Int J Clin Exp Pathol 2017;10(12):11679-11684
www.ijcep.com /ISSN:1936-2625/IJCEP0065684

Original Article

Dynamaic changes of proliferation and apoptosis in rat retina development

Weibo Shi[1*], Ran Wang[2*], Shiba Niu[1], Yingmin Li[1], Chunling Ma[1], Guozhong Zhang[1], Bin Cong[1]

[1]Department of Forensic Medicine, Hebei Medical University, Hebei Key Laboratory of Forensic Medicine, Collaborative Innovation Center of Forensic Medical Molecular Identification, Shijiazhuang, China; [2]Department of Psychiatry, The First Hospital of Hebei Medical University, Shijiazhuang, China. [*]Equal contributors.

Received September 15, 2017; Accepted October 24, 2017; Epub December 1, 2017; Published December 15, 2017

Abstract: Precise coordination of cell proliferation and apoptosis is essential for proper organ morphogenesis and function during mammalian development. The retina is a part of the central nervous system, with a function in capture and conversion of light particles into nerve signals. However, the dynamical changes of cell proliferation and apoptosis during the retinal development is unclear. Thus, this study was to observe the changes of proliferating cell nuclear antigen expression and apoptosis during the retina development in rats. Rats from different developmental stages (embryonic days 14-20 (E14-20), postnatal days 0-15 (P0-15) and adult (P36)) were used. The eyeballs were removed and fixed immediately in formalin. The tissues were embedded in paraffin. Sections were prepared for immunohistochemical staining and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). PCNA expression appeared in large quantities in rat retina from E14 to P7, decreased obviously since P9, and could not be observed since P15. Apoptotic cells appeared in small quantities since E18. And then apoptotic cells dramatically increased during the retina development, and peaked at P7. As maturation continued, these cells decreased gradually. Apoptotic cells could not be found in the mature retina. Cell proliferation and apoptosis shows orderly and dynamic changes during the retina development, which is essential for the development of normal structure and function of the retina.

Keywords: Retina, development, proliferating cell nuclear antigen, proliferation, apoptosis

Introduction

Cell proliferation, apoptosis and the balance between them are essential for proper tissue morphogenesis, homeostasis and function [1]. Both cell proliferation and apoptosis are required for sel-renewal, maintenance and adaptation of tissues to physiological stimuli by allowing an organism to control cell number and tissue volume [2, 3]. The retina, at the back of the eye, is a part of the central nervous system, with a function in capture and conversion of light particles into nerve signals. Previous studies have indicated that the retina development is a significant and orderly spatial-pattern development process [4-7]. However, it is difficult to observe the whole process of the human retina development, thus systematic investigating cell proliferation, differentiation and apoptosis in rat retina development will be an effective approach, which contribute to better understand the mechanism of retina development.

Proliferating cell nuclear antigen (PCNA) [8-10], belonging to the DNA sliding clamp family, is the auxiliary protein of DNA-poly-merase δ [11]. It increases during the late part of the G1 phase of the cell-division cycle, peaks during the S phase, and then decreases during the G2 and M phases. These changes occur at the same rate as the changes in DNA synthesis. Therefore, PCNA is considered as an essential factor for cell proliferation and is used as an index of cell proliferation [12, 13]. TdT-mediated dUTP nick-end labeling (TUNEL) is a simple, sensitive and reliable method that can selectively mark apoptotic cells in tissues. For the present study, we investigated the dynamic changes of PCNA and apoptotic cells in different durations of retinal development to provide morphological evidence for better understanding the mechanism of

retinal development and the diseases induced by abnormal retinal development.

Materials and methods

Animals

Healthy and pregnant Wistar rats (Experimental Animal Center, Hebei Medical University, China) were adapted to a 12/12 h light/dark cycle, with free access to food and water. This study was approved by the Institutional Review Board for Animal Experiments at Hebei Medical University. Every attempt was made to reduce the number of animals and to minimize pain and suffering. Rats from different developmental stages [embryonic days 14-20 (E14-E20), postnatal days 0-15 (P0-15) and adult (P36)] were used in experiments. The rats were deeply anesthetized by inhalation of diethyl ether (Tianjin Precision Chemical Reagent Factory, China) and were sacrificed by cervical dislocation.

