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Prevention of diabetic nephropathy by treatment with astaxanthin in diabetic db/db mice

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Abstract. Oxidative stress is implicated as an important mechanism by which diabetes causes nephropathy. Astaxanthin, which is found as a common pigment in algae, fish, and birds, is a carotenoid with significant potential for antioxidative activity. In this study, we examined whether chronic administration of astaxanthin could prevent the progression of diabetic nephropathy induced by oxidative stress in mice. We used female db/db mice, a rodent model of type 2 diabetes, and their non-diabetic db/m littermates. The mice were divided into three groups as follows: non-diabetic db/m, diabetic db/db, and diabetic db/db treated with astaxanthin. Blood glucose level, body weight, urinary albumin, and urinary 8-hydroxydeoxyguanosine (8-OHdG) were measured during the experiments. Histological and 8-OHdG immunohistochemical studies were performed for 12 weeks from the beginning of treatment. After 12 weeks of treatment, the astaxanthin-treated group showed a lower level of blood glucose compared with the non-treated db/db group; however, both groups had a significantly high level compared with the db/m mice. The relative mesangial area calculated by the mesangial area/total glomerular area ratio was significantly ameliorated in the astaxanthin-treated group compared with the non-treated db/db group. The increases in urinary albumin and 8-OHdG at 12 weeks of treatment were significantly inhibited by chronic treatment with astaxanthin. The 8-OHdG immunoreactive cells in glomeruli of non-treated db/db mice were more numerous than in the astaxanthin-treated db/db mice. In this study, treatment with astaxanthin ameliorated the progression and acceleration of diabetic nephropathy in the rodent model of type 2 diabetes. The results suggested that the antioxidative activity of astaxanthin reduced the oxidative stress on the kidneys and prevented renal cell damage. In conclusion, administration of astaxanthin might be a novel approach for the prevention of diabetes nephropathy.

Keywords: Astaxanthin, diabetic nephropathy, 8-hydroxydeoxyguanosine, oxidative stress

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1. Introduction

Diabetic nephropathy is characterized by the enlargement of glomerular mesangium due to the accumulation of extra-cellular matrix proteins, and is a leading cause of end-stage renal disease [1,2]. Clinical studies in subjects with type 1 and type 2 diabetes clearly link hyperglycemia to vascular complications, including diabetic nephropathy [3,4]. Hyperglycemia is responsible for the development and progression of diabetic nephropathy through metabolic derangements, including increased oxidative stress, renal polyol formation, activation of protein kinase C-mitogen-activated protein kinases, and accumulation of advanced glycation end products, as well as such hemodynamic factors as systemic hypertension and increased intraglomerular pressure [5]. However, the definite mechanism of mesangial cell activation by high ambient glucose has not been investigated.

We postulated that increased oxidative stress by high glucose is important in the pathogenesis of diabetic nephropathy. Studies that have used natural and synthetic antioxidants have provided convincing evidence that glomerular hypertrophy and accumulation of collagen and transforming growth factor (TGF)-β by high glucose is largely mediated by reactive oxygen species (ROS) [6–9]. Under diabetic conditions, ROS are produced by the non-enzymatic glycation reaction of proteins, mitochondria, and protein kinase C-dependent activation of NAD(P)H oxidase in mesangial cells, infiltrated inflammatory cells, and endothelial cells [10,11]. In addition, the persistence of hyperglycemia has been reported to increase the production of ROS through glucose auto-oxidation, abnormal metabolism of prostaglandins, and high polyol pathway flux. Recent study using a suppression-subtractive hybridization has demonstrated that high glucose induces actin cytoskeleton regulatory genes in mesangial cells, and that the induction is dependent on mitochondria-induced ROS and is independent of protein kinase C and TGF-β [12].

