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Morphological and migratory alterations in retinal Müller cells during early stages of hypoxia and oxidative stress☆

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Abstract

In the present study, retinal Müller cells were cultured *in vitro* and treated with hydrogen peroxide (oxidative stressor) and cobalt chloride (hypoxic injury). Following 24 hours of culture, compensatory hypertrophy was observed and cellular apoptosis increased. Hypoxia enhanced the migration ability of retinal Müller cells and induced the expression of α-smooth muscle actin. Oxidative stress altered the morphology of Müller cells when compared with hypoxia treatment.

Key Words: retina; Müller cells; hypoxia; oxidative stress; migration; glutamine synthetase; α-smooth muscle actin; neural regeneration

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INTRODUCTION

Diabetes mellitus induces glucose and glycated protein autoxidation, resulting in a large amount of oxygen-derived free radicals[1-3]. During this process, glycometabolism produces 5, 10-methylenetetrahydrofolate reductase (NADPH) and activates cytochrome P450[4], inverts the ratio of NADPH/methylenetetrahydrofolate dehydrogenase (NADP)[5-6], activates NADPH oxidase, induces production of acetone bodies and acetic ether[7-8], and antioxidase is saccharified[9-10]. These reactions induce cell hypoxia and oxidative stress. In particular, oxidative stress may cause further damage to larger molecules or alter cell signal transduction systems by enhancing cell membrane lipid peroxidation, thereby leading to apoptosis[11-13].

Retinal Müller cells are a type of glial cell mainly found in the retina. Their functions resemble that of astrocytes and oligodendrocytes[14-16]. Müller cell injury can result in the accumulation of glutamic acid, an excitatory neurotransmitter, which is toxic to ganglion cells and can hinder vascular regulation, leading to blood-retina barrier damage[17-18]. The morphology and physiological function of retinal Müller cells has been shown to change prior to retinal vasculopathy onset[19-20]. The present study sought to investigate the early influence of hypoxia and oxidative stress on retinal Müller cells using cobalt chloride ($CoCl_2$) and hydrogen peroxide (H_2O_2), respectively.

RESULTS

In vitro cell culture and identification of retinal Müller cells

A few cells outgrew retinal tissue blocks in primary cultures of retinal Müller cells maintained for 2–3 days. The majority of *in vitro* cultured Müller cells appeared similar to epithelial cells, with a flat, polygonal shape, while a few cells appeared similar to nerve fiber cells, with an irregular shape and long processes (supplementary Figure 1 online). *In vitro* cultured retinal Müller cells expressed glutathione synthetase (GS) and vimentin. GS was expressed in the nuclei and cytoplasm, and positive expression was observed in more than 80% of cells. Vimentin was expressed only in the cytoplasm, and positive expression was observed in more than 90% of cells.

Influence of hypoxia and oxidative stress on the morphology of retinal Müller cells

Normal Müller cells appeared with a flat and polygonal shape. However, the number of floating Müller cells increased following 24 hours of oxidative stress. Cell bodies of Müller cells became large, the number of processes increased, and filiform fibers were observed in the cytoplasm after 24 hours of hypoxia (Figure 1, supplementary Figure 2 online).

Influence of hypoxia and oxidative stress on GS and α-smooth muscle actin (α-SMA) expression in retinal Müller cells

Fluorescence microscopy revealed that normal retinal Müller cells expressed GS, but did not express α-SMA. Oxidative stress inhibited GS expression, but did not change α-SMA expression, while retinal Müller cells exposed to hypoxia were negative for GS expression, but strongly positive for α-SMA expression (Figure 2).

Influence of hypoxia and oxidative stress on retinal Müller cell proliferation and apoptosis

Flow cytometric analysis showed that 24 hours of oxidative stress or hypoxia reduced the number of Müller cells in the G_1 phase, but increased the number of Müller cells in the S and G_2/M phases when compared with normal retinal Müller cells (Figure 3). In addition, the percentage of cells undergoing apoptosis was low in normal retinal Müller cells, while 24 hours of oxidative stress or hypoxia increased the percentage of apoptotic cells (Figure 4).

