DNA aptamer raised against AGEs blocks the progression of experimental diabetic nephropathy

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ABSTRACT

Advanced glycation end products (AGEs) and their receptor (RAGE) play a role in diabetic nephropathy. We screened DNA aptamer directed against AGEs (AGEs-aptamer) in vitro, and examined its effects on renal injury in KKAy/Ta mice, an animal model of type 2 diabetes. Eight week-old male KKAy/Ta or C57BL/6J mice received continuous intraperitoneal infusion of AGEs- or control-aptamer for 8 weeks. AGEs-aptamer was detected and its level was increased in the kidney for at least 7 days. The elimination half-lives of AGEs-aptamer in the kidney were about 7 days. Compared with C57BL/6J mice, glomerular AGEs levels were significantly increased in KKAy/Ta mice, which were blocked by AGEs-aptamer. Urinary albumin and 8-hydroxy-2’-deoxy-guanosine levels were increased, and glomerular hypertrophy and enhanced extracellular matrix accumulation were observed in KKAy/Ta mice, all of which were prevented by AGEs-aptamer. Moreover, AGEs-aptamer significantly reduced gene expression of RAGE, monocyte chemoattractant protein-1, connective tissue growth factor or type-IV collagen both in the kidney of KKAy/Ta mice and in AGEs-exposed human cultured mesangial cells. Our present data suggest that continuous administration of AGEs-aptamer could protect against experimental diabetic nephropathy by blocking the AGEs-RAGE axis and may be a feasible and promising therapeutic strategy for the treatment of diabetic nephropathy.

KEY WORDS; aptamer, AGEs, diabetic nephropathy, RAGE
INTRODUCTION

Diabetic nephropathy is a leading cause of end-stage renal disease, which accounts for disability and high mortality rate in patients with diabetes (1, 2). The development of diabetic nephropathy is characterized by glomerular hypertrophy and inflammatory cell infiltration, followed by extracellular matrix (ECM) accumulation in mesangial area and an increased urinary albumin excretion (UAE) rate (3). Diabetic nephropathy ultimately progresses glomerular sclerosis associated with renal dysfunction (4). Large-scale clinical studies have shown that intensive glycemic and blood pressure control reduce the risk of diabetic nephropathy (5). However, management of diabetic nephropathy is far from satisfactory, because strict control of blood glucose and/or pressure is often difficult to maintain and may increase the risk of hypoglycemia or hypotension. Further, despite intensive management of classical cardiovascular risk factors using blood pressure-lowering and lipid-lowering agents, a substantial number of diabetic patients still have experienced end-stage renal disease. Therefore, to develop novel therapeutic strategies that specifically target diabetic nephropathy is urgently needed.

Reducing sugars can react non-enzymatically with the amino groups of proteins to initiate a complex series of rearrangements and dehydrations, and then to
produce a class of irreversibly cross-linked moieties termed advanced glycation end products (AGEs) (6-8). The formation and accumulation of AGEs in various tissues have been shown to progress at an accelerated rate under hyperglycemic conditions (9-11). There is accumulating evidence that AGEs and RAGE (receptor for AGEs) interaction stimulates oxidative stress generation and subsequently evokes inflammatory reactions, thereby causing progressive alteration in renal architecture and loss of renal function in diabetes (12-14). Indeed, inhibitors of AGEs formation have been shown to attenuate the increase in albuminuria and prevent the development and progression of experimental diabetic nephropathy (15, 16). Further, RAGE-overexpressing diabetic mice showed progressive glomerulosclerosis with renal dysfunction, while diabetic homozygous RAGE null mice failed to develop significantly increased mesangial matrix expansion or thickening of the glomerular basement membrane (14, 17). These observations suggest that the inhibition of the AGEs-RAGE axis could be a novel therapeutic target for diabetic nephropathy.

Aptamers are short, single-stranded DNA or RNA molecules that can bind with high affinity and specificity to a wide range of target proteins (18). Recently, numerous aptamers have been developed and used in the clinical fields as a tool for modulating various protein function (19-21). Pegaptanib, an RNA aptamer directed against vascular
endothelial growth factor_{165} (VEGF_{165}) isoform, has been shown to be effective in treating choroidal neovascularization in patients with age-related macular degeneration (19). In addition, ARC1779, a DNA-aptamer raised against the A1 domain of von Willebrand factor, has also been reported to inhibit its pro-thrombotic function \textit{in vivo}, and phase II clinical trials of ARC1779 are currently underway for the treatment of thrombotic thrombocytopenic purpura and cerebral embolism after carotid endarterectomy (20, 21).

Therefore, in this study, we screened a high-affinity DNA aptamer directed against AGEs (AGEs-aptamer) using a combinatorial chemistry \textit{in vitro}, and examined its effects on experimental diabetic nephropathy. For this, we chose KKAy/Ta mice because they are an animal model of obese and type 2 diabetes associated with ECM accumulation, inflammatory cell infiltration and sclerotic changes within the glomerular areas, whose characteristics closely resemble those in human diabetic nephropathy (22). Furthermore, we investigated the effects of AGEs-aptamer on mesangial cell damage \textit{in vitro}.

**RESEARCH DESIGN AND METHODS**

**Animals.** Male 8-week old KKAy/Ta (DM) and 8-week old C57BL/6J (Ctr) mice were
purchased from CLEA Japan (Tokyo, Japan). These mice were divided into two groups respectively, and received continuous intraperitoneal infusion of either AGEs-aptamer or control-aptamer (Ctr-aptamer) (0.136 µg/day) by an osmotic mini pump (Alzet osmotic pumps, model 1004, Cupertino, CA) [Ctr-aptamer-treated Ctr mice (Ctr-Ctr-aptamer) (n=8), AGEs-aptamer-treated Ctr mice (Ctr-AGEs-aptamer) (n=9), Ctr-aptamer-treated DM mice (DM-Ctr-aptamer) (n=11) and AGEs-aptamer-treated DM mice (DM-AGEs-aptamer) (n=10) group]. Eight weeks after the continuous intraperitoneal infusion, blood pressure was measured by a tail-cuff sphygmomanometer using an automated system with a photoelectric sensor (BP-98A; Softron, Tokyo, Japan), and mice were transferred to metabolic cages for 24 hr for urinalysis. Then, mice were killed and blood and kidney samples were obtained. All experimental procedures were conducted in accordance with the National Institutes Health Guide for Care and Use of Laboratory Animals and were approved by the ethnical committee of Kurume University School of Medicine.