The oxidation products of biological components are generally accepted as markers of oxidative stress, and these products have been measured in many clinical specimens, including lipid peroxides, malondialdehyde, and 4-hydroxy-2-nonenal (all of which are indices of lipid peroxidation); isoprostane (a product of oxidation of arachidonic acid); 8-hydroxydeoxyguanosine (8-OHdG) and thymine glycol (both are markers of DNA oxidation); and protein carbonyl, hydroxyleucine, hydrovaline, and nitrotyrosine (products of oxidation of protein and amino acids) [13]. Increased urinary 8-OHdG levels have been reported in patients with diabetes and in diabetic rodents [14–16], and Hinokio et al. [17] have reported a direct association between oxidative DNA damage and the complication of diabetes by measuring the 8-OHdG levels in urine and the blood mononuclear leukocytes of patients with type 2 diabetes.

Astaxanthin, which is found as a common pigment in algae, fish, and birds, is a carotenoid that has many highly potent pharmacological effects, such as antioxidative activity [18–20], immunomodulating actions [21,22], anticancer activity [23], and anti-inflammation action [24,25]. Recently, astaxanthin has been made available for in vivo study because astaxanthin esters biosynthesized in the unicellular microalga Haematococcus pluvialis have been obtained by culture of the algae [20,26]. A randomized clinical trial revealed that 6 mg of astaxanthin per day from a Haematococcus pluvialis algal extract can be safely consumed by healthy adults [27]. We have reported the potential usefulness of astaxanthin treatment for reducing glucose toxicity using db/db mice, a rodent model of type 2 diabetes, in which the function of islet cells to secrete insulin function determined by the intraperitoneal glucose tolerance test was preserved in the astaxanthin-treated group, although histologic study of the pancreas revealed no significant differences in the β-cell mass between astaxanthin-treated and -untreated db/db mice [28].

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Kaneto et al. [9] also demonstrated that anti-oxidant treatment can exert beneficial effects in db/db mice with preservation of in vivo β-cell function. The db/db mouse exhibits clinical and histological features of diabetic nephropathy that track the human disease. This animal exhibits hyperglycemia and renal insufficiency by 16 weeks of age. The kidneys show the characteristic histological lesions of diabetic nephropathy, including mesangial matrix expansion and glomerular basement membrane thickening [29]. However, the effects of astaxanthin on oxidative damage and the development of diabetic renal complication have not been investigated. We examined whether chronic administration of astaxanthin could prevent the glomerular mesangial expansion in a preclinical model of diabetes, and whether astaxanthin could ameliorate oxidative injury in renal mesangial cells.

2. Materials and method

2.1. Animals and experimental design

Female db/db mice, a rodent model of type 2 diabetes, and their non-diabetic db/m littermates were purchased from the Clea Japan Co. Ltd. (Tokyo, Japan). They were kept under controlled conditions with a 12-h light:dark cycle and at 21–25°C. All mice were fed commercial CE-2 (Clea Japan, Tokyo, Japan) with free access to water for 1 week to adapt to the new environment. The control diet, CE-2, contained (g/100g): moisture 8.9, protein 25.4, fat 4.4, fiber 4.1, ash 6.9, and carbohydrate 50.3, and sufficient vitamins and minerals to maintain the health of the mice. The mice were divided into three groups as follows: non-diabetic db/m, diabetic db/db, and diabetic db/db treated with astaxanthin. Each of the groups contained 5 mice. The diet for the astaxanthin supplementation group was prepared by mixing CE-2 powder with astaxanthin (Fuji Chem. Industry Co., Ltd, Toyama, Japan) at 0.02%. The food intake was measured daily for 12 weeks before dissection. Body weight of the mice was measured every 7 days. Maintenance of animals and experimental procedures were carried out in accordance with the US National Institutes of Health Guidelines for the Use of Experimental Animals. All procedures were approved by the Animal Care Committee of the Kyoto Prefectural University of Medicine (Kyoto, Japan).

