

DNA aptamer raised against advanced glycation end products (AGEs) prevents abnormalities in electroretinogram of experimental diabetic retinopathy

Satoshi Maeda^{a,b}, Takanori Matsui^b, Ayako Ojima^b, Mika Suematsu^{b,c}, Kuniyoshi Kaseda^c, Yuichiro Higashimoto^d, Ryoji Yamakawa^a, Sho-ichi Yamagishi^b

^aDepartment of Ophthalmology, ^bDepartment of Pathophysiology and Therapeutics of Diabetic Vascular Complications, ^dDepartment of Medical Biochemistry, Kurume University School of Medicine, Kurume, Japan

^cSaravio Central Institute, Saravio Cosmetics Ltd. Beppu 874-0840, Oita, Japan

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Address reprint requests to: Dr. Sho-ichi Yamagishi, Department of Pathophysiology and Therapeutics of Diabetic Vascular

Complications, Kurume University School of Medicine, 67
Asahi-machi, Kurume 830-0011, Japan; Telephone Number,
+81-942-31-7873; Fax Number, +81-942-31-7895; E-mail
address, shoichi@med.kurume-u.ac.jp

Abstract

Purpose: Abnormalities in electroretinogram (ERG), such as reduced amplitudes and delayed implicit times of a-, b-wave and oscillatory potentials (OPs), are one of the earliest features of diabetic retinopathy prior to obvious vascular changes in the diabetic retinas. We have previously shown that serum levels of advanced glycation end products (AGEs) are correlated with delayed latency of OPs in type 2 diabetic rats. However, the pathological role of AGEs in ERG abnormalities remains unclear. We examined here whether high-affinity DNA aptamer directed against AGEs (AGE-aptamer) prevents ERG abnormalities in experimental type 1 diabetic retinopathy.

Methods: Streptozotocin-induced diabetic rats or control rats received continuous intraperitoneal infusion of either AGE-aptamer or control-aptamer by an osmotic mini pump for 16 weeks. Anthropometric, metabolic and hemodynamic variables were measured, and ERG was performed.

Results: Although AGE-aptamer did not affect body weight, fasting and random blood glucose, HbA1c, blood pressure or lipid parameters, it completely prevented the increase in serum AGE levels as well as the reduction in a- and b-wave and OP amplitudes in diabetic rats.

Conclusion: The present study demonstrated for the first time that AGE-aptamer prevented abnormalities of ERG in experimental diabetic retinopathy probably by

blocking the harmful effects of AGEs.

Introduction

Sugars, such as glucose or glyceraldehyde can react non-enzymatically with the amino groups of proteins, lipids and nucleic acids to form reversible Schiff bases, and then Amadori products [1-3]. These early glycation products undergo further complex reactions such as rearrangement, dehydration and condensation to become irreversibly cross-linked, heterogeneous fluorescent derivatives called “advanced glycation end products (AGEs)” [1-3]. The formation and accumulation of AGEs in various tissues have been known to progress at an extremely accelerated rate under diabetes [1,2]. There is a growing body of evidence that AGEs and their receptor (RAGE) interaction elicits oxidative stress generation and evokes inflammation and thrombogenic reactions, thereby being involved in the development and progression of diabetic retinopathy, a leading cause of acquired blindness among the people of occupational age [4-8].

Accumulating evidence has suggested that retinal neurodegeneration is the earliest characteristic feature of diabetic retinopathy prior to obvious vascular changes in the diabetic retinas [9-12]. Since abnormalities in electroretinogram (ERG), such as reduced amplitudes and delayed implicit times of a-, b-wave and oscillatory potentials (OPs), have been shown to represent retinal neuronal cell damage, ERG is considered to be an useful tool for diagnosing early phase of diabetic retinopathy [9-12]. We have previously shown that peak latency of OPs is prolonged in obese type 2 diabetic rats

compared to control rats and that serum levels of AGEs are correlated with the delay in implicit times of OPs [10]. However, the pathological role of AGEs in ERG abnormalities in early diabetic retinopathy remains to be elucidated. Therefore, in this study, we examined whether high-affinity DNA aptamer directed against AGEs (AGE-aptamer) could block the effects of AGEs and resultantly prevent ERG abnormalities in experimental type 1 diabetic retinopathy.

Materials and Methods

Materials

Bovine serum albumin (BSA) (essentially fatty acid free and essentially globulin free, lyophilized powder) was purchased from Sigma (St. Louis, MO, USA). D-glyceraldehyde from Nakalai Tesque (Kyoto, Japan).