Reagents

Mouse monoclonal anti-PCNA antibody was purchased from Abcam (UK, CN: ab29), mouse monoclonal anti-β-actin antibody was purchased from Abcam (UK, CN: ab8229), the immunohistochemical kit was purchased from Zhongshan Goldenbridge Biotech (China, CN: SP901), the second antibody of immunoblot was purchased from Rockland (USA), and the TUNEL kit was purchased from Promega (USA, CN: #G7132).

Immunohistochemistry

The eyeball tissue was harvested and fixed immediately in 10% formalin. The tissue was subsequently dehydrated in graded ethanol series and embedded in paraffin. Deparaffinized sections were pretreated using microwave antigen retrieval, followed by incubation in 3% H_2O_2 in cold methanol for 30 min, and goat serum for 30 min. Next, the tissues were incubated with monoclonal antibodies specific for mouse PCNA (1:100) overnight at 4°C. The tissues were then incubated for 1 h with biotinylated secondary antibody and subsequently with horseradish peroxidase (HRP)-conjugated biotin for 30 min. Finally, 3,3'-diaminobenzidine (DAB) was used as the chromagen. The tissues were counterstained with hematoxylin to visualize locations in the sections.

Immunoblotting

Five rats from different development stages were used for immunoblotting. Tissue extracts (50 μg of protein/lane) were loaded, separated by SDS-PAGE and transferred to PVDF membranes. The membranes were incubated overnight at 4°C with anti- PCNA (1:150) and β-actin antibodies. Then, incubation with horseradish peroxidase-conjugated goat anti-mouse IgG was performed. For the X-ray film exposure, the enhanced chemiluminescence system was employed. The optical densities of the bands were measured using LabWorks 4.5 software. PCNA expression was quantified with respect to the signals of the corresponding β-actin band.

TUNEL assay

The TUNEL assay was used assess the extent of apoptosis in the treated cells. The slides were washed with PBS and fixed using 4% paraformaldehyde. The slides were stained by diaminobenzidine to nicked ends of DNA and then visualized with a microscope.

Positive cell counting

Five rats from each group were used for morphological observation. Using the serial section technique, we took one out of every five sections and selected a total of three sections for each rat. The number of apoptotic cells in each section was counted (per 400× field of view) in five view fields. Two independent observers who were blinded to the experimental conditions performed counts and calculated the average number of positive cells.

Statistical analysis

Statistical analysis was performed by one-way ANOVA. Student's *t*-test was used when comparisons were restricted to two experiment groups. The results are presented as mean ± SEM. The threshold for statistical significance was defined as $P<0.05$.

Results

PCNA immunohistochemical staining

PCNA-positive cells are proliferating cells during the cell cycle. PCNA, which is expressed in the nucleus, was stained brown by immunohis-

