Regulation of Cell Cycle Length in Progenitor Cells of the Developing Vertebrate Retina

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Introduction

Progenitor cells exist in two distinct phases during development with different forms of division: preneurogenic and neurogenic. Preneurogenic progenitor cells produce two cells that divide again while neurogenic progenitor cells divide to produce one or both cells that withdraw from the mitotic cycle and differentiate. Preneurogenic and neurogenic cells are fundamentally different in that only preneurogenic progenitor cells express the Notch ligand, Delta 1, and only neurogenic progenitor cells express the bHLH transcription factor, E2A (Yang et. al., 2009). Additional differences in these cell types are not well understood. The vertebrate retina is an effective model system for studying these differences in progenitor cells because it is a part of the central nervous system that buds off from the brain during early development. As the retina develops, it matures from the center to the periphery with differentiated cells towards the center and progenitor cells replicating without differentiation in the periphery (Dutting et al., 1983). The neurogenic front marks the border between preneurogenic and neurogenic progenitor cells. Ganglion cells are the first postmitotic, differentiated cells to be generated at the neurogenic front, which is present for several days in chick embryo retina.

It is well documented that as development progresses, cell cycle length increases (Takahashi et. al. 1995). It is unknown whether this is a gradual increase throughout development or if it is related to a change in cell type. In this study we explore the question, is the change in progenitor cell mode from preneurogenic to neurogenic accompanied by a change in cell cycle

length? The retina will be used to answer this question because preneurogenic and neurogenic progenitor cells can be studied in the same animal during several days of early development.

To determine if there is a difference in cell cycle length between these differentiated, neurogenic progenitor cells in the central retina and the undifferentiated, preneurogenic progenitor cells in the periphery during development, we used two BrdU injections. These thymidine analogs are incorporated into the cell's DNA during S phase of the cell cycle and we detected them using immunohistochemistry. Immunohistochemistry with RA4 was also used to determine the location of the most peripheral ganglion cell, which marks the neurogenic front. The total number of cells and the number identified by each BrdU injection were counted in both preneurogenic and neurogenic retina. We used an equation considering the percentage of cells that incorporated only one of the thymidine analogs and the survival times after each injection to determine the total cell cycle length. Here we show that the cell cycle of neurogenic progenitor cells is longer than that of preneurogenic progenitor cells, indicating that this is a fundamental difference in the cell types and not due to a gradual increase in cell cycle length throughout development.

Methods

Animals. Pathogen-free, fertilized While Leghorn chicken eggs were obtained from Hy-line (Spencer, Iowa). Eggs were incubated at 37°C, 98% relative humidity.

BrdU injections and tissue preparation. After 4.5d incubation, embryos were staged according to Hamburger and Hamilton (1951) and double injected with thymidine analogues that is incorporated into the DNA during S phase of the cell cycle (Gratzner, 1982). 35 to 40 μl of 10 mg/ml IDU in saline (MP Biomedicals) was injected into the vein of the embryo using Picospritzer II. The embryo was incubated for 3 hours at which time the embryo was injected with 35 to 40 μl of 10 mg/ml CldU in saline (MP Biomedicals). The embryo was incubated for

either 30 minutes or one hour after which time it was harvested and fixed in 4% paraformaldehyde/ 0.1M phosphate buffer, pH 7.4 for 2 hours. Embryos were cryoprotected in 20% sucrose/ 0.1M phosphate buffer, pH7.4, overnight at 4° C, heads were removed from the body and embedded in O.C.T. compound (from Electron Microscopy Sciences, Hatfield). 12 μm thick frozen sections of the retina were prepared on Superfrost Plus glass slides using at cryostat (from Fisher Scientific, Pittsburgh).

Adapted immunohistochemistry. To detect the thymidine analogues IDU and CldU injected into the embryo, immunohistochemistry was performed. Tissue preparations were re-hydrated in Phosphate buffered saline (PBS), fixed in 4% paraformaldehyde/ PBS for 10 minutes, rinsed with PBS and treated with 3 μg/ml proteinase K in PBS for 3 minutes. Tissue was again fixed in 4% paraformaldehyde/ PBS for 5 minutes, rinsed in PBS, acetylated in triethanolamine-HCl, pH 8.0 for 10 minutes, and permealized in 1% TritonX-100/PBS for 30 minutes. After being rinsed with PBS, tissue preparations were prehybridized in hybridization solution overnight at 65°C. Slides were then immersed in 50% formamide/ 1XSSC, washed in 0.5% Tween-20/ PBS and nonspecific antibody binding was blocked using 10% normal donkey serum/ 0.5% Tween-20/ PBS for 15 minutes. Tissue was incubated overnight at 4°C in 1:50 concentration of mouse anti-BrdU (Idu detecting) antibody in 10% NDS/ PBS/ 0.5% Tween-20. Tissue was then washed in 0.5% Tween-20/ PBS and incubated overnight at 4°C in 1:120000 concentration of rat anti-BrdU (CldU detecting) antibody in 10%NDS/ PBS/ 0.5% Tween-20. Tissue was washed in 0.5% Tween-20/ PBS and incubated at 4°C for 2 hours in 1:500 concentration of affinity purified and minimal cross reactivity antibodies Cy3-anti-rat, Cy2-anti-mouse and Dylight 488-anti-mouse. Tissue was stained with a 1:2000 concentration of DAPI/ PBS for 1 minute and rinsed in 0.5% Tween-20/ PBS. Slides were wet cover slipped using PVA DABCO.

Adjacent sections were processed as controls in which no primary antibodies were used or only one was used.

