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Brief Communication

Blockade by phosphorothioate aptamers of advanced glycation end products-induced damage in cultured pericytes and endothelial cells

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ABSTRACT

Advanced glycation end products (AGEs) not only inhibit DNA synthesis of retinal pericytes, but also elicit vascular hyperpermeability, pathological angiogenesis, and thrombogenic reactions by inducing vascular endothelial growth factor (VEGF) and plasminogen activator inhibitor-1 (PAI-1) through the interaction with the receptor for AGEs (RAGE), thereby being involved in the pathogenesis of diabetic retinopathy. In this study, we screened novel phosphorothioate-modified aptamers directed against AGEs (AGEs–thioaptamers) using a combinatorial chemistry in vitro, and examined whether these aptamers could inhibit the AGE-induced damage in both retinal pericytes and human umbilical vein endothelial cells (HUVECs). We identified 11 AGEs–thioaptamers; among them, clones #4, #7s and #9s aptamers had higher binding affinity to AGEs–human serum albumin (HSA) than the others. Surface plasmon resonance analysis revealed that K_D values of #4s, #7s and #9s were 0.63, 0.36, and 0.57 nM, respectively. Furthermore, these 3 clones dose-dependently restored the decrease in DNA synthesis in AGE-exposed pericytes. AGEs significantly increased RAGE, VEGF and PAI-1 mRNA levels in HUVEC, all of which were completely blocked by the treatment with 20 nM clone #4s aptamer. Quartz crystal microbalance analysis confirmed that #4s aptamer dose-dependently inhibited the binding of AGEs–HSA to RAGE. Our present study demonstrated that AGEs–thioaptamers could inhibit the harmful effects of AGEs in pericytes and HUVEC by suppressing the binding of AGEs to RAGE. Blockade by AGEs–thioaptamers of the AGEs–RAGE axis might be a novel therapeutic strategy for diabetic retinopathy.

Introduction

Diabetic retinopathy is one of the most miserable complications in diabetes and is a leading cause of acquired blindness among the people of occupational age (L'Esperance et al., 1990). Development of diabetic retinopathy is characterized by loss of pericytes, increased vascular permeability and acellular capillaries, followed by microvascular thrombus formation in the retinas (Orlidge and D'Amore, 1987; Pfister et al., 2010; Yamagishi and Imaizumi, 2005a). Diabetic retinopathy ultimately progresses to proliferative changes associated with neovascularization (Yamagishi et al., 1993). Non-enzymatic modification of proteins by reducing sugars, a process also known as the Maillard reaction, has progressed at an extremely accelerated rate under diabetes, leading to the formation of advanced glycation end products (AGEs) (Yamagishi and Imaizumi, 2005b). There is accumulating evidence that AGEs are implicated in the development and progression of many pathological sequelae of diabetes- and age-associated disorders (Barile and Schmidt, 2007; Rahbar and Figarola, 2003; Sourris and Forbes, 2009; Sun et al., 2011; Yamagishi and Imaizumi, 2005b; Zong et al., 2011), including diabetic retinopathy. Indeed, we have previously shown that AGEs not only inhibit DNA synthesis, but also induce apoptotic cell death in cultured retinal pericytes, the earliest histopathological hallmark of diabetic retinopathy (Yamagishi and Imaizumi, 2005a). Moreover, we have found that AGEs evoke vascular hyperpermeability and

Abbreviations: AGEs, advanced glycation end products; EC, endothelial cells; VEGF, vascular endothelial growth factor; PAI-1, plasminogen activator inhibitor-1; RAGE, receptor for AGEs; SELEX, systematic evolution of ligands by exponential enrichment; AGEs–thioaptamers, phosphorothioate aptamers directed against AGEs; HUVECs, human umbilical vein endothelial cells; HSA, human serum albumin; BSA, bovine serum albumin; ssDNA, single-stranded DNA; PCR, polymerase chain reactions; dATP(αS), 2′-deoxyadenosine-5′-O-(1-thiotriphosphate); dTTP(αS), 2′-deoxythymidine-5′-O-(1-thiotriphosphate); dsDNA, double-stranded DNA; ELISA, enzyme-linked immunosorbent assay; SPR, surface plasmon resonance; K_D, dissociation constant; FBS, fetal bovine serum; RT-PCR, real-time reverse transcription PCR; vRAGE, v-domain of RAGE; QCM, quartz crystal microbalance.

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thrombogenic reactions in endothelial cells (ECs) by inducing vascular endothelial growth factor (VEGF) and plasminogen activator inhibitor-1 (PAI-1) through the interaction with the receptor for AGEs (RAGE), thereby being involved in diabetic retinopathy (Matsui et al., 2010; Ojima et al., 2012, Yamagishi et al., 1998, 2006, 2008). These observations suggest that blockade of AGEs–RAGE axis in retinal vasculatures may be a novel therapeutic target for diabetic retinopathy.