2.2. Urinary albumin and 8-OHdG analysis

Well-mixed 24-h urine was collected from mice in metabolic cages, and after it was centrifuged at 3,000 g for 10 min, 1 ml of supernatant was stored frozen at -80°C until analyzed. 8-OHdG concentrations in urine were determined by a competitive enzyme-linked immunosorbent assay (ELISA) kit (8-OHdG Check; Japan Institute for the Control of Aging, Fukuroi, Japan) according to the manufacturer's instruction. Absorbance of each well was read at 450 nm by a microplate reader (MPR-A4i; Tosoh, Tokyo, Japan). The determination range was 0.5–200 ng/ml. The urinary 8-OHdG was expressed as total amounts excreted in 24 h. Urinary albumin levels were measured by a competitive ELISA (Albuwell M, Exocell Inc., Philadelphia, PA, USA) according to the manufacturer's instruction.

2.3. Histological and morphometric analysis of kidneys

For morphometric analysis of the glomeruli, half of the middle portion of each left kidney was fixed with 10% buffered formalin and embedded in paraffin. Four-micrometer-thick slices were stained with periodic acid-Schiff (PAS). In each PAS-stained section of the renal cortex, the glomerular tuft area

was determined by the total glomerulare area minus the urinary space area and the urinary recesses, as previously described [30]. Sections were coded and read by an observer unaware of the experimental protocol applied. More than 30 glomeruli were counted per kidney, and the average was used for analysis. The mesangial area analysis was performed with NIH Image Software. The digitalized microscopic image of glomeruli was imported into NIH Image with Photoshop (Adobe Systems, San Jose, CA, USA), and we calculated the areas of mesangial expansion and glomeruli. The relative mesangial area was calculated as the ratio of the mesangial area to the glomerular area.

2.4. Immunohistochemical analysis

To analyze the inhibitory effects of astaxanthin on the amount of 8-OHdG, we took renal samples of db/db mice at the end of the study protocol. Renal samples were removed and fixed overnight in Bouin's solution. We used the avidin-biotin complex method described previously, with a slight modification [31]. After deparaffinization of kidney tissue with xylene and ethanol, normal rabbit serum (diluted to 1 : 75; Dako, Kyoto, Japan) for the inhibition of nonspecific binding of secondary antibody, monoclonal antibody N45.1 for 8-OHdG (0.5 μg/ml; Japan Institute for the Control of Aging, Fukuroi, Japan), biotin-labeled rabbit anti-mouse IgG serum (diluted to 1 : 300; Dako), and avidin-biotin-alkaline phosphatase complex (diluted to 1 : 100; Dako) were sequentially applied. Substrate for alkaline phosphatase was obtained from Vector Laboratories (CA, USA). To confirm the specificity of immunostaining, we conducted 8-OHdG absorption tests by incubating an excess of 8-OHdG (final concentration, 500 μM; Wako, Osaka, Japan) with the primary antibody on glass slides. Alternatively, to produce a negative control, we omitted the first antibody from the staining procedure.

2.5. Quantitative analysis of astaxanthin

Astaxanthin content in each tissue was quantified by HPLC. Tissues were homogenized with acetone. After centrifugation (3,000 rpm, 10 min), supernatants were evaporated to dryness. The residue was dissolved in 200 μl of acetone and filtered through a 0.45-μm polytetrafluoroethylene membrane filter; then 20 μl of solution was subjected to HPLC on a Shimadzu SPD-6AV spectorometer (Shimadzu, Kyoto, Japan) set at 460 nm. The column used was a Wakosil $5C_{18}$ (ODS) column (250 mm length $\times$ 4.6 mm internal diameter) with a mobile phase of methyl alcohol. A low rate was used (1.0 ml/min). Astaxanthin was quantified relative to calibration with a standard sample (F. Hoffman-La Roche, CA, USA).

2.6. Statistics

All values in the figure and text are expressed as mean $\pm$ SE. The data were compared by two-way analysis of variance (ANOVA), and differences were analyzed by Scheffe's multiple-comparison test. Simple regression analysis was used to test the correlations between albumin level and 8-OHdG concentration in urine. Differences between the groups were considered significant if the p value was less than 0.05. All analyses were performed using the Stat View 5.0-J program (Abacus Concepts, Berkeley, CA, USA) with a Macintosh computer.

