J Pharmacol Sci **99**, 287 – 293 (2005)

Journal of Pharmacological Sciences

Full Paper

Increase in the Deposition of Aggregated Protein in the Glomeruli of Spontaneously Diabetic Mice

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Received November 25, 2004; Accepted September 29, 2005

Abstract. The aim of this study was to investigate the disposal of aggregated protein in the glomeruli of spontaneously diabetic mice. Diabetic mice, KK-A^y and db/db, and age-matched ICR mice were injected intravenously with aggregated bovine serum albumin (a-BSA) at 0.6 mg/g, and the glomeruli and the blood were obtained. Diabetic mice had larger amounts of a-BSA in their glomeruli than the ICR mice, threefold in KK-A^y and twofold in db/db, at 3 h after the a-BSA injection. Additionally, the disappearance of a-BSA was retarded in the diabetic glomeruli. KK-A^y displayed a-BSA in the glomeruli 24 h after the a-BSA injection and db/db did after 12 h, while the ICR did by 8 h. In spite of increases of insulin to similar degrees in both strains of diabetic mice after the a-BSA injection, blood glucose levels markedly decreased in KK-A^y compared with db/db. There were no histopathological alterations in the glomeruli of the diabetic mice. Depositions of a-BSA were confirmed to be higher in the diabetic glomeruli by the immunofluoresnce technique, and KK-A^y displayed higher depositions of a-BSA than did db/db. The present study suggests that hyperglycemia is involved in the increased deposition of aggregated protein in the glomeruli and that the degradation of aggregated protein is retarded in diabetic glomeruli.

Keywords: diabetic mice, diabetic nephropathy, glomeruli, aggregated protein, hyperglycemia

Introduction

Half of the patients, who had recently started hemodialysis, suffer from diabetic nephropathy. Additionally, the number of dialysis patients is considered to surely increase in the future. Medical intervention to lower the blood glucose levels is crucial to prevent chronic complications in diabetic patients. Diabetic nephropathy, however, develops in about 40% of the patients in spite of their taking anti-diabetic remedies. Therefore, the establishment of medical treatments to maintain renal function in diabetic patients is urgently needed, and the attention of investigators should be focused on the underlying mechanisms of development of diabetic nephropathy (1 – 3). It has been documented that chronic hyperglycemia induces advanced glycation end-products (AGE) (4) and that a significant increase in both glycated albumin and immunoglobulin is determined in diabetic patients (5). Histological alterations, that is, an expanded mesangial matrix and thickening of the capillary wall, in the glomeruli are observed in normal rats that are administered AGE (6, 7). In addition to AGE, depositions of immunoglobulins and albumin are immunohistologically detected in the glomeruli of experimental models of diabetes (8 – 11). There have been, however, no investigations regarding the ability to degrade these substances in the diabetic glomeruli. We hypothesize that if AGE interfere with the degradation of aggregated protein in the glomeruli in diabetic mice, obviously more aggregated protein should accumulate in the glomeruli when diabetic mice are intravenously administered with a large quantity of exogenous aggregated protein.

Aggregated bovine serum albumin (a-BSA) is used

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to estimate the degree of protein degradation in the glomeruli (12 – 14). a-BSA is considered (1) to be drained from the glomeruli to the lymph vessel through the mesangial matrix channels and (2) to be degraded by the mesangial cells and resident macrophages in the glomeruli in vivo. The KK-A^y mice and db/db mice spontaneously develop diabetic symptoms, that is, hyperglycemia, elevation of insulin levels in the plasma, abnormal tolerance to glucose, and reduction of insulin susceptibility. These mice should be a useful animal model for research on the pathophysiology of type 2 diabetes mellitus and for the screening of new medicines for the treatment of type 2 diabetes mellitus (1, 2, 15 – 17).

In this study, we investigated the disposal of aggregated protein in the glomeruli of the KK-A^y mice and the db/db mice to clarify the influence of hyperglycemia and hyperinsulinemia on this process. We demonstrated that diabetic mice exhibited remarkable deposition and retarded degradation of aggregated protein in the glomeruli due to hyperglycemia in diabetes mellitus, but these mice displayed no histological alterations in the glomeruli. An intervention that accelerates the retarded processing of aggregated protein in the glomeruli will prevent the development of diabetic nephropathy.