Preparation of AGEs

AGE-BSA was prepared as described previously [4]. In brief, BSA was incubated under sterile conditions with D-glyceraldehyde for 7 days. Then, unbounded sugars were removed by dialysis against phosphate-buffered saline.

Enzyme-linked immunosorbent assay (ELISA) for AGEs

Monoclonal antibody directed against AGEs was prepared according to the standard

method described previously [13]. Serum levels of AGEs were measured with ELISA as described previously [14].

Library and polymerase chain reactions (PCR)

A random combinatorial single-stranded DNA library with normal phosphate ester backbone oligonucleotides (80-mer) was synthesized (Greiner bio-one, Tokyo, Japan): 5'-AGCTCAGAATGGATCCAAAC-[N]₄₀-CATGAGAATTCGGCCGGATC-3' where N is a randomized nucleotide with equal proportion of A, G, C, and T. The library with phosphorothioate backbone substituted at A and T positions was then synthesized by PCR amplification of the template as described previously [15].

Selection of AGEs-thioaptamers

Selection of DNA aptamers raised against AGEs was performed as described previously [15]. Sequences of AGE-aptamer and control DNA aptamer (control-aptamer) were as follows.

AGE-aptamer; 5'-
TGTAGCCCGAGTATCATTCTCCATCGCCCCCAGATACAAG-3', control-aptamer;
5'-GTTATCTGTCATAGGAACAGTCAGACTCAGCGTCGCAGTTCAGGGCACTTT
AGCAC-3'.

Animal experiments

Six week-old male Wistar rats received single 60 mg/kg intraperitoneal injection of streptozotocin (STZ) (Sigma) in 10 mM citrate buffer (pH 4.5). Non-diabetic control rats received citrate buffer alone. Animals with blood glucose levels greater than 200 mg/dl 48 hours later were considered diabetic. Non-diabetic control or STZ-induced diabetic rats received continuous intraperitoneal infusion (6 ng/body weight/day; body weight at 6 weeks old and 22 weeks old were about 200g and 340g, respectively) of either AGE-aptamer or control-aptamer by an osmotic mini pump (Alzet osmotic pumps, model 1004, Cupertino, CA, USA) for 16 weeks as described previously [16]. 2 U insulin (2 times/week) (Humalin N, Eli Lilly, Indianapolis, IN, USA) was subcutaneously injected to diabetic rats to maintain life, while it was not enough to consistently reduce blood glucose levels. At baseline and 16 weeks after treatment, animals were housed in metabolic cages to collect urine for measurement of urinary excretion levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), and then body weight, heart rates, systolic and diastolic blood pressure, fasting and random blood glucose, glycated hemoglobin (HbA1c) and blood biochemistry were measured. Blood pressure was monitored by a tail-cuff sphygmomanometer (BP-98A; Softron, Tokyo, Japan). Blood biochemistry was determined as described previously [17]. Body weight, fasting and random blood glucose was measured every other week. Urinary excretion levels of 8-OHdG were determined with a commercially available ELISA kit (Japan Institute for the Control of Aging NIKKEN SEIL Co., Ltd., Shizuoka, Japan). 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 ethical committee of Kurume University School of Medicine.

ERG

ERG was monitored as described previously [18]. In brief, rats were anesthetized with an intramuscular injection of ketamine hydrochloride (50 mg/kg) and xylazine (10mg/kg), and then the pupils were maximally dilated with a solution of 0.5% tropicamide and 0.5% phenylephrine hydrochloride (Mydrin-P, Santen, Japan). After inducing mydriasis, the animals were adapted to the dark for more than 30 min in a dark room. A built-in LED contact lens electrode (Mayo, Aichi, Japan) was placed on the right eye, and stimulus was controlled by a white LED stimulator (Mayo). The retinal signals were amplified with a bandpass 1-1000 Hz for scotopic responses.

Statistical analysis

All values were presented as mean \pm standard error. One-way ANOVA followed by the Tukey's test was performed for statistical comparisons; $p < 0.05$ was considered significant.

Results

During the 16-week experimental periods, body weight was significantly lower, and fasting and random blood glucose levels were higher in STZ-induced diabetic rats received control-aptamer (STZ-rats) compared with non-diabetic rats received control-aptamer (Control rats) (Fig. 1). Moreover, as shown in Table 1, HbA1c and urinary excretion levels of 8-OHdG were significantly higher, while serum creatinine was lower in 22-week old STZ-rats than those of Control rats at the same age. Although AGE-aptamer treatment for 16 weeks did not affect body weight, fasting and random blood glucose levels, HbA1c, blood pressures, lipid parameters or urinary 8-OHdG in STZ-induced diabetic rats at 22-week old (Table 1), it completely prevented the elevation in serum AGE levels in diabetic rats (Fig. 2).

