

# Involvement of pro-apoptotic and anti-apoptotic factors in the early development of the human pituitary gland

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**Summary.** The spatial and temporal pattern of appearance of pro-apoptotic caspase-3 and p53 proteins, and anti-apoptotic bcl-2 protein was investigated in the developing pituitary gland of 6 human embryos 5-8-weeks old, using morphological and immunohistochemical techniques. Their dynamic appearance was analyzed in the Rathke's pouch (future adenohypophysis), mesenchyme, and in the developing neurohypophysis. In the 5th and 6th week, caspase-3 positive cells appeared in the Rathke's pouch (5%) and stalk (11%), in the mesenchyme, but not in the neurohypophysis. In the 6th and 7th week, apoptotic cells were more numerous in the caudal part of the Rathke's pouch due to its separation from the oral epithelium. Pro-apoptotic p53 protein was detected in all parts of the pituitary gland throughout the investigated period. Nuclear condensations characterized cells positive to caspase-3 and p53 proteins. Apoptotic cells displayed condensations of nuclear chromatin on an ultrastructural level as well. While caspase-3 dependent pathway of cell death participated in morphogenesis of the adenohypophysis and associated connective tissue, p53-mediated apoptosis most likely participates in morphogenesis of all parts of the gland, including neurohypophysis. The anti-apoptotic bcl-2 protein was also detected in all parts of the developing gland. With advancing development, the positivity to bcl-2 protein increased in the cells of the adenohypophysis, while it decreased in the neurohypophysis. Bcl-2 protein probably prevented cell death in all parts of the gland and enhanced cell differentiation. The described pattern of appearance of the investigated pro-apoptotic and anti-apoptotic factors might be important for normal

morphogenesis and function of the pituitary gland.

**Key words:** Human embryos, Caspase-3, p53, Bcl-2, pituitary gland

## Introduction

The pituitary gland contains three lobes and is of dual embryonic origin. The anterior and intermediate lobes derive from Rathke's pouch, an invagination of the oral ectoderm, while the posterior lobe develops from the infundibulum, a stalk derived from the region of the ventral diencephalon (Moore and Persaud, 1993; Rizzoti and Lovell-Badge, 2005). The apposition of Rathke's pouch and the diencephalon is maintained throughout the early stages of pituitary organogenesis, thus suggesting that inductive tissue interactions are involved in the process of pituitary development (Takuma et al., 1998; Bazina et al., 2007). Dubois et al. (1997) reported that interactions between the adenohypophysis and neighboring tissues exist as early as the open neurula stage. The expression of a given phenotype by the committed cells is coordinated by a number of differentiation and transcription factors (Dubois et al., 1997). In this context, gene activation and expression of the controlling processes of cell proliferation, and the apoptosis and appearance of pituitary-specific transcription factors, allows the formation of a gland containing six cell types (Burgess et al., 2002). Expressed in a tightly regulated fashion, these factors ensure the dorso-ventral migration of Rathke's pouch, control cell determination and differentiation and maintenance of cell function (Sheng et al., 1997; Ericson et al., 1998; Takuma et al., 1998; Mullis, 2000; Rosenfeld et al., 2000; Dasen and Rosenfeld, 2001; Sbrogna et al., 2003; Asa and Ezzat, 2004).

The bcl-2 family members, caspase-3 and p53 proteins are only some of many factors that are involved in cell proliferation and apoptosis, as well as in the regulation of differentiation and growth processes. Expression of these genes appears to be important for growth and differentiation of different tissues during human development, as their occurrence is not only restricted to specific tissues, but also to specific stages of development (Miosge et al., 1997; Lichnovsky et al., 1998, 1999).

Apoptosis is a type of cell death in which cells die in a controlled and programmed manner. It is regulated by a number of different genes (Rezvani et al., 2000). It is characterized by a specific pattern of morphological features, which can be detected by electron microscopy (Anilkumar et al., 1992), DNA and biochemical assays or by *in situ* labelling techniques (Kapranos et al., 2004). Caspases are a large family of proteases that play an important role in cell cycle regulation, including caspase-3, which is the key effector caspase in the apoptotic process (Hengartner, 2000). The role of apoptosis and genes involved in its regulation during the development of different organs in human embryos and foetuses was studied by several authors (Koseki et al., 1992; James, 1994; Lichnovsky et al., 1998, 1999; Kim et al., 2000; Rezvani et al., 2000; Ketola et al., 2003; Bozanic and Saraga-Babic, 2004; Prochazkova et al., 2004; Carev et al., 2006; Vilovic et al., 2006). Apoptosis was also shown during the development of the mice immune and nervous system (Motoyama et al., 1995; Fujita et al., 2000; Klocke et al., 2002). However, there are no data on the role of apoptosis during normal development and growth of the human pituitary gland. On the other hand, apoptosis has been shown to be an important process in pituitary oncogenesis (Ozer et al., 2003) and in pituitary neoplastic lesions (Green et al., 1997; Kulig et al., 1999; Sambaziotis et al., 2003; Kontogeorgos, 2005, 2006).

Protection from apoptotic cell death is often associated with bcl-2 protein, an integral mitochondrial, nuclear and endoplasmatic reticulum membrane protein. Positive relationships between proliferation cell activity and bcl-2 presence in some tissues of developing human embryos and foetuses indicate its role in cell survival and control of cell division (Lichnovsky et al., 1998). In human embryos, bcl-2 expression was described in many developing organs, including the gastrointestinal tract, kidneys, central nervous system (Lichnovsky et al., 1999; Carev et al., 2006; Vilovic et al., 2006), cardiovascular system (Kim et al., 2000) and in testicular development (Ketola et al., 2003), but not in the pituitary gland.

