ROLE OF APOPTOSIS AND PROLIFERATION IN DIFFERENTIATION OF HUMAN RETINA, REGIO OLFACTORIA AND REGIO RESPIRATORIA

Michaela Kubíniová*, Eva Pospíšilová

Department of Histology and Embryology, Medical Faculty, Palacký University, Hněvotínská 3, 775 15 Olomouc, Czech Republic, e-mail: kubinyi@centrum.cz

Received: November 21, 2001

Key words: Development / Bcl-2 protein / PCNA protein / KI-67 protein / TUNEL / Apostain / Immunohistochemistry

Apoptosis and proliferation are very important processes during central nervous system development. In our study we concentrated on human fetus retina, regio respiratoria and olfactoria. The aims of our work were to evaluate a proliferative activity proved by PCNA and KI-67 gene expressions, to measure an expression of anti-apoptotic gene bcl-2, and finally to compare levels of apoptosis and proliferation in the areas investigated. Altogether we studied 29 fetuses between the 5th to 13th week of gestation (WG). Parallel sections were elaborated by using standard methods of immunohistochemistry for the detection of proteins PCNA, Bcl-2 and Ki-67. Apoptotic cells were detected by two methods: TUNEL (for the detection of DNA fragmentation) and Apostain (for evidence of ssDNA). The results of semiquantitative evaluation show that expressions of proteins PCNA and KI-67 begins after the 6th WG, from the 8th to 13th WG they are very high in all investigated areas. Occurrence of apoptotic cells in regio respiratoria is sporadic between the 5th to 9th WG, while later it is widely spread. Apoptosis in regio olfactoria appears after the 9th WG and it is also very extensive. In the retina we found a higher frequency of apoptotic cells only in the 11th to 13th WG. The results of both methods for detection of apoptosis were concordant. Anti-apoptotic gene bcl-2 was detectable in all examined areas, but it followed no orderly pattern. We conclude, that the expression of apoptosis increases with the age from the 7th to 13th WG, and that both apoptosis and proliferation participate in the morphogenesis of fetuses between the 5th to 13th WG.

INTRODUCTION

PCNA and Ki-67 antigens are endogenous markers of proliferative activity mostly used in the world. Especially PCNA, an auxiliary protein of DNA-polymerase delta, offers reliable information of proliferation. It participates in synthesis and – if there is – in a reparation of DNA. Ki-67 is a protein with short biological half – life time which is a disadvantage for the evaluation.

Apoptosis (as one of major ways of programmed cell death) is an active, genetically controlled process leading to a cell death, characterized by typical morphological and functional changes^{1–6}. These were described by J. F. Kerr, A. H. Willie and A. R. Currie in 1972⁴ as shrinkage of the dying cell's nucleus, rippling of its membrane (zeiosis) while preserving its integrity, loss of volume of the cytoplasm and cell disintegration into apoptotic bodies subsequently phagocyted by surrounding cells without any inflammatory reaction.

After the initiation of apoptosis follows the activation of the cell's proteolytic system with subsequent breakdown of structural proteins (e. g. lamin B, cyto-keratin 18) and transfer of cell membrane phosphatidylserine from the inner to the outer surface serving as a signal for phagocytic cells. The process continues by the DNA cleavage executed by specific endonucleases giving birth to fragments of mono- to poly- nucleosomal

length and the outcome is the creation of apoptotic bodies.

Apoptosis may be triggered by various stimuli. It may be a physiological termination of a cell's life as well as response to a particular pathological process. The apoptosis is intricate, multilevel, genetically regulated process. Specific proapoptotic (bax, p53)⁸ and anti-apoptotic (bcl-2)^{9–11} genes as well as a cascade of cytoplasmic proteases – caspases are involved. Apoptosis in a damaged or old cell is very fast and leaves no traces.

Apoptosis and proliferation are processed maintaining the organism's homeostasis. As mentioned above, apoptosis means elimination of cells without inflammatory reaction, i. e. without damage of the surroundings.