Materials and Methods

Animals

ICR/Crj/CD-1 (ICR; Charles River Japan, Inc., Yokohama), KK-A^y/Ta Jcl (KK-A^y; Clea Japan, Inc., Tokyo), and C57BL/KsJ-db/db Jcl (db/db; Clea Japan, Inc.) were used in the present study. These animals were male and 9-week-old. The animals had free access to food and water. The experiments were performed according to the The Japanese Pharmacological Society and the National Institute of Health Guide for the Care and Use of Laboratory Animal.

Reagents

The following reagents were used in this study: crystallized bovine serum albumin (Seikagaku Co., Tokyo), iron oxide (Fe_3O_4; Aldrich Chemical Company, Milwaukee, WI, USA), SEA BLOCK™ (Techno Chemical Co., Tokyo), monoclonal anti-bovine serum albumin antibody (clone BSA-33; Sigma Chemical Co., St. Louis, MO, USA), biotin-conjugated anti-mouse immunoglobulin G antibody (Sigma Chemical Co.), streptavidin-horseradish peroxidase (Zymed Laboratories, Inc., San Francisco, CA, USA), and ABTS peroxidase substrate and ABTS stop solution (KPL, Gaithersburg, MD, USA). The swine serum, rabbit anti-bovine serum albumin antibody and fluorescent isothiocyanate (FITC)-conjugated swine anti-rabbit immunoglobulin G antibody were purchased from Dako (Glostrup, Denmark).

Preparation of a-BSA

a-BSA was prepared according to the previously reported procedure (18). Briefly, BSA was dissolved in physiological saline at 30 mg/ml. The solution was alkalinized to pH 10 with 0.2 N NaOH and then incubated at 70°C for 20 min and at 79°C for 15 min. After allowing the solution to cool to room temperature, the solution was neutralized with 0.2 N HCl and centrifuged at 3500 rpm for 30 min at 4°C. The supernatant was stored in a freezer until use.

Measurement of a-BSA in the glomeruli

a-BSA was injected into the tail vein at 0.6 mg/g body weight (19). The kidneys were removed after perfusion with 1 mg/ml of iron oxide in phosphate-buffered saline via the abdominal aorta (20). Then, the kidneys were cut into small pieces. The minced tissue was filtered through a 90-μm mesh screen and collected on a 38-μm mesh screen. The glomeruli containing iron oxide were collected from the suspension of glomeruli and renal tubules with a magnet (Magical Trapper; Toyobo, Osaka). The purity of the glomeruli was more than 95% as observed under a light microscope. The concentration of the glomeruli was adjusted to 3000 glomeruli/ml with PBS. After destroying the glomeruli with ultrasonic waves, the amount of a-BSA in the glomeruli was determined by enzyme-linked immunosorbent assay (ELISA) as previously reported (18).

Measurement of glucose and insulin levels in plasma

Blood samples were collected via the orbital plexus vein immediately before and after the a-BSA injection at 1, 3, 6, 8, 12, 18, and 24 h. Glucose and insulin levels were determined with a glucose measuring kit (Glucose C-II Test; Wako Pure Chemical Industries, Osaka) and an insulin enzyme-linked immunosorbent assay kit (Mouse Insulin ELISA KIT; Shibayagi, Gunma).

Histopathological examination of glomeruli

The kidneys were removed under anesthesia with pentobarbital (5 mg/ml) at 3 and 6 h after the a-BSA injection and were fixed with 10% neutral buffered formalin. The tissues were then embedded in the paraffin and sectioned into 3- to 4-μm-thick slices. The sections were stained routinely with Hematoxylin-Eosin (H.E.) and Periodic acid-Schiff (PAS). The pathology of the glomeruli was observed under a light microscope by another investigator blinded to the results.