3. Results

3.1. Effect of astaxanthin on body weight and blood glucose level

The db/db mice, a model for type 2 diabetes, exhibited hyperglycemia associated with obesity as compared with their nondiabetic db/m littermates at 6 weeks of age. Throughout the experimental periods (6–18 weeks of age), blood glucose levels were consistently higher in db/db mice than in db/m mice. At 18 weeks of age, the blood glucose levels of mice in the astaxanthin-treated group (338.0 $\pm$ 43.8 mg/dl) were lower than those of mice in the non-treated db/db group (417.6 $\pm$ 13.7 mg/dl), but the levels continued to be high (more than 300 mg/kg) throughout the experiment period compared with those of db/m mice (111.1 $\pm$ 3.4 mg/dl at 18 weeks of age). The mean body weight of db/db mice at 18 weeks of age (47.6 $\pm$ 0.6 g) was greater than that of db/m mice (29.5 $\pm$ 1.1 g). There were no differences in body weight and food intake between astaxanthin-treated and non-treated db/db mice.

3.2. Effect of astaxanthin on diabetic nephropathy

Albumin levels in urine of db/db mice significantly ($p < 0.05$) increased compared with those of db/m mice at 18 weeks of age (Fig. 1). The increase in urinary albumin at 18 weeks of age was significantly inhibited by chronic treatment with astaxanthin ($p < 0.01$). Kidney weights were slightly, but not significantly, increased in db/db mice as compared to db/m mice. There are no differences in the histology of renal tubles between db/m and db/db mice. In contrast, the glomerular histology in db/db mice showed accelerated mesangial expansion characterized by an increase in PAS-positive mesangial matrix area relative to that observed in db/m mice at 18 weeks of age (Fig. 2). On the other hand, therapy with astaxanthin for 12 weeks partially reversed the mesangial matrix accumulation that had been established by 18 weeks of age. The glomerulus contained less PAS-positive matrix material and the capillary loops were more widely open following astaxanthin therapy. Mesangial expansion was further quantitated by a morphometrical analysis. The relative mesangial area calculated by the mesangial area/total glomerular area ratio was increased by 250% ($p < 0.01$) in db/db mice as compared with db/m mice. Administration of astaxanthin significantly ameliorated the increase in the relative mesangial area in db/db mice (Fig. 3).

3.3. Effects of astaxanthin on urinary and renal levels of 8-OHdG

The urinary 8-OHdG level in db/db mice was significantly higher than that in db/m mice at 6 weeks of age (Fig. 4a). In non-treated db/db mice, the increase in urinary 8-OHdG levels was significantly enhanced at 18 weeks of age (Fig. 4a), but the enhancement was significantly inhibited by the treatment with astaxanthin (astaxanthin-treated db/db mice, 166.8 $\pm$ 44.6 ng/day; non-treated db/db mice, 336.1 $\pm$ 68.7 ng/day). Figure 4b shows the correlation between the levels of 8-OHdG and albumin in urine of db/db mice. 8-OHdG levels closely paralleled the increase in albumin levels in urine ($r = 0.754$, $p < 0.05$ by the simple regression analysis).

To evaluate the damage caused by oxidative stress, we performed 8-OHdG immunostaining in the kidneys of mice in each group. The results revealed that the 8-OHdG immunoreactive cells in glomeruli of non-treated db/db mice were more numerous than those in astaxanthin-treated db/db mice (Fig. 5). The number of 8-OHdG-positive cells in db/db mice was significantly increased compared with that of db/m mice. The increase in the number of 8-OHdG-positive cells was significantly inhibited by the treatment with astaxanthin (Fig. 6). The number of 8-OHdG-positive cells in db/db mice was also increased in the renal tubles compared with that of db/m mice, however this increase was not reduced by the treatment with astaxanthin.

