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Research Report

Effect of neurotrophic factors on neuronal apoptosis and neurite regeneration in cultured rat retinas exposed to high glucose

Toshiyuki Oshitari, Natsuyo Yoshida-Hata, 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

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ABSTRACT

The purpose of this study was to investigate the effect of brain-derived neurotrophic factor (BDNF), neurotrophin-4 (NT-4), and citicoline on neuronal apoptosis and neurite regeneration in cultured rat retinas exposed to high glucose (HG). The retinas of six adult Sprague–Dawley rats were studied. After the rats were euthanized, the retinas were isolated and cultured in serum-free medium. One group of explants was cultured in normal glucose (NG) and another group in HG medium (HGM). BDNF, NT-4, or citicoline were added to the HGM. After 7 days, the number of regenerating neurites was counted. Then, the explants were fixed, cryosectioned, and stained by TdT-dUTP terminal nick-end labeling (TUNEL), and also immunostained for the active-forms of caspase-3 and -9. The numbers of TUNEL-positive and caspase-3 and -9-immunopositive cells in the ganglion cell layer (GCL) were significantly higher, and the number of regenerating neurites was significantly lower in retinas cultured in HGM than in NG medium. Retinas incubated in HGM supplemented with BDNF, NT-4, or citicoline had significantly lower numbers of TUNEL-positive and caspase-3 and -9-immunopositive cells in the GCL, and the numbers of regenerating neurites were significantly higher than in HGM without these factors. We conclude that the increase in the number of apoptotic cells and decrease the number of regenerating neurites in the HGM indicate that HG is toxic to RGCs. The decrease in the number of apoptotic cells in the HGM containing BDNF, NT-4, or citicoline is correlated with the suppression of the caspase-9 and -3 activities.

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1. Introduction

Diabetic retinopathy is a major complication in diabetic patients and can lead to blindness. Despite extensive research, the precise mechanism for the development and progression of diabetic retinopathy has not been determined (Danis et al., 2001; Lorenzi and Gerhardinger, 2001). The results of several studies have indicated that neuronal abnormalities such as retinal ganglion cell (RGC) death are associated with the pathogenesis of diabetic retinopathy (Barber et al., 1998;

* *Corresponding author*. Fax: +81 43 224 4162.
E-mail address: Tarii@aol.com (T. Oshitari).

Abbreviations: RGCs, Retinal ganglion cells; BDNF, brain-derived neurotrophic factor; NT-4, neurotrophin-4; citicoline, cytidine 5′-diphosphocholine; VEGF, vascular endothelial growth factor; DAPI, 4, 6-diamidino-2-phenyl indole; GCL, ganglion cell layer

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Takano et al., 1999; Asnaghi et al., 2003; Martin et al., 2004; Oshitari and Roy, 2005; Oshitari et al., 2008a). This is important because once RGCs die by apoptosis, visual function cannot be recovered because retinal neurons do not regenerate (Negishi et al., 2001; Whitmore et al., 2005; Oshitari and Roy, 2007; Oshitari et al., 2008a).

Thus, the focus of research has turned to determining whether neuroprotective procedures can protect the function of RGCs and their long axons in diabetic retinas (Takano et al., 1999; Oshitari and Roy, 2005). Because diabetes is associated with high levels of glucose in the serum, the biochemical effect of neuronal cell death and regeneration under high-glucose (HG) or diabetic conditions have been studied (Takano et al., 1999; Oshitari and Roy, 2005). It has been reported that exposure of retinal explants to advanced glycation end products can induce neuronal degeneration by caspase-3 activation (Lecleire-Collet et al., 2005). Another study showed that exposure of neurons to a HG medium can enhance intracellular Ca^{2+} responses that may be responsible for retinal cell death in the early stage of diabetic retinopathy (Pereira et al., 2010).

Evidence has been accumulation that endogenous neurotrophic factors can function as neuroprotective agents (Takano, 1996; Böcker-Meffert et al., 2002; Mo et al., 2002; Oshitari et al., 2002b; Oshitari and Adachi-Usami, 2003; Seki et al., 2004; Adibhatla and Hatcher, 2005; Parrilla-Reverter et al., 2009). Thus, brain-derived neurotrophic factor (BDNF) has been shown to have neuroprotective properties in both diabetic and physical injured models (Mo et al., 2002; Oshitari and Adachi-Usami, 2003; Seki et al., 2004). Neurotrophin-4 (NT-4) has similar neuroprotective effects in the survival and regeneration of RGCs in vitro and in vivo (Takano, 1996; Parrilla-Reverter et al., 2009). Cytidine 5′-diphosphocholine (citicoline, CDP-choline) is prescribed for brain injury, Alzheimer's, and Parkinson's disorders in Japan and Europe. In addition, oral and intramuscular administration of citicoline significantly improves retinal and cortical responses in glaucoma patients (Parisi et al., 2008). Exogenous citicoline is involved in the synthesis of the phospholipids of cell membranes and stabilizes the intracellular conditions of neuronal cells (Adibhatla and Hatcher, 2005). Our laboratory has demonstrated that citicoline reduces RGC death and promotes neurite regeneration in cultured retinas of mice (Oshitari et al., 2002b). An earlier study showed that vascular endothelial growth factor (VEGF) can promote neurite regeneration in postnatal rats (Böcker-Meffert et al., 2002).