**Measurement of clinical variables.** Albuminuria was determined with commercially available enzyme-linked immunosorbent assay (ELISA) kit (Exocell, Philadelphia, PA, USA). Blood was collected, centrifuged and plasma and serum were obtained, and
stored at –40°C. Total cholesterol and transaminase levels were determined by commercially available kits (Wako, Osaka, Japan). Plasma creatinine levels were measured by an enzymatic method (SRL Inc., Tokyo, Japan). Blood urea nitrogen levels were measured by an auto-analyzer (Nihondenshi Co., Tokyo, Japan). Plasma glucose was measured by a glucose oxidase method (Shionotest Co., Tokyo, Japan). HbA1c was determined by latex coagulation method (TFB Co., Tokyo, Japan). Urinary 8-hydroxy-2’-deoxy-guanosine (8-OHdG) levels were measured with ELISA system (Japan Institute for the Control of Aging, Fukuroi, Shizuoka, Japan). Urinary N-acetyl-β-D-glucosaminidase (NAG) was measured with a fractional colorimetric determination method (Shionogi Pharma, Inc., Osaka, Japan). Intra- and interassay coefficients of variation were 6.2 and 8.8%, respectively.

**Preparation of AGEs-human serum albumin (AGEs-HSA) and AGEs-bovine serum albumin (AGEs-BSA).** AGEs-modified proteins were prepared as described previously (23).

**Immobilizing AGEs-HSA on agarose beads.** AGEs-HSA was covalently coupled to indoacetyl groups on SulfoLink Coupling Gel (Pierce, Rockford, IL, USA) as described
previously (24).

Screening and modification of AGEs-aptamer (Systematic Evolution of Ligands by EXponential enrichment (SELEX)). Preparation and selection of DNA aptamers were performed as described previously (24). Sequences of AGEs- and Ctr-DNA aptamers are below. AGEs-aptamer;

5’-CCGAAACCAGACCACCCACCAAGGCCACTCGTCGAACCGCCAACACTCACCCCA-3’,

CTR-aptamer;

5’-GTATCTGTAGGAACGTCGACTCAGCAGCTCGTCTTCAGGGTCACGT-3’.

DNA aptamers are susceptible to degradation by nucleases. This will limit their applications for real samples, such as blood and tissues. To solve this issue, we modified aptamers with phosphorothioate as described previously (25).

Binding affinity of AGEs-aptamer to AGEs-HSA or AGEs-HSA to RAGE. The binding affinity of the selected AGEs-aptamer to AGEs-HSA or AGEs-HSA to extracellular AGEs-binding V-domain of RAGE (vRAGE) was measured using sensitive 27-MHz Quarts crystal microbalance (QCM) (Affinix Q; Initium, Tokyo, Japan) according to the method of Okahata et al (26). In brief, AGEs-HSA or vRAGE
was immobilized on an avidin-bound QCM surface. After adding Ctr-aptamer or AGEs-aptamer to a reaction vessel in the presence or absence of 70 ng/ml AGEs-HSA, the time course of the frequency decrease of bound AGEs-HSA or bound vRAGE on the QCM was monitored. The binding affinity of AGEs-aptamer to AGEs-HSA or AGEs-HSA to vRAGE was calculated from curve fitting to the QCM frequency decrease. Human vRAGE (residues 23–121) was prepared as described previously (27, 28). SDS-PAGE analysis of purified vRAGE proteins revealed a single band, which showed positive reactivity with polyclonal antibody raised against RAGE (data not shown).

**Distribution and kinetics of \( [\gamma-^{32}P]ATP\)-labeled AGEs-aptamer.** Aptamer was radioactively labeled by a 5'-end-labeling technique using T4 polynucleotide kinase (T4 PNK; Promega Corporation, WI, USA) and \( [\gamma-^{32}P]ATP\) (PerkinElmer Japan Co., Ltd., Kanagawa, Japan) according to the manufacture's protocol. Male 8-week old Ctr mice received continuous intraperitoneal infusion of \( [\gamma-^{32}P]ATP\)-labeled AGEs-aptamer (0.136 µg/day) by an osmotic mini pump, and killed at 0 hr, 1 hr, 6 hr, 1 day, 2 days and 7 days after the infusion. At 0, 3, 7 and 14 days after stopping the infusion, mice were killed, and blood and organs were obtained. The radioactivity of AGEs-aptamer was
measured by Cherenkov counting using LS-6500 scintillation counter (Beckman Coulter, San Francisco, CA, USA).

**Turnover rate of aptamer-bound AGEs by macrophages.** Human THP-1 monocyctic leukemia cells were differentiated to macrophages as described previously (29). One hundred μg/ml AGEs-BSA was added to the culture medium in the presence or absence of 2 μM AGEs-aptamer where differentiated THP-1 macrophages were grown or ungrown. After 4 hr, the supernatant was collected, concentrated with Amicon Ultra centrifugal filter (Millipore Co., Billerica, MA), separated by SDS-PAGE and transferred to polyvinylidene fluoride membrane as described previously (30). Membranes were probed with rabbit polyclonal antibodies raised against AGEs, and then immune complexes were visualized with an enhanced chemiluminescence detection system (ECL; Amersham Bioscience, Buckinghamshire, UK).

**Morphological analysis.** Three-micrometer paraffin sections were stained with periodic acid-schiff and Masson’s trichrome for light microscopic analysis as described previously (31).
**Measurement of serum AGEs.** Measurement of serum AGEs levels were performed with a competitive enzyme-linked immunosorbent assay (ELISA) as described previously (32).

**Immunostaining.** Specimens of kidney cortex were fixed with 4% paraformaldehyde, embedded in paraffin, sectioned at 4-µm intervals and mounted on glass slides. The sections were incubated in 0.3% hydrogen peroxide methanol for 30 min, and incubated overnight at 4°C with rabbit polyclonal antibodies raised against AGEs and synaptopodin (PROGEN Biotechnik GmbH, Heidelberg, Germany) as described previously (32). Immunoreactivity in 10 different fields (x 600) in each sample was measured by an image analysis software (Optimas version 6.57; Media Cybernetics, Silver Spring, MD, USA).