Blockade of apoptosis provides conditions for the development of tumour process: e. g. an accumulation of lymphocytes leads to a development of a lymphoma. Failure of apoptosis can be also seen in autoimmune diseases (rheumatoid arthritis, autoimmune diabetes, systemic lupus erythematoses etc.). Pathologically increased occurrence of apoptosis was observed in AIDS where apoptosis is caused by CD4+ receptor stimulation by viral protein. Myelodysplastic syndrome, thalassemia, ischemic disease and neurodegenerative diseases (e. g. Alzheimer's disease) are also based on inadequate stimulation of receptors triggering apoptosis.

One of major roles of apoptosis is its contribution to morphogenetic processes in the course of embryonic

development. It is known that apoptosis occurs in the course of differentiation of cell layers within the retina, but the function of this phenomenon has not been cleared yet satisfactorily. Olfactory cells represent a unique model of neurons the number of which is maintained by the dynamic process of cell death and proliferation.

MATERIAL AND METHODS

The research was performed on 29 human embryos aged 5–13 weeks. The material was fixed in methacarn and embedded in paraffin. For the detection of PCNA, Ki-67 and Bcl-2 proteins standard indirect 3-step immunohistochemical method was used. The studied protein was being subsequently bound to primary monoclonal mouse antibody PC-10 (Masaryk Institute of Oncology), or MIB-1 (Bio-Genex), or anti Bcl-2 Oncoprotein (Bio-Genex), then to secondary rabbit biotinilated anti-mouse antibody (Bio-Genex), creating conjugate with streptavidine-peroxidase complex (Bio-Genex). Visualization was performed after addition of hydrogen peroxide (H_2O_2) by diaminobenzidine. Positive reaction was marked by brown colouring of cytoplasm (Bcl-2 and Bax) or nucleus (PCNA and Ki-67).

For detection of apoptosis TUNEL and Apostain methods were used. TUNEL (Terminal dNTP tranferase mediated dUTP nick end labeling)⁷. The method is based on the proof of numerous DNA fragments in apoptotic cells. After the removal of masking nucleoproteins by microwave activity in acid environment (pH 6,0) fluoresceinated nucleotides are transferred by TdT to free 3'OH termini of all the fragments. Then fluorescein bounds anti-fluorescein antibody/alkaline phosphatase conjugate. After incubation with chromogenic substrate consisting of oxidant and the substrate for alkaline phosphatase itself (NBT/BCIP) the nuclei are stained dark blue in case of positivity.

The other method for evaluation of apoptosis was Apostain. It is also a 3-step immunohistochemical method based on the detection of single-stranded DNA in cell nuclei. It proves the earlier phases of apoptosis than TUNEL. The primary and secondary substances were mouse anti-human F7-26 (Alexis Biochemicals) and biotinilated anti-mouse Ig (Bio-Genex), respectively. Incubation was performed using streptavidine – alkaline phosphatase conjugate and visualization was reached by incubation with substrate buffer containing NBT/BCIP and levamisole as a inhibitor of endogenic phosphatases. Again, the nuclei of apoptotic cells were stained dark blue in case of positivity.

The sections were evaluated using Olympus BX 40 microscope. For description of results the semiquantitative evaluation was used as follows:

- + sporadic occurrence of positive cell or nuclei
- ++ groups of positive cells or nuclei
- +++ massive occurrence of positive cells or nuclei

RESULTS

The results of semiquantitative evaluation are listed in Tables 1–4 (see appendix). Evaluation criteria are as stated above.

1. Retina proliferation (see Table 1) starts from week 7 of the fetus development when the occurrence was observed in groups only (++) but starting from week 8 it is massive (+++). Apoptosis in retina was first observed in the future ganglion layer (GCL-ganglion Cell Layer) in week 7. In week 13 apoptosis is still present in GCL but also in the area of rods and cones (INL-Inner Nuclear Layer): (+++). Anti-apoptotic protein Bcl-2 was only sporadic in retina during weeks 7-9 and is more frequent in week 10.