Semi-quantitative analysis of deposition of a-BSA

The paraffin sections were then subjected to immunofluorescence staining regarding deposition of a-BSA in the glomeruli. Briefly, sections were deparaffined and incubated with anti-BSA antibody (1:100) at room temperature for 60 min, followed by incubation with FITC-conjugated anti-rabbit immunoglobulin G antibody (1:200) at room temperature for 60 min. The extent of the deposition of a-BSA in the glomeruli was observed under a fluorescence microscope. Thirty of the glomeruli were selected by the criteria of whether it has the vascular pole and a certain size. The extent of deposition of a-BSA was graded from negative (–) to severe (+++). The glomeruli were sorted out according to their extent and counted by another investigator blinded to the identity of sections. The percentage was calculated for the glomeruli with each category of a-BSA deposition: negative, slight, moderate, and severe. Deposition index was obtained by the following formula: Deposition index = $(0.1 \times n_0) + (1 \times n_1) + (2 \times n_2) + (3 \times n_3)$, where n represents the number of the glomeruli in each category of deposition.

Statistical analyses

Results were expressed as means ± S.E.M. Statistical analyses were performed with a statistical software package, SAS clinical package Version 5.0 Stand Alone version (SAS Statistical analysis system, Tokyo). The data were analyzed using the F test for the variance ratio. When the variances were homogeneous, Student's *t*-test was used to analyze the differences. If the variances were heterogeneous, the Aspin-Welch's *t*-test was used to analyze the differences. Differences were considered to be significant at $P<0.05$.

Results

Time course of a-BSA levels in the glomeruli

The amount of a-BSA in the glomeruli was measured each time after the a-BSA injection (Fig. 1). In ICR mice, a-BSA levels were 55.6 ng after 6 h. Then a-BSA levels declined gradually and were not detected at 12 h. In KK-A^y mice, the a-BSA levels reached 155.3 ng after 3 h, which was higher than that in the ICR mice, and were 33.1 ng even 24 h after the a-BSA injection. Glomerular a-BSA levels increased in the db/db mice to 100.8 ng 3 h after the a-BSA injection. However, they decreased to 0.3 ng 18 h after the a-BSA injection and became negligible at 24 h.

Plasma glucose levels in diabetic mice

Glucose levels in the plasma were measured each time after the a-BSA injection (Fig. 2). In KK-A^y mice, the plasma glucose levels decreased to half of the pre-injection levels 3 h after the a-BSA injection (239.2 mg/dl and 517.1 mg/dl). The plasma glucose then remained at lower levels and was 354.7 mg/dl 24 h after the injection. In the db/db mice, the plasma glucose levels were 470.2 and 493.9 mg/dl at 3 and 6 h after the a-BSA injection, respectively. The levels were lower

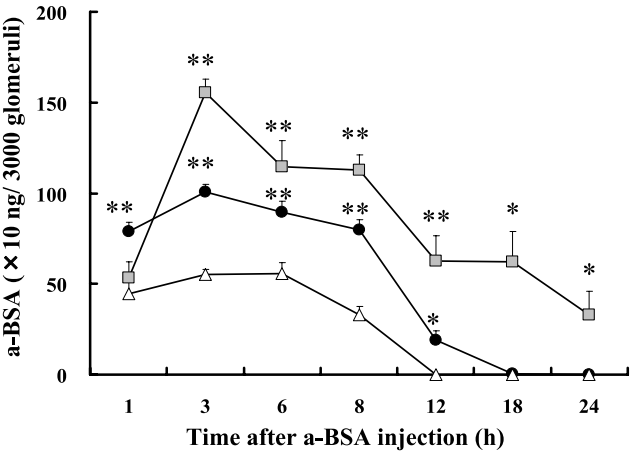

Fig. 1. Time course of glomerular a-BSA in ICR, KK-A^y, and db/db mice. The kidneys were isolated under anesthesia with pentobarbital sodium at 1, 3, 6, 8, 12, 18, and 24 h after a-BSA injection. Mice had blood drawn from the orbital plexus vein and had their plasma glucose levels measured prior to the experiments. Each group consisted of 8 mice. All groups had similar glucose levels. Values are the means ± S.E.M. Significant difference from the ICR group at *$P<0.05$ and **$P<0.01$ (Student's *t*-test). Triangle: ICR mice, square: KK-A^y mice, circle: db/db mice.