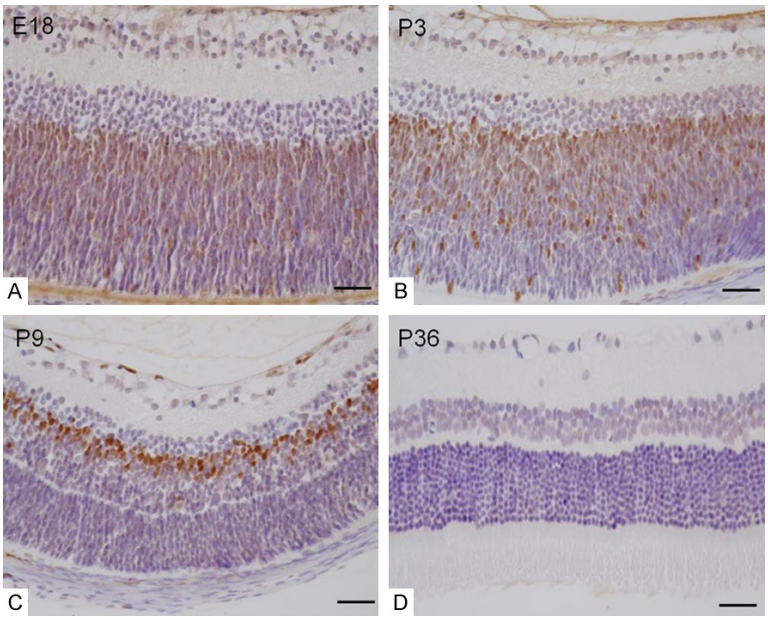

Figure 1. PCNA expression in retina development. (A-D) Show the PCNA expression in retina development at E18, P3, P9 and P36, respectively. PCNA expression occurred at high levels in the early stages of development (A and B). PCNA expression decreased obviously from P9 (C) and could not be observed at P36 (D). Bars =25 μm.

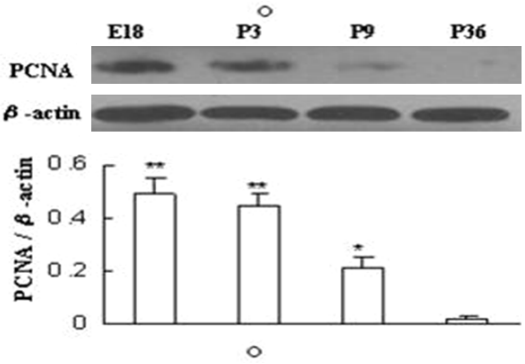

Figure 2. Quantitative analysis of PCNA protein. With the retina development, PCNA expression was gradually decreased. And only a little of PCNA was detected at P9 and P36. The results are shown as the mean ± SEM, $^{**}P<0.01$, $^{*}P<0.05$ vs. P36 (n=5).

tochemical staining. PCNA expression occurred at high levels in the early stages of development (E14-P7) (**Figure 1A**, **1B**). As cells matured, it showed significant dynamic changes. The number of PCNA decreased obviously from P9 (**Figure 1C**), and could not been found from P15 to mature stages (**Figure 1D**).

Quantitative analysis of PCNA protein

PCNA expression was gradually decreased during the retina development. And only a little of PCNA was detected from P9 to P36 (**Figure 2**).

TUNEL staining

Apoptotic cells were first observed in small quantities in rat retina at E14 (**Figure 3A**). Then, with the retina development, the number of apoptotic cells increased. Apoptotic cells significantly upregulated at P5 (**Figure 3B-E**), and peaked at P7 (**Figure 3F**). As the cells matured, apoptotic cells gradually decreased. There was no obvious apoptotic cells from P15 to P36 (**Figure 3G-I**). Besides, no obvious regional distribution in apoptotic cells, they could be found in the ganglion cell layer, inner nuclear layer and outer nuclear layer.

Quantification of apoptotic cells

Apoptotic cells appeared dynamic changes during the retina development, and the number of apoptotic cells peaked at P7 (**Figure 4**).

Discussion

During organogenesis, tissue architecture and cell number are tightly controlled by balancing proliferation and cell death [2, 3]. Cells are initially organized into a loose pattern, and then selective programmed death removes undesired cells, just as a sculptor removes some material to reveal the hidden image. The retina, as a part of the central nervous system, is also tightly controlled by cell proliferation and apoptosis during its development.

PCNA, as a significant regular factor in cell division and proliferation, increases during the late part of the G1 phase, peaks during the S phase and then decreases during the G2 and M phase, which is consisted with the rate of DNA synthesis. Thereby, it is regarded as a routine indicator of cell proliferation status [12, 13]. In the present study, PCNA-positive cells first appeared in the early embryo, and exhibited a trend of increasing during the development of retinal nerve in rats. However, with the gradual differentiation and maturation of the retinal cells the PCNA expression decreased. These

