BRAIN RESEARCH 1534 (2013) 33–45

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Brain Research

Research Report

Neurite regeneration in adult rat retinas exposed to advanced glycation end-products and regenerative effects of neurotrophin-4

Guzel Bikbova, Toshiyuki Oshitari, Shuichi Yamamoto*

Department of Ophthalmology and Visual Science, Chiba University Graduate School of Medicine, Inohana 1-8-1, Chuo-ku, Chiba 260-8670, Chiba, Japan

ARTICLE INFO

Article history:
Accepted 15 August 2013
Available online 21 August 2013

Keywords:
AGE
Apoptosis
Regeneration
NT-4
Caspase-9
AIF

ABSTRACT

The purpose of this study was to determine the effect of low concentrations of advanced glycation end-products on neurite regeneration in isolated rat retinas, and to determine the effects of neurotrophin-4 on regeneration in advanced glycation end-products exposed retinas. Retinal explants of 4 adult Sprague-Dawley rats were cultured on collagen gel and were incubated in; (1) serum-free control culture media, (2) glucose-advanced glycation end-products–bovine serum albumin media, (3) glycolaldehyde-advanced glycation end-products–bovine serum albumin media, (4) glyceraldehyde-advanced glycation end-products–bovine serum albumin media, (5) glucose-advanced glycation end-products+neurotrophin-4 media, (6) glycolaldehyde-advanced glycation end-products+neurotrophin-4 media, or (7) glyceraldehyde-advanced glycation end-products+neurotrophin-4 supplemented culture media. After 7 days, the number of regenerating neurites from the explants was counted. Then, explants were fixed, cryosectioned, and stained for TUNEL. The ratio of TUNEL-positive cells to all cells in the ganglion cell layer was determined. Immunohistochemical examinations for the active-form of caspase-9 and apoptosis-inducing factor were performed. In retinas incubated with advanced glycation end-products containing media, the number of regenerating neurites were fewer than in retinas without advanced glycation end-products, and the number of TUNEL-positive cells and caspase-9- and apoptosis-inducing factor-immunopositive cells was significantly higher than in control media. Neurotrophin-4 supplementation increased the numbers of regenerating neuritis, and the number of TUNEL-positives, caspase-9-, and apoptosis-inducing factor-immunopositive cells were significantly fewer than that in advanced glycation end-products without neurotrophin-4 media. Low doses of advanced glycation end-products impede neurite regeneration in the rat retinas. Neurotrophin-4 significantly enhances neurite regeneration in retinas exposed to advanced glycation end-products.

Abbreviations: AGEs, advanced glycation end-products; NT-4, neurotrophin-4; BSA, bovine serum albumin; AIF, apoptosis-inducing factor; ANOVA, analysis of variance; TUNEL, TdT-dUTP terminal nick-end labeling; DAPI, 4,6-diamidino-2-phenyl indole; RAGE, receptor for AGE; VEGF, vascular endothelial neurotrophic factor; RGCs, retinal ganglion cells; SD, Sprague-Dawley; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer

*Corresponding author. Fax: +81 43 224 4162.
E-mail addresses: Tarii@aol.com, oshitari@faculty.chiba-u.jp (T. Oshitari).

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http://dx.doi.org/10.1016/j.brainres.2013.08.027

1. Introduction

Advanced glycation end-products (AGEs) are a heterogeneous group of molecules formed from the nonenzymatic reactions of reducing sugars with free amino groups of proteins, lipids, and nucleic acids (Peppa et al., 2003). In 1912, Maillard reported the formation of brown products upon heating mixtures of amino acids and sugars. The Maillard reaction begins with the formation of a Schiff base between glucose and -amino groups of the terminal valine in hemoglobin (Bailey et al., 1998). The forward reactions give rise to additional irreversible protein-bound compounds collectively termed the AGEs (Stitt, 2010).

A key characteristic of certain reactive or precursor AGEs is their ability to form covalent crosslinks between proteins which alters their structure and function as occurs in cellular matrices, basement membranes, and vessel-wall components (Peppa et al., 2003). Other major features of AGEs are related to their interactions with a variety of cell-surface AGE-binding receptors, leading either to their endocytosis and degradation or to cellular activation and pro-oxidant, pro-inflammatory events. The pathogenetic importance of the AGEs is found in most of the diabetes complications which are grouped into micro- or macroangiopathies (Brownlee et al., 1988; Sell and Monnier, 1989; Dyer et al., 1991; Ruderman et al., 1992; Wautier et al., 1994). AGEs affected cells from three main perspectives: as adducts occurring on modified serum proteins, as endogenous adducts formed as a consequence of glucose metabolism, and as extracellular matrix-immobilized modifications on long-lived structural proteins (Stitt, 2010).