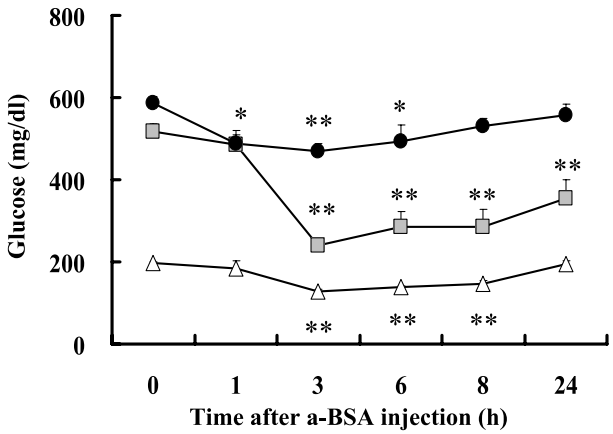

Fig. 2. Time course of plasma glucose levels after a-BSA injection in ICR, KK-A^y, and db/db mice. Mice had blood drawn from the orbital plexus vein and had their plasma glucose levels measured prior to the experiments. Each group consisted of 8 mice. All groups had similar glucose levels. Triangle: ICR mice, square: KK-A^y mice, circle: db/db mice. Values represent the means ± S.E.M. Significant difference from 0 h at *$P<0.05$ and **$P<0.01$ (Student's *t*-test or Aspin-Welch's *t*-test).

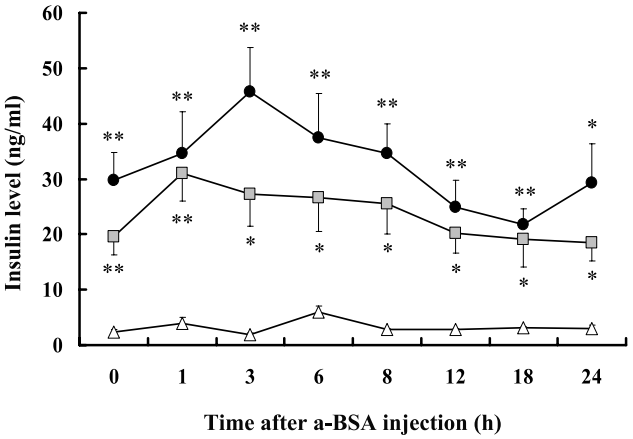

Fig. 3. Time course of insulin levels after a-BSA injection in ICR, KK-A^y, and db/db mice. Mice had blood drawn from the orbital plexus vein and had their plasma glucose levels measured prior to the experiments. Each group consisted of 8 mice. All groups had similar glucose levels. Triangle: ICR mice, square: KK-A^y mice, circle: db/db mice. Values represent the means ± S.E.M. Significant difference from the ICR mice at *P<0.05 and **P<0.01 (Student's t-test or Aspin-Welch's t-test).

than that before the a-BSA injection (585.7 mg/dl) and recovered to the level before the a-BSA injection by 24 h. In the ICR mice, the plasma glucose levels slightly decreased to 126.8 mg/dl at 3 h after the a-BSA injection, and the lower levels continued until 8 h.

Plasma insulin levels in diabetic mice

Insulin levels in the plasma were measured each time after the a-BSA injection (Fig. 3). In the KK-A^y mice, the insulin levels were increased by about 40% by the a-BSA injection (19.5 ng/ml before the a-BSA injection, 31.0 ng/ml at 1 h, and 25.6 ng/ml at 8 h) and then returned to the level before the a-BSA injection by 12 h. In the db/db mice, the insulin levels were 34.6 ng/ml at 1 h after the a-BSA injection and were higher than that before the injection (29.7 ng/ml). In particular, the insulin levels in the db/db mice were elevated to 45.7 ng/ml at 3 h after the a-BSA injection and then recovered to the level before the a-BSA injection by 12 h. In the ICR mice, 6 h after the injection, the insulin levels were elevated to 5.9 from 2.4 ng/ml, which was the level before the injection, and the levels were restored by 24 h.

Light microscopy and immunofluorescent microscopy of the glomeruli in diabetic mice

Under light microscopy, no histological alterations, such as a thickening of glomerular capillary wall and the expansion of the mesangial matrix, were observed in the glomeruli in the KK-A^y and the db/db mice (Fig. 4a). However, with immunofluorescent microscopy, depositions of a-BSA in the glomeruli were located in the mesangial area in 3 groups of mice 3 h after the a-BSA injection (Fig. 4b). The positive area of a-BSA was much greater in the glomeruli of the KK-A^y and the db/db mice than that in the ICR mice, and mesangial a-BSA depositions were the most abundant in the KK-A^y mice (Fig. 4b).

