Neurobiology of Aging 34 (2013) 1902–1912

Contents lists available at SciVerse ScienceDirect

Neurobiology of Aging

journal homepage: www.elsevier.com/locate/neuaging

Advanced glycation end-products disrupt the blood–brain barrier by stimulating the release of transforming growth factor–β by pericytes and vascular endothelial growth factor and matrix metalloproteinase–2 by endothelial cells in vitro

Fumitaka Shimizu, Yasuteru Sano, Osamu Tominaga, Toshihiko Maeda, Masa-aki Abe, Takashi Kanda*

Department of Neurology and Clinical Neuroscience, Yamaguchi University Graduate School of Medicine, Ube, Japan

ARTICLE INFO

Article history:
Received 18 September 2012
Received in revised form 14 January 2013
Accepted 20 January 2013
Available online 19 February 2013

Keywords:
Diabetic encephalopathy
Advanced glycation end-products
Basement membrane hypertrophy
Blood-brain barrier

ABSTRACT

Diabetic encephalopathy is now accepted as an important complication of diabetes. The breakdown of the blood–brain barrier (BBB) is associated with dementia in patients with type 2 diabetes mellitus (T2DM). The purpose of this study was to identify the possible mechanisms responsible for the disruption of the BBB after exposure to advanced glycation end-products (AGEs). We investigated the effect of AGEs on the basement membrane and the barrier property of the BBB by Western blot analysis, using our newly established lines of human brain microvascular endothelial cell (BMEC), pericytes, and astrocytes. AGEs reduced the expression of claudin-5 in BMECs by increasing the autocrine signaling through vascular endothelial growth factor (VEGF) and matrix metalloproteinase–2 (MMP-2) secreted by the BMECs themselves. Furthermore, AGEs increased the amount of fibronectin in the pericytes through a similar up-regulation of the autocrine transforming growth factor (TGF)–β released by pericytes. These results indicated that AGEs induce basement membrane hypertrophy of the BBB by increasing the degree of autocrine TGF-β signaling by pericytes, and thereby disrupt the BBB through the up-regulation of VEGF and MMP-2 in BMECs under diabetic conditions.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a common metabolic disorder among elderly individuals. Recently, central nervous system complications, which comprise what are called diabetic encephalopathy, have been increasingly recognized (Sima, 2010; van den Berg et al., 2010). Several studies have shown an association between T2DM and a mild-to-moderate cognitive impairment and an increased risk of dementia (Arvanitakis et al., 2004; Ott et al., 1999). Population-based studies show a lower score on cognitive screening tests, such as the Mini-Mental State Examination (MMSE) in T2DM patients than in nondiabetic patients matched for age, sex, and education (Hiltunen et al., 2001; Nguyen et al., 2002). Longitudinal studies showed an accelerated decline in the cognitive function over time in diabetic patients (van den Berg et al., 2010; Hassing et al., 2004). In addition, T2DM is also associated with abnormalities on brain magnetic resonance imaging (MRI). Lacunar infarcts are commonly found in patients with T2DM, and microbleeds may also be more prevalent in these individuals (Gouw et al., 2008). A cross-sectional study using brain MRI showed T2DM to be associated with a moderate degree of cerebral atrophy and a higher volume of white matter hyperintensity (WMH) (de Bresser et al., 2010). However, the molecular mechanisms underlying the observed cognitive impairment associated with T2DM has not yet been adequately explained.

As in other so-called microvascular complications, including retinopathy and nephropathy, the microvascular damage to the brain that occurs under diabetic conditions is considered to be associated with cognitive decline and dementia in patients with T2DM (Biessels et al., 2008; Hawkins et al., 2007; Kamada et al., 2007; Starr et al., 2003). For diabetic neuropathy, microangiopathy of the endoneurium is thought to be a contributing factor in the development of these diseases. The blood–nerve barrier (BNB) is localized in the microvessels of the endoneurium, and the BNB consists of peripheral nerve microvascular endothelial cells (PnMECs), pericytes of endoneurial microvascular origin, and the basement membrane (Abbott et al., 2006; Poduslo et al., 1994). The hypertrophy of the basement membrane and the breakdown of the BNB are both characteristic features of diabetic neuropathy (Giannini and Dyck, 1995; Shimizu et al., 2011a,b; Vinik and Mehrabyan, 2004). Similar alterations could take place at the blood–brain barrier (BBB) under diabetic conditions, because a loss of the integrity of the BBB has already been reported during diabetic stages with changes in the CNS blood capillary density and

* Corresponding author at: Department of Neurology and Clinical Neuroscience, Yamaguchi University Graduate School of Medicine, 1-1-1, Minamikogushi, Ube, Yamaguchi 7558505, Japan. Tel.: +81 8 3622 2714; fax: +81 8 3622 2364.
E-mail address: tkanda@yamaguchi-u.ac.jp (T. Kanda).

0197-4580/$ – see front matter © 2013 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.neurobiolaging.2013.01.012

basement membrane thickening (Bouchard et al., 2002; Johnson et al., 1982; Junker et al., 1985; Mukai et al., 1980). Although advanced glycation end-products (AGEs) are the late products of nonenzymatic glycation, and because their accumulation of proteins in the microvasculature appears to be a key factor in the development of diabetic neuropathy (Goldin et al., 2006), the molecular mechanism by which AGEs induce hypertrophy of the basement membrane and breakdown of the BBB during diabetic condition remains unclear.

The purpose of the present study is to identify the possible mechanisms responsible for increasing the basement membrane thickness and for impairing the barrier function of the BBB after exposure to AGEs, using our newly established human immortalized brain microvascular endothelial cells (BMECs), pericytes, and astrocytes.

