Aphanothece sacrum (Sur.) Okada Prevents Cataractogenesis in Type 1 Diabetic Mice

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Summary Aphanothece sacrum (Sur.) Okada is a species of cyanobacteria found in Japan. Although it has been used in local cuisine in Kyushu, Japan, for 250 years, little is known about its beneficial effect as food. The daily intake of health beneficial phytochemicals is believed to be useful for preventing lifestyle-related diseases, such as diabetic cataracts. In this study, the inhibitory effect of freeze-dried A. sacrum (Asa) on the formation of diabetic cataracts (DCs) was evaluated. Type 1 diabetes was induced in mice using streptozotocin (STZ). The mice were divided into two groups: one was fed a normal diet (DM-control group) and the other was fed a diet containing 1% Asa (DM-Asa group). During the study, changes in blood glucose levels and the amount of food and water consumed were measured. After 3 mo, the amount of Nε-(carboxymethyl)lysine (CML), an oxidative stress marker, in the lens was measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Although the blood glucose levels (p=0.91) and food consumption did not significantly change in any group, the oral administration of Asa tended to suppress CML accumulation (p=0.15) and significantly inhibited the progression of cataractogenesis in the diabetic lens compared with that reported for the normal diet (p=0.009). These results suggested that the daily intake of A. sacrum prevents the pathogenesis of cataracts, and indicated that may reduce the number of DC patients.

Key Words A. sacrum, AGEs, cataract, diabetes mellitus

The prevalence of diabetes in adults is increasing worldwide, and the number of adults with diabetes is expected to reach 472 million by 2030 (1). It is believed that the risk of eye diseases such as retinopathy and diabetic cataract (DC) increases with the pathogenesis of diabetes. DC is a serious disease because approximately 20% of diabetic patients develop DC (2), and cataracts account for a majority of the visual impairment cases globally (3). As lens proteins are known to be only slightly metabolized during the lifetime, cataracts are difficult to reverse once they have progressed. Therefore, preventive medicine is important to reduce the pathogenic risk of cataracts. Daily intake of natural compounds that suppress the progression of lifestyle-related complications is a promising preventive strategy. Aphanothece sacrum (Sur.) Okada is a species of cyanobacteria found in Japan. It is found as a lump of green-brown agar-like substance. A. sacrum was protected and nurtured as a part of the local cuisine in Kyushu, Japan, before it was scientifically recognized (4). Although people have consumed A. sacrum for more than 250 years, little is known about the physiology, ecology, or function of A. sacrum owing to its limited distribution and difficulty in obtaining the experimental material. In this study, we evaluated the inhibitory effect of A. sacrum on DC for its functional effect in vivo.

Oxidative stress, which is enhanced by hyperglycemia, is reported as one of the factors causing cataracts (5). Chronic hyperglycemia enhances the formation of advanced glycation end products (AGEs) (5). AGEs are generated from a non-enzymatic reaction between reducing sugars and proteins called the Maillard reaction. Previous studies have reported an increased accumulation of AGEs during normal aging (6), which increased during the development of diabetic complications, such as retinopathy, neurosis, and atherosclerosis (7, 8). In addition, the accumulated AGEs are actively involved in the development of tissue damage in these pathologies.

Nε-(carboxymethyl)lysine (CML) is a determinant of AGE antigen, and it accumulates in crystalline proteins in an age-dependent manner (9). CML is generated by the oxidative cleavage of Amadori products by hydroxyl radicals (10), peroxynitrite (11), and hypochloric acid (12). Therefore, CML is an important biological marker.
of oxidative stress in vivo.

Our previous study demonstrated that the CML level in the serum is increased by the pathogenesis of diabetes in rats (13). Therefore, the inhibitory effect of A. sacrum on the accumulation of CML, an oxidation marker, was also measured using streptozotocin (STZ)-induced diabetic mice.

**MATERIALS AND METHODS**

**Preparation of A. sacrum.** A. sacrum (Sur.) Okada was cultured in Kumamoto, Japan, where the average temperature for a half year was 15.6°C, which provided a suitable condition to grow A. sacrum (4). After harvesting and washing (Fig. 1), A. sacrum was freeze-dried and used as a rodent chow diet additive (Freeze-dried A. sacrum: Asa).

**Animal experiments.** Animal experiments were performed according to the methods of Shirakawa et al. (14). All animal experiments were approved by Tokai University (approval number: 131088), and the study was undertaken in compliance with the Guidelines for the Care and Use of Animals for Scientific Purposes at Tokai University (established on April 1, 2007). Ddy (Deutschland, Denken, and Yoken) mice were purchased from Kyudo (Kumamoto, Japan). They were housed in a pathogen-free barrier facility (12:12 h light-dark cycle) and fed a normal rodent chow diet (CLEA Japan Inc., Tokyo, Japan). It has been reported that STZ induces type 1 (15) and type 2 (16) diabetes mellitus (DM) in a concentration-dependent manner (17). Therefore, the administration of a low dose of STZ (~100 mg/kg BW) induces type 2 DM and the blood glucose levels gradually

Fig. 1. Semblance of Aphanothece sacrum (Sur.) Okada. Washed A. sacrum (A). A. sacrum cell (B). Scale bar = 1 cm (A) and 10 μm (B).