Table 1. Semiquantitative evaluation of observed reactions in retina

Age:	PCNA	Ki-67	TUNEL	Apostain	Bcl-2
5/6 w.	0	0	0	+	0
7 w.	++	0	+	++	+
8 w.	+++	++	+	++	+
9 w.	+++	+++	0	++	+
10 w.	+++	++		0	++
11 w.	+++	+++	+++	+++	0
12 w.	+++				
13 w.	+++	+++	0	+++	+

2. In regio respiratoria (Table 2) the onset of proliferation is in week 7 and it stays constantly high (++/+++) until week 13. Apoptosis is detectable as early as in week 5, with sporadic frequency (+) unchanged until week 10. From week 11 on the occurrence is more massive (+++). During the whole observed period anti-apoptotic protein Bcl-2 was only sporadically present (+/+ +).

Table 2. Semiquantitative evaluation of observed reactions in regio respiratoria

Age:	PCNA	Ki-67	TUNEL	Apostain	Bcl-2
5/6 w.	0	0	+	+	+
7 w.	++	0	++	+	+
8 w.	+++	+	+	+	++
9 w.		+	+	+	0
10 w.	+++	++	++	+	+
11 w.	++	++	+++	+++	++
12 w.	+++	++	+++	+++	
13 w.	++	++		+++	+

3. In regio olfactoria (Table 3) proliferation is observed from week 7 and the frequency fluctuates between ++ and +++ until week 13. The onset of apoptosis is as late as in week 10 with high frequency (+++). The occurrence of anti-apoptotic protein Bcl is mostly sporadic (+) with the exception of week 9 (++).

Table 3. Semiquantitative evaluation of observed reactions in regio olfactoria

Age:	PCNA	Ki-67	TUNEL	Apostain	Bcl-2
5/6 w.	0	0	0	+	0
7 w.	++	0	0	0	0
8 w.	+++	+	0	0	+
9 w.		++	+	+	++
10 w.	+++	++	+++	0	0
11 w.	++	+++	+++	+++	+
12 w.	+++	++	+++	+++	
13 w.	++	++		+++	+

4. In the lens (Table 4) the proliferation is seen from week 8, mainly in the frontal epithelium. Apoptosis is not very frequent and can be proved especially using Apostain method: sporadic (+) in weeks 5–10, more frequent from week 11 (++/+ + +). Apoptosis is also localized in the frontal epithelium of the lens. Bcl-2 protein was not proved (0).

Table 4. Semiquantitative evaluation of observed reactions in lens

Age:	PCNA	Ki-67	TUNEL	Apostain	Bcl-2
5/6 w.	0	0	0	+	0
7 w.		0	+		
8 w.	+++	+		+	
9 w.		++	0	+	
10 w.					
11 w.	+++	++		++	
12 w.					
13 w.				+++	

N. B.: Empty cells in the tables mean that suitable material was not available for the given reaction at particular age. The reactions were not evaluated.

DISCUSSION

Proliferation in observed areas begins in week 7 of the intrauterine development and stays intensive until the end of observed period, i. e. week 13. It contributes to differentiation of human retina, lens, regio respiratoria and regio olfactoria.

High proliferative activity is confirmed by protein Ki-67 with lower sensitivity (shorter biological half time) to proliferation compared with PCNA protein and yet can be proven.

Proliferation is not equally frequent in all areas of CNS. Unlike Dana Kylarová (a student at the Faculty of Medicine, Palacký University in Olomouc, a work called Proliferative activity, apoptosis and their regulation mechanisms in the development of human fetus CNS) we found out that e. g. in the spinal cord coat and germinal layers proliferation is highest in weeks 7–8 of the intrauterine development and its frequency subsequently declines to zero (week 18). We assume that proliferation process is not analogous in different CNS areas.