Immunohistochemistry. Adjacent sections to the sections used for adapted immunohistochemistry were processed for immunohistochemistry as described previously (Waid and McLoon, 1995). Differentiating ganglion cells were identified using RA4 monoclonal antibody at 1:100 concentration (McLoon and Barnes, 1989) and Cy3-anti-mouse at 1:500 concentration. The ganglion cell phenotype is expressed when a cell is postmitotic, so preneurogenic tissue was identified as having no RA4 positive cells while neurogenic tissue did have RA4 positive cells. This was used to locate the neurogenic front.

Microscopy. Fluorescence signals were viewed with an Olympus Fluoview TM FV1000 confocal microscope. The sections processed for immunohistochemistry with RA4 were used to locate the neurogenic front as indicated by the detection of differentiated ganglion cells. The neurogenic front was then located on the nearby section processed with adapted immunohistochemistry by using an identifying cell or feature of the tissue. An area of the retina 100 microns in length was located 50 microns from the neurogenic front on the both the preneurogenic and neurogenic sides. All of the cells present in this area were counted using the DAPI staining, as well as the number of cells labeled with Cy3, indicating CldU incorporation and the number labeled with Cy2, indicating Idu incorporation. The following equation was used to determine cell cycle time in preneurogenic and neurogenic retina. This was performed for both nasal and temporal neurogenic fronts and the calculated cell cycle time from three tissue sections was averaged per eye.

$$T = \frac{D_1 - D_2}{P_3}$$

$$S = P_1 T - D_1 - 2M$$

D_1= incubation time after Idu injection

D_2= incubation time after CldU injection

P_3=Percent of cells with only Idu incorporated

T= total cell cycle length

M=minimum time required for cells to incorporate a label= 10 minutes

Results

We used the above equation to calculate cell cycle length for preneurogenic and neurogenic progenitor cells to determine if there was a difference as development progresses. In order to determine the number of cells labeled by the two injected thymidine analogs, we first located the neurogenic front by identifying the most peripherally positioned ganglion cell as visualized using immunohistochemistry with RA4 (Waid and McLoon, 1995). Portions of 100 μm of preneurogenic and neurogenic retina located 50 μm from the neurogenic front were counted in both eyes of a tissue preparation and from both nasal and temporal sides of the retina. Three counts were performed of different tissue preparations in each retina and cell cycle length was calculated using the above equation. These values were averaged to find the cell cycle length of preneurogenic and neurogenic progenitor cells. The average cell cycle length in nasal retina of all eyes counted was 12.84 hours for preneurogenic progenitor cells and 20.54 hours for neurogenic progenitor cells. The average length of the cell cycle in progenitor cells in temporal preneurogenic retina was 12.19 hours and 20.11 hours in neurogenic retina. We consistently found that the cell cycle was longer in neurogenic progenitor cells than in preneurogenic progenitor cells. There was no significant difference in cell cycle length in nasal versus temporal retina.

We then asked if this observed difference in cell cycle length was due to a gradient of increasing length as development progresses peripherally in retina or if it is a fundamental

difference in preneurogenic and neurogenic progenitor cells. We explored this by counting the number of cells labeled with BrdU in another 100μm portion of preneurogenic and neurogenic retina located 50μm from the previously counted portion, therefore 200μm from the neurogenic front, and calculating cell cycle length. There was no significant difference in cell cycle length for either preneurogenic or neurogenic progenitor cells between these portions of retina farther from the neurogenic front and those closer. These findings suggest that cell cycle length is fundamentally different in preneurogenic and neurogenic progenitor cells.

The validity of the equation used in this study was confirmed by varying survival times after the second injection with CldU. After being injected with CldU, embryos were harvested after either 30 minutes or one hour. There was consistently a larger percentage of cells that only incorporated the first label, IdU, and not the second, CldU, when the embryos were harvested 30 minutes after the second injection than those harvested after one hour. Calculation of cell cycle length resulted in similar values for both preneurogenic and neurogenic progenitor cells at both survival times after injection two.

Discussion

Progenitor cells are present in the early developing retina as either preneurogenic, in which they divide without generating postmitotic cells, or neurogenic, which do generate differentiated, postmitotic cells. These two types of progenitor cells are found in the early developing retina on either side of the neurogenic front, with development progressing peripherally. The fundamental differences between preneurogenic and neurogenic progenitor cells have not been completely determined. It has previously been shown that cell cycle length increases throughout development, but we asked if cell cycle length was intrinsically different in preneurogenic verses neurogenic progenitor cells. In this study we injected two thymidine analogs into a developing chick embryo and detected them using immunohistochemistry to

determine cell cycle length in preneurogenic and neurogenic retina. We located the neurogenic front using immunohistochemistry with RA4 and counted the number of unlabeled, single and double labeled cells in portions of preneurogenic and neurogenic retina. We used a novel equation and found that the cell cycle is significantly longer in neurogenic progenitor cells than in preneurogenic progenitor cells.

The method used in this study provides an effective way to measure cell cycle length. Cell cycle length can be calculated by injecting cells twice with BrdU and subsequently performing an adapted version of immunohistochemistry to detect the cells that have incorporated the labels into their DNA during S phase. Counting the number of cells labeled with the first but not the second BrdU label and the total number of cells and then using the equation presented in this study results in cell cycle length. The survival times each after injection of BrdU can be varied to further explore cell cycle length.

The data in this study provides evidence that cell cycle length is fundamentally different in preneurogenic progenitor cells and neurogenic progenitor cells. Neurogenic progenitor cells have a significantly longer cell cycle than preneurogenic progenitor cells. This is consistent with previous findings that cell cycle length increases as development progresses.

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