The p53 is a negative regulator of G1-S phase transition in the cell cycle and may stimulate cell death in response to DNA damage. In human embryonic development p53 was detected in the kidney, intestine and lungs, liver, pancreas, heart and embryonic osteoblasts. In the central nervous system and kidneys of the same embryos, p53 was co-expressed with bcl-2

(Lichnovsky et al., 1998; Carev et al., 2006). Co-expression of p53 with c-erb-B2 or c-fos oncoproteins was found in specific tissues and at specific stages of human embryonic and foetal development (Miosge et al., 1997).

The present study is the first to report the pattern of appearance of apoptotic and anti-apoptotic factors during the earliest stages of human pituitary gland development. The aim of our study was to determine the spatial and temporal distribution pattern of bcl-2, caspase-3 and p53 antigens in correlation with important developmental processes of cell proliferation and apoptosis, which seem to be important for early morphology and differentiation of the human pituitary gland.

## Material and methods

### *Human material*

Human conceptuses between 5th and 8th week of development were collected after spontaneous abortions from otherwise healthy mothers in the Department of Gynaecology and Obstetrics, Clinical Hospital Centre of Split, Croatia, or after tubal pregnancies from the Department of Pathology, Clinical Hospital Centre of Split. The embryos and foetuses were examined macroscopically and measured. Only normal conceptuses, without any sign of abnormality, intrauterine death or macerations were used in our study. The embryonic tissues were treated as post mortem material with permission of the Ethics and Drug Committee of the Clinical Hospital Split in accordance with the 1964 Helsinki Declaration. The post-ovulatory age was estimated on the basis of menstrual data, ultrasonographic examination performed by a gynaecologist and by crown-rump length (CRL) measurements, which were afterwards correlated with Carnegie stages (O'Rahilly and Gardner, 1971) (Table 1). Every 10th slide was additionally stained by Haematoxylin and Eosin and examined under microscope to confirm the developmental stage (week) of each embryo.

### *Immunohistochemical staining*

#### a) Embedding and sectioning

Cranial parts of embryos containing developing pituitary gland were dissected. Tissue samples were

**Table 1.** Age and number of human conceptuses analysed in this study.

Age (weeks)	CRL (mm)	Carnegie stage	No.
5	8	15	1
6	14	17	1
7	23	20-22	2
8	23-32	21-23	2

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fixed in 4% paraformaldehyde in phosphate buffer and dehydrated in 100% ethanol. They were embedded in paraffin wax, serially sectioned as 4-6  $\mu\text{m}$  thick sections, mounted on glass slides, and examined with an Olympus BX51 light microscope (Olympus, Tokyo, Japan).

### b) Staining with bcl-2 and active caspase-3 antibodies

The paraffin was removed with xylene, and the sections were rehydrated in ethanol and water. In order to quench endogenous peroxidase activity, sections were incubated for 30 min in 0.1%  $\text{H}_2\text{O}_2$ .

Sections for immunohistochemical staining with bcl-2, p53 and caspase-3 antigens were treated with  $\text{H}_2\text{O}_2$ , washed in phosphate-buffered saline (PBS), and then incubated in sodium citrate buffer or EDTA (bcl-2 sections) for 10 min at 95°C. After being cooled to room temperature, they were incubated with rabbit anti-human/mouse active caspase-3 primary antibody (dilution 1:250; AF835, R&D Systems, Minneapolis, Minn., USA) and mouse anti-human bcl-2 oncoprotein primary antibody (dilution 1:50; M 0887, DAKO) overnight at 4°C in a humidified chamber. After being washed in PBS, the bcl-2, and caspase-3 sections were incubated with biotinylated secondary antibody (mouse and rabbit UniTect ABC Kit, Oncogene, Boston, Mass., USA) for 30 min at room temperature. They were then washed again in PBS and incubated with avidin biotinylated horseradish peroxidase complex (ABC) for 30 min, washed again with PBS, and then stained with diaminobenzidine tetrahydrochloride solution (DAB). Finally, the sections were rinsed in distilled water, counter-stained with haematoxylin, and dehydrated in ethanol and xylol.

### c) Staining with p53 antibody

The paraffin sections were incubated with mouse anti-human p53 protein (dilution 1:50; M 7001, DAKO) for 45 min. The primary p53 antibodies were detected using a streptavidin-biotin peroxidase system (K0690, DakoCytomation, Carpinteria, Calif., USA) as recommended by the manufacturer. The p53 sections were later washed with PBS, stained with DAB, counter-stained with haematoxylin and dehydrated as described above (Vojtesek et al., 1992; Huppertz et al., 1999).

Depending on the intensity of staining, the immunoreactivity of the pituitary cells was selected into 4 classes: strong staining (++), moderate staining (+), weak staining ( $\pm$ ) and absence of staining (-).

### Controls

Sections without primary antibodies incubation were used as negative controls. Positive controls were other tissues known to label specifically with primary antibodies (each section, besides pituitary gland, contained other organs such as brain, skull bones, cranial

ganglia, eye or ear primordial etc.).