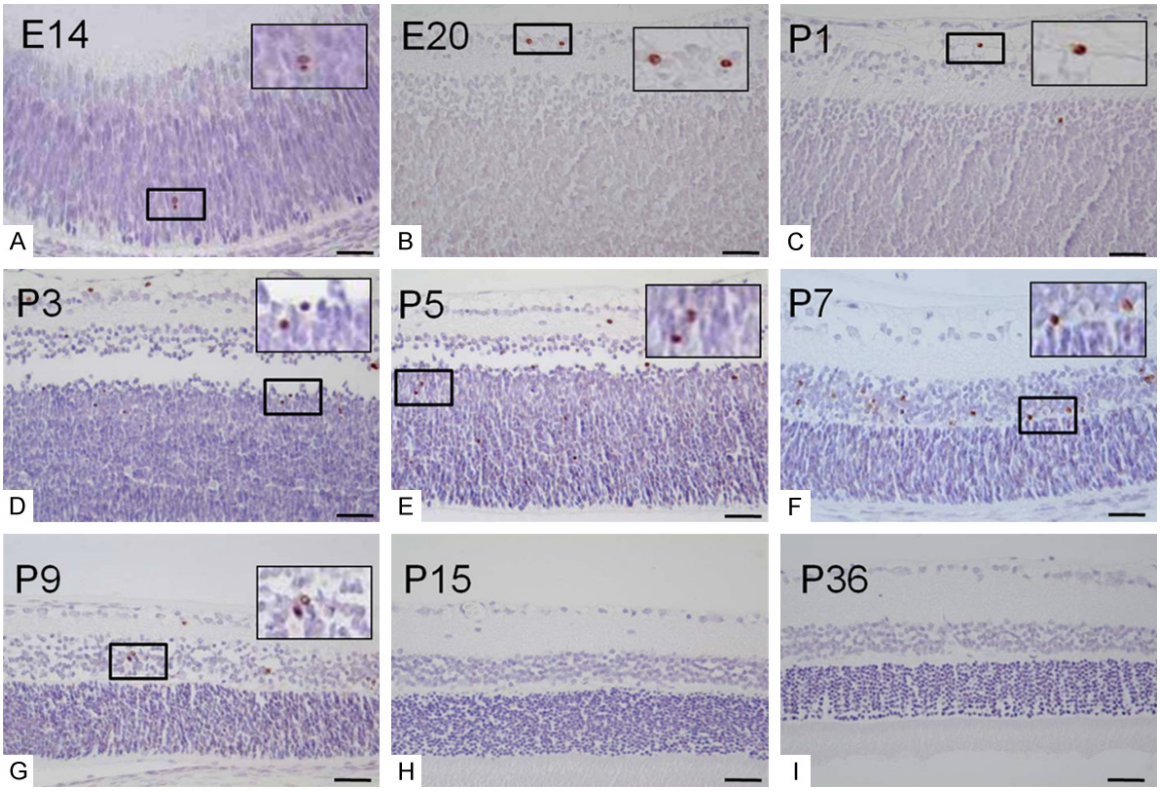

Figure 3. TUNEL staining. (A-I) Show the expression of apoptotic cells in retina development at E14, E20, P1, P3, P5, P7, P9, P15 and P36, respectively. The images in the upper right corner are magnification of the area in the boxes. With the retina development, apoptotic cells were significantly increased and peaked at P7 (A-F). As the cells matured, apoptotic cells gradually decreased, and could not be found in mature retinal cells (G-I). Bars =25 μm.

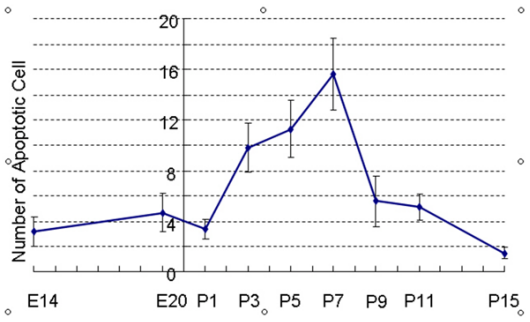

Figure 4. Quantification of apoptotic cells in retina development at different times. Apoptotic cells appeared dynamic changes during the retina development, and the number of apoptotic cells peaked at P7. The results are shown as the mean ± SEM (n=5).

