inflammatory stimulation and at the time of necropsy and the individual scores of hepatic and splenic AA deposition (Fig. 3). But, in propolis-treated groups, the correlation coefficients were lower than those in the control groups.

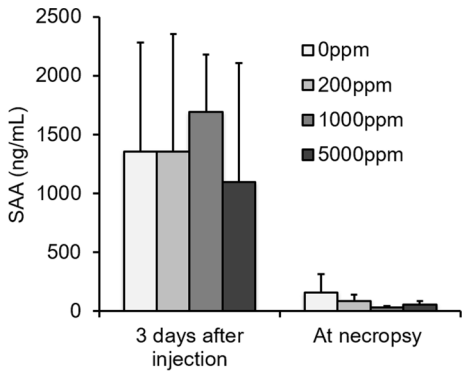

Fig. 2. Serum SAA concentrations in the experiment. There were no significant differences in serum SAA concentration between mouse groups.

mRNA transcript expression of inflammatory markers and antioxidant markers in the liver

The mRNA levels of IL-6 and IL-1β were suppressed in the 5,000 ppm group, while the mRNA level of MCP-1 was significantly reduced in the 1,000 and 5,000 ppm groups. The mRNA levels of CAT, GPx1, Prdx1, SOD1, and SOD2 were significantly elevated in the 5,000 ppm group, but that of GSR was not found to be affected by propolis (Table 1).

Discussion

In this study, AA deposition in the spleen and liver of a mouse model of AA amyloidosis was inhibited by propolis administration. Propolis administration elevated the expression of antioxidant-related genes, CAT, GPx1, Prdx1, SOD1, and SOD2. Additionally, it was previously reported that propolis also induced the expression of antioxidant-related genes such as Heme oxygenase-1, Glutamine-cysteine ligase catalytic subunit, Glutamine-cysteine ligase modifier subunit, and thioredoxin reductase-1 and inhibited ROS production in vitro[19]. All of these antioxidant-related genes were under-controlled by NF-E2-related factor 2 (NRF2), and activation of NRF2 led to elevated antioxidant activity[20]. Therefore, propolis may have enhanced the expression of antioxidant-related genes, and antioxidant activity may have increased in accordance with propolis administration. Generally, a high serum SAA concentration reflects the severity of amyloid deposition[2]. In this study, the serum SAA levels in the control group were strongly correlated with the degree of AA deposits both in the acute phase and at necrop-