### Quantification of apoptotic cells

The number of apoptotic cells was evaluated quantitatively by two independent investigators and classified as negative (no stained cells) and positive (stained cells) cells. Counts were made along the length of the pituitary gland separately for Rathke's pouch and stalk (5th and 6th week embryos) and adenohypophysis (7th and 8th week embryo). We used DP-SOFT version 3.1 software to divide each chosen part of pituitary gland section in squares of 50  $\mu\text{m}$  x 50  $\mu\text{m}$  at 40x magnification, and then we counted positive cell profiles in squares that were completely covered with cells. All together, there were 715 such squares. The cells below the left and upper border of squares were not taken into account, only those on the right and lower border. To avoid counting the same cell twice, we used every other consecutive section. The examination was performed on an Olympus BX51 microscope equipped with a DP11 digital camera and using DP-SOFT version 3.1 software. Quantitative analysis for caspase-3 was performed for three developmental periods (5-6, 7 and 8 weeks) according to the corresponding age of the embryo (Table 1). In each chosen area of the pituitary gland (50  $\mu\text{m}$  x 50  $\mu\text{m}$ ) the percentage of caspase-3 positive cells was calculated and expressed as mean  $\pm$  SD. Data were analysed by the Mann-Whitney test. Significance was accepted at  $p < 0.05$ .

### Light and electron microscopy

The cranial parts of 2 human conceptus (5 and 7 developmental weeks old) were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2), for 24 h at 4°C, post fixed in 2% osmium tetroxide and embedded in LX 112 Embedding Kit (Ladd Research, Williston, USA). Semi-thin sections, 1  $\mu\text{m}$  thick were stained with toluidine blue; adjacent ultrathin sections were stained with uranyl acetate and lead citrate (Reynolds, 1963). Possible early signs of morphological differences between cells were analysed in the adenohypophysis, using Olympus BX51 microscope. Electron micrographs were obtained with a Jeol 1200 EX microscope.

### Detection of apoptotic cells by TUNEL method

DNA fragmentation in apoptotic cells was detected with terminal deoxynucleotidyl transferase (TdT)-mediated nick-end labelling (TUNEL) method binding of fluorescein-labelled and unlabelled deoxynucleotides using the Fluorescein FragEL™ DNA Fragmentation Detection Kit (Calbiochem®, USA). Sections were deparaffinized and rehydrated and then pretreated with proteinase K for 20 min. Afterwards, sections were treated with equilibration buffer for 30 min, all at room temperature. The sections were later covered with

working TdT-labelling reaction mixture and incubated in a humidified chamber at 37°C for 1.5 h. After that procedure the sections were briefly stained with haematoxylin and mounted with the mounting media provided in the TUNEL kit. We replaced the block control with labeling safe buffer, and used the positive control section provided by the kit.

Labelled nuclei were examined with a standard fluorescein filter (465-495 nm). Microphotographs were captured with a SPOT Insight QE camera (Diagnostic Instruments, USA) mounted on an Olympus BX51 microscope using the SPOT software.

## Results

### *Anatomical relationship between developing Rathke's pouch and neurohypophysis*

In the 5th and 6th week of development, the Rathke's pouch was still connected to the pharyngeal epithelium by the epithelial strand (craniopharyngeal epithelial stalk). The cranial end of Rathke's pouch became wider and came into close vicinity to the developing neurohypophysis, represented by evagination of diencephalic wall called the infundibulum. Part of the head mesenchyme surrounding the pituitary primordium formed the source of future capsula.

### *Immunohistochemical (caspase-3) and morphological features of apoptotic cells and their quantification*

Caspase-3 positive cells were only occasionally seen at the cranial end of the Rathke's pouch and could be seen only at higher magnifications (Fig. 1A).

The most prominent morphological finding of developing pituitary gland during the 6th and 7th development week was separation of the Rathke's pouch from the primitive oral cavity. In association with this

process, numerous apoptotic cells were seen in the region of detachment of the Rathke's pouch from the pharyngeal epithelium, as well as in the part of the gland (epithelial stalk) that disappears during further development (11%) (Fig. 4), thus allowing the separation of the adenohypophysis from the oral cavity. The distal part of the Rathke's pouch (future adenohypophysis) contained a significantly lower number of apoptotic cells (5%) (Fig. 4) than the stalk area (Mann-Whitney,  $p=0.03$ ). When stained with apoptotic marker to caspase-3, numerous caspase-3 positive cells were detected around the presumptive lumen of the Rathke's pouch and inside its walls (Fig. 1B; Table 2). During the same developmental stage, most of the cells forming the walls of Rathke's pouch displayed ultrastructural features of vital cells: pale nuclei had prominent nucleoli, while the cytoplasm contained glycogen accumulations. Among vital cells, apoptotic cells with dark-staining nuclei or nuclear fragmentations could be seen as well (Fig. 1C).

During the 7th developmental week, part of the Rathke's pouch proliferated in a ventral direction and formed strands of epithelial cells separated by the mesenchymal tissue, thus forming the glandular-like primordium of pars distalis. Cells surrounding the remaining lumen of the Rathke's pouch mostly contributed to the pars intermedia. Several rows of mesenchymal cells around the gland condensed to form future connective tissue capsule. Caspase-3 positive cells or cell fragments were seen scattered within glandular cells, at the border of the residual lumen of the Rathke's pouch, as well as in the nearby mesenchyme (Fig. 1D, Table 2). During the 8th developmental week, the apoptotic cells were detected in the pars distalis, pars intermedia, and in the mesenchyme, while they were not present in the neurohypophysis (Table 2). The number of apoptotic cells decreased in the developing adenohypophysis from 5% in the 5th and 6th week to 3% in the 7th and 8th developmental week (Fig. 5).