Semi-quantitative analysis of a-BSA in the glomeruli

We analyzed semi-quantitatively 30 glomeruli per each section regarding deposition of a-BSA by the immunofluorescence technique at 3 and 6 h after the injection of a-BSA. In the ICR mice most of the glomeruli showed negative or slight deposition of a-BSA (Fig. 5a). Among the KK-A^y mice, 75% of the glomeruli had severe deposition of a-BSA and 1% of the glomeruli had slight deposition 3 h after a-BSA injection (Fig. 5b). Among the db/db mice, 65% had glomeruli with severe deposition and 10% had glomeruli with slight deposition at 3 h after the a-BSA injection (Fig. 5c). After 6 h, severe deposition was observed in 60% of the KK-A^y mice and 40% showed moderate deposition (Fig. 5b). Conversely, the db/db mice showed a percentage of severe deposition less than that of moderate deposition (Fig. 5c). The diabetic mice demonstrated significantly higher score than the ICR mice with Deposition index, but we failed to find any significant difference between the diabetic mouse groups (Fig. 5d).

Discussion

In this study, we demonstrated that the depositions of a-BSA in the glomeruli of diabetic mice were remarkably higher than those in the control mice. We confirmed it both with ELISA and an immunofluorescence analysis. As demonstrated by the present results, depositions of aggregated protein in the diabetic glomeruli can be considered to result from 1) a delay of degradation of aggregated protein in the glomeruli and 2) a pathological microcirculation environment.

Deposition of immune complex is demonstrated in the glomeruli of nephritic patients, and deposition of albumin and/or immunoglobulin in diabetic patients as well. We reported that the glomeuli increase the expression of cyclooxygenase 2 and produce more prostaglandin E_2 by the aggregated protein (19). We consider that prostaglandin E_2 stimulates mesangial cells in an autocrine and/or paracrine manner to activate proteasome through EP4 receptors, a prostaglandin E receptor, and then the proteasomes breakdown the aggregated protein (14). Because we reported that aggregated protein stays

