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Increased Hyaluronidase Activity in the Kidney of Streptozotocin-Induced Diabetic Rats

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We investigated changes in renal hyaluronidase activity in streptozotocin (STZ)-induced diabetic rats during the progression of diabetes. Prior to the study, we characterized rat renal hyaluronidase activity to find that its optimum pH is 3.5 and that it consists of two isomers of 73 and 63 kDa, as detected by zymography. Hyaluronidase activity was traced in one whole kidney and in the cortex and medulla of the other kidney up to the 18th week after STZ injection. Whole kidney hyaluronidase activity started to increase on day 3 and reached a maximum level 2.4 times that of the controls in the 3rd week. Cortical hyaluronidase showed a similar tendency to that of whole kidney hyaluronidase, while medullary hyaluronidase activity continued to increase until the 8th week, suggesting their different involvements in the progression of diabetic nephropathy. In zymography, the intensities of the two isomer bands increased with the progression of diabetes, but the intensity ratio did not change significantly and no new isomer band appeared. Renal HAase activity increased only in STZ-induced diabetic rats, but not in spontaneously diabetic Goto-Kakizaki rats still without remarkable renal disorder. Based on these findings, increased renal HAase activity may serve as a useful marker for diabetic nephropathy.

Key words: diabetes, hyaluronidase, kidney, zymography.

Abbreviations: DMAB, *p*-dimethylaminobenzaldehyde; ECM, extracellular matrix; GAG, glycosaminoglycan; GBM, glomerular basement membrane; GK rat, Goto-Kakizaki rat; GlcNAc, *N*-acetyl-D-glucosamine; GlcUA, D-glucuronic acid; HA, hyaluronic acid; HAase, hyaluronidase; STZ, streptozotocin.

Hyaluronic acid (HA), a major component of the extracellular matrix (ECM), is a negatively charged, high molecular weight polysaccharide composed of repeating disaccharide units of D-glucuronic acid (GlcUA) and *N*-acetyl-D-glucosamine (GlcNAc). HA is implicated in many important biological processes such as the stabilization of loose connective tissue, maintenance of water and protein homeostasis, cell proliferation and locomotion, and modulation of the inflammatory reaction (*1*).

In the normal kidney, HA exists at a relatively high concentration in the medulla and at a very low concentration in the cortex (*2*). In the medulla, HA is present in particularly high concentration in the inner medulla, that is the papilla, and regulates water balance (*3*, *4*). In the cortex, HA is present in the glomerular basement membrane (GBM) with sulfated glycosaminoglycans (GAGs) mainly consisting of heparan sulfate, forming anionic sites, and contributes to GBM permeability of biomolecules as a size or charge barrier (*5*–*7*). In various states such as aging (*8*), diabetes (*9*) and different body hydration conditions (*3*), the composition and molecular weight of GAGs, including HA, change in the kidney, but few studies have been performed on the metabolic enzymes involved in these changes.

Regarding hyaluronidase (HAase) *i.e.*, hyaluronate 4-glycanohydrolase (hyaluronoglucosaminidase: EC 3.2.1.35), which is responsible for the degradation of HA in mammalian tissues, six HAase(-like) genes have recently been identified in humans and mice (*10*). Four types of protein product (Hyal-1, Hyal-2, Hyal-3, PH-20) are known to function as HAases in humans, and characterization is in progress. The kidney is considered to be the most important tissue responsible for the degradation of HA next to the liver, and the presence of renal HAase activity has long been recognized (*11*). It has recently been reported that mRNA transcripts for lysosomal-type HAases, Hyal-1 and Hyal-2, and also a testicular-type HAase, PH-20, are constitutively expressed in mouse kidney (*12*).

Very recently, we have traced changes in serum HAase activity with the progression of diabetes using streptozotocin (STZ)-induced diabetic rats and spontaneously diabetic Goto-Kakizaki (GK) rats as models of type 1 and type 2 diabetes mellitus, respectively, and clarified that serum HAase activity increases significantly with increases in blood glucose from an early stage in diabetic rats (*13*). In this study, we investigated changes in renal HAase activity in order to study the relationship between a complication of diabetes, diabetic nephropathy, and renal HAase.

MATERIALS AND METHODS

Materials—STZ, saccharic acid 1,4-lactone, and Alcian blue 8GX were obtained from Sigma Chemical (MO, USA). Sodium hyaruronate from *Streptococcus zooepidemicus*, GlcNAc, potassium tetraborate, *p*-dimethylaminobenzaldehyde (DMAB), and all reagents for the polymerization of electrophoretic gels were from Wako Pure

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Chemical Industries (Tokyo). Actinase E was from Kaken Pharmaceutical (Tokyo). All other chemicals were of reagent grade.