**Table 2.** Immunoreactivity to specific antibodies in the human pituitary gland during the 5-8 developmental weeks.

Primary antibody	Age of embryos (weeks)			Parts of the pituitary gland	
	5 and 6		7		8
Caspase 3	++ c	+d	++	+	RP/AH M D/NH
	-	+	+	+	
	/	-	-	-	
p53	+		+	+	RP/AH M D/NH
	+		+	+	
	/		/	/	
Bcl-2	±		++	++	RP/AH M D/NH
	/		+	+	
	/		++	+	

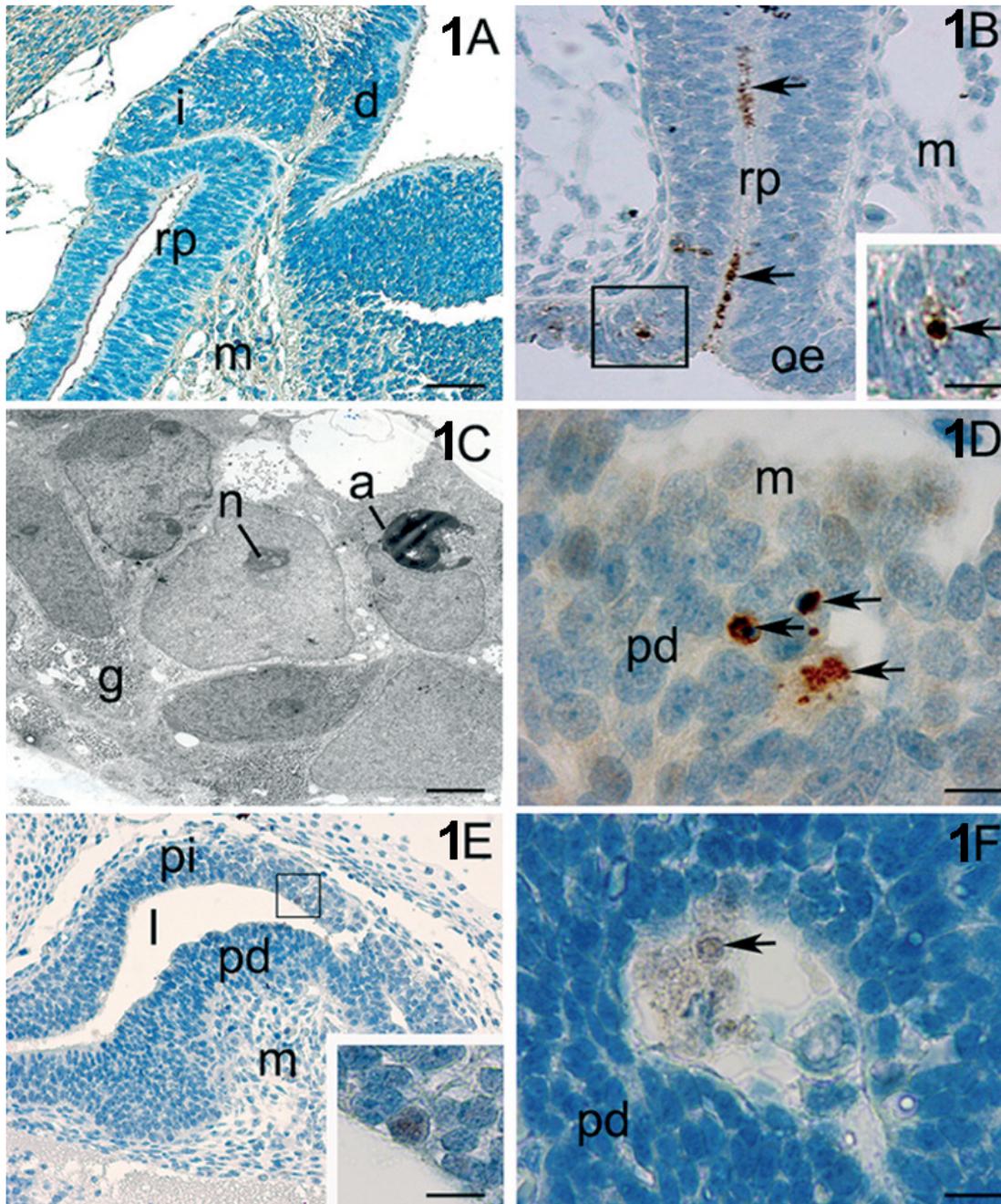
RP/AH: Rathke's pouch/adenohypophysis; M: mesenchyme; D/NH: diencephalon/neurohypophysis; ++: strong staining; +: moderate staining; ±: weak staining; -: absence of staining; /: absence of structure in the section, c-caudal part of pituitary gland (craniopharyngeal stalk), d-distal part of pituitary gland.

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*Immunohistochemical detection of p53 positive cells*

The p53 positive cells that appeared in the Rathke's pouch for the first time in the 6th developmental week

were found only occasionally, and were mostly localized at the luminal border of Rathke's pouch and in the pars distalis. At higher magnifications, the nuclei of p53 positive cells contained nuclear condensations, e.g. they



**Fig. 1.** Distribution of apoptotic factors caspase-3 and p53 in developing pituitary gland. **A.** Sagittal section through the 6-week human pituitary gland: cranial part of Rathke's pouch (rp), mesenchyme (m), infundibulum (i), diencephalon (d). Apoptotic cells are seen only occasionally and at higher magnification. Immunostaining to caspase-3, x 20. Scale bar 50  $\mu$ m. **B.** Sagittal section through the 6-week human pituitary gland: craniopharyngeal stalk of the Rathke's pouch (rp), mesenchyme (m), oral epithelium (oe). Note numerous brown-stained nuclei of apoptotic cells (arrows) along the closing lumen of the caudal Rathke's pouch, and at places of detachment from the oral epithelium. Immunostaining to caspase-3, x 40. Scale bar, 25  $\mu$ m. Inset: apoptotic cell inside oral epithelium, x 100. **C.** Ultrastructure of the Rathke's pouch in the 6th developmental week: most of the vital cells have pale nuclei (n) with prominent nucleoli and accumulations of cytoplasmic glycogen (g). Apoptotic cell (a) is characterized by condensed dark-staining nucleus of half-moon shape. x 2000. Scale bar, 0.5  $\mu$ m. **D.** Sagittal section through the 8-week human pituitary gland: pars distalis (pd) mainly contains vital cells with pale euchromatic