**Cells.** Human mesangial cells were maintained in basal medium supplemented with 5% fetal bovine serum according to the supplier’s instructions (Clonetics Corporation, San Diego, California, USA). AGEs treatment was carried out in a medium containing 0.5% fetal bovine serum. Mesangial cells less than 6 passages were used for the experiments. Mesangial cells were treated with 100 µg/ml AGEs-BSA or non-glycated BSA for 4 and
24 hr in the presence or absence of 0.2 or 2 µM AGEs-aptamer.

**Real-time reverse-transcription PCR (RT-PCR).** Three to six µg of total RNA extracted from each kidney cortex were used to synthesize cDNA with the Superscript First Strand synthesis system for RT-PCR (Invitrogen Co., Carlsbad, CA, USA). Quantitative real-time RT-PCR was performed using Assay-on-Demand and TaqMan 5 fluorogenic nuclease chemistry (Applied Biosystems, Foster city, CA, USA) according to the supplier’s recommendation. IDs of primers and probe for mice monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-alpha (TNF-α), connective tissue growth factor (CTGF), type-IV collagen, vascular endothelial growth factor (VEGF), AGE-receptor 3 (AGE-R3) and RAGE gene were Mm00441242_ml, Mn00443258_m1, Mm01192933_g1, Mm01210125_m1, Mm01281449_m1, PN4331348 and Mm00545815_m1, respectively (Applied Biosystems, Foster city, CA, USA). TaqMan Ribosomal RNA Control Reagents (18S) was used as an endogenous control (Applied Biosystems, Foster city, CA, USA). Total RNAs were extracted from cultured human mesangial cells and THP-1 cells with RNA queous-4 PCR kit (Ambion Inc., Austin, TX, USA), and quantitative real-time RT-PCR was performed. IDs of primers and probe for human MCP-1, TNF-α, CTGF, RAGE and
glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were Hs00234140_m1, Hs00170014_m1, Hs00174128_m1, Hs00153957_m1 and Hs99999905_m1, respectively (Applied Biosystems, Foster city, CA, USA).

**Measurement of reactive oxygen species (ROS) generation.** The intracellular formation of ROS was detected using the fluorescent probe CM-H$_2$DCFDA (Molecular Probes, Inc., Eugene, OR, USA) as described previously (33).

**Statistical analysis.** All data are presented as means ± standard error of mean. Analysis of variance (ANOVA) followed by the Turkey’s or Games-Howell post-hoc test was performed for all studied parameters for statistical comparisons; p<0.05 was considered significant. All statistical analyses were performed with SPSS 19 system.

**RESULTS**

**Isolation and characterization of DNA aptamers directed against AGEs-modified proteins.** DNA aptamers specific for AGEs-HSA were isolated by an *in vitro* selection process, SELEX, from a pool of ~10$^{15}$ different nucleic acid sequences as described previously (24). In this study, 35 clones were sequenced from the pool of selected
single-stranded DNAs to obtain 15 unique sequences, indicating that some of the sequences among the 35 clones were identified and that multiple selection of the same clone occurred. Structural analysis revealed that all of the aptamers had bulge-loop structure with cytosine-rich sequences. We have previously shown that although all the clones significantly inhibit the AGEs-induced decrease in DNA synthesis in cultured pericytes, a counterpart of mesangial cells in the kidney, clone 1 had the strongest effect (24). So, we modified clone 1 with phosphorothioate for generating nuclease-resistant aptamer and used it for the following experiments. The structure of phosphorothioate AGEs-aptamer used here is shown in Fig. 1A.

**Binding affinity of phosphorothioate AGEs-aptamer to AGEs-HSA or AGEs-HSA to vRAGE.** We first examined the binding affinity of phosphorothioate AGEs-aptamer to AGEs-HSA *in vitro*. For this, AGEs-HSA was immobilized on an avidin-bound QCM surface, and then Ctr- or AGEs-aptamer was added to a reaction vessel. AGEs-aptamer bound to AGEs-HSA with a dissociation constant of $1.38 \times 10^{-6}$ M, whereas Ctr-aptamer did not bind to AGEs-HSA at all (Fig. 1B). Further, as shown in Fig. 1C, the binding of AGEs-HSA to vRAGE was dose-dependently inhibited by the co-treatment with AGEs-aptamer.
**Turnover rate of aptamer-bound AGES-BSA.** Compared with unbound AGES-BSA, turnover rate of aptamer-bound AGES by differentiated THP-1 macrophages were more increased (Fig. 1D). On the contrary, under the conditions without THP-1 cells, presence of AGES-aptamer did not affect the turnover rate of AGES-BSA (Fig. 1E).

**Distribution and kinetics of infused AGES-aptamer.** To examine the kinetics of injected AGES-aptamer, we labeled the aptamer with $[^{32}\text{P}]$ATP and infused it into the peritoneal cavity of 8-week old C57BL/6J (Ctr) mice by an osmotic pump. As shown in Fig. 2A, when AGES-aptamer was continuously administrated up to 7 days, it was distributed mainly in the kidney, aorta and muscle. The levels of AGES-aptamer were increased in the kidney, liver and blood of 8-week old Ctr mice for at least 7 days following continuous 0.136 µg/day-intraperitoneal infusion (Fig. 2B). After stopping the injection, AGES-aptamer levels were gradually decreased. But the aptamer was still detected in the kidney, liver and blood at day 14 after the removal of an osmotic pump (Fig. 2B). The elimination half-lives of AGES-aptamer in the kidney were about 7 days.

**Characteristics of animals.** Clinical characteristics of each group are shown in Table 1.
Compared with Ctr-Ctr-aptamer mice, body weight, plasma glucose, HbA1c and total cholesterol levels were significantly increased in DM-Ctr-aptamer mice (p<0.05). Treatment with AGEs-aptamer did not affect these parameters in mice. Plasma levels of blood urea nitrogen and creatinine were tended to be higher and kidney-to-body weight ratio was significantly increased in DM-Ctr-aptamer mice, which were ameliorated by the treatment of AGEs-aptamer (p<0.05). There were no significant differences of clinical variables between Ctr-Ctr-aptamer and Ctr-AGEs-aptamer mice.