Further, as shown in Fig. 3, compared with Control rats, a- and b-wave amplitudes evoked by flash intensity at equal or more than 1 candle (cd)/sec/m² as well as OP₂₋₄ amplitudes were significantly reduced in STZ-rats, all of which were completely prevented by the treatment of AGE-aptamer. There were no significant differences of OP, a-wave or b-wave implicit times among the groups (data not shown).

Discussion

Numerous animal and human studies have shown that electrophysiologic changes in ERG, such as reduction in a- and b-wave amplitudes and prolongation of peak latency

of OPs are the earliest characteristic features of diabetic retinopathy, which could be observed before the appearance of ophthalmoscopically obvious lesions [9-12]. Furthermore, there is accumulating evidence that neuronal cell damage detected by ERG may participate in microvascular abnormalities in diabetic retinopathy [19-21]. Therefore, ERG abnormalities are thought to be a novel biomarker for the progression of diabetic retinopathy.

We, along with others, have previously reported that serum levels of AGEs are correlated with delay in peak latencies of OPs [10] and that blockade of the AGE-RAGE axis ameliorates abnormalities in OP implicit times in type 2 diabetic animals [22]. These observations may suggest the pathological involvement of AGEs in retinal neuronal cell injury in early phase of diabetic retinopathy. However, it remains unclear whether inhibition of AGE-RAGE axis could prevent ERG abnormalities in experimental type 1 diabetic retinopathy.

To address this issue, we used an AGE-aptamer as a tool to block the AGE-RAGE axis because the AGE-aptamer used here has been shown to bind in a specific manner to AGEs and subsequently prevent the interaction of AGEs with RAGE in cell culture models [15]. In this study, we found for the first time that although AGE-aptamer treatment for 16 weeks did not affect anthropometric, metabolic or hemodynamic parameters, it completely prevented the increase in serum AGE levels as well as the reduction in a-, b-wave and OP₂₋₄ amplitudes in ERG of STZ-induced diabetic rats at

22-week old. Therefore, our present findings support the pathological role of AGEs in ERG abnormalities in diabetic retinas, thus suggesting that blockade of the AGE-RAGE axis by AGE-aptamer may be a novel therapeutic strategy for preventing neuronal cell damages in early phase of diabetic retinopathy. We have previously shown that degradation of aptamer-bound AGEs by macrophages is accelerated compared with that of unbound AGEs [16]. This is one possible mechanism by which AGE-aptamer decreased serum levels of AGEs in our model.

Limitations

We had several limitations in the present study. First, AGE-aptamer did not reduce urinary excretion levels of 8-OHdG, a well-established marker of oxidative stress in our diabetic model. Effects of AGE-aptamer on retinal 8-OHdG levels may have to be examined. Second, we found that AGE-aptamer prevented the reduction in a-, b-wave and OP amplitudes in type 1 diabetic rats, but it remains unclear whether AGE-aptamer could actually prevent neurodegeneration and subsequently inhibit microvascular lesions in diabetic retinas. It would be interesting to investigate the effects of AGE-aptamer on apoptosis and reactive gliosis in the diabetic retinas, two hallmarks of retinal neurodegeneration in diabetic retinopathy. Third, although no toxicities related to AGE-aptamer were observed following the intraperitoneal injection here, this will have to be affirmed before widespread clinical use of the AGE-aptamer is entertained. Finally,

since experimental animal model does not completely mimic human diabetes, further longitudinal study is needed to clarify whether AGE-aptamer might have clinical relevance in the prevention of early phase of human diabetic retinopathy.

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Figure Legends

Fig. 1. Body weight (A), fasting (B) and random (C) blood glucose levels in Control rats (N=6), STZ-rats (N=7) and STZ-induced diabetic rats received AGE-aptamer (STZ+AGE-aptamer rats) (N=5). **p<0.01 compared with Control rats.

Fig. 2. Effects of AGE-aptamer or control-aptamer on serum levels of AGEs in Control rats (N=6), STZ-rats (N=6) and STZ+AGE-aptamer rats (N=5). Serum AGE levels were measured by ELISA. **p<0.01 compared with STZ-rats.

Fig. 3. Effects of AGE-aptamer or control-aptamer on representative ERG (A), a- and b-wave (B and C) and OP amplitudes (D) in Control rats (N=6), STZ-rats (N=6) and STZ+AGE-aptamer rats (N=6). *p<0.05, **p<0.01 compared with Control rats. #p<0.05, ##p<0.01 compared with STZ-rats.