nuclei, and mesenchyme (m) with blood vessels. Several apoptotic bodies are seen among the vital cell (arrows). Immunostaining to caspase-3, x 100. Scale bar, 8  $\mu$ m. **E.** Sagittal section through a 7-8 week human pituitary gland: pars distalis (pd), pars intermedia (pi), residual lumen of the Rathke's pouch (l), mesenchyme (m). P53 positive cell (squared) is seen at the luminal border of the pars intermedia. Inset: higher magnification of the p53-positive cells showing also nuclear condensations. Immunostaining to p53, x 20. Scale bar, 50  $\mu$ m. **F.** Sagittal section through an 8-week human pituitary gland-pars distalis (pd). Note p53-positive cells (arrow) in the follicle-like nest of the epithelial cells. Immunostaining to p53, x 100. Scale bar, 10  $\mu$ m.

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showed morphological features of cell death (apoptosis) (Fig. 1E). At later stages, some of the dead cells were found in the lumen of the follicle-like nests of the epithelial cells, among the glandular-like primordium of the pars distalis, as well as in the mesenchyme penetrating the gland (Table 2, Fig. 1F). Some p53 positive cells were seen in the neurohypophysis as well.

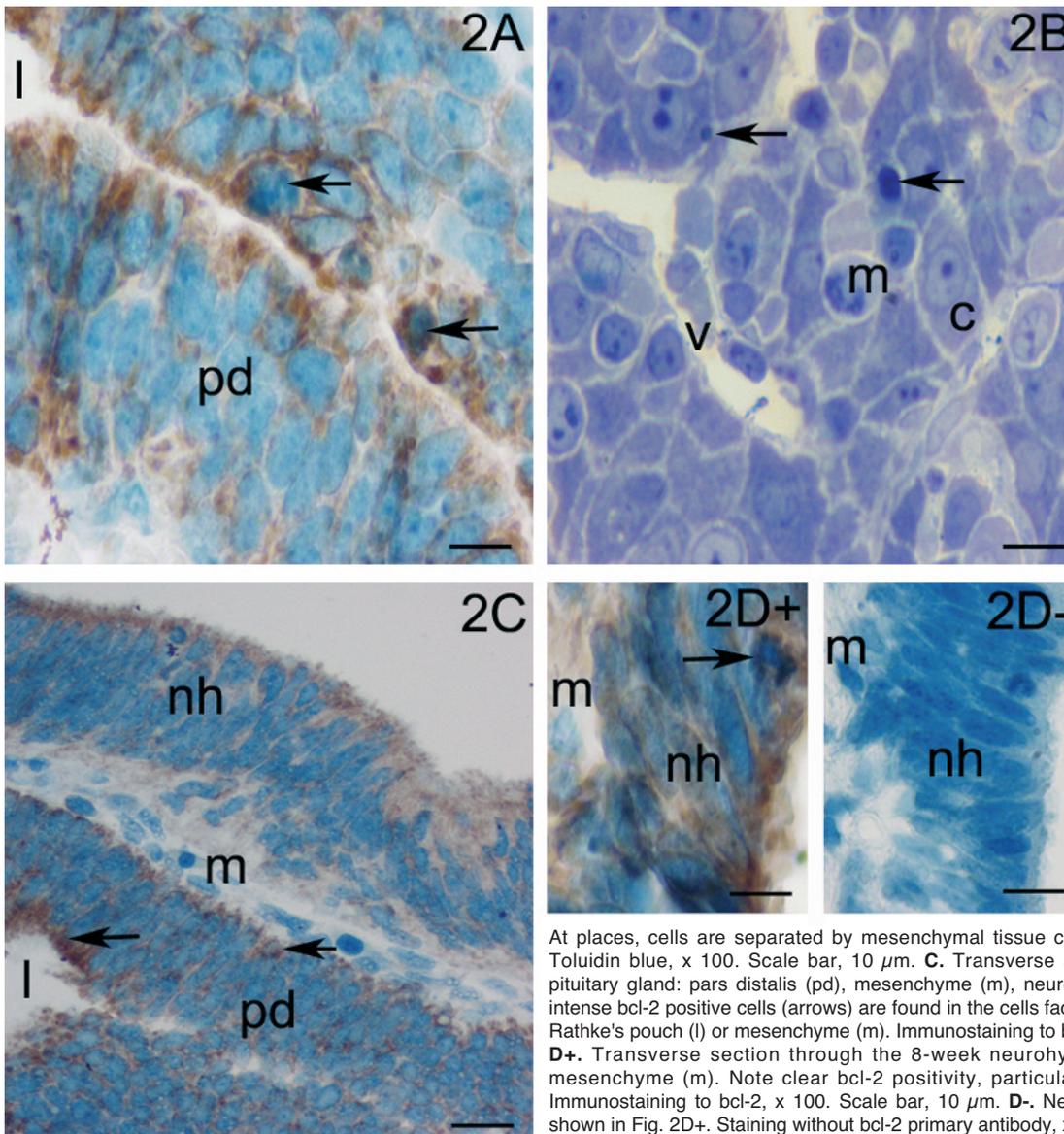
*Immunohistochemical and morphological detection of bcl-2 positive cells*

In the 5th and 6th week of development, only very weak bcl-2 positivity could be seen in some cells of Rathke's pouch epithelium (not shown). First, clearly bcl-2 positive cells appeared in all parts of the

developing gland during the 7th developmental week.