Effect of AGEs-aptamer on serum and renal levels of AGEs in mice. We investigated whether treatment with AGEs-aptamer could reduce serum and renal levels of AGEs in KKAy/Ta mice. As shown in Fig. 3A, compared with Ctr-Ctr-aptamer mouse, serum levels of AGEs were significantly increased to about 3-fold in DM-Ctr-aptamer mouse (p<0.05), which were not affected by the treatment with AGEs-aptamer. However, immunohistochemical analysis revealed that AGEs levels in the glomeruli of DM-Ctr-aptamer mice were significantly higher than those of Ctr-Ctr-aptamer mice, which was prevented by the treatment with AGEs-aptamer (Fig. 3B-F). AGEs-aptamer itself did not affect serum or renal levels of AGEs in Ctr mice.
Treatment with AGEs-aptamer decreased UAE levels in DM mice. We examined the effect of AGEs-aptamer on UAE levels in mice. As shown in Fig. 4, UAE levels were gradually and significantly elevated in DM-Ctr-aptamer mice compared with Ctr-Ctr-aptamer mice (p<0.01). Administration of AGEs-aptamer for 8 weeks significantly decreased UAE levels in DM mice (p<0.01). AGEs-aptamer itself did not affect UAE levels in Ctr mice.

Treatment with AGEs-aptamer prevented glomerular hypertrophy and ECM protein accumulation in the kidney of DM mice. Glomerular hypertrophy and ECM accumulation are one of the characteristic features of diabetic nephropathy (3). So, we investigated whether AGEs-aptamer treatment could prevent the structural changes in the kidney of DM mice. As shown in Fig. 5A-E, periodic acid-schiff staining revealed that diabetes was associated with glomerular hypertrophy, which was significantly ameliorated by the administration of AGEs-aptamer. Further, ECM accumulation assessed by Masson’s trichrom staining was significantly increased in DM-Ctr-aptamer mice, which was also prevented by the treatment with AGEs-aptamer (Fig. 5F-J). AGEs-aptamer itself did not affect glomerular hypertrophy or ECM accumulation in Ctr mice. Further, immunohistochemical analysis revealed that synaptopodin levels, a
marker of podocytes were reduced in DM-Crl-aptamer mice, which were significantly restored by the treatment with AGEs-aptamer (Fig. 5K-O). There was no significant difference of renal VEGF expression, tubulointerstitial fibrosis (data not shown) or NAG levels (Table 1) among the groups.

Treatment with AGEs-aptamer decreased urinary excretion levels of 8-OHdG in DM mice. We next examined whether AGEs-aptamer treatment could decrease urinary excretion levels of 8-OHdG, a marker of oxidative stress, in DM mice. Compared with Ctr-Ctr-aptamer mice, urinary 8-OHdG levels were significantly increased in DM-Ctr-aptamer mice to about 2.5-fold (p<0.05). Administration of AGEs-aptamer significantly reduced urinary excretion levels of 8-OHdG in DM mice (p<0.05) (Fig. 5P).

Treatment with AGEs-aptamer decreased inflammatory and fibrotic gene expression in the kidney of KKAy/Ta mice. AGEs induce a variety of inflammatory and fibrotic gene expression, thus being involved in diabetic nephropathy (16, 33, 34). So, we studied the effects of AGEs-aptamer on MCP-1, TNF-α, CTGF, type-IV collagen and RAGE gene expression in KKAy/Ta mice. As shown in Fig. 6A-D, renal MCP-1, TNF-α, CTGF and type-IV collagen gene expression was significantly
increased in DM-Ctr-aptamer mice, which were prevented by the administration of AGEs-aptamer. Moreover, although there were no significant differences in gene expression levels of RAGE and other AGEs scavenging receptor, AGE-R3 between Ctr-Ctr-aptamer and DM-Ctr-aptamer mice, treatment with AGEs-aptamer significantly reduced RAGE and AGE-R3 mRNA levels in the kidney of DM mice (Fig. 6E and 6F).

**AGEs-aptamer decreased ROS generation and inflammatory and fibrotic gene expression in AGEs-exposed human mesangial cells.** We next examined whether AGEs-aptamer could inhibit AGEs-elicited ROS generation and RAGE, MCP-1, and CTGF gene expression in human mesangial cells. One hundred µg/ml AGEs-BSA for 4 hr significantly increased ROS generation (Fig. 7A). RAGE gene expression was increased at 4 and 24 hr after the treatment with AGEs-BSA, whereas MCP-1 and CTGF mRNA levels were elevated at 24 hr only (Fig. 7B-D). AGEs-aptamer significantly prevented these harmful effects of AGEs although they did not have any toxic effects on BSA-treated cells. Furthermore, AGEs-BSA for 4 hr increased gene expression of TNF-α, but not RAGE, MCP-1 or CTGF in THP-1 cells, which was also blocked by AGEs-aptamer (data not shown).
DISCUSSION

In the present study, we demonstrated for the first time that although infusion of in vitro-selected DNA-aptamer raised against AGEs-HSA did not affect glucose, HbA1c or blood pressure levels, it not only inhibited glomerular hypertrophy and ECM protein accumulation, but also decreased urinary excretion levels of albumin and 8-OHdG in DM mice. In addition, administration of AGEs-aptamer significantly decreased plasma levels of blood urea nitrogen and creatinine, thus preventing renal dysfunction in DM mice. Moreover, treatment with AGEs-aptamer significantly suppressed MCP-1, TNF-α, CTGF, type-IV collagen and RAGE gene expression in the kidney of DM mice, whereas AGEs-aptamer completely blocked the AGEs-induced up-regulation of RAGE, MCP-1, and CTGF mRNA levels in mesangial cells. In this study, we found that levels of AGEs-aptamer were increased in the kidney of 8-week old Ctr mice for at least 7 days following the continuous intraperitoneal infusion. Further, at day 14 after stopping the injection, AGEs-aptamer was still detected in the kidney. These findings suggest that phosphorothioate AGEs-aptamer used in these experiments may be resistant to nuclease attack and quite stable, therefore suitable as a therapeutic agent. Given the fact that no toxicities related to AGEs-aptamer were observed following the intraperitoneal injection, our present observations indicate that
continuous infusion of AGEs-aptamer may be a safe and effective therapeutic strategy for preventing the development and progression of diabetic nephropathy. However, this will have to be affirmed before widespread clinical use of the AGEs-aptamer is entertained. To compare the efficacy of AGEs-aptamer with other means to reduce the load of AGEs would also be interesting because the latter is mostly delivered orally.