results suggested that the related proteins that affect cell proliferation have achieved dynamic balance and the proliferation ability of cells has decreased significantly in the later stage of retinal neurodevelopment. As broadly documented in the literature, many regulatory proteins and signaling pathways are involved in regulating the proliferation of retinal nerve cells in rats. Pax6, as the master factor of eye morphogenesis control, takes part in the proliferation and differentiation of the retinal [14]. Pou4f2 and Isl1, controlled by intrinsic and extracellular factors, precisely regulate the retinal proliferation [15]. In addition, the Hippo pathway and Wnt/Frizzled pathway have a critical role in the different structures of the eye. These ways are involved in many processes, such as cellular proliferation and differentiation, especially in the central nervous system [16-18]. Combined with previous researches and the present study, we can easily get a conclusion that because of co-regulation in all kinds of signal pathways, proliferative related regulation proteins are always maintain in a state of dynamic balance, and the retinal nerve cells display obviously dynamic changes during its development.

The apoptosis of nerve cells has a unique biological significance in the development of retina. From one simple neural plate to the exqui-

sitely constructed and finely functional retina, the proliferation, migration and cell apoptosis play a critical role. Once the dynamic balance between proliferation and death in cells is disordered, the development of the retina is abnormal. In the present study, we observed the apoptotic changes from E14 to P36 of the development of retina in detail. Apoptotic cells could be found at E14 in retinal nerve cells, and with the development of retina, apoptotic cells exhibited a trend of first increasing and then decreasing, although in mature retina, no obviously apoptotic cells were observed. These changes basically corresponds to a previous mouse brain research, which indicated that apoptotic cells peaked at eight day after birth and almost no apoptotic rate was found at two weeks [19]. Of course, a variety of proapoptotic stimuli, including extracellular and intracellular signals, can result in apoptotic cell death. The extracellular signals include tumor necrosis factor (TNF) [20], Fas ligands (FasL) [21], and the absence of trophic factors [22]. DAN damage and endoplamic reticulum stress (ERS) [23] are examples of intracellular proapoptotic signals. These specific signals activate the intracellular pathways, leading to biochemical and morphological changes in retinal nerve cells.

In a conclusion, this study systematically observed the dynamic changes of cell proliferation and apoptosis in rat retinal development in detail from the perspective of morphology, which contribute to better understand the mechanism of retina development and pathological changes. The orderly balance between proliferation and apoptosis is the basis of normal retinal structure and function, and those regulation factors directly involved in regulating balance will be the focus of our further studies.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (81571850) and the Natural Science Foundation of Hebei Province (H2017206231).

Disclosure of conflict of interest

None.

Address correspondence to: Drs. Bin Cong and Guozhong Zhang, Department of Forensic Medicine, Hebei Medical University, Hebei Key Laboratory of Forensic Medicine, Collaborative Innovation Center of Forensic Medical Molecular Identification, Shijiazhuang 050017, China. Tel: 86 311 86266406; E-mail: cong6406@126.com (BC); Tel: 86 311 86261004; E-mail: zhanggz3@126.com (GZZ)

References

[1] Biebl M, Cooper CM, Winkler J, Kuhn HG. Analysis of neurogenesis and programmed cell death reveals a self-renewing capacity in the adult rat brain. Neurosci Lett 2000; 291: 17-20.

[2] Karl J Wahlin, Raymond A Enke, John A Fuller, Giedrius Kalesnykas, Donald J Zack, Shannath L Merbs. Epigenetics and cell death: DNA hypermethylation in programmed retinal cell death. PLoS One 2013; 8: e79140.

[3] Raquet M, Brun C, Exbrayat JM. Patterns of apoptosis and proliferation throughout the biennial reproductive cycle of viviparous female typhlonectes compressicauda (Amphibia, Gymnophiona). Int J Mol Sci 2017; 18: 16.

[4] Elena Vecino and Arantxa Acrea. Development and programmed cell death in the mammalian eye. Int J Dev Biol 2015; 59: 63-71.

[5] Browne AW, Arnesano C, Harutyunyan N, Khuu T, Martinez JC, Pollack HA, Koos DS, Lee TC, Fraser SE, Mats RA, Aparicio JG, Cobrinik D. Structural and functional characterization of human stem-cell-derived retinal organoids by live imaging. Invest Ophthalmol Vis Sci 2017; 58: 3311-3318.

[6] Lonfat N, Cepko C. Epigenomics of retinal development in mice and humans. Neuron 2017; 94: 420-423.