Cells strongly positive to anti-apoptotic bcl-2 protein were seen in the regions of the pituitary gland with high mitotic activity e.g. near the luminal border of Rathke's pouch and in the neurohypophysis. Less numerous bcl-2 positive cells were seen throughout the epithelium of glandular like formation of pars distalis, as well as in the adjacent mesenchyme (Fig. 2A, Table 2).

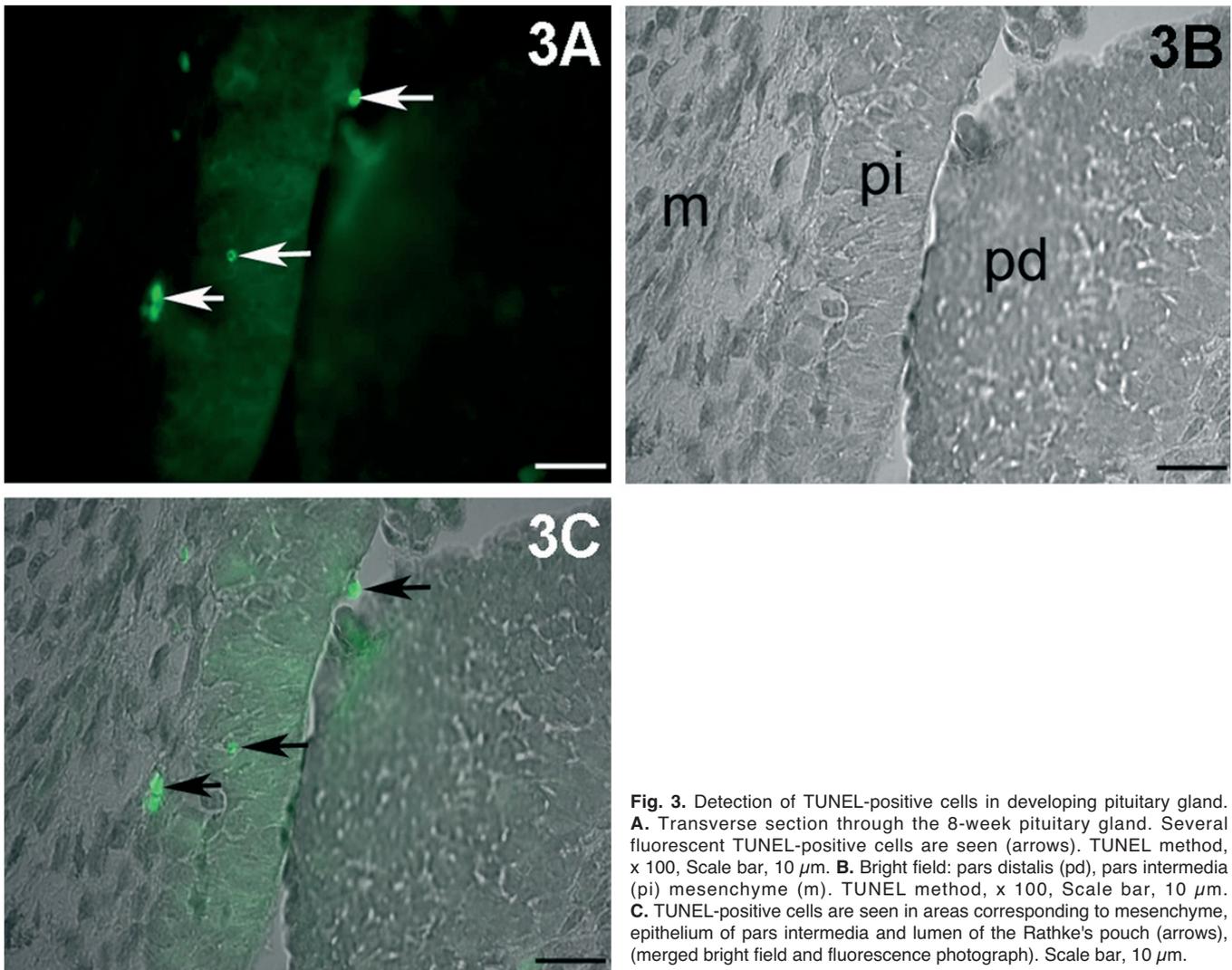
In the semi thin-sections of a 7-week embryo, in the distal part of the pituitary gland some cells showed mitotic figures, while others had condensed nuclei or formed apoptotic bodies. However, most of the cells had pale nuclei with prominent nucleoli, indicating that they are in the active phase. Cells were separated by mesenchymal tissue containing blood vessels (Fig. 2B).