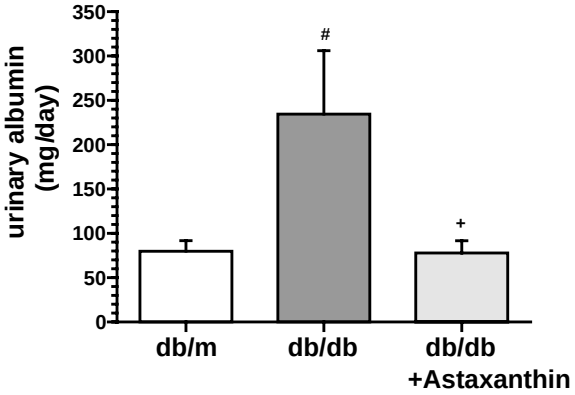

Fig. 1. Effect of astaxanthin on the urinary albumin excretion rate of diabetic db/db mice. A 24-h urine sample for each mouse was collected in metabolic cages 12 weeks after the start of this experiment. Urine samples were processed to measure urinary albumin concentration using a competitive ELISA. Data shown are the mean ± SE from 5 mice. $^{\#}p < 0.01$ vs. db/m mice and $^{+}p < 0.05$ vs. db/db mice.

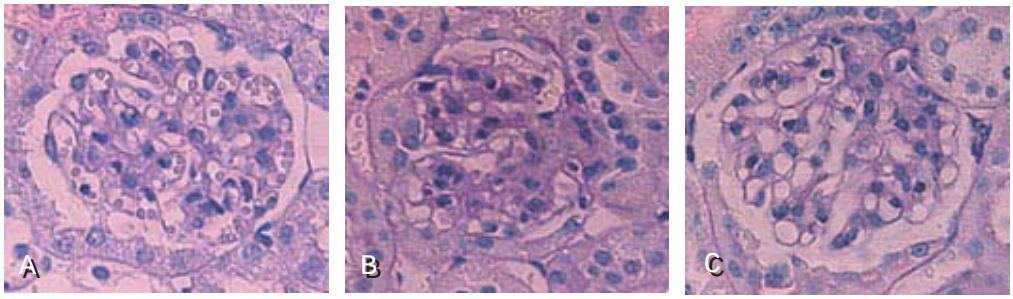

Fig. 2. Effect of astaxanthin on accelerated mesangial expansion in db/db mice. Paraffin-embedded sections of the renal cortex were stained with periodic acid-Schiff (PAS). Representative light micrographs (magnification: 400 x) from each of the mouse groups are shown. (A) Normal glomerulus from a non-diabetic db/m mouse at 18 weeks. (B) Glomerulus from an untreated db/db mouse at 18 weeks of age, showing hypertrophy and mesangial matrix expansion. (C) Glomerulus from a db/db mouse treated with 12 weeks of astaxanthin until 18 weeks of age, depicting partial reversal of mesangial matrix expansion.

Table 1
Astaxanthin content in tissues

	Content (μg/g of tissue)	
	Astaxanthin diet	Control diet
Kidney	29.4	n.d.
Heart	46.7	n.d.
Liver	270.3	n.d.

Values are means for six mice receiving an astaxanthin or control diet for 3 weeks.
n.d.: not determined.

3.4. Astaxanthin content in tissues

Astaxanthin was clearly accumulated not only in the liver but also in the kidney of mice after 3 weeks of a diet containing 0.02% astaxanthin. It was not detected in the tissues of the rats fed a normal meal (Table 1).