Experimental Animals—Male Wistar and GK rats (Japan Laboratory Animals, Tokyo), 8 weeks old, were used. Wistar rats were separated into two groups, control and STZ groups. To induce type 1 diabetes mellitus, a single injection of STZ (60 mg/kg body weight) dissolved in normal saline buffered with sodium citrate (0.10 M, pH 4.5) was administered *via* the tail vein of the STZ group rats. Control group rats received an equivalent volume of the dissolving buffer alone. Control, STZ, and GK rats were housed 3 or 4 per cage and fed *ad libitum* regular chow and tap water from 8 to 26 weeks of age. All experiments were performed in accordance with the Guidelines for Animal Experiments at Hoshi University.

Three rats from each group were decapitated, and the kidneys were excised after blood collection. The kidneys were immediately washed with physiological saline, and one kidney was kept whole and the other was divided into the medulla and cortex. Two tissue-weight volumes of physiological saline were added to these specimens, and the specimens were homogenized in ice using a polytron homogenizer. The homogenates were centrifuged at 18,000 ×*g* at 4°C for 30 min, and the supernatants were obtained as extracts.

Determination of Serum Glucose Level—Trunk blood was collected and, after standing for 30 min at 4°C, centrifuged at 500 ×*g* for 15 min to obtain serum. Serum glucose levels were determined by a glucose oxidase method with a kit (Glucose CII-Test Wako) supplied by Wako Pure Chemical Industries.

Assay of HAase Activity—HAase activity was determined by the fluorimetric Morgan-Elson assay method as recently described by us (*14*). Briefly, 120 μl of substrate solution (1.5 mg/ml HA in 0.1 M formate buffer, pH 3.9, containing 0.1 M NaCl and 1.5 mM saccharic acid 1,4-lactone) was mixed with 10 μl of kidney extract and digested at 37°C for 40 min (for whole kidney and cortex extracts) or 100 min (for medulla extract). After heating to terminate the enzyme reaction, the Morgan-Elson reaction was started by the addition of 25 μl of 0.8 M potassium tetraborate reagent (pH 10.4) and subsequent heating for 3 min in a boiling water bath. Then, 0.75 ml of DMAB reagent was added and the mixture was incubated at 37°C for 20 min. After centrifugation at 18,000 ×*g* at 4°C for 10 min, the fluorescence (excitation, 545 nm; emission, 604 nm) of the clear supernatant was measured against that of a blank, which was prepared in the same way except that the enzyme reaction mixture was incubated for 0 min. One unit of HAase activity was defined as the amount of enzyme required to produce 1 μmol of reducing terminal GlcNAc per min under the specified conditions.

To determine the pH optimum, HAase activity was measured according to the above fluorimetric Morgan-Elson method, except that 0.1 M formate buffers of various pHs from pH 2.0 to pH 5.0, each containing 0.1 M NaCl and 1.5 mM saccharic acid 1,4-lactone, were used and the HA concentration was 0.5 mg/ml.

Zymography of HAase Activity—HAase activity was detected by HA zymography (*15*) as described by us (*13*). Rat kidney extracts were diluted with 0.15 M NaCl to a concentration of 10 mg/ml protein. Rat and human sera were diluted with 9 volumes of 0.15 M NaCl. These diluted samples and undiluted rat urine were mixed with an equivalent volume of Laemmli's sample buffer (*16*) containing 4% SDS and no reducing reagent. After incubation for 1 h at 37°C, without heating, the mixtures were applied to 7% SDS-polyacrylamide gels containing 0.17 mg/ml HA. After electrophoresis at 25 mA for approximately 70 min at 4°C, the gels were rinsed with 2.5% Triton X-100 for 80 min at room temperature and incubated with 0.1 M sodium formate buffer (pH 3.5) containing 0.03 M NaCl for 18 h at 37°C on an orbital shaker. The gels were then treated with 0.1 mg/ml Actinase E in 20 mM Tris-HCl buffer (pH 8.0) for 2 h at 37°C. To visualize the digestion of HA, gels were stained with 0.5% Alcian blue in 25% ethanol–10% acetic acid. After destaining, the gels were counterstained with Coomassie Brilliant Blue R-250.