Fig. 2. Changes in body weight and blood glucose levels of mice. Diabetes was induced in mice using streptozotocin and changes in blood glucose levels were measured (A). Body weight was also measured (B). Normal: Normal group (n=6), DM-C: diabetic group (n=9), DM-Asa: diabetic group treated with 1% Asa (n=10). The data are presented as mean±SE. *p<0.05 vs. DM-C; **p<0.01 vs. DM-C.
increase in a time-dependent manner, whereas a high dose of STZ (~150 mg/kg BW) rapidly increases blood glucose levels and is used for induction of type 1 DM (17). In the present study, diabetes was induced based on the protocol for induction of type 1 DM (17) in 6-wk-old male mice (body weight approximately 40 g) by a single intravenous (tail vein) injection of STZ (150 mg/kg body weight) in 200 μL of 0.05 M saline-citrate buffer (pH 4.5). One week after the induction of diabetes, the mice (blood glucose ≥ 300 mg/dL) were randomly...
divided into an untreated diabetic group (n=9), which was fed a normal rodent chow diet, and a treated diabetic group (n=10), which was fed 1.0% Asa in the normal rodent chow diet. Powdered normal rodent chow was kneaded with 20% water in the presence or absence of 1% Asa. The cut and shaped chow was baked at 80°C for 3 h. The normal group (non-diabetic group; n=6) was fed a normal rodent chow diet. Food and water consumption as well as body weights were measured every 2 wk, and the blood glucose levels were measured every month. Twelve weeks after diabetes induction, the animals were euthanized under pentobarbital anesthesia, and the lenses were extirpated from the mice.

Measurement of opacity of crystal lens proteins. Cataracts were graded on a 0 to +3 scale by placing the lenses on a grid sheet, as described previously (18, 19). The lens samples were photographed, and the images captured were analyzed using ImageJ (National Institutes of Health). The quantitative evaluation of opacity of a crystal lens was expressed as the percent of pixels in the lesion area.

Measurement of CML by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The lens samples were homogenized in 400 μL of 1 mM diethylenetriamine pentaacetic acid (DTPA) using a Shake-Master Ver 1.2 (Bio Medical Science, Tokyo, Japan). Lens protein concentration was measured using bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). The CML content in the lens and serum were measured using LC-MS/MS as described previously (13). The lens (0.3 mg) and serum (5 μL) samples were reduced with sodium borohydride (NaBH₄; 2 μL of 1 m NaBH₄ in 0.1 N NaOH) in 20 μL of 200 mM sodium borate buffer (pH 9.1) at 25°C for 4 h. Standard [²H₂]CML (PolyPeptide Laboratories, Strasbourg, France) and [¹³C₆]lysine (Cambridge Isotope Laboratories, Inc., Tewksbury, MA) were added to the lens pellets, which were then hydrolyzed with 1 mL of 6 N HCl at 100°C for 18 h. The dried samples were resuspended in 1 mL of distilled water and passed over a Strata-X-C column (Phenomenex, Torrance, CA), which was pre-washed with 1 mL of methanol and equilibrated with 1 mL of distilled water. The column was then washed with 3 mL of 2% formic acid and eluted with 3 mL 7% ammonia. The pooled elution fractions were dried and resuspended in 1 mL 20% acetonitrile containing 0.1% formic acid. The samples were subjected to LC-MS/MS assay using a TSQ Vantage triple stage quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA). LC was conducted on a ZIC®-HILIC column (150×2.1 mm, 5 μm; Merck Millipore, Billerica, MA). The mobile phase consisted of solvent A (distilled water containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid). The flow rate was 0.2 mL/min, and the column was maintained at 40°C. The retention times for CML and lysine were approximately 12 and 13 min, respectively. CML, lysine, and [²H₂]CML were detected by electrospray ionization and positive ion mass spectrometric multiple reaction monitoring. The parent ions of CML and [²H₂]CML were 205 (m/z) and 207 (m/z), respectively. Fragment ions of 130 (m/z) from each parent ion were measured for the analysis of CML and [²H₂]CML in the lens and serum samples.

Effects of Asa extracts on CML formation in vitro. Crude extracts of Asa were obtained using 20% ethanol (EtOH), 100% EtOH, acetone and hot water. The inhibitory effect of Asa extracts on CML formation in vitro was determined by ELISA as described previously (20). Briefly, ribose-gelatin was prepared by incubating 2 mg per mL of gelatin with 30 mM ribose in 200 mM of phosphate buffer (pH 7.2) at 37°C for 7 d in the presence of Asa extracts (0.1 mg/mL), followed by the determination of CML formation using a noncompetitive ELISA.

Statistical analysis. Data were expressed as mean±SE (Fig. 2, Fig. 4C and Fig. 5) or median±max and minimum (Fig. 4B). The differences in blood glucose, body weights, and CML contents between the groups were examined for statistical significance using the one-way analysis of variance (ANOVA; normal vs. DM-C, DM-C vs. DM-Asa). Student’s t-test and subsequent post-hoc analysis (Sidak adjustment method) were used to correct for multiple comparisons. Statistical analysis of the opacity of crystal lens was conducted by the Kruskal-Wallis test followed by the Mann-Whitney U test and post-hoc analysis (Sidak adjustment method).