Thus, we hypothesize that the apoptosis and reduced neurite regeneration of retinal neurons induced by exposure to HG can be neutralized by neurotrophic factors. We also hypothesized that the mechanisms causing cell death after long term HG exposure are similar to the cell death mechanisms after physical injuries to the retinas. To test these hypotheses, we used three-dimensional retinal culture system that allow us to study both neuronal cell death and neurite regeneration (Takano, 1996; Takano et al., 1999; Oshitari et al., 2002a,b; Oshitari and Adachi-Usami, 2003; Oshitari and Roy, 2005). Isolated retinal explants were exposed to HG medium, and we counted the number of apoptotic cells and the number of regenerating neurites. In addition, we determined the correlations between caspase-9 and -3 activation and the neuronal apoptosis in the retinas exposed HG medium. We also examined the neuroprotective and regenerative effects of BDNF, NT-4, citicoline, $VEGF_{120}$, and $VEGF_{164}$ under HG conditions.

2. Results

2.1. *In situ detection of apoptosis in ganglion cell layer (GCL) of retinal explants*

To assess the effect of HG medium and neurotrophic factors on neuronal cell death, we counted the number of TdT-dUTP terminal nick-end labeling (TUNEL)-positive cells in the GCL under each condition. Because all of the RGCs were axotomized to isolate the retina, the majority of the apoptotic cells were observed in the GCL (Oshitari et al., 2002a,b; Oshitari and Adachi-Usami, 2003; Oshitari et al., 2003; Oshitari and Roy, 2005).

In retinas cultured in HG medium, the number of TUNEL-positive cells in the GCL was significantly higher than that in retinas cultured in normal glucose (NG) and in NG+D-mannitol (NG+M) medium (40.7±7.7% vs. 28.1±11.9%; 40.7±7.7% vs. 29.3±9.6%; mean±standard deviation (SD); $P=0.0011$, 0.003, respectively; Figs. 1A–C and 2). The number of TUNEL-positive cells in the GCL was significantly higher in retinas incubated with than without BDNF (27.6±10.8% vs. 40.7±7.7%; $P=0.002$), NT-4 (22.5±11.1 vs. 40.7±7.7%; $P=0.0003$), citicoline (26.4±7.4% vs. 40.7±7.7%; $P=0.0005$), $VEGF_{120}$ (31.5±9.2% vs. 40.7±7.7%; $P=0.005$), or $VEGF_{164}$ (31.7±8.7% vs. 40.7±7.7%; $P=0.0024$) in the HG media (Figs. 1C–H and 2).

2.2. *Caspase-9 immunopositivity in GCL of retinal explants*

We immunostained for the active-form of caspase-9 to determine whether caspase-9 activation was associated with the apoptosis under our experimental conditions. In retinas cultured in HG medium, the number of cells that were immunopositive to the active-form of caspase-9 in the GCL was significantly higher than that in the NG medium and the NG+M medium (25.3±5.3% vs. 15.9±5.9%; 25.3±5.3% vs. 16.3±6.3%; the mean±SD, $P=0.0127$, 0.0213, respectively; Figs. 3A–C and 4). In retinas incubated in HG medium supplemented with BDNF, NT-4, or citicoline, the number of cells immunopositive to caspase-9 in the GCL was significantly lower than that in the HG medium without BDNF, NT-4, or citicoline (16.2±7.7% vs. 25.3±5.3%; $P=0.035$, 15.7±11% vs. 25.3±5.3%; $P=0.0253$, or 16.8±6.9% vs. 25.3±5.3%; $P=0.0407$, respectively; Figs. 3C–F and 4). The differences in the number of cells that were immunopositive to caspase-9 in the HG media with and without $VEGF_{120}$ or $VEGF_{164}$ were not significant (21.5±9.0% vs. 25.3±5.3% or 21.5±8.5 vs. 25.3±5.3%, respectively; Figs. 3G, H and 4). In all groups of retinal explants, the majority of active-form of caspase-9 signals in the GCL was expressed in the nuclei (Fig. 5). These results indicated that the active-form of caspase-9 in the cytosol was translocated into the nucleus during apoptosis.

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