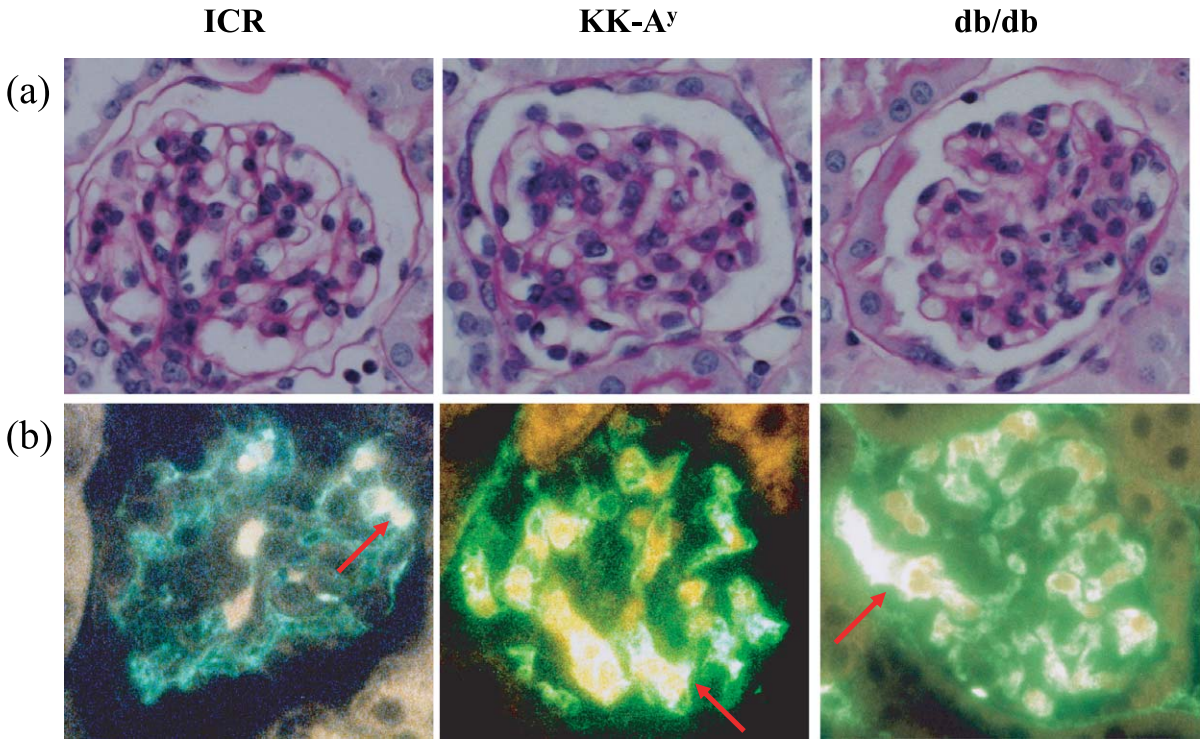

Fig. 4. The kidneys were removed 3 h after a-BSA. a: PAS staining, b: fluorescent staining. Magnification: ×400. Each group consisted of 3 mice. We observed 30 glomeruli in each section. Photographs are the representative glomeulus in ICR, KK-A^y, and db/db mice, respectively. The arrow indicates positive area of a-BSA in the glomerulus.

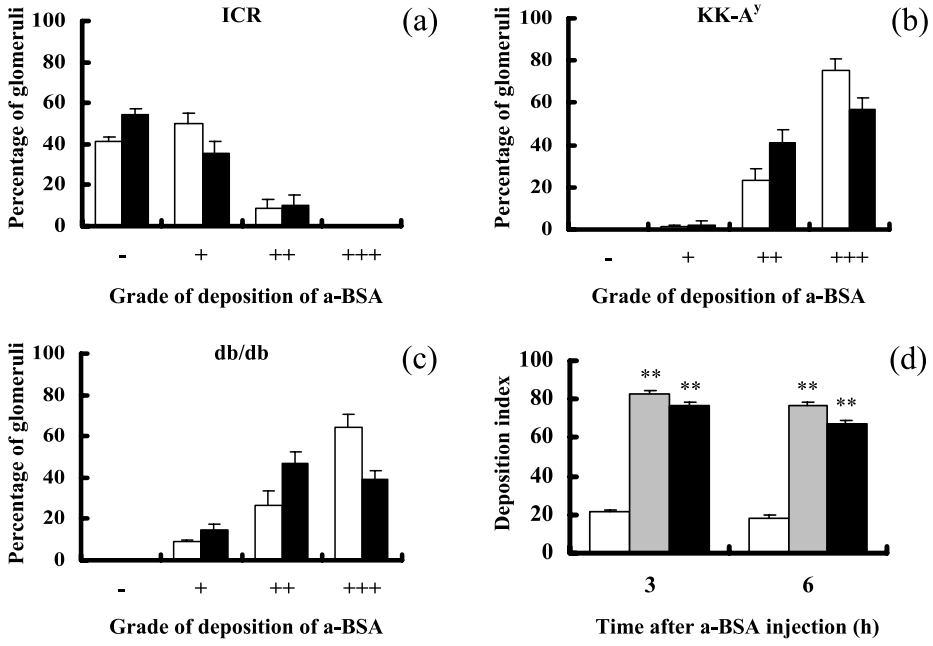

Fig. 5. The kidneys were removed 3 and 6 h after a-BSA injection in ICR, KK-A^y, and db/db mice. Each group consisted of 3 mice. We observed 30 glomeruli in each section. The extent of deposition of a-BSA was graded from negative to severe. The percentage of the glomeruli was calculated with each category in ICR mice (a), KK-A^y mice (b), and db/db mice (c). White column: 3 h after a-BSA injection, black column: 6 h after a-BSA injection. Deposition index was obtained by following formula: Deposition index = $(0.1 \times n_0) + (1 \times n_1) + (2 \times n_2) + (3 \times n_3)$ (d). n represents the number of glomeruli in each category: negative, slight, moderate, and severe. White column: ICR mice, gray column: KK-A^y mice, black column: db/db mice. Values represent the means ± S.E.M. Significant difference from the ICR group at $^{**}P<0.01$ (Student's *t*-test).