Protein Determination—Protein concentrations were determined by the bicinchoninic acid assay (Pierce Chemical, Rockford, IL, USA) according to the manufacturer's protocol using BSA as a standard.

Statistical Analysis—Results are expressed as means ± SD. Student's two-sided *t*-test for paired samples and ANOVA for independent groups, followed by Fisher's protected least significant difference (PLSD) test, were used. The significance level is indicated for each experiment.

RESULTS AND DISCUSSION

Characterization of Rat Renal HAase—Prior to studying changes in rat renal HAase activity with the progression of diabetes, we investigated some properties of rat renal HAase.

(a) Optimum pH: We first examined the pH-activity profiles of rat renal HAase and also serum HAase, since the activity of the latter enzyme has been shown to increase in diabetic rats by us (*13*) and others (*17*). As shown in Fig. 1a, the optimum pH was 3.5 for both rat HAases. HAase activity is known to be more readily affected by assay conditions and co-existing substances than general enzymes (*18*). Therefore, for comparison, we also measured the pH-activity profile of human serum HAase under the same conditions, for which many researchers have reported different optimum pH values of 3.7–4.2 under different conditions (*19–21*), and found its optimum pH to be 3.9. It appears that the optimum pH of rat serum HAase, which may be Hyal-1 type as is human serum HAase, is more acidic than that of human serum HAase and thus the pH optima of both rat serum and renal HAases are the most acidic among the reported pH optima of various HAases. As seen in the pH-activity profile curve, rat renal HAase exhibits no activity above pH 5, indicating that the activity of PH-20 type HAase with a neutral optimum pH is, if present, lower than the detection limit in rat kidney.

(b) Zymography of rat renal HAase: In the preceding report (*13*), we analyzed rat serum and urinary HAases by zymography. In this study, we analyzed rat renal HAase by zymography in comparison with these rat HAases and also human serum HAase (Fig. 1b). As previously reported, a major band of Hyal-1 type HAase (73 kDa), corresponding to human serum Hyal-1 (59 kDa),

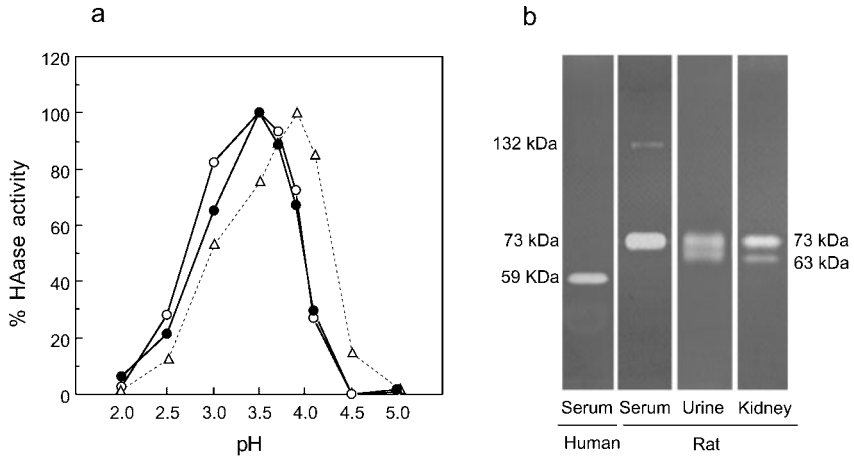

Fig. 1. **Determination of pH optimum (a) and zymography (b) of rat renal HAase.** (a) The pH-activity profiles of rat renal HAase, as well as rat serum HAase, were obtained in comparison with the profile of human serum HAase. HAase activity was determined by the fluorimetric Morgan-Elson assay method as described in "MATERIALS AND METHODS." The samples used were 10 μl of rat kidney extract, 10 μl of diluted rat serum with three volumes of 0.15 M NaCl, and 5 μl of human serum. The incubation time was 60 min for the kidney extract and rat serum and 120 min for human serum. Closed circles, rat kidney; open circles, rat serum; open triangles, human serum. (b) Zymography was carried out as described in "MATERIALS AND METHODS." Diluted human serum, rat serum, and whole kidney extract and undiluted rat urine were mixed with an equivalent volume of Laemmli's sample buffer and incubated for 1 h at 37°C. The samples loaded were 0.5 μl of human serum, 0.2 μl of rat serum, 7.5 μl of rat urine and 5 μl (50 μg protein) of kidney extract. The numbers on each side represent molecular mass in kDa.