Influence of hypoxia and oxidative stress on retinal Müller cell migration

The transwell cell migration test revealed that the number of migrating cells was 1.2 ± 0.8 in the control group. Oxidative stress did not significantly influence the number of migrating cells (4.0 ± 1.6; $P > 0.05$), however, the number of migrating cells following 24 hours of hypoxia significantly increased (69.4 ± 10.4; $P < 0.05$; supplementary Figure 3 online) when compared to the control and oxidative stress groups.

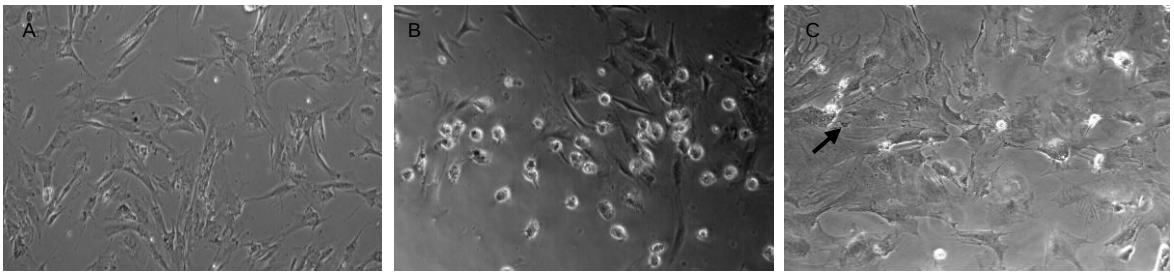

Figure 1 Morphology of retinal Müller cells exposed to oxidative stress and hypoxia (× 200).

(A) Normal group cells were unified and had a fusiform shape. Following oxidative stress.

(B) Some cells disintegrated, were irregular in shape and had a disorderly arrangement.

(C) Hypoxia treatment resulted in enlargement of cell bodies, and some cells became fused to form blocks (arrow), with a disorderly alignment.

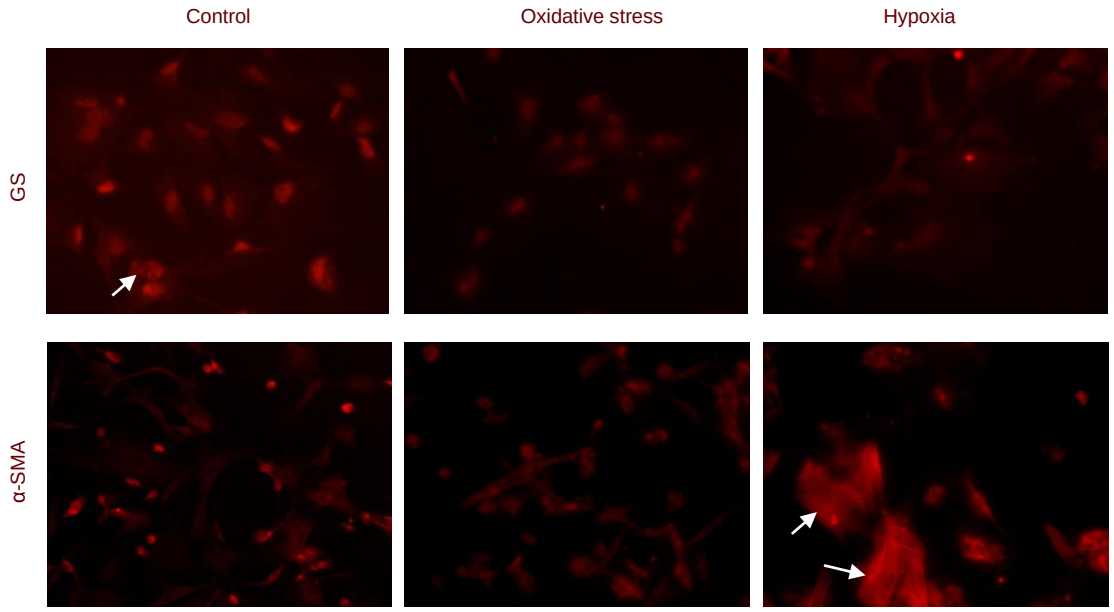

Figure 2 Glutathione synthetase (GS) and α-smooth muscle actin (α-SMA) expression in retinal Müller cells (× 200).