longer in the nephritic glomeruli than in the normal glomeuli (18), dysfunction of the degradation process on aggregated protein may occur in the glomeruli of diabetic mice due to hyperglycemia. Besides the glomeruli, aggregated protein such as immune complexes or glycosylated proteins are vigorously eliminated from the circulation by monocytes, macrophages, and Kupffer cells. When these phagocytic cells fail to eliminate the aggregated protein from the circulation, the aggregated protein is trapped in the glomeruli. Thus, it was not excluded that the phagocytic function of such cells may be affected by hyperglycemia.

In the present study, comparing the amount of a-BSA in the glomeruli between the KK-A^y mice and the db/db mice, a-BSA was demonstrated in the glomeruli of the KK-A^y mice more than the db/db mice. Intriguingly, the blood glucose levels fell remarkably in the KK-A^y mice in response to the a-BSA injection, but not in the db/db mice, although the insulin levels increased in both of them. Because both strains of diabetic mice are known to be an diabetic animal model with insulin resistance (21, 22), increase of insulin levels hardly explains why KKA^y mice had lower blood glucose level after the injection of a-BSA. Bhatwadekar and Ghole found that rapid glycation of BSA occurred by incubation at 50°C in 0.5 M glucose solution (23). Therefore, we speculated that in the KK-A^y mice, the Maillard reaction could easily occur in a short time with a-BSA and that this glycosylated a-BSA is favorably deposited to the glomeruli. Although we did not show the data on plasma lipid levels, the KK-A^y mice had higher levels of lipid than the db/db mice. This hyperlipidemia would accelerate this reaction in the circulation. Further investigation is necessary to clarify the possible mechanism.

In the present study we did not observe any histopathological alterations in the diabetic glomeruli in light microscopy. Advanced glycation end products (AGE) induce the disturbance of endothelial cells and the infiltration of macrophages in the glomeruli in diabetes mellitus (24). It was also reported that AGE augments the production of the extra cellular matrix via the receptor for advanced glycation end products (RAGE) in the mesangial cells (25) and that the excessive expression of RAGE accelerates the progress of diabetic nephropathy (26). Therefore, it is likely that if glycosylated protein is deposited for a longer period in the glomeuli, these proteins would activate resident macrophages and the mesangial cells. It is also speculated that the cells activated by aggregated protein induce excessive expression of RAGE. Because macrophages take up more glycosylated proteins via RAGE and produce TNF-α and IL-1β, which cause injury to the glomeruli (27), it is likely that the mesangial cells produce proinflammatory cytokines by aggregated protein as well. Therefore, it is important to remove AGE and glycosylated proteins from the glomeruli to prevent the development of diabetic nephropathy.

Hyperfiltration is a well-known phenomenon in the glomeruli in diabetes mellitus (28, 29). The production of nitric oxide and the expression of endothelial cell nitric oxide synthase were demonstrated in the glomeruli of streptozotocin-induced diabetic rats, and nitric oxide is considered to be involved in the pathogenesis of glomerular hyperfitration in early experimental diabetic nephropathy (30, 31). Deposition of immunoglobulin and albumin is detected immunohistologically in the glomeruli of experimental models of diabetes (10, 11). It was also reported that the hyperfiltration of the glomeruli arises after protein ingestion (32). Therefore, the possibility is not excluded that the injection of a-BSA induces hyperfiltration in the glomeruli, and the hyperfiltration could create the circumstances for glycosylated a-BSA to easily deposit in the diabetic glomeuli.

In this study, the extraordinary deposition of the aggregated protein was observed in the glomeruli in both diabetic mice at a younger age before the appearance of histological alterations, suggesting that the excess deposition of aggregated protein in the glomeruli is a very early manifestation of the malfunction in diabetic nephropathy. Additionally, the present investigation provides an experimental method that is available for the screening of agents preventing the development of diabetic nephropathy.

Acknowledgment

A part of the cost of this research was supported from the Scientific Frontier Project of the Japanese Ministry of Education, Culture, Sciences, Sports and Technology.

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