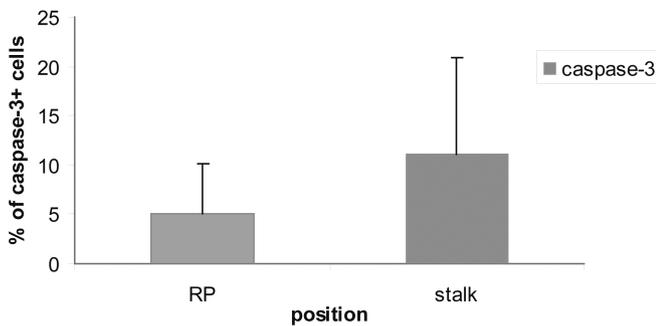
**Fig. 2.** Distribution of anti-apoptotic bcl-2 protein in developing pituitary gland. **A.** Transversal section through the pars distalis (pd) of a 7-week human pituitary gland. Bcl-2 positive cells are seen throughout the epithelium, but are the most numerous (arrows) near the luminal border (l) of the Rathke's pouch. Immunostaining to bcl-2, x 100. Scale bar, 10  $\mu$ m. **B.** Semi-thin section through the distal part of a 7-week pituitary gland. Most of the cells (c) have pale-stained nuclei with prominent nucleoli: some cells are in the stage of mitosis (m) and others show condensed nuclei or apoptotic bodies (arrows). Immunostaining to bcl-2, x 100. Scale bar, 10  $\mu$ m. **C.** Transverse section through the 8-week pituitary gland: pars distalis (pd), mesenchyme (m), neurohypophysis (nh). The most intense bcl-2 positive cells (arrows) are found in the cells facing the residual lumen of the Rathke's pouch (l) or mesenchyme (m). Immunostaining to bcl-2, x 40. Scale bar, 25  $\mu$ m. **D+.** Transverse section through the 8-week neurohypophysis (nh) and nearby mesenchyme (m). Note clear bcl-2 positivity, particularly in mitotic cell (arrow). Immunostaining to bcl-2, x 100. Scale bar, 10  $\mu$ m. **D-.** Negative control of the section shown in Fig. 2D+. Staining without bcl-2 primary antibody, x 100. Scale bar, 10  $\mu$ m.

At places, cells are separated by mesenchymal tissue containing blood vessels (v). Toluidin blue, x 100. Scale bar, 10  $\mu$ m. **C.** Transverse section through the 8-week pituitary gland: pars distalis (pd), mesenchyme (m), neurohypophysis (nh). The most intense bcl-2 positive cells (arrows) are found in the cells facing the residual lumen of the Rathke's pouch (l) or mesenchyme (m). Immunostaining to bcl-2, x 40. Scale bar, 25  $\mu$ m. **D+.** Transverse section through the 8-week neurohypophysis (nh) and nearby mesenchyme (m). Note clear bcl-2 positivity, particularly in mitotic cell (arrow). Immunostaining to bcl-2, x 100. Scale bar, 10  $\mu$ m. **D-.** Negative control of the section shown in Fig. 2D+. Staining without bcl-2 primary antibody, x 100. Scale bar, 10  $\mu$ m.

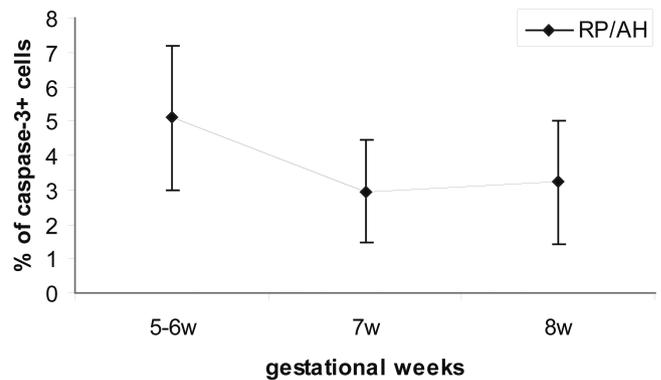
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**Fig. 3.** Detection of TUNEL-positive cells in developing pituitary gland. **A.** Transverse section through the 8-week pituitary gland. Several fluorescent TUNEL-positive cells are seen (arrows). TUNEL method, x 100, Scale bar, 10 μm. **B.** Bright field: pars distalis (pd), pars intermedia (pi) mesenchyme (m). TUNEL method, x 100, Scale bar, 10 μm. **C.** TUNEL-positive cells are seen in areas corresponding to mesenchyme, epithelium of pars intermedia and lumen of the Rathke's pouch (arrows), (merged bright field and fluorescence photograph). Scale bar, 10 μm.



**Fig. 4.** Percent of apoptotic (caspase-3 positive) cells in Rathke's pouch and stalk during development (5th and 6th week). Data are presented as mean ± standard deviation. The difference between Rathke's pouch and stalk is a statistically significant (Mann-Whitney, p=0.03).



**Fig. 5.** Percent of apoptotic cells in developing adenohypophysis during development (5-8 weeks). Data are presented as mean ± standard deviation.

In the 7th and 8th week of development, more glandular-like formations were seen in the pars distalis, and were separated by the mesenchymal (connective tissue) strands. Cells surrounding the remaining lumen of Rathke's pouch, as well as cells facing the nearby mesenchyme showed the most numerous mitotic figures, clearly recognized by characteristically arranged chromosomes. Intense proliferation (mitotic figures) and positive reaction to bcl-2 antigen co-localized in the cell of the observed regions. Less numerous bcl-2 positive cells were found in glandular-like part of the pars distalis, in the mesenchyme penetrating the gland, as well as in the connective tissue capsula (Fig. 2C). Diencephalon and the developing neural part of the pituitary gland showed cells with moderate immunoreactivity to bcl-2 antigen, being most numerous at the ventricular border of the diencephalon (Fig. 2D).

#### *TUNEL-method*

The internucleosomal fragmentation of cells, indicating that they were undergoing apoptosis, was confirmed by using TUNEL-method. TUNEL-positive cells displayed clear fluorescence in the dark field. After merging with the same bright-field image, it was possible to detect the position of apoptotic cells: in the mesenchyme, inside the epithelium of pars distalis and intermedia, and at the luminal border of the Rathke's pouch (Fig. 3C).

#### **Discussion**

The developmental period investigated in our study included several steps of pituitary gland formation: from dorso-ventral migration of the Rathke's pouch and separation from the oral ectoderm, to differentiation and formation of the glandular-like structures and the primordia of three lobes in the future gland. During the described developmental events, adjustment of cell number executed through cell death (apoptosis), as well as distribution and intensity of cell proliferation participated in the described early pituitary organogenesis.