2. Methods

2.1. Reagents

The culture medium for the cells consisted of Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) containing 100 U/mL penicillin (Sigma), 100 μg/mL streptomycin (Sigma), 25 ng/mL amphotericin B (Invitrogen, Grand Island, NY), and 10% fetal bovine serum (FBS) (Sigma). Polyclonal anti−tissue inhibitor of metalloproteinase (TIMP)−1 (sc-5538), anti-fibronectin (sc-6953), anti−matrix metalloproteinases (MMP)-2 (sc-10736), anti-MMP-9 (sc-6840), and anti−receptor of AGEs (RAGE) (sc-5563) antibodies were obtained from Santa Cruz (Santa Cruz, CA). Polyclonal anti-claudin-5 (34−1600) and ant-occludin (71−1500) antibodies were purchased from Zymed (San Francisco, CA). Polyclonal anti-collagen type IV was obtained from American Research Products (03-10760, Belmont, CA). AGEs-BSA was purchased from Calbiochem (121800; Darmstadt, Germany). Polyclonal anti−transforming growth factor (TGF)−β1 (MAB240) and anti−vascular endothelial growth factor (VEGF) (MAB293) antibodies were purchased from R&D Systems (Minneapolis, MN). Recombinant human TGF-β1 (100-21) and VEGF (100-20) were purchased from Peprotech EC (London, UK). The broad-spectrum MMPs inhibitor GM6001 was purchased from Chemicom (CC1100, Temecula, CA). Human astrocytes were purchased from Lonza (Walkersville, MD).

2.2. Cell culture

The BMECs and human brain pericytes were generated as described previously (Sano et al., 2010; Shimizu et al., 2011a,b). The cell lines were isolated from human brain tissue, which harbored a temperature-sensitive SV40 large T-antigen (tsA58) protein. Human primary astrocytes were also immortalized via transfection by retroviral vectors harboring the tsA58 gene. All analyses were performed 3 days after the temperature shift from 33 °C to 37 °C. The cultures were maintained at 37 °C in 5% CO_2/air, and the DMEM medium was replaced every 3 days.

2.3. Western blot analysis

Protein samples (10−20 μg) were separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad), and then were transferred to the nitrocellulose membrane (Amersham, Chalfont, UK). The membranes were treated with blocking buffer (5% skim milk in 25 mmol/L Tris-HCl pH 7.6,125 nmol/L NaCl, 0.5% Tween 20) for 1 hour at room temperature and then were incubated with relevant primary antibodies (dilution 1:100) for 2 hours at room temperature. Thereafter, the membrane was incubated with a secondary antibody, and was visualized by enhanced chemiluminescence detection (ECL-prime, Amersham, UK). A densitometric analysis was performed using the Quantity One software program (Bio-Rad, Hercules, CA).

2.4. Analysis of the effect of TGF-β1 or VEGF on the expression of basement membrane-related genes

Brain pericytes were either left untreated or were treated with human TGF-β1 (10 ng/mL) or VEGF (10 ng/mL). The total protein was collected 2 days later.

2.5. Treatment of human BMECs and brain pericytes with AGEs-BSA

BMECs and brain pericytes were cultured in DMEM with unmodified BSA (100 μg/mL) and AGEs-modified BSA (100 μg/mL, 200 μg/mL, 400 μg/mL). AGEs-BSA contained <0.2 ng/mL of endotoxin. The cells were cultured for 2 days before the collection of total protein.

2.6. VEGF or TGF-β1 inhibitory study

Brain pericytes were cultured with AGEs-BSA containing 2.0 μg/mL antibody against VEGF or TGF-β1, or normal rabbit IgG. BMECs were also cultured with AGEs containing 2.0 μg/mL antibody against VEGF or normal rabbit IgG. The total protein was obtained 2 days later.

2.7. Treatment of BMECs with conditioned medium from pericytes after AGEs exposure

The brain pericytes were cultured in DMEM with AGEs-modified BSA (200 μg/mL). After 12 hours, the conditioned medium of the treated brain pericytes (PCTCM AGEs) was collected and stored at −20 °C until the analysis. BMECs were cultured with PCTCM AGEs containing 2.0 μg/mL antibody against TGF-β1 or normal rabbit IgG. The total protein was obtained 1 day after the addition of the antibody/IgG.

2.8. Treatment with a MMP inhibitor

A broad-spectrum MMPs inhibitor, GM6001 (Chemicom, Temecula, CA) was prepared for the inhibition study. BMECs were cultured with AGEs containing 25 μmol/L of GM6001. The TEER values were determined and a permeability study was carried out 24 hours later, and the total proteins were obtained the next day.

2.9. siRNA transfection

For siRNA transfection, 1×10^6 cells were seeded on 60-mm collagen-coated dishes. After 24 hours, cells were transfected with scramble or RAGE-specific siRNA (sc-36374; Santa Cruz) according to the manufacturer's instructions. AGEs treatment was performed 36 hours after transfection.

2.10. Transendothelial electrical resistance study

For the transendothelial electrical resistance (TEER) study, transwell inserts (pore size, 0.4 μm, effective growth area 0.3 cm^2; BD Bioscience, NJ) were coated by rat-tail collagen type 1 (BD Bioscience). The TEER values of cell layers were measured with a Millicell electrical resistance apparatus (Endohm-6 and EVOM; World Precision Instruments, Sarasota, FL). The BMECs were seeded (1×10^6 cells/insert) on the upper compartment, and cultured for 24 hours. To estimate the effect of AGEs against BMEC, the upper