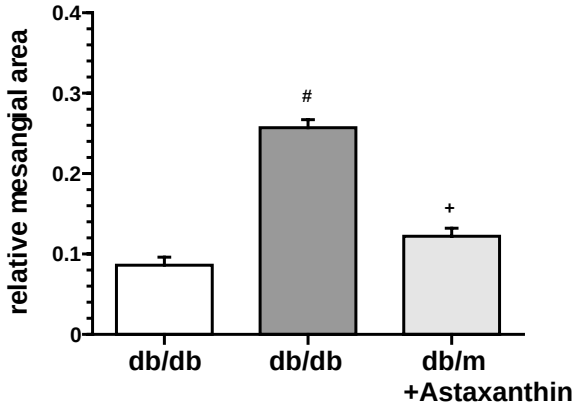

Fig. 3. Effect of astaxanthin on relative mesangial area shown as the ratio of mesangial area/glomerular area. Data shown are the mean ± SE from 5 mice. $^{\#}p < 0.01$ vs. db/m mice and $^{+}p < 0.05$ vs. db/db mice.

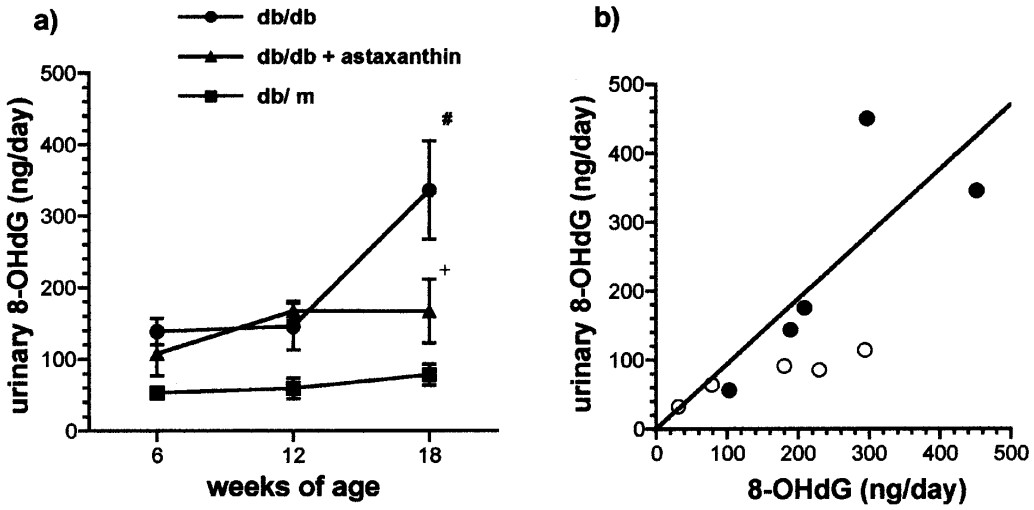

Fig. 4. Effect of astaxanthin on the urinary 8-hydroxydeoxyguanosine (8-OHdG) excretion rate of db/db mice (a), and the correlation between 8-OHdG levels and albumin level in the urine in db/db mice (b). Data in (a) are the mean ± SE from 5 mice. $^{\#}p < 0.01$ vs. db/m mice and $^{+}p < 0.05$ vs. db/db mice. The closed circles indicate the untreated-db/db mice, and the open circles show db/db mouse treated with 12 weeks of astaxanthin until 18 weeks of age. 8-OHdG levels closely paralleled the increase in albumin levels in urine ($r = 0.754$, $p < 0.05$ by the simple regression analysis).

4. Discussion

Two important findings were observed in the present study. First, 8-OHdG, an index of oxidative stress, was increased in the urine and kidneys of diabetic db/db mice, a model of type 2 diabetes. More importantly, it was clearly demonstrated that long-term oral treatment with astaxanthin, a strong natural antioxidant, reduced not only an increase in albuminuria but also glomerular histological changes in db/db mice with little effect on blood glucose levels, the effect being accompanied by decreased urinary excretion of 8-OHdG and reduced 8-OHdG expression in mesangial cells.