and a high molecular weight band (132 kDa) with trace intensity were detected in rat serum, and a Hyal-1–derived doublet band (centered at 71 kDa) was detected in rat urine. In the rat kidney, a major 73-kDa band and a 63-kDa band with trace intensity were detected. Among Hyal-1, Hyal-2, and PH-20 type HAases that may be expressed in rat kidney as in mouse kidney (*12*), Hyal-1 and PH-20 type HAases are detectable by this zymographic technique, but a Hyal-2 type HAase is not detectable because it cleaves HA only to intermediates of about 20 kDa (*22*). Since the enzyme reaction in zymography was performed at pH 3.5, the pH of the enzyme reaction was changed to neutral to detect PH-20 type HAase with a neutral pH optimum, but no new bands were detected (data not shown). It appears that in rat kidney, a PH-20 type HAase is, if expressed, under the detection limit of even zymography with high sensitivity. The two HAase bands detected, therefore, may be multiple isomers of the Hyal-1 type HAase. It is now unclear whether the major renal HAase of 73 kDa is completely identical to the serum HAase of 73 kDa. It should be noted that rat renal HAase isomers are not as easily denatured by SDS as human serum HAase and take longer (>20 min at 37°C) to denature completely, as is the case with rat serum and urinary HAases (*13*).

Characteristics of STZ-Induced Diabetic Rats—The body weights of the STZ-injected rats increased only slightly compared to the control rats. The blood glucose level started to increase on day 3 after injection, and a level about 4-fold higher than in the control rats persisted until the 13th week (Fig. 2a). The kidney weight started to increase in the 1st week in the STZ group compared with that of the control group, and reached a 1.4-fold higher weight in the 13th week ($p < 0.05$) (Fig. 2b). Since body weight was 3/5 that of the control rats at this time point, the kidney weight per body weight was 2.3-fold higher. Since renal hypertrophy, one of the morphological renal changes that take place in diabetic nephropathy, occurs very shortly after STZ induction in rats (*23*, *24*), renal injury may have occurred in the STZ group in this study.

Changes in Renal HAase Activity in STZ-Induced Diabetic Rats—HAase activities in whole kidney, cortex, and medulla extracts were measured, and the activity time-courses after STZ induction were compared until the 13th week (Fig. 3). HAase activities in whole kidney, cortex, and medulla at the start of the experiment (8 weeks

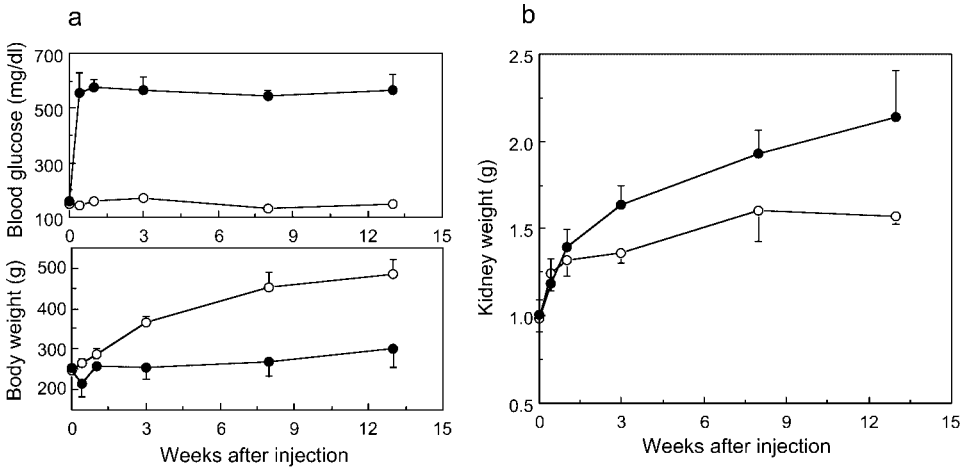

Fig. 2. **Changes in blood glucose level and body weight (a) and kidney weight (b) in STZ and control rats.** Each point represents mean ± SD of 3 experiments. Closed circles, STZ rats; open circles, control rats.