Aptamers have the following advantages over antibodies for blocking the function of targeted proteins; [1] production of aptamers does not rely on biological systems, and can be easily selected and constructed from oligonucleotide library with low-cost and time-saving *in vitro*; [2] aptamers are quite thermally stable and can be denatured and renatured multiple times without loss of activity and specificity; [3] aptamers do not have immunogenicity over antibodies; [4] small size allows more efficient entry into biological compartments (35, 36). There is accumulating evidence that AGEs play a role in various disorders such as Alzheimer’s disease, cancers and cardiovascular disease (37-39). Increased formation and accumulation of AGEs could link the increased risks for these disorders to diabetes (37-39). Since feasibility and efficacy of insulin pump therapy has been already established in diabetic patients (40) and that various clinical trials with AGEs inhibitors have been terminated because of the safety concern (41), continuous pump infusion of insulin plus AGEs-aptamer may be
promising for preventing various life-threatening AGEs-related disorders in diabetes.

In the present study, we demonstrated that continuous infusion of AGEs-aptamer for 8 weeks dramatically decreased AGEs levels in the glomeruli of DM mice to basal levels. Engagement of RAGE with AGEs stimulates ROS generation in a variety of cells, which could in turn promote the formation and accumulation of AGEs, thus forming a positive feedback loop between RAGE-downstream signaling and AGEs generation (16, 42). Indeed, aortic AGEs accumulation has been suppressed in RAGE-deficient diabetic apoE knockout mice (42). In the present study, AGEs-aptamer directly bound to AGEs and resultantly blocked the binding of AGEs to RAGE (Fig. 1B and 1C). These observations suggest that AGEs-aptamer could decrease the glomerular accumulation of AGEs via the blockade of AGEs binding to RAGE in the kidney. Further, we found here that turnover rate of aptamer-bound AGEs by THP-1 macrophages was increased compared with that of unbound AGEs (Fig. 1D). This is another possible mechanism by which AGEs-aptamer decreased the glomerular accumulation of AGEs. Since treatment with AGEs-aptamer did not affect serum AGEs levels in DM mice, AGEs-aptamer may be preferentially distributed and accumulated in the kidney and vessels, locally suppress the actions of AGEs, and finally eliminated from the body via the increased turnover by macrophages. Under non-diabetic normal
conditions, oxidative stress, RAGE expression and AGEs accumulation are largely suppressed. This is a possible reason why AGEs-aptamer did not reduce renal AGEs levels of Ctr mice.

AGEs-RAGE interaction elicits inflammatory and fibrotic reactions in the kidney cells via oxidative stress generation (13). In this study, we found that treatment with AGEs-aptamer decreased urinary 8-OHdG and albumin levels and reduced inflammatory and fibrotic gene expression (MCP-1, TNF-α, CTGF and type-IV collagen) in the kidney of DM mice. MCP-1 is a specific chemokine that recruits and activates monocytes from circulation to inflammatory sites (43). Increased MCP-1 expression associated with monocyte infiltration in mesangial areas has been observed in early phase of diabetic nephropathy (44). Plasma MCP-1 level was positively correlated with UAE in type 1 diabetic patients (44). Further, administration of AGEs has been shown to cause glomerular hypertrophy and ECM accumulation in the rat kidney via induction of CTGF and type-IV collagen expression (45). Since AGEs-aptamer completely blocked the AGEs-induced up-regulation of RAGE, MCP-1 and CTGF mRNA levels in human mesangial cells, the present findings suggest that blockade of the AGEs-RAGE axis by AGEs-aptamer could decrease albuminuria and fibrotic reactions in the diabetic kidney via suppression of ROS. It would be interesting
to examine the effects of AGEs-aptamer on nitrotyrosine levels, another marker of oxidative stress in the diabetic kidney. Further, although decreased synaptopodin levels were restored by AGEs-aptamer, to examine the effects of aptamer on podocyte apoptosis and injury using cell culture system may be more helpful.

If we assume that volume of distribution for the aptamer is extracellular fluid and that total body water is about 60% of body weight, the concentration of aptamer in mice is estimated to be $1.59 \times 10^9$ M. Therefore, the estimated delivered dose of aptamer seems to be 880 times less than the measured KD of AGEs-aptamer ($1.38 \times 10^6$ M) at a glance. We may not be able to reconcile the large discrepancy in the delivered dose of AGEs-aptamer and the KD. However, in this study, AGEs-aptamer was more accumulated in the kidney compared with the circulating blood; after 7 day-continuous injection, levels of AGEs-aptamer in the kidney were about 8-fold higher than those in the blood (Fig. 2A and 2B). Furthermore, AGEs-aptamer was continuously administrated for 8 weeks. So, although the cumulative effect of dosing may plateau or increase in a non-linear fashion after several weeks of dosing and that we did not measure the kidney concentration of the AGEs-aptamer after 8 weeks of treatment, it could be estimated that there is a 64-fold (8x8) preferential distribution of AGEs-aptamer in the glomeruli compared with the blood. In addition, AGEs-aptamer
dramatically suppressed the glomerular accumulation of AGEs; the levels in the kidney were decreased to about 1/20 of Ctr-aptamer-treated DM mice (Fig. 3F). These findings suggest that the delivered dose of AGEs-aptamer used here (0.136 µg/day) may not be necessarily insufficient for suppressing the actions of AGEs. We have found in the preliminary animal experiments that intravenous administration of about 70 times higher dose of AGEs-aptamer every other day up to two months causes severe diarrhea. This is a rationale why we chose the original relatively lower dose of AGEs-aptamer in the present experiments.

In the present study, AGEs-aptamer not only inhibited the inflammatory and fibrotic gene expression in both kidney and mesangial cells, but also prevented the development of histologic and physiologic parameters associated with experimental diabetic nephropathy. Further, Ctr-aptamer alone did not have any specific actions in either the kidney or mesangial cells. These observations suggest the biological relevance of changes in gene expression although the differences among the groups are modest. In this study, there were no significant differences in gene expression levels of RAGE and AGE-R3 between Ctr-Ctr-aptamer and DM-Ctr-aptamer mice. However, we performed RT-PCR analysis for RAGE and AGE-R3 gene using whole kidney. So, the gene expression in response to AGEs may be differently regulated in mesangial cells and
whole kidney, because the latter is mainly composed of tubular cells.

Moreover, it would be helpful to determine whether a long-term administration of AGEs-aptamer could stabilize the excretion of albuminuria and resultantly prevent the progression of diabetic nephropathy.