During the early phases of pituitary gland formation, the most prominent morphological finding was separation of the Rathke's pouch from the primitive oral cavity. In accordance with this developmental event, numerous apoptotic cells were detected in the region of their detachment (11%), as this is the part of the gland (craniopharyngeal stalk) destined to disappear by the end of the 8th week (Carlson, 2004). In the distal part of the gland, which gives rise to the adenohypophysis, the ratio of apoptosis was significantly lower (5%). These cells showed clear caspase-3 positive reaction, indicating involvement of caspase-3 mediated apoptosis in the separation of the adenohypophysis from the place of its origin in the oral cavity. With progression of development, the number of apoptotic cells decreased in the region of the adenohypophysis (decline to 3%).

Additionally, the presence of caspase-3 positive cells in the nearby mesenchyme suggest that the same type of cell death might be involved in the formation of the connective tissue strands inside the gland, as well as the capsule on its surface. On the other hand, caspase-3 dependent apoptosis was not observed in the developing neurohypophysis. It was shown that if the process of separation of Rathke's pouch from the stomodeal epithelium is disturbed, the remaining tissue might be normal, and in that case is called a pharyngeal hypophysis. If the residual tissue becomes neoplastic, it forms hormone-secreting tumors called craniopharyngiomas (Carlson, 2004). Indeed, in several studies of craniopharyngiomas the location of these tumors was found along the path of the craniopharyngeal stalk (Chen, 2001; Deutsch et al., 2001; Falavigna and Kraemer, 2001; Kachhara et al., 2002). Thus, change in this clearly temporally and spatially defined apoptotic process could be associated with persistence of remnants of Rathke's pouch or the craniopharyngeal duct and their possible neoplastic transformation (Prabhu and Brown, 2005).

Until now, morphological features, molecular regulation and significance of apoptosis were investigated in non-tumorous pituitaries, pituitary adenomas, carcinomas and in pituitary hyperplasia, in both transgenic animals and adult humans (Kulig et al., 1999; Sambaziotis et al., 2003; Kapranos et al., 2004), but not during human pituitary development. It was found that apoptosis mostly occurred in functioning pituitary adenomas, or was used for evaluation of drug effects or for defining adenoma subtypes (Kontogeorgos, 2005). In experimental animals, the connection between the expression of certain genes and change in apoptotic process was detected. In mice, reduced expression of LIM-homeobox gene *Lhx3* caused impaired growth and differentiation of Rathke's pouch, and increased cell apoptosis during pituitary development (Zhao et al., 2006). It was shown that pituitary hypoplasia in *Lhx4* mutant mice resulted from increased cell death (Raetzman et al., 2002).

In our study, the p53 positive cells appeared in Rathke's pouch for the first time in the 6th developmental week, and were distributed inside its epithelium or at its luminal border, but were present also in the surrounding mesenchyme. Later, they were detected in the lumen of the follicle-like nests of the epithelial cells in the pars distalis, in the mesenchyme and in the developing neurohypophysis as well. Since we found that p-53 positive cells showed morphological signs of cell death (nuclear condensations), we can speculate that this protein might control the elimination of cells in the developing gland by inducing p-53 mediated apoptosis. It is known that p53 is a negative regulator of G1-S phase transition in the cell cycle and that it may stimulate cell death in response to DNA damage. Thus, Lichnovsky et al. (1998) did not exclude the role of DNA-damage in accumulation of p53 protein in different tissues during the early stages of human

embryogenesis. On the other hand, the appearance of this protein in Rathke's pouch cells could be considered as a part of normal pituitary morphogenesis. Additionally, in our study p53 protein was co-expressed with bcl-2 protein during normal pituitary gland formation. Thus, we can speculate that in regions of intense cell division, p53 expression might be necessary to prevent inadequate and uncontrolled proliferation, which is usually related to serious disturbances in cell alignment, as well as with the appearance of different disorders (Lichnovsky et al., 1998). While Ozer et al. (2003) suggested a significant relationship between the apoptosis-related proteins bcl-2, bax and p53 and hormone function in pituitary adenomas, Green et al. (1997) found no significant association between apoptosis and p53 protein expression and pituitary adenomas.

In our study, cells positive to anti-apoptotic bcl-2 protein were mostly seen in the regions of the pituitary gland with high mitotic activity: around the residual lumen of Rathke's pouch, as well as at the side facing the mesenchyme. Less numerous bcl-2-positive cells were observed in the glandular-like formations of the pars distalis. In our previous study, the Ki-67 positive cells predominated in the region of the most intense cell proliferation, while they were rarely seen in the gland-like structures (Bazina et al., 2007). Taniguchi et al. (2002) also described that in the rat pituitary gland, cells in active proliferation and differentiation contradicted each other (Taniguchi et al., 2002a,b). Location of the bcl-2 positive cells, found in the neural part of the pituitary gland and in the diencephalon, also coincided with previously described high cell proliferation activity detected by Ki-67 in the same regions (Bazina et al., 2007). At later stages, when the cells of the infundibulum have mostly differentiated into pituicytes, immunoreactivity to bcl-2 was also less intense. Thus, our findings support the idea that bcl-2 protein not only protects cells from apoptotic death, but also enhances cell survival and control of cell division.

In the described study, all investigated pro-apoptotic and anti-apoptotic factors had spatially and temporally restricted patterns of appearance in the developing human pituitary gland. Their balance and interplay seem to be involved in early normal pituitary formation, primarily in the control of cell number, cell survival and cell death. Changes in their expression patterns might be associated with serious disturbances in gland morphogenesis and function, as shown in the cases of tumor transformation of the pituitary gland.

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