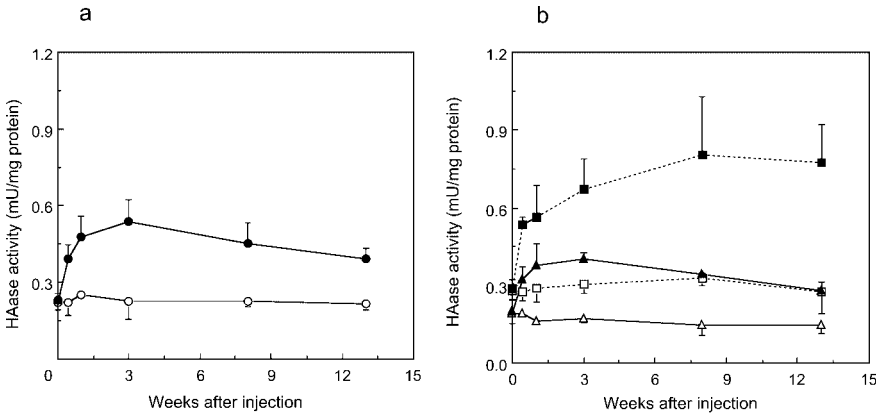

Fig. 3. **Changes in renal HAase activity in extracts of whole kidney (a) and cortex and medulla (b) from STZ and control rats.** HAase activity was determined by the fluorimetric Morgan-Elson assay method as described in "MATERIALS AND METHODS." Each point represents mean ± SD of 3 experiments. (a) Whole kidney: closed circles, STZ rats; open circles, control rats. (b) Cortex: closed triangles, STZ rats; open triangles, control rats. Medulla: closed squares, STZ rats; open squares, control rats.

old) were 0.22 ± 0.03, 0.19 ± 0.05, and 0.28 ± 0.04 mU/mg protein, respectively, with the medullary activity being the highest. As shown in Fig. 3a, little change with age was detected in the whole kidney HAase activity in the control group. In the STZ group, the activity increased significantly to a level 1.8-fold higher than that of the control group on day 3 ($p < 0.05$) and to a maximum level 2.4-fold higher than the control group in the 3rd week ($p < 0.01$), then decreased slightly; the activity in the 13th week was 1.7-fold higher than that of the control rats. The medullary and cortical HAase activities also started to increase on day 3 (Fig. 3b). The pattern of the increase in HAase activity in the cortex, occupying most of the kidney, was almost the same as that in the whole kidney (peaking in the 3rd week), while the medullary HAase activity peaked slightly later, in the 8th week. As previously reported (*13*), serum HAase activity in the STZ group started to increase on day 3 with an increase in blood glucose, and, in this study, the renal HAase activity started to increase in the early stage of diabetes, consistent with the increase in blood glucose, although there was a slight difference between the medulla and cortex. These findings suggest that renal HAase may be involved in the progression of diabetes accompanied by renal injury.

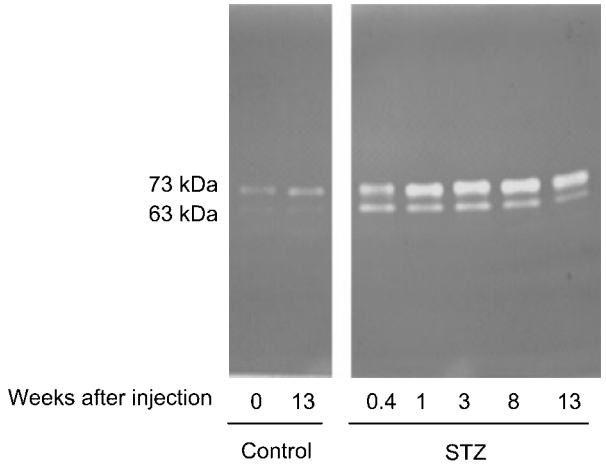

Fig. 4. **Changes in renal HAase activity in STZ and control rats as analyzed by zymography.** Two point five microliters (25 μg protein) of kidney extract were applied to HA-impregnated gels as described in "MATERIALS AND METHODS." The numbers on the left side represent molecular mass in kDa.

Zymographic Analysis of Increased Renal HAase Activity in STZ-Induced Diabetic Rats—Next, we used zymography to investigate whether the increase in renal HAase activity during the progression of diabetes is accompanied by qualitative changes (Fig. 4). The intensities of both bands of rat kidney HAase increased with the progression of the pathological state, but no new HAase isomer appeared, and no significant change was observed in the intensity ratio of the two bands (data not shown). The kidney was divided into the medulla and cortex, and the ratio of the intensities of the two HAase isomers was traced in the medulla and cortex, but again no significant changes were observed (data not shown), clarifying that the increase in renal HAase activity with the progression of the pathological state is due to nearly identical increases in the activities of the two isomers, not to the appearance of a new HAase isomer(s) or a change in the ratio of the two pre-existing HAase isomers.

