SCRIPTA MEDICA (BRNO) – 73 (4): 245–250, *October 2000*

APOPTOSIS AND EXPRESSION OF BCL-2 AND BAX DURING EARLY HUMAN EMBRYOGENESIS

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A b s t r a c t

Apoptosis is an important process that plays a fundamental role in embryonic development. The aim of this study was to detect apoptotic cells in the tissues and organs of human embryos during early embryogenesis and to correlate the level of apoptosis with the expression of BCL-2 and BAX as proteins which are known to regulate this vital process.

Tissue samples from 42 human embryos, aged 4 to 30 weeks of intrauterine development, were studied using the TUNEL technique. The standard, three-step, indirect immunohistochemical method was used for the detection of BCL-2 and BAX.

A high level of apoptosis was observed at the following sites: anlages of the vertebral bodies, interdigital zones of limb anlages, left lobe of the liver anlage, spongious layer of the primitive myocardium and apical parts of the primitive intestinal villi. The relationship between BCL-2 expression and the level of apoptosis and BAX expression appeared to be inversely proportional.

In other issues, i.e., skeletal muscles and lungs, the described relationship was not so convincing.

K e y w o r d s

apoptosis, human embryo, regulating genes

INTRODUCTION

Apoptosis plays an important role in the maintenance of homeostasis and is also needed for a proper development of organs and tissues during embryogenesis. It destroys the cells that represent a threat to the integrity of an organism (*1, 2*), e. g., cells infected with viruses, immunologically autoreactive cells, cytotoxic T-lymphocytes and cells with damaged DNA.

This work was designed to shed light on the role of apoptosis during human embryonic differentiation, with an intention to make a correlation between our findings and the expression of BCL-2 and BAX proteins (products of apoptosis regulating genes). BCL-2 is an integral, membrane-associated protein with antiapoptotic and perhaps antioxidative effects. It may also regulate intracellular concentrations of Ca^{2+} ions. BAX is another member of the family of BCL-2-related proteins. It has an extensive aminoacid homology with BCL-2. Both proteins homodimerise and heterodimerise with each other. Whether the

cell will live or die may depend on the level of either protein; while BCL-2 prevents death, BAX is a death promotor (*3, 4*).

MATERIAL S AND METHODS

A total number of 42 human embryos, aged 4 to 30 weeks of intrauterine development, were used to the study. They were processed by routine methods (fixed in methacarn or Baker's fluid and embedded in paraffin), pre-treated by exposure to microwaves or Proteinase K and studied with the use of a Boehringer-Mannheim Company kit for the TUNEL technique (**T**dT-mediated X-d**U**TP **n**ick **e**nd **l**abelling) and an immunohistochemical assay. With the TUNEL technique, breaks of DNA strands, which occur in the early stages of apoptosis, are detected by the terminal deoxynucleotidyl transferase-mediated labelling of the free 3'-OH termini with fluorescein-modified nucleotides. Apoptotic nuclei were visualised by means of anti-fluorescein antibody conjugated with alkaline phosphatase which dissociates a yellow-coloured substrate (NBT/BCIP) to produce a blue-coloured precipitate. Intact nuclei were labelled by nuclear red. The positive control of each sample is based on DNase treatment which produces artificial 3'-OH termini. TdT was not added to the reaction mixture of a negative control.

The standard, three-step, indirect immunohistochemical method was employed for the detection of BCL-2 and BAX proteins, using commercially accessible primary antibodies, i.e., anti-BCL-2 (BioGenex) and polyclonal antibody P 19 (Santa Cruz Biotechnol.) for BAX. The primary antibodies were omitted from negative controls.

RESULTS

In the majority of structures examined we proved the occurrence of cells whose growth was ceased due to apoptosis and which were substituted by cells of a higher developmental level. The other cells were protected by the effