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NOTCH SIGNALING PATHWAY REGULATES PROLIFERATION AND DIFFERENTIATION OF IMMORTALIZED MÜLLER CELLS UNDER HYPOXIC CONDITIONS *IN VITRO*

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Abstract—Previous studies have indicated that Müller glia in chick and fish retinas can re-enter the cell cycle, express progenitor genes, and regenerate neurons via the Notch signaling pathway in response to retinal damage or growth factors. Here, we investigated the role of Notch signaling and the effect of hypoxia, as a means to induce retinal damage, on the proliferation of an immortalized Müller cell line (rMC-1 cells). Our data showed that rMC-1 cells expressed Müller glia and neural and retinal progenitor markers but did not express neuronal or retinal markers. Hypoxia increased rMC-1 cell proliferation by activating the positive cell-cycle regulators, cyclins A and D1, as well as the neural and retinal progenitor markers, Notch1, Hes1, nestin, Sox2, Msi1, Pax6, and NeuroD1. However, hypoxia did not significantly influence the expression of Müller glial markers GS, CRALBP, and cyclin D3 or the death of the rMC-1 cells. The increase in cell proliferation induced by hypoxia was greatly attenuated by blocking Notch signaling with the inhibitor DAPT, resulting in the reduced expression of positive cell-cycle regulators (cyclins A and D1) and neural and retinal progenitor markers (Notch1, Hes1, Sox2, Pax6, and NeuroD1). Blockade of the Notch signaling pathway by DAPT after hypoxia promoted the differentiation of rMC-1 cells to neurons, as demonstrated by the induction of neural marker (Tuj1), retinal amacrine (Syntaxin1), and retinal ganglion cell (Brn3b) markers, although the expression of the latter marker was low. Taken together, our data indicate that Notch signaling is required for proliferation under hypoxic conditions either by activating the positive cell-cycle regulators or by skewing their de-differentiation towards a neural progenitor lineage. These findings indicate that the Notch signaling pathway regulates hypoxia-induced proliferation and differentiation of Müller glia. © **2012 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: Müller cells, hypoxia, proliferation, differentiation, Notch.

INTRODUCTION

Several studies have revealed that retinal Müller glial cells have the potential to re-enter the cell cycle, express progenitor marker genes, and regenerate new retinal neurons (primarily amacrine cells, especially in mammals) under certain circumstances (species-dependent). This can occur in response to retinal damage or growth factors through the reactivation of a molecular program normally expressed in retinal progenitors (Fischer and Reh, 2003; Raymond et al., 2006; Yurco and Cameron, 2005; Hayes et al., 2007).

Hypoxia/ischemia is a type of pathological damage that can occur in neural and glial cells. Hypoxia has been implicated in the proliferation of neural stem cells (NSCs) or progenitors (referred to as neural progenitors) in both the rodent and human brain, and newly divided cells can differentiate into neurons to participate in the regenerative response to this type of insult (Sun et al., 2003; Felling et al., 2006; Lichtenwalner and Parent, 2006; Bürgers et al., 2008). Experimental stroke has been shown to enhance neurogenesis in the subventricular zone (SVZ) and subgranular zone (SGZ) of the hippocampus dentate gyrus in rodents and primates (Zhang et al., 2006; Chen et al., 2010). Similarly, Chen et al. (2010) suggested that hypoxia/ischemia can stimulate neural progenitor cell proliferation through the activation of the c-Jun N-terminal protein kinase (JNK) signaling pathway and expression of cyclin D1 in the rat brain. In addition, increasing evidence suggests that the responses of neural progenitors to various types of brain injury in the adult may be mediated by Notch signaling, since Notch signaling is up-regulated in proliferating cells of the adult anterior SVZ (aSVZ) or the hippocampal CA1 region after cortical injury or global ischemia (Givogri et al., 2006; Oya et al., 2009).

Notch signaling is a key pathway that regulates the proliferation and differentiation of neural progenitors in developing and adult brains (Hitoshi et al., 2002). Notch signaling activation maintains the progenitor cells in an undifferentiated state during neurogenesis, promotes the proliferation of neural progenitors, and inhibits the

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Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; CRALBP, cellular retinaldehyde-binding protein; DAPI, 4′,6-diamidino-2-phenylindole; DAPT, *N*-[*N*-(3, 5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester; ERK, extracellular signal-regulated kinase; FGF2, fibroblast growth factor 2; GFAP, glial fibrillary acidic protein; GS, glutamine synthetase; Msi1, musashi-1; NMDA, *N*-methyl-D-aspartic acid; NSCs, neural stem cells; PKC, protein kinase C; Sox2, SRY (sex determining region Y)-box containing gene 2; TdT, terminal deoxynucleotidyl transferase; TUNEL, terminal dUTP nick end labeling.

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differentiation of neural progenitors into neurons (Yoon and Gaiano, 2005; Oya et al., 2009). Recently, several studies have provided evidence that Müller glia exhibit the functional and phenotypic features of retinal progenitor cells (Das et al., 2006; Nickerson et al., 2008), and Notch signaling has been shown to play important roles during the development of retinal Müller glia (Hayes et al., 2007). More specifically, Hayes et al. (2007) suggested that Notch signaling plays 2 distinct roles during retinal regeneration: induction of Müller glia cell proliferation and inhibition of newly generated progenitor cell differentiation. However, the effect of hypoxia on the regulation of mammalian retinal Müller glia proliferation via Notch signaling has not yet been investigated.

Previous studies have primarily focused on *in vivo* paradigms; however, an *in vitro* approach could simplify matters and provide insight into the complexities inherent in the *in vivo*-damaged retina. Here, we used a transformed rat Müller glial cell line (rMC-1) to investigate the effect of hypoxia on the regulation of Müller glia proliferation by Notch signaling and to explore a possible link between Notch signaling and the proliferation and differentiation of Müller glial cells under hypoxic conditions.

EXPERIMENTAL PROCEDURES

Cell culture

rMC-1, an SV40 large T antigen-transformed immortalized cell line exhibiting the characteristics of rat Müller glia, was kindly provided by Dr. Sarthy's laboratory (Sarthy et al., 1998). According to their method, rMC-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1× antibiotic/antimycotic, and 1× GlutaMAX (Invitrogen, Carlsbad, CA, USA) at 37 °C in normoxic conditions with 95% air/5% CO_2, which is referred to as normal condition (NC) in this report.

Characterization of rMC-1

To characterize the rMC-1 cell line, immunocytochemistry and reverse transcription (RT)-PCR were performed to determine the specific markers of Müller glia, retinal neuronal cells, and neural and retinal progenitors.

Hypoxia/reoxygenation assay

Hypoxia was induced in rMC-1 cells by placing them in a humidified microaerophilic incubation system under conditions of 2.0% O_2/93% N_2/5% CO_2 at 37 °C. The oxygen concentration was monitored by the incubator system. rMC-1 cells were incubated under hypoxic conditions for different periods of time after which they were transferred to normoxic conditions for an additional number of days based on the experimental plan. The control group was kept under normoxic conditions throughout the experiment.

Proliferation of rMC-1 cells under hypoxia

To determine cell viability, rMC-1 cells were plated onto 96-well microplates at a concentration of 5×10^3 cells per well and cultured for 2 days under normoxic conditions. Cells were then placed in the hypoxic incubator for 12 or 24 h, after which they were cultured further for 1 or 3 days under normoxic conditions. Cell proliferation was measured using the Cell Proliferation ELISA, BrdU Kit (Roche, Mannheim, Germany), according to the manufacturer's instructions. In brief, the cells were incubated with 10 μM 5-bromo-2′-deoxyuridine (BrdU) for 2 h. FixDenat was added to fix the cells and denature the DNA. Anti-BrdU-POD was then added to quantify the amount of BrdU incorporated into the newly synthesized DNA. Absorbance was measured in an enzyme-linked immunosorbent assay (ELISA) reader at 370 nm. Each experiment was performed in triplicate on different days.

For immunocytochemical labeling of BrdU (Sigma, St. Louis, MO, USA) incorporation, rMC-1 cells were plated on poly-D-lysine-coated slides and cultured under hypoxic conditions for 12 or 24 h, followed by 3 days of normoxia. Then, 10 μM BrdU was added to label the dividing cells during the subsequent 48-h period. The cells were fixed with 4% paraformaldehyde, and immunocytochemical analysis was performed to quantify the incorporated BrdU as previously described (Das et al., 2006; Wang et al., 2011).

To determine the expression of hypoxia-induced cell-cycle regulators (cyclin A, cyclin D1, and p27), neural and retinal progenitor markers (Sox2, Notch1, Hes1, Pax6, and NeuroD1), and the Müller glial markers (GS, CRALBP, and cyclinD3), rMC-1 cells were cultured for 2 days under normoxic conditions and transferred to hypoxic conditions for 24 h followed by 3 days of reoxygenation. RNA was then isolated, and RT-PCR was performed.

Effects of blocking Notch signaling on the proliferation, differentiation, and retinal regeneration of rMC-1 cells under hypoxic conditions

rMC-1 cells were pre-treated with or without 10 μM of the γ-secretase inhibitor *N*-[*N*-(3, 5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT; Sigma) for 2 h to inhibit Notch signaling followed by hypoxic treatment for 24 h. Cell proliferation was then determined by BrdU ELISA, and the expression of cell-cycle regulators cyclin A, cyclinD1, and p27 was determined by real-time PCR.

rMC-1 cells were also pre-treated with or without 10 or 20 μM of DAPT for 2 or 12 h to inhibit Notch signaling; this was followed by the hypoxic treatment or pre-treatment with hypoxia for 12 h and then, the addition of DAPT and cultured under normoxic conditions. The expression of neuronal, retinal neuronal, and glial markers, as well as neural progenitor markers, was determined by real-time PCR.

RT-PCR and real-time quantitative RT-PCR

Real-time RT-PCR was performed as previously described (Sugano et al., 2003). Total RNA was isolated from the cultured cells using Trizol (Sigma), and cDNA was synthesized with the PrimeScript RT Reagent Kit and gDNA Eraser (Perfect Real Time; Takara, Shiga, Japan). KOD Dash (Toyobo, Osaka, Japan) was used for normal RT-PCR amplification. The expression level of each gene was calculated after normalization to β-actin. SYBR Premix Ex Taq (Perfect Real Time; Takara) was used for real-time PCR, and the specific transcripts were amplified with Smart Cycler (Takara). Primers used in the experiment are listed in Table 1.

Immunocytochemistry

Immunocytochemical analysis was performed as previously described (Sugano et al., 2005; Tomita et al., 2005; Das et al., 2006). Briefly, cells were fixed with 4% paraformaldehyde for 10 min at room temperature. After permeabilization with 0.3% Triton X-100 in phosphate-buffered solution (PBS) for 10 min, slides were incubated with 3% bovine serum albumin (BSA) and 5% blocking serum for 30 min at room temperature. For BrdU staining, cells were treated with 2 N HCl for 30 min to denature the DNA and neutralized with 0.1 M boric acid. Slides were incubated with primary antibodies overnight at 4 °C, followed by incubation with secondary antibodies conjugated to Alexa Fluor 594 (red) or