Comparison of the Changes in Renal HAase Activity between STZ-Induced Diabetic Rats and GK Rats—To investigate whether an elevation of renal HAase activity also occurs in a model of type 2 diabetes (GK rats), the renal HAase activities of STZ rats in the 18th week and age-matched GK rats (26 weeks old) were compared (Fig. 5). In the STZ group, HAase activity was significantly

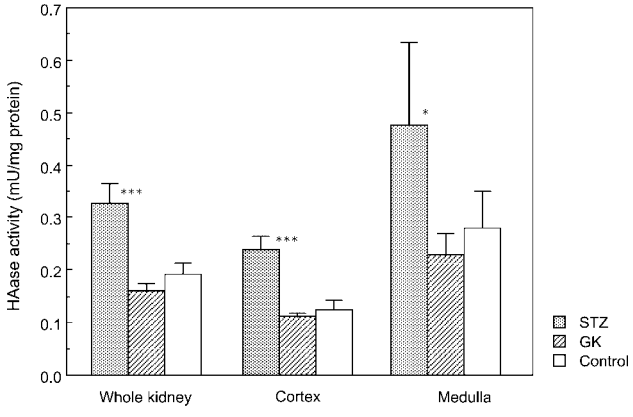

Fig. 5. **Comparison of the changes in renal HAase activity between STZ-induced diabetic rats and GK rats.** Age (26 weeks)-matched STZ-induced diabetic rats and GK rats were used. Data represent means ± SD of 3 experiments. Significant differences from values for the control group are indicated by $*p < 0.05$ and $***p < 0.001$.

higher, 1.7- to 1.9-fold higher than that in the control group, in the whole kidney, cortex, and medulla, but no significant changes relative to the control group were observed for the GK group. Although not as high as in the STZ group, the blood glucose level in the GK group was 2.6 times higher and the serum HAase activity was 1.5 times higher, respectively, than those in the control group, while the renal HAase activity did not change. It has been reported that although thickening of the GBM is present in GK rats of the same age as used in the present experiment (26 weeks old), it has not yet caused renal disorders such as microalbumiuria (*25*). In agreement with this report, our preceding paper (*13*) demonstrated that albumin excretion in the GK group (26 weeks old) was not larger than that in control rats, whereas albumin excretion in age-matched STZ rats was about 9 times greater than that in the control group. Since, in the present work, increased renal HAase activity was observed only in STZ rats with renal disorder but not in GK rats without any remarkable renal disorder, increased renal HAase activity may serve as a useful marker of renal dysfunction.

Earlier Silberberg *et al*. (*26*) reported that in the articular cartilage of STZ-induced diabetic rats, the activities of both GAG-degrading enzymes and synthetic enzymes are increased, with the activities of the formers increasing more than the activities of the latters, and that the implantation of pancreatic islets reduces these enzymatic activities to near-normal levels. Chajara *et al*. (*17*) also reported that an increase in serum HAase activity observed in STZ-induced diabetic rats was abolished by insulin treatment. Therefore, the increase in renal HAase activity observed in the present study in STZ-induced diabetic rats seems to be involved in diabetes.

This study, for the first time, clarifies that renal HAase activity increases from the early stages of diabetic nephropathy in STZ-induced diabetic rats. Based on reports that anionic sites on the GBM decrease in diabetic patients (*27*) and in STZ-induced diabetic rats (*28*), the increase in HAase activity in the cortex may contribute to one development step in diabetic nephropathy, microalbumiuria, by acting on the glomerular size and/or charge barrier and enhancing GBM permeability. Furthermore, lower molecular weight HA (HA fragments) produced by HAase, unlike high molecular weight HA, has been reported to induce the expression of adhesion molecules in mouse cortical tubular epithelial cells and inflammatory cytokines in macrophages (*29–31*). Most recently, it has been reported that the mRNA levels of Hyal-2, which generates HA fragments with such proinflammatory effects, doubles in the rat kidney cortex with post-ischemic injury or nephrotoxic damage (*32*). Based on these reports, it is likely that an increased HAase activity contributes to the aggravation of nephropathy, although it is unclear whether a Hyal-2 type activity was included in the increased cortical HAase activity in this study. On the other hand, since the involvement of renal HAase in the action mechanism of antidiuretic hormone has been suggested (*33–35*), and HAase activity was shown to increase in the medulla in this study, HAase may be closely involved in the regulation of HA function in the medulla. The difference between the patterns of increase of the cortical and medullary HAase activities attracts attention because it may reflect functional differences; however, a more detailed investigation is necessary to confirm this.

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