In 1990s, an in vitro-selection process called systematic evolution of ligands by exponential enrichment (SELEX) was developed to screen single stranded nucleic acid molecules that bind specific ligands from random pool of library (Ellington and Szostak, 1990; Tuerk and Gold, 1990). These classes of single stranded molecules are referred as "aptamers". Aptamers have possessed a high binding affinity and specificity to target proteins, whose property is more than or equal to that of monoclonal antibodies. In addition, its small size, non-immunogenicity and ease of modification compared to conventional monoclonal antibodies make aptamers a more attractive tool for therapeutic application (Jayasena, 1999). One major problem that arises in the therapeutic application of aptamers is their instability; they are susceptible to enzymatic nuclease attack in the cellular and serum fluids (Agrawal et al., 1995). To circumvent this, aptamers are further chemically modified through addition of amino, fluoro, or O-methyl in the 2′-position of ribose sugar and/or conjugation to polyethylene glycol or cholesterol (Wang et al., 2011; Yang et al., 2011). However, these post-SELEX modification processes are time consuming and sometimes might lead to decrease the binding affinity of aptamers (Wang et al., 2011; Yang et al., 2011). Therefore, it is better to screen aptamers directly from modified oligonucleotide-containing library. Indeed, phosphorothioate aptamers have the advantages of their enhanced affinity, specificity, and higher stability due to the sulfur backbone modifications (Yang et al., 2006). In this study, we screened novel phosphorothioate aptamers directed against AGEs (AGEs–thioaptamers) using a combinatorial approach involving the construction and screening of a phosphorothioate DNA library, and investigated if AGEs–thioaptamers could actually block the binding of AGEs to RAGE. We further examined the effects of AGEs–thioaptamers on DNA synthesis in AGE-exposed bovine retinal pericytes and studied whether they also could inhibit the AGE-induced RAGE, VEGF and PAI-1 gene expression in human umbilical vein EC (HUVEC).

Materials and methods

Materials

D-glyceraldehyde was purchased from Nakalai Tesque (Kyoto, Japan), and [^{3}H]thymidine from GE Healthcare (Buckinghamshire, UK). Other chemicals were purchased from Sigma (St Louis, MO, USA).

Preparation of AGE-modified proteins

AGE-modified proteins were prepared as described previously (Yamagishi et al., 2002a). In brief, human serum albumin (HSA) or bovine serum albumin (BSA) was incubated under sterile conditions with D-glyceraldehyde for 7 days. Then, unincorporated sugars were then removed by dialysis against phosphate-buffered saline. Control non-glycated HSA or BSA was incubated under the same conditions except for the absence of reducing sugars.

Immobilizing AGEs–HSA on agarose beads

AGEs–HSA was covalently coupled via sulfhydryl groups to iodoacetyl groups on SulfoLink Coupling Gel (Pierce, Rockford, IL, USA) as described previously (Higashimoto et al., 2007).

Library and polymerase chain reactions (PCR)

A random combinatorial single-stranded DNA (ssDNA) library with normal phosphate ester backbone oligonucleotides (80-mer) was synthesized (Greiner Bio-One, Tokyo, Japan): 5′-AGCTCAGAATGGATCC AAAC-[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 using *Ex Taq* polymerase (Takara Bio, Otsu, Japan) and a mixture of 2′-deoxyadenosine-5′-O-(1-thiotriphosphate) (dATP(αS)), 2′-deoxythymidine-5′-O-(1-thiotriphosphate) dTTP(αS) (BIOLOG Life Institute, Bremen, Germany), dGTP and dCTP. The PCR condition for amplification of the starting random library (4^{40} sequences) includes 200 μM each of dATP (αS), dTTP(αS), dGTP, and dCTP, 4 mM $MgCl_2$, 740 nM 80-mer random template, 50 units of *Ex Taq* polymerase, and 2.4 μM each primer in a total volume of 0.1 ml. PCR was performed to amplify with 5′ primer (5′-AGCTCAGAATGGATCCAAAC-3′) and biotin-conjugated 3′ primer (biotin-5′-GATCCGGCCGAATTCTCATG-3′) (Greiner Bio-One) under the following conditions: 95 °C for 2 min; 30 cycles at 95 °C for 1 min, 52 °C for 30 s, and 72 °C for 15 s.

Selection of AGEs–thioaptamers

The 80-nucleotide PCR product was applied to 20 μl of a Streptavidin Mag Sepharose (GE Healthcare) bead matrix suspended in binding/washing buffer (2 M NaCl, 1 mM ethylenediaminetetraacetic acid, 10 mM Tris–HCl, pH 7.5). After equilibration of binding of the biotinylated double-stranded DNA (dsDNA) to streptavidin beads, unbound dsDNA was removed with binding/washing buffer, and matrix-bound dsDNA was denatured. Then ssDNA was mixed with AGEs–HSA beads, and bound ssDNA was isolated as described previously (Higashimoto et al., 2007). To remove the ssDNA that could bind to non-glycated HSA, isolated ssDNA was passed through non-glycated HSA-immobilized agarose beads. The recovered ssDNA was amplified by PCR and used as the input DNA for the next selection. The sequences of the ssDNA cloned after repeating the SELEX procedure fifteen times were determined as described previously (Higashimoto et al., 2007).

Enzyme-linked immunosorbent assay (ELISA)

Each well was coated with polyclonal antibodies raised against AGEs overnight. The wells were incubated with 10 μg/ml AGEs–HSA for 30 min, and then with 5′-biotin-labeled 250 nM AGEs–thioaptamers (Tanaka et al., 2009). After 30 min, horseradish peroxidase-streptavidin was added, and absorbance at 450 nm was measured.

Surface plasmon resonance (SPR)

AGEs–HSA and non-glycated HSA were immobilized via the amino groups to CM5 sensor chip (GE Healthcare) with the aid of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and *N*-hydroxysuccinimide, respectively. The association and dissociation phases were monitored in a BIAcore 1000 (GE Healthcare). Chemically synthesized AGEs–thioaptamers were injected into the flow cell at concentrations of 5 and 10 nM at a flow rate of 10 μl/min. The sensor chip was regenerated with pulses of 20 mM Tris–HCl buffer (pH 8.0) containing 6 M urea to the baseline level, followed by an extensive washing with the running buffer. Control experiments were performed with ligand-free channel on the same sensor chip. From the assay curves obtained, the control signals, reflecting the bulk effect of buffer, were subtracted using BIA-evaluation 4.1 software (GE Healthcare). Equilibrium dissociation constant (K_D) was determined using the equation for 1:1 Langmuir binding.