It has been reported that the AGEs enhanced the apoptosis of retinal pericytes, corneal endothelial cells, neuronal cells, and renal mesangial cells (Kasper et al., 2000; Kaji et al., 2003). Kase et al. (2011) found that the immunoreactivity for AGEs was strongly detected in the stromal vessels of the iris and ciliary body, in the choriocapillaris, large choroidal vessels, and peripheral retinal vessels (Kase et al., 2011). AGEs were also detected in the walls of the central retinal artery and vein located on the optic nerve head of diabetic eyes (Kase et al., 2011).

Recent studies have shown that the glycation-associated damage is not limited to patients with diabetes. Although it does not cause rapid or severe cell damage, glycation in conjunction with oxidation accompanies every fundamental process of cellular metabolism (Tezel et al., 2007). The alterations affect the physiological aging process because AGEs accumulate in various tissues during aging (Bailey et al.,1998; Thornalley, 1998; Schmidt et al., 2000). Because of their association with oxidative stress, AGEs have also been implicated in many neurodegenerative diseases, such as Alzheimer disease, amyotrophic lateral sclerosis, and Huntington disease (Yan et al., 1996; Stitt, 2001; Ma and Nicholson, 2004). As in other age-dependent neurodegenerative diseases of the brain, oxidative stress-associated age-dependent pathogenic processes are not unexpected in glaucoma because this disease is also more common in the elderly (Quigley and Vitale, 1997). Tezel et al. (2007) showed the importance of AGE/receptor of AGE (RAGE)-mediated cytotoxicity in glaucomatous neurodegeneration.

Growing evidence indicates that neuronal abnormalities including neuronal cell death and vascular abnormalities are associated with the pathogenesis of early diabetic retinopathy. Studies of human retinas indicate that mitochondrial- and caspase-dependent cell death pathways are associated with retinal neuronal cell degeneration in patients with diabetes (Oshitari et al., 2008). Oshitari et al. (2003, 2010) used three-dimensional retinal culture to examine the effect of several neurotrophic and/or survival factors on retinal neuronal cell death and regeneration. Brain-derived neurotrophic factor, NT-4, citicoline, vascular endothelial growth factor $(VEGF)_{120}$, $VEGF_{164}$, and taurine-conjugated ursodeoxycholic acid were tested. All factors had a survival effect on damaged retinal neurons induced by diabetic stress. Oshitari et al. (2010) also found that NT-4 had the best neuroprotective and regenerative effect under high glucose conditions.

The results of an earlier study indicated that the maximum rescue ratio of caspase-1, -3, -8, and -9 inhibitors in cultured retinas was 60% in damaged retinal ganglion cells (RGCs) (Oshitari and Adachi-Usami, 2003). Thus, at least 40% of neuronal cell death in damaged RGCs in cultured retinas should be related to caspase-independent cell death mechanisms. However, no reports focus on caspase-independent cell death pathways under diabetic stress including AGE exposure.

Apoptosis-inducing factor (AIF) was the first mitochondrial protein shown to mediate cell death independent of caspase (Susin et al., 1996, 1999). It was initially characterized as a mitochondrial protein confined in the intermembrane space of healthy cells. After permeabilization of the mitochondrial outer membrane, a feature of most if not all, apoptotic pathways (Green and Kroemer, 2004), AIF is released from the mitochondria and is translocated to the nucleus where it mediates the nuclear features of apoptosis such as chromatin condensation and large-scale (~50 kb) DNA degradation (Susin et al., 1996, 1999). In healthy cells, AIF is a mitochondrial flavin adenine dinucleotide which is dependent on oxidoreductase that plays roles in oxidative phosphorylation and redox control (Modjtahedi et al., 2006).

The aim of this study was to determine the effect of low dose AGE on neurite regeneration in isolated rat retinas and to determine the regenerative effects of NT-4 in AGE exposed retinas. In addition, we performed immunohistochemistry to examine whether a caspase-independent cell death factor, AIF and active-forms of caspase-9 expression were related to the neuronal cell death induced by AGE exposure in cultured retinas.

2. Results

2.1. *Detection of apoptosis*

To determine the effect of AGEs and NT-4 on the retinas in culture, the number of TUNEL-positive cells in the ganglion cell layer (GCL) was counted. The majority of the TUNEL-positive cells was detected in the GCL because all of the RGCs were axotomized to isolate the retina (Oshitari et al., 2002a, 2002b, 2003, 2010, 2011; Oshitari and Adachi-Usami, 2003; Oshitari and Roy, 2005). In retinas cultured in low-dose