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REFERENCES

1. Krolewski AS, Warram JH, Valsania P, Martin BC, Laffel LM, Christlieb AR: Evolving natural history of coronary artery disease in diabetes mellitus. Am J Med 1991;90:56S-61S

2. Maisonneuve P, Agodoa L, Gellert R, Stewart JH, Buccianti G, Lowenfels AB, Wolfe RA, Jones E, Disney AP, Briggs D, McCredie M, Boyle P: Distribution of primary renal diseases leading to end-stage renal failure in the United States, Europe, and Australia/New Zealand: results from an international comparative study. Am J Kidney Dis 2000;35:157-165

3. Mauer SM, Lane P, Hattori M, Fioretto P, Steffes MW: Renal structure and function in insulin-dependent diabetes mellitus and type I membranoproliferative glomerulonephritis in humans. J Am Soc Nephrol 1992;2:S181-S184

4. Sharma K, Ziyadeh FN: Hyperglycemia and diabetic kidney disease. The case for transforming growth factor-beta as a key mediator. Diabetes 1995;44:1139-1146

5. Skyler JS, Bergenstal R, Bonow RO, Buse J, Deedwania P, Gale EA, Howard BV, Kirkman MS, Kosiborod M, Reaven P, Sherwin RS, Association AD, Foundation ACoC, Association AH: Intensive glycemic control and the prevention of cardiovascular events: implications of the ACCORD, ADVANCE, and VA Diabetes Trials: a position statement of the American Diabetes Association and a Scientific Statement of the American College of Cardiology Foundation and the American Heart Association. J Am Coll Cardiol 2009;53:298-304

6. Brownlee M, Cerami A, Vlassara H: Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. N Engl J Med 1988;318:1315-1321

7. Grandhee SK, Monnier VM: Mechanism of formation of the Maillard protein cross-link pentosidine. Glucose, fructose, and ascorbate as pentosidine precursors. J Biol Chem 1991;266:11649-11653

8. Dyer DG, Blackledge JA, Thorpe SR, Baynes JW: Formation of pentosidine during nonenzymatic browning of proteins by glucose. Identification of glucose and other carbohydrates as possible precursors of pentosidine in vivo. J Biol Chem 1991;266:11654-11660