Recent data support that 8-OHdG is a good marker to evaluate renal oxidative stress in several models of diseases, such as cisplatin-induced toxicity and ageing [32–34]. In 1994, Ha et al. [35] first

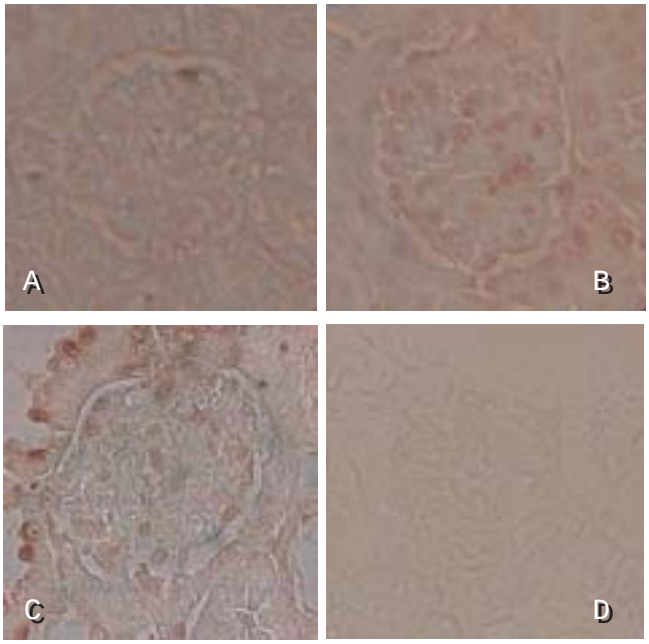

Fig. 5. Effect of astaxanthin on the expression of 8-hydroxydeoxyguanosine (8-OHdG) in the kidney of db/db mice. A representative result for immunostaining for 8-OHdG performed on glomerulus of non-diabetic db/m mice at 18 weeks of age (A) and diabetic db/db mice non-treated (B) and treated with astaxanthin (C). (D) Negative control without the first antibody.

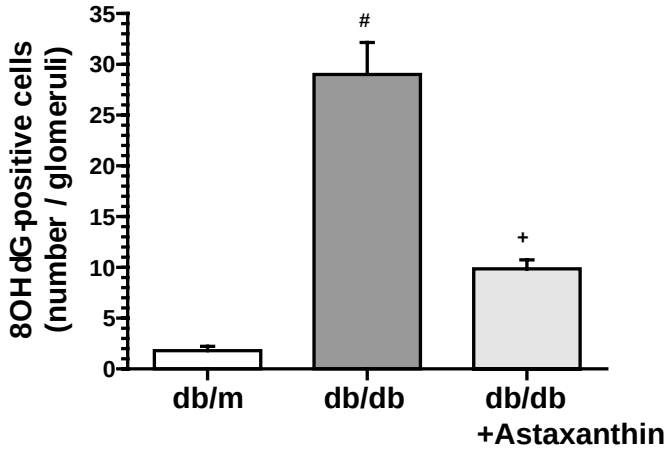

Fig. 6. Effect of astaxanthin on the number of 8-hydroxydeoxyguanosine (8-OHdG)-positive cells per glomeruli in db/db mice. Data shown are the mean $\pm$ SE from 5 mice. $^{\#}p < 0.01$ vs. db/m mice and $^{+}p < 0.05$ vs. db/db mice.

demonstrated that the formation of 8-OHdG is closely related to the process of diabetic nephropathy in experimental diabetic rodents. They showed that the 8-OHdG levels in both rat renal cortex and papilla were significantly increased compared to those of controls after streptozotocin (STZ) administration, and that daily injection of insulin after STZ treatment significantly reduced both urinary albumin excretion and 8-OHdG formation, which suggests that these are associated with the diabetic state induced by STZ rather than a direct nephrotoxic effect of the drug [35]. Recent clinical evaluation indicates that 8-OHdG

in urine is a useful clinical marker not only for detecting micro- and macro-vascular complications [16] but also for predicting the development of diabetic nephropathy in diabetic patients [36]. In the present study, the urinary albumin levels, one of the early-stage indices of renal damage, increased in db/db mice paralleled with the increase in urinary 8-OHdG levels. In addition, renal immunostaining of 8-OHdG revealed an increase of positive cells in non-treated db/db mice, and increased levels of 8-OHdG in urine and tissue were both attenuated by oral astaxanthin treatment. These results indicate that the urinary level of 8-OHdG reflects renal oxidative damage in diabetes mellitus and might be the biomarker of diabetic nephropathy. The administration of dietary astaxanthin improved renal dysfunction in diabetic mice through its antioxidant function, and the urinary and tissue 8-OHdG data support these results.