RESULTS

Biochemical parameters in the animal experiments. During the experimental period, the blood glucose levels were measured every 2 wk. The blood glucose levels in all diabetic mice were >300 mg/dL and significantly higher than those in the normal mice were (Fig. 2A). Although blood glucose levels increased slightly during the study period, it did not change with or without the administration of Asa. Body weight in all groups increased significantly over time, and the value in the normal group was higher than that in the diabetic group (Fig. 2B). Furthermore, intake of food (Fig. 3A) and of water (Fig. 3B) in the diabetic group were higher than those in the normal group. However, food and water intake did not change with the addition of Asa.

Cataract progression. Cataractogenesis was determined based on the four phases of grading as described previously (18, 19), and quantitative evaluation by ImageJ as described in “Materials and Methods.” The development of cataracts, which was estimated by transparency, was enhanced in mice with diabetes compared to that in normal mice (p = 0.04), whereas Asa administration inhibited the progression of cataracts to a nearly normal level (p = 0.13) (Fig. 4B). Furthermore, quantitative evaluation by ImageJ demonstrated that opacity was enhanced by pathogenesis of diabetes (p = 0.006), whereas the progression of cataractogenesis was significantly inhibited by Asa administration (p = 0.009) (Fig. 4C).

CML accumulation in lens proteins and serum. Accumulation of CML, an oxidation-dependent AGE, in the lens was measured because post-translational modification of crystallines by AGEs is thought to underlie the insolubilization of lens proteins and devel-
opment of cataracts. As shown in Fig. 5A, the presence of diabetes enhanced the accumulation of CML (p=0.03), and the administration of Asa tended to suppress the accumulation of CML (p=0.15). Furthermore, CML accumulation was found to be correlated with the opacity level (|r|=0.36) (p<0.08) (Fig. 5B). The CML concentration in serum was also measured since CML in rat serum increased with the pathogenesis of diabetes (13). However, it did not change between administration of Asa or its lack (data not shown).

Inhibitory effect of Asa on CML formation in vitro

This study was performed to estimate what component from Asa has inhibitory effects on CML. However, not all the extracts displayed an inhibitory effect on CML formation (data not shown).

**DISCUSSION**

Protein glycation is closely related to denaturation (21). The side chain of each amino acid is an important factor in the formation of tertiary protein structures. For instance, the epsilon amino residues of lysine possess positive charges, whereas carboxymethylation of the residues that form CML leads to an increase in the net negative charge, followed by inactivation and denaturation of proteins. This study provided the first evidence that oral administration of Asa to diabetic mice showed a tendency to inhibit CML accumulation (Fig. 5A) in the lens. In addition, the pathogenesis of cataracts was ameliorated (Fig. 4B). Because CML accumulation was weakly correlated with the opacity level (|r|=0.36) (p<0.08) (Fig. 5B), the effect of Asa on preventing cataracts may be partially correlated with amelioration of CML accumulation. A previous study reported that A. sacrum has an anti-inflammatory effect (22). CML is a major antigenic AGE, and it is produced not only under hyperglycemic conditions, but also by inflammatory reactions in vivo (12). Asa ameliorated the pathogenesis of cataracts by inhibiting inflammation-dependent oxidative stress because Asa administration tended to inhibit CML accumulation. It is speculated that megamolecular polysaccharide in Asa possibly prevents the absorption of AGEs in food. The AGE contents were measured by LC-MS/MS not only in lens proteins but also in serum. Although the CML level was inhibited in lens proteins, the level of five AGEs in serum did not change whether Asa was administered or not (Matsuda et al., unpublished observation). We speculated that Asa did not affect serum AGE levels because the blood samples were obtained after fasting for 6 h. The oral administration of Asa to diabetic mice did not result in any detectable side effects, such as weight loss or changes in blood glucose (Fig. 2). Because the daily intake of A. sacrum prevents the pathogenesis of cataracts, it may reduce the number of DC patients. Although there was no significant difference observed in AGE levels with and without Asa treatment in diabetic mice because of the sample size, we would expect to see an inhibitory effect of Asa on AGE formation if the study was conducted using a large number of subjects. Lutein is a food carotenoid present in the human lens, known to inhibit the development of cataracts (23). Asa contains zeaxanthin, a structural isomer of lutein. Therefore, we measured the inhibitory effect of several Asa extracts on CML by established assay with an enzyme-linked immunosorbent assay (ELISA) (20). However, not all the extracts displayed an inhibitory effect on CML formation (Matsuda et al., unpublished observation). The previous study reported that citric acid inhibited N\(^\text{3}\)-(carboxyethyl)lysine (CEL), one of the AGEs in vivo, but it did not inhibit that one in vitro (15). Taken together, these results suggested that the inhibitory effect of compounds on AGE formation under physiological conditions is different from that in vitro. Therefore, further study will be required to establish an assay system that imitates physiological environments and to connect an in vitro observation to animal experiments.

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