9. Genuth S, Sun W, Cleary P, Sell DR, Dahms W, Malone J, Sivitz W, Monnier VM, Group DSCAS: Glycation and carboxymethyllysine levels in skin collagen predict the risk of future 10-year progression of diabetic retinopathy and nephropathy in the diabetes control and complications trial and epidemiology of diabetes interventions and complications participants with type 1 diabetes. Diabetes 2005;54:3103-3111
10. Thomas MC, Forbes JM, Cooper ME: Advanced glycation end products and diabetic nephropathy. Am J Ther 2005;12:562-572
11. Nożyński J, Zakliczyński M, Konecka-Mrowka D, Zielinska T, Zakliczynska H, Nikiel B, Mlynarczyk-Liszka J, Mrowka A, Zembala-Nozynska E, Pijet M, Rdzanowska K, Lange D, Przybylski R, Zembala M: Advanced glycation end product accumulation in the cardiomyocytes of heart failure patients with and without diabetes. Ann Transplant 2012;17:53-61
12. Beisswenger PJ, Drummond KS, Nelson RG, Howell SK, Szwegold BS, Mauer M: Susceptibility to diabetic nephropathy is related to dicarbonyl and oxidative stress. Diabetes 2005;54:3274-3281
13. Fukami K, Yamagishi S, Ueda S, Okuda S: Role of AGEs in diabetic nephropathy. Curr Pharm Des 2008;14:946-952
14. Coughlan MT, Thorburn DR, Penfold SA, Laskowski A, Harcourt BE, Sourris KC, Tan AL, Fukami K, Thallas-Bonke V, Nawroth PP, Brownlee M, Bierhaus A, Cooper ME, Forbes JM: RAGE-induced cytosolic ROS promote mitochondrial superoxide generation in diabetes. J Am Soc Nephrol 2009;20:742-752
15. Tsuchida K, Makita Z, Yamagishi S, Atsumi T, Miyoshi H, Obara S, Ishida M, Ishikawa S, Yasumura K, Koike T: Suppression of transforming growth factor beta and vascular endothelial growth factor in diabetic nephropathy in rats by a novel advanced glycation end product inhibitor, OPB-9195. Diabetologia 1999;42:579-588
16. Yamagishi S, Imaizumi T: Diabetic vascular complications: pathophysiology, biochemical basis and potential therapeutic strategy. Curr Pharm Des 2005;11:2279-2299
17. Yamamoto Y, Kato I, Doi T, Yonekura H, Ohashi S, Takeuchi M, Watanabe T, Yamagishi S, Sakurai S, Takasawa S, Okamoto H, Yamamoto H: Development and prevention of advanced diabetic nephropathy in RAGE-overexpressing mice. J Clin Invest 2001;108:261-268
18. Bock LC, Griffin LC, Latham JA, Vermaas EH, Toole JJ: Selection of single-stranded DNA molecules that bind and inhibit human thrombin. Nature 1992;355:564-566
19. Gragoudas ES, Adamis AP, Cunningham ET, Feinsod M, Guyer DR, Group VISiONCT: Pegaptanib for neovascular age-related macular degeneration. N Engl J Med 2004;351:2805-2816
20. Jilma-Stohlalwetz P, Gilbert JC, Gorczyca ME, Knöbl P, Jilma B: A dose ranging phase I/II trial of the von Willebrand factor inhibiting aptamer ARC1779 in patients with congenital thrombotic thrombocytopenic purpura. Thromb Haemost
21. Markus HS, McCollum C, Imray C, Goulder MA, Gilbert J, King A: The von Willebrand inhibitor ARC1779 reduces cerebral embolization after carotid endarterectomy: a randomized trial. Stroke 2011;42:2149-2153
22. Hagiwara S, Makita Y, Gu L, Tanimoto M, Zhang M, Nakamura S, Kaneko S, Itoh T, Gohda T, Horikoshi S, Tomino Y: Eicosapentaenoic acid ameliorates diabetic nephropathy of type 2 diabetic KKAy/Ta mice: involvement of MCP-1 suppression and decreased ERK1/2 and p38 phosphorylation. Nephrol Dial Transplant 2006;21:605-615
23. Takeuchi M, Makita Z, Bucala R, Suzuki T, Koike T, Kameda Y: Immunological evidence that non-carboxymethyllysine advanced glycation end-products are produced from short chain sugars and dicarbonyl compounds in vivo. Mol Med 2000;6:114-125
24. Higashimoto Y, Yamagishi S, Nakamura K, Matsu T, Takeuchi M, Noguchi M, Inoue H: In vitro selection of DNA aptamers that block toxic effects of AGE on cultured retinal pericytes. Microvasc Res 2007;74:65-69
25. King DJ, Ventura DA, Brasier AR, Gorenstein DG: Novel combinatorial selection of phosphorothioate oligonucleotide aptamers. Biochemistry 1998;37:16489-16493
26. Okahata Y, Niikura K, Sugiura Y, Sawada M, Morii T: Kinetic studies of sequence-specific binding of GCN4-bZIP peptides to DNA strands immobilized on a 27-MHz quartz-crystal microbalance. Biochemistry 1998;37:5666-5672
27. Yamagishi S, Inagaki Y, Amano S, Okamoto T, Takeuchi M, Makita Z: Pigment epithelium-derived factor protects cultured retinal pericytes from advanced glycation end product-induced injury through its antioxidative properties. Biochem Biophys Res Commun 2002;296:877-882
28. Suga T, Iso T, Shimizu T, Tanaka T, Yamagishi S, Takeuchi M, Imaizumi T, Kurabayashi M: Activation of receptor for advanced glycation end products induces osteogenic differentiation of vascular smooth muscle cells. J Atheroscler Thromb 2011;18:670-683
29. Inagaki Y, Yamagishi S, Amano S, Okamoto T, Koga K, Makita Z: Interferon-gamma-induced apoptosis and activation of THP-1 macrophages. Life Sci 2002;71:2499-2508
30. Ojima A, Ishibashi Y, Matsu T, Maeda S, Nishino Y, Takeuchi M, Fukami K, Yamagishi S: Glucagon-like peptide-1 receptor agonist inhibits asymmetric dimethylarginine generation in the kidney of streptozotocin-induced diabetic rats by blocking advanced glycation end product-induced protein arginine methyltransferase-1 expression. Am J Pathol 2013;182:132-141
31. Takamiya Y, Fukami K, Yamagishi SI, Kaida Y, Nakayama Y, Obara N, Iwatani R,
Ando R, Koike K, Matsui T, Nishino Y, Ueda S, Cooper ME, Okuda S: Experimental diabetic nephropathy is accelerated in matrix metalloproteinase-2 knockout mice. Nephrol Dial Transplant 2013;28:55-62
32. Matsui T, Nishino Y, Takeuchi M, Yamagishi S: Vildagliptin blocks vascular injury in thoracic aorta of diabetic rats by suppressing advanced glycation end product-receptor axis. Pharmacol Res 2011;63:383-388
33. Fukami K, Ueda S, Yamagishi S, Kato S, Inagaki Y, Takeuchi M, Motomiya Y, Bucala R, Iida S, Tamaki K, Imaizumi T, Cooper ME, Okuda S: AGEs activate mesangial TGF-beta-Smad signaling via an angiotensin II type I receptor interaction. Kidney Int 2004;66:2137-2147
34. Matsui T, Yamagishi S, Takeuchi M, Ueda S, Fukami K, Okuda S: Irbesartan inhibits advanced glycation end product (AGE)-induced proximal tubular cell injury in vitro by suppressing receptor for AGEs (RAGE) expression. Pharmacol Res 2010;61:34-39
35. Osborne SE, Matsumura I, Ellington AD: Aptamers as therapeutic and diagnostic reagents: problems and prospects. Curr Opin Chem Biol 1997;1:5-9
36. Famulok M, Hartig JS, Mayer G: Functional aptamers and aptazymes in biotechnology, diagnostics, and therapy. Chem Rev 2007;107:3715-3743
37. Takeuchi M, Kikuchi S, Sasaki N, Suzuki T, Watai T, Iwaki M, Bucala R, Yamagishi S: Involvement of advanced glycation end-products (AGEs) in Alzheimer's disease. Curr Alzheimer Res 2004;1:39-46
38. Abe R, Yamagishi S: AGE-RAGE system and carcinogenesis. Curr Pharm Des 2008;14:940-945
39. Yamagishi S, Nakamura K, Matsui T, Ueda S, Noda Y, Imaizumi T: Inhibitors of advanced glycation end products (AGEs): potential utility for the treatment of cardiovascular disease. Cardiovasc Ther 2008;26:50-58
40. Bergenstal RM, Tamborlane WV, Ahmann A, Buse JB, Dailey G, Davis SN, Joyce C, Peoples T, Perkins BA, Welsh JB, Willi SM, Wood MA, Group SS: Effectiveness of sensor-augmented insulin-pump therapy in type 1 diabetes. N Engl J Med 2010;363:311-320
41. Fukami K, Yamagishi S, Ueda S, Okuda S: Novel therapeutic targets for diabetic nephropathy. Endocr Metab Immune Disord Drug Targets 2007;7:83-92
42. Soro-Paavonen A, Watson AM, Li J, Paavonen K, Koitka A, Calkin AC, Barit D, Coughlan MT, Drew BG, Lancaster GI, Thomas M, Forbes JM, Nawroth PP, Bierhaus A, Cooper ME, Jandeleit-Dahm KA: Receptor for advanced glycation end products (RAGE) deficiency attenuates the development of atherosclerosis in diabetes. Diabetes
43. Wenzel UO, Abboud HE: Chemokines and renal disease. Am J Kidney Dis 1995;26:982-994
44. Banba N, Nakamura T, Matsumura M, Kuroda H, Hattori Y, Kasai K: Possible relationship of monocyte chemoattractant protein-1 with diabetic nephropathy. Kidney Int 2000;58:684-690
45. Zhou G, Li C, Cai L: Advanced glycation end-products induce connective tissue growth factor-mediated renal fibrosis predominantly through transforming growth factor beta-independent pathway. Am J Pathol 2004;165:2033-2043
FIGURE LEGENDS

Fig. 1
(A) Predicted secondary structure of AGEs-DNA aptamer. AGEs-aptamer was modified with phosphorothioate. S; phosphorothioate. C; cytosine, G; guanine, A; adenine, T; thymine. (B) Binding affinity of Ctr- or AGEs-aptamer to AGEs-HSA. Dashed line; Ctr-apptamer, solid line; AGEs-aptamer. n=4. (C) Binding affinity of AGEs-HSA to vRAGE. Solid line; AGEs-aptamer 0 nM, dashed line; AGEs-apptamer 20 nM, dotted line; AGEs-aptamer 100 nM. n=3. (D) and (E) One hundred µg/ml AGEs-BSA was added to the culture medium in the presence or absence of 2 µM AGEs-aptamer where differentiated THP-1 macrophages were grown (D) or ungrown (E). After 4 hr, AGEs levels in the supernatant were determined with western blots.