Although there is no doubt that hyperglycemia is a major contributor to oxidative stress, there has been debate about the association between the glycemic control and levels of 8-OHdG. First, Leinonen et al. [14] showed a positive association of HbA1c, an index of glycemic control, and urinary 8-OHdG in diabetic patients. Kouda et al. [37] demonstrated the positive correlation between 8-OHdG and pentosidine, a marker of nonenzymatic glycation and oxidation damage, in the urine of patients with hypercholesterolemia and/or hypertension. However, in our previous study, there was no significant correlation between HbA1c and urinary 8-OHdG levels in patients with diabetes, although their urinary levels of 8-OHdG were significantly higher that those of control patients [38]. In this study, urinary and kidney 8-OHdG levels exhibited marked reduction by the treatment with astaxanthin in association with reduction of albuminuria, in spite of the high levels of blood glucose during the treatment. These results suggest that astaxanthin might directly attenuate the diabetic oxidative damage even if the slight decrease of blood glucose level could partly contribute to the attenuation of oxidative damage. In addition, our results might provide further insight into therapeutic strategies for diabetic kidney disease.

The protective effect that antioxidants have on certain aspects of nephropathy in diabetic animals has been reported. Recently, it was reported that antioxidant treatment with vitamin E, probucol, alpha-lipoic acid, or taurine normalized not only diabetes-induced renal dysfunction such as albuminuria and glomerular hypertension but also glomerular pathologies [7]. Ueno et al. [39] have also reported that dietary glutathione can exert beneficial effects on diabetic complications in STZ-induced diabetic rats. Astaxanthin is reported to be more effective than other antioxidants such as vitamin E and β-carotene in preventing lipid peroxidation in solution and various biomembrane systems [18,19]. Goto et al. [40] reported that the efficient antioxidant activity of astaxanthin could be due to the unique structure of its terminal ring moiety. Astaxanthin traps radicals not only at the conjugated polyene chain but also in its terminal ring moiety, in which the hydrogen atom at the C3 methine is suggested to be a radical trapping site. Furthermore, our data demonstrate that the esterified astaxanthin used in this study is efficiently absorbed and is transported into the kidney. Esterified astaxanthin is known to be more effectively absorbed from the small intestine compared to non-esterified astaxanthin, and is mainly metabolized in the liver. The diabetes-induced changes observed in the present study were markedly prevented by the potent antioxidant astaxanthine, which is quite consistent with the effects of this compound in other non-diabetic models of oxidative stress [41]. The unique characteristics of astaxanthin could contribute to the decrease of oxidative stress in these models.

The limited data available suggest that antioxidants help protect against diabetic nephropathy in humans, and a combined daily treatment with 680 I.U. vitamin E and 1,250 mg vitamin C for 4 weeks reduced the urinary albumin excretion rate by 19% in a crossover study of 30 patients with type 2 diabetes [42]. We have just started to administer astaxanthin ester biosynthesized in Haematococcus pluvialis algae to humans. Recent data in a randomized clinical trial have shown that 6 mg of astaxanthin per day can be safely consumed by healthy adults [27]. Further studies should help more clearly define

the role of astaxanthin in enhancing the quality of life of individuals with diabetes. In conclusion, our present results reveal for the first time that astaxanthin can exert beneficial effects on renal mesangial cells in diabetic db/db mice. Thus, astaxanthin might be a novel approach for the prevention of diabetes nephropathy.

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