Retinal Müller cells stained positive for GS in the control group (arrow), but were negative for GS in the oxidative stress and hypoxia groups. Retinal Müller cells stained negative for α-SMA expression in the control and oxidative stress groups, but were strongly positive for α-SMA in the hypoxia group (arrows).

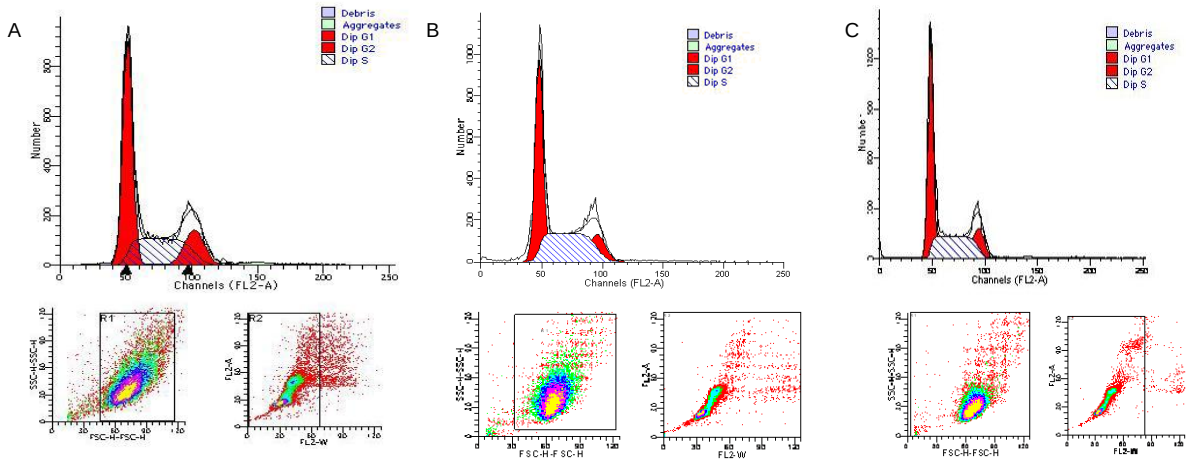

Figure 3 Cell cycle analysis in each group as detected by flow cytometry.

(A) The percentage of cells in the G_1 and S phases was 51.40% and 32.54%, respectively, for the control group.

(B) The percentage of cells in the G_1 and S phases was 48.46% and 38.46%, respectively, in the oxidative stress group.

(C) The percentage of cells in the G_1 and S phases was 50.66% and 35.49%, respectively, in the hypoxia group.

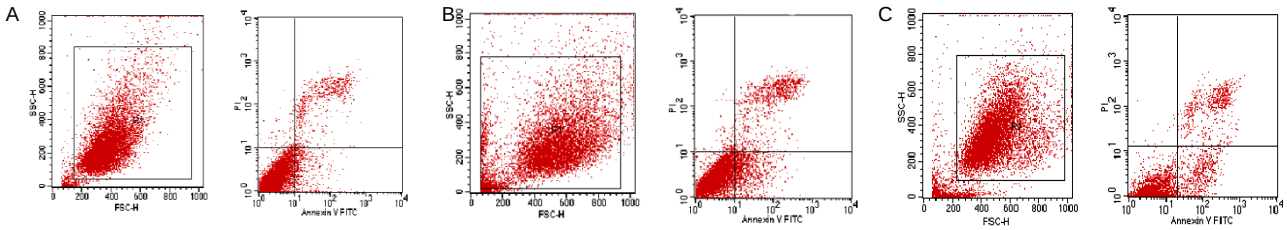

Figure 4 Apoptotic cells in each group as detected by flow cytometry.