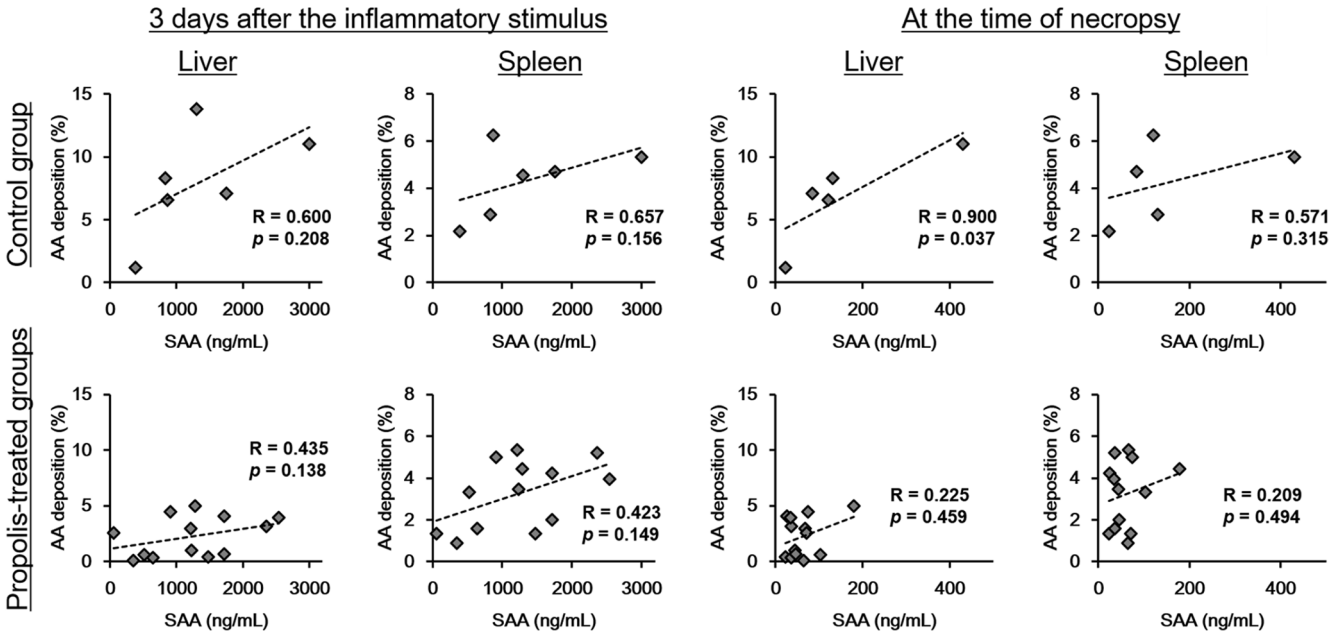

Fig. 3. Correlation analysis between serum SAA concentration and degree of AA deposition. In the control group, there were moderate to strong positive correlations between the serum SAA concentration and the degrees of hepatic and splenic AA deposition both in the acute phase (3 days after the inflammatory stimulus) and at necropsy. In contrast, the correlation coefficient decreased in propolis-treated groups.

Table 1. Comparisons of Transcript Expression of Inflammatory Markers and Antioxidant Markers among Groups

		Propolis (ppm)			
		0 (Control)	200	1000	5000
Inflammatory markers	IL-6	1.00 ± 0.52	0.56 ± 0.21	0.72 ± 0.38	0.49 ± 0.19
	IL-1β	1.00 ± 0.34	0.81 ± 0.41	0.98 ± 0.47	0.61 ± 0.31
	MCP-1	1.00 ± 0.35	0.60 ± 0.34	0.54* ± 0.15	0.31** ± 0.09
Antioxidant markers	CAT	1.00 ± 0.17	2.13** ± 0.43	1.80* ± 0.34	2.11** ± 0.39
	GPx1	1.00 ± 0.31	1.49 ± 0.30	1.37 ± 0.35	1.68* ± 0.34
	Prdx1	1.00 ± 0.26	1.77* ± 0.49	1.49 ± 0.43	1.98** ± 0.30
	SOD1	1.00 ± 0.28	1.47 ± 0.32	1.58 ± 0.93	2.66** ± 0.92
	SOD2	1.00 ± 0.34	1.62 ± 0.42	1.33 ± 0.53	2.51** ± 0.46
	GSR	1.00 ± 0.39	1.08 ± 0.27	1.04 ± 0.15	1.11 ± 0.45

Data are presented as the average ± SD. *p<0.05 and **p<0.01 vs. 0 ppm by Dunnett's test.

sy, but these correlations were weaker in the propolis-treated groups. Further, there were no changes in SAA levels upon intake of propolis. These results suggest that the propolis had antioxidant effects rather than anti-inflammatory effects in this model. Furthermore, it is possible that the decrease in AA levels was caused not so much by anti-inflammatory effects as by the antioxidant activity of propolis.

The expression levels of MCP-1 were significantly reduced by propolis. MCP-1 is a chemokine produced by macrophages under the influence of an inflammatory stimulus, and in promotes the chemotaxis and activation of monocytes[21]. Although the detailed mechanisms are not clear, macrophages are known to be involved in both the formation and resolution of AA fibrils[22]. Furthermore, in AA amyloidosis, it was reported that lipoperoxidation was detected in macrophages present around amyloid deposits[14]. The observed decrease in MCP-1 levels in amyloid-reduced mice also suggests that activated macrophages may contribute to amyloid formation.

In conclusion, we showed that administration of propolis resulted in inhibition of AA amyloidosis. Detection of inhibitory effects on AA amyloidosis caused by antioxidants points towards the development of therapeutic strategies to prevent or treat AA amyloidosis.

Disclosure of Potential Conflict of Interest: The authors declare that they have no conflicts of interest.

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