Apoptosis in retina and regio respiratoria occurs in earlier phase of the development (week 7) while in regio olfactoria as late as in week 9. The occurrence is markedly higher in week 11 and stays very high.

Both methods proving apoptosis provide almost identical results.

Anti-apoptotic protein Bcl-2 expression was proved in the eye retina, mainly in the layer of light sensitive elements and the future layer of ganglion cells as well as in the epithelium of both areas of nasal cavity.

Our results concerning apoptosis in differentiating human retina are comparable to those in a similar study performed in quails¹².

Apoptosis is important for the differentiation of human retina, lens, regio respiratoria and regio olfactoria.

ACKNOWLEDGEMENTS

We would like to thank our collaborator Iva Ambrosova, who provided us with the material and then to thank Pavel Kurfürst, who translated this article to English.

REFERENCES

- Rosenbaum, P. S., Gupta, H., Savitz, S. I., Rosenbaum, D. M. (1997): Apoptosis in the Retina. *Clinical Neuroscience* 4, 224–232.
- Georges, P., Madigan, M. C., Provis, J. M. (1999): Apoptosis During Development of the Human Retina: Relationship to Foetal Development and Retinal Synaptogenesis. *The Journal of Comparative Neurology* 413, 198–208.
- Gut J. (1997): Apoptóza – Nový termín lékařského slovníku. *Praktický lékař* 77.
- Kerr, J. F. R., Wyllie, A. H., Currie, A. R. (1972): Apoptosis: a basic biological phenomenon with wide ranging implication in tissue kinetics. *Br. J. Cancer* 26, 239–257.
- Blaschke, A. J., Weiner, J. A., Chun, J. (1998): Programmed Cell Death Is a Universal Feature of Embryonic and Postnatal Neuroproliferative Regions Throughout the Central Nervous System. *The Journal of Comparative Neurology* 396, 39–50.
- Majno, J., Goris, I. (1995): Apoptosis, oncosis, and necrosis. *Am. J. Pathol.*, 146, 3–15.
- Gavrieli, Y., Sherman, Y., Ben-Sasson, S. A. (1992): Identification of programmed cell death in situ via specific labelling of nuclear DNA fragmentation. *J. Cell Biol.* 119, 493–501.
- Middleton, G., Nunez, G., Davies, A. M. (1996): Bax promotes neuronal survival and antagonises the survival effects of neurotrophic factors. *Development* 122, 695–701.
- Marry, D. E., Veis, D. J., Hickey, W. F., Korsmeyer, S. J. (1994): bcl-2 protein expression is widespread in the developing nervous system and retained in the adult PNS. *Development* 120, 301–311.
- Gonzales-Garcia, M., Garcia, I., Ding, L., O'Shea, S., Boise, L. H., Thompson, C. B., Nunez, G. (1995): bcl-x is expressed in embryonic and postnatal neural tissues and functions to prevent neuronal cell death. *Neurobiology* 92, 4304–4308.
- Novack, D. V., Korsmeyer, S. J. (1994): Bcl-2 protein expression during murine development. *Am. J. Pathol.* 145 (1), 61–73.
- Mar'in-Teva, J. L., Cuadros, M. A., Calvente, R., Almendros, A., Navascués, J. (1999): Naturally occurring cell death in migration of microglial precursors in the quail retina during normal development. *J. Comp. Neurol.* 19, 255–75.
- Berger, J. (2000): Biologie buněk. Nakladatelství Kopp, České Budějovice.

Fig. 1 Reaction for the detection of protein Bcl-2. All of studied areas. Fetus – week 8. Extension 100×.

Fig. 3 Apoptosis in a frontal epithelium of lens. Apostain. Fetus – week 11. Extension 200×.

Fig. 2 Detail of vomeronasal organ and regio respiratoria. Fetus – week 8. Extension 200×.

Fig. 4 Apoptosis in epithelium of nasal cavity. Apostain. Fetus – week 8. Extension 200×.