The percentage of cells undergoing apoptosis in the (A) control, (B) oxidative stress and (C) hypoxia groups was 4.00%, 11.38% and 6.92%, respectively.

DISCUSSION

The vertebrate retina includes three types of glial cells: Müller cells, astrocytes and microglia, with Müller cells being the most predominant cell type[21]. The present study purified Müller cells using the mechanical pipetting method. GS, a key enzyme used to convert glutamic acid into glutamine, is expressed in Müller cells and is therefore a useful marker for these cells[22-23]. In addition, vimentin, an intermediate filament protein found in mammalian retinal glial cells, can be used as a marker for retinal glial cells[24-26]. Therefore, vimentin can label both Müller cells and astrocytes. In the present study, more than 80% of cells stained positive for GS, and more than 90% of cells labeled positive for vimentin.

Müller cells may participate in the occurrence and progression of proliferative diabetic retinopathy[27-28]. In the present study, the number of Müller cells in the G_1 phase reduced, however, following hypoxia, the number of cells in the S and G_2/M phases increased. These results indicated that during early stages of hypoxia, the function of Müller cells is damaged, resulting in compensatory hyperplasia, demonstrating a protective role of Müller cells during early stages of hypoxia. After Müller cells were exposed to 100 μM H_2O_2 for 24 hours, GS expression reduced, and cell morphology was altered. Moreover, Müller cell apoptosis significantly increased following exposure to H_2O_2, indicating that oxidative stress produces greater cytotoxicity when compared to compensatory hyperplasia at early stages. After 24 hours of hypoxia, GS expression was completely absent. Moreover, the percentage of apoptotic cells increased, indicating hypoxia can damage the morphology and function of Müller cells. Müller cells also stained positive for α-SMA, and the transwell cell migration test indicated enhanced cell migration following hypoxia treatment. Our results revealed that hypoxia can change the phenotype of Müller cells into muscle fiber-like cells while changing the migratory and contractile ability of cells.

Our results revealed that oxidative stress and hypoxia can detrimentally alter the morphology and function of Müller cells. In addition, oxidative stress inhibited GS expression, and hypoxia induced α-SMA expression and transwell chamber migration of Müller cells. Therefore, we conclude that hypoxia may induce the participation of Müller cells in the formation of proliferative diabetic retinopathy.

MATERIALS AND METHODS

Design

A comparative observation of cytology.

Time and setting

The experiment was performed at the Medical Experimental Center of Xi'an Jiaotong University Medical College, China, from December 2009 to December 2010.

Materials

A total of 12 healthy Sprague Dawley rats, 7 days old, of either gender, were provided by the Animal Center of Xi'an Jiaotong University Medical College (No. SCXK (Shaan) 2007-001). They were housed in 12-hour day/night cycle at 22 ± 20°C with a humidity of 60 ± 10%. The experimental protocols were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[29].

Methods

In vitro culture and identification of retinal Müller cells

Rats were anesthetized by intraperitoneal injection with 10% (w/v) chloral hydrate (3 mL/kg) and sacrificed. The eyeballs were removed under sterile conditions, and the retina was separated, trypsinized (0.25% (w/v); Sigma, St. Louis, MO, USA) at 37°C for 30 minutes. Approximately 60% of floating cells were observed under the microscope (Olympus, Tokyo, Japan), seeded in 96-well culture plates (Corning Inc, Now York, USA), and incubated in a humidified incubator (Sheldon Manufacturing, Cornelius, NC, USA) containing 5% CO_2 at 37°C. When cells were 80% confluent, they were digested and passaged, pipetted, centrifuged at 800 r/min for 5 minutes, resuspended in complete culture medium (Dulbecco's modified Eagle's medium containing 100 U/mL penicillin, 100 μg/mL streptomycin and 10% (v/v) fetal bovine serum; Sigma) and passaged at a ratio of 1: 3.