[7] Chavarría T, Baleriola J, Mayordomo R, de Pablo F, de la Rosa EJ. Early neural cell death is an extensive, dynamic process in the embryonic chick and mouse retina. ScientificWorldJournal 2013; 2013: 627240.

[8] Burgers PM. Mammalian cyclin/PCNA (DNA polymerase delta auxiliary protein) stimulates processive DNA synthesis by yeast DNA polymerase III. Nucleic Acids Res 1988; 16: 6297-6307.

[9] Moldovan GL, Pfander B, Jentsch S. PCNA, the maestro of the replication fork. Cell 2007; 129: 665-679.

[10] Dieckman LM, Freudenthal BD, Washington MT. PCNA structure and function: insights from structures of PCNA complexes and post-translationally modified PCNA. Subcell Biochem 2012; 62: 281-299.

[11] Li J, Holzschu DL and Sugiyama T. PCNA is efficiently loaded on the DNA recombination intermediate to modulate polymerase delta, eta, and zeta activities. Proc Natl Acad Sci U S A 2013; 110: 7672-7677.

[12] Park HR, Park YK. Expression of p53 protein, PCNA, and Ki-67 in osreosarcomas of bone. J Korean Med Sci 1995; 10: 360-367.
[13] Li Y, Qi Q, Cong B, Shi W, Liu X, Zhang G, Ma C. Expression patterns of Nurr1 in rat retina development. J Mol Histol 2012; 43: 633-9.
[14] Carla S. Lopes, Fernando Casares. Eye selector logic for a coordinated cell cycle exit. PLoS Genet 2015; 11: e1004981.
[15] Wu F, Kaczynski TJ, Sethuramanujam S, Li R, Jain V, Slaughter M, Mu X. Two transcription factors, Pou4f2 and Isl1, are sufficient to specify the retinal ganglion cell fate. Proc Natl Acad Sci U S A 2015; 112: E1559-E1568.
[16] Asaoka Y, Hata S, Namae M, Furutani-Seiki M, Nishina H. The Hippo pathway controls a switch between retinal progenitor cell proliferation and photoreceptor cell differentiation in zebrafish. PLoS One 2014; 9: e97365.
[17] Ang SJ, Stump RJ, Lovicu FJ, McAvoy JW. Spatial and temporal expression of Wnt and Dickkopf genes during murine lens development. Gene Expr Patterns 2004; 4: 289-295.
[18] Liu H, Thurig S, Mohamed O, Dufort D, Wallace VA. Mapping canonical Wnt signaling in the development and adult retina. Invest Ophthalmol Vis Sci 2006; 47: 5088-5097.
[19] Cheng XS, Du YW, Jiang QY, Hu YQ, Li MS, Yu DM, Deng JB. Neuroapoptosis of cerebellar cortex during the development in mouse. Acta Anatomica Sinica 2008; 39: 470-474.
[20] Zhang Q, Jiang Y, Miller MJ, Peng B, Liu L, Soderiand C, Tang J, Kern TS, Pintar J, Steinle JJ. IGFBP-3 and TNF-α regulate retinal endothelial cell apoptosis. Invest Ophthalmol Vis Sci 2013; 54: 5376-84.
[21] Lin CH, Wu MR, Li CH, Cheng HW, Huang SH, Tsai CH, Lin FL, Ho JD, Kang JJ, Hsiao G, Cheng YW. Editor's highlight: periodic exposure to smartphone-mimic low-luminance blue light induces retina damage through Bcl-2/BAX-dependent apoptosis. Toxicol Sci 2017; 157: 196-210.
[22] Von Bartheld CS, Johnson JE. Target-derived BDNF (brain-derived neurotrophic factor) is essential for the survival of developing neurons in the isthmo-optic nucleus. J Comp Neurol 2001; 433: 550-64.
[23] Yan Q, Zhu H, Wang FH, Feng JY, Wang WQ, Shi X, Zhou YP, Zhang X, Sun XD. Inhibition of TRB3 protects photoreceptors against endoplasmic reticulum stress-induced apoptosis after experimental retinal detachment. Curr Eye Res 2016; 41: 240-8.