Fig. 2
(A) Biodistribution of [γ-32P]ATP-labeled AGEs-DNA aptamer. C57BL/6J mice received continuous intraperitoneal infusion of [γ-32P]ATP-labeled AGEs-aptamer for 7 days. Then blood, urine and several organs were obtained. [γ-32P]ATP-labeled AGEs-aptamer was detected by Cherenkov counting. Results were presented as mole per gram of tissue. n=3. (B) Time course kinetics of [γ-32P]ATP-labeled AGEs-aptamer (0.136 µg/day). C57BL/6J mice received continuous intraperitoneal infusion of [γ-32P]ATP-labeled AGEs-aptamer for 7 days. Then blood, liver and kidney were obtained. Results are presented as mole per gram of tissue. n=2.

Fig. 3
Serum and renal levels of AGEs in each animal. (A) Serum levels of AGEs were measured with ELISA. n=4-5 per group. Representative photographs of AGEs immunostaining in the kidney. (B) Ctr-Ctr-aptamer mice, (C) Ctr-AGEs-aptamer mice, (D) DM-Ctr-aptamer mice, (E) DM-AGEs-aptamer mice. (F) Quantitative data of glomerular staining for AGEs. n=4-5 per group. Magnification, x600.

Fig. 4
Effect of AGEs-aptamer on UAE levels (µg/g creatinine) in each animal. n=8-10 per group. ** p<0.01 vs Ctr-Ctr-aptamer mice. ## p<0.01 vs DM-Ctr-aptamer mice. Open triangle; Ctr-Ctr-aptamer, open square; Ctr-AGEs-aptamer, open circle; DM-Ctr-aptamer, closed circle; DM-AGEs-aptamer.
Fig. 5
Representative photographs of glomerular hypertrophy. Glomerular hypertrophy was evaluated by measuring glomerular area of cross section in the distal cortex. (A) Ctr-Ctr-aptamer mice, (B) Ctr-AGEs-aptamer mice, (C) DM-Ctr-aptamer mice, (D) DM-AGEs-aptamer mice. (E) Quantitative data of glomerular area. n=4-5 per group. Magnification, x600. Effect of AGEs-aptamer on glomerular ECM accumulation in each animal. Glomerular ECM accumulation was evaluated by the intensity of Masson’s trichrome staining in the glomeruli. Representative photographs of the kidney in (F) Ctr-Ctr-aptamer mice, (G) Ctr-AGEs-aptamer mice, (H) DM-Ctr-aptamer mice, (I) DM-AGEs-aptamer mice. (J) Quantitative data of ECM accumulation. n=3-5 per group. Synaptopodin levels in the glomeruli. Representative photographs of the kidney in (K) Ctr-Ctr-aptamer mice, (L) Ctr-AGEs-aptamer mice, (M) DM-Ctr-aptamer mice, (N) DM-AGEs-aptamer mice. (O) Quantitative data of synaptopodin expression. n=3-5 per group. Magnification, x600. (P) Effect of AGEs-aptamer on urinary 8-OHdG levels (ng/day) in each animal group. Urinary 8-OHdG levels were measured by ELISA. n=8-11 per group.

Fig. 6
Effect of AGEs-aptamer on cortical (A) MCP-1, (B) TNF-α, (C) CTGF, (D) type-IV collagen, (E) RAGE and (F) AGE-R3 gene expression in each animal group. Total RNAs were transcribed and amplified by real-time PCR. Data were normalized by the intensity of 18S rRNA-derived signals and then related to the value obtained with Ctr-Ctr-aptamer mice. n=10-16 per group.

Fig. 7
Effect of AGEs-atamer on (A) ROS generation and (B) RAGE, (C) MCP-1 and (D) CTGF gene expression in human mesangial cells. Mesangial cells were treated with 100 µg/ml AGEs-BSA or non-glycated BSA for 4 and 24 hr in the presence or absence of 0.2 or 2 µM AGEs-aptamer. Total RNAs were transcribed and amplified by real-time PCR. Data were normalized by the intensity of GAPDH mRNA-derived signals and then related to the value obtained with non-glycated BSA. n=3 per group.
Table 1. Characteristics of animals

| Group                        | Ctr-Ctr-aptamer | Ctr-AGEs-aptamer | DM-Ctr-aptamer | DM-AGEs-aptamer |
|------------------------------|-----------------|------------------|----------------|----------------|
| Number                       | 8               | 9                | 11             | 10             |
| Body weight (g)              | 24.9±1.5        | 25.1±1.1         | 45.5±1.2*      | 43.2±1.2*      |
| Systolic blood pressure (mmHg) | 99.7±3.9      | 107.0±2.7        | 104.5±2.5     | 102.6±2.5     |
| Plasma glucose (mg/dl)       | 177.0±9.9       | 185.4±9.8        | 297.9±42.4*   | 328.2±53.3*   |
| HbA1c (%)                    | 4.0±0.05        | 4.0±0.08         | 6.0±0.4*      | 6.3±0.3*      |
| Total cholesterol (mg/dl)    | 70.6±2.8        | 66.1±3.9         | 103.5±13.9*   | 88.4±5.3*     |
| Blood urea nitrogen (mg/dl)  | 34.3±2.1        | 31.4±2.4         | 33.3±1.6      | 28.0±1.2*     |
| Creatinine (mg/dl)           | 0.14±0.01       | 0.12±0.01        | 0.15±0.01     | 0.10±0.02*    |
| Aspartate aminotransferase (IU/L) | 158.4±60.6  | 149.8±39.9       | 273.0±105.7   | 99.5±15.4     |
| Alanine aminotransferase (IU/L) | 6.8±3.0       | 6.5±1.6          | 29.4±8.0      | 24.5±11.9     |
| Kidney/body weight ratio     | 13.6±0.7        | 13.7±0.4         | 20.3±2.5*     | 14.8±0.7*     |
| Urinary N-acetyl-β-D-glucosaminidase (U/day) | 0.22±0.06   | 0.10±0.01        | 0.34±0.06     | 0.26±0.02     |

Values are shown as mean ± SEM, * p<0.05 vs Ctr-Ctr-aptamer, # p<0.05 vs DM-Ctr-aptamer.
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