After primary culture for 5 days, the cell slide was washed with phosphate buffered saline (PBS), fixed with 100% acetone (Beijing Biosynthesis Biotechnology, Beijing, China) and incubated with 20% (v/v) goat serum (Beijing Biosynthesis Biotechnology) at room temperature for 30 minutes. Cells were incubated with either rabbit anti-mouse GS or anti-vimentin primary antibodies (1: 100; Santa Cruz Biotechnology Inc, Santa Cruz, California, USA) overnight at 4°C, followed by fluorescein-coupled goat anti-rabbit IgG (1: 200; Beijing Biosynthesis Biotechnology) at 37°C for 1 hour in the dark. Cells were viewed using a fluorescence microscope (Olympus).

Hypoxia and oxidative stress treatment

A third passage of retinal Müller cells were placed in slide-coated 24-well culture plates and cultured for 15 hours. Cells at 70% confluency were randomly assigned to control (no treatment), oxidative stress (100 μM H_2O_2 solution (Sigma)) and hypoxia (500 μM $CoCl_2$ solution (Sigma)) groups for 24 hours.

Morphology of Müller cells

Following oxidative stress or hypoxia for 24 hours, Müller cells were fixed with pre-cooled acetone for 20 minutes, washed with PBS three times and observed using an inverted microscope (Olympus).

Immunohistochemistry for GS and α-SMA expression in Müller cells

Following oxidative stress or hypoxia for 24 hours, three wells were randomly selected from each group, washed three times with PBS for 5 minutes each, incubated with 20% (v/v) goat serum at room temperature for 30 minutes, followed by incubation in rabbit anti-rat GS antibody (1: 100; Santa Cruz Biotechnology) or rabbit anti-rat α-SMA antibody (1: 100; Beijing Biosynthesis Biotechnology) overnight at 4°C. Fluorescein-coupled goat anti-rabbit IgG (1: 200; Beijing Biosynthesis Biotechnology) was added to cells at 37°C for 1 hour in the dark and cells were mounted using 20% (v/v) glycerol. Cells were observed using a fluorescence microscope (Olympus).

Flow cytometric analysis for cell proliferation and apoptosis

Following oxidative stress or hypoxia for 24 hours, Müller cells were trypsinized (0.25% (w/v)) for 15 minutes and centrifuged at 1 000 r/min. Cells (100 μL) were collected from each group at room temperature, incubated with 50 mg/L propidium iodide (Sigma) and 100 mg/L RNase A (Sigma) at room temperature for 30 minutes in the dark. Cells were detected using FACSort (Becton Dickson, San Diego, USA), and the percentage of cells in different phases was analyzed using CELLQuest software (Becton Dickson). Cells (100 μL) were collected from each group at room temperature, incubated with 10 μL fluorescein isothiocyanate-Annexin-V (Sigma) and 5 μL propidium iodide at room temperature for 15 minutes in the dark. Apoptosis was detected using flow cytometry.

Transwell chamber cell migration test for cell migration

Müller cells were passaged in transwell chambers (Corning Incorporated, New York, USA) for 15 hours, exposed to oxidative stress or hypoxia for 24 hours, and fixed with acetone. Cells that had migrated to the back of the filter membrane through the wells were stained with hematoxylin for 10 minutes. Five fields of view were randomly selected under 200 × magnification and the mean number of migrated cells was quantified by microscopy (Olympus).

Statistical analysis

Results were analyzed using SAS 9.1 software (SAS Institute Inc, Cary, USA). The number of migrating cells was analyzed using two-way analysis of variance, and the Student Newman-Keuls and Fisher's test. A value of $P < 0.05$ was considered statistically significant.

Author contributions: Xiaohui Zhang and Zhaohui Feng designed this study. Xiaohui Zhang, Zhaohui Feng and Chunhua Li conducted the experiments and collected the data. All authors contributed to the evaluation of this study. Xiaohui Zhang wrote the manuscript. Zhaohui Feng revised the manuscript.

Conflicts of interest: None declared.

Ethical approval: This study received permission from the Animal Ethics Committee of Xi'an Jiaotong University Medical College, China.

Supplementary information: Supplementary data associated with this article can be found in the online version, by visiting www.nrronline.org, and entering Vol. 7, No. 1, 2012 after selecting the "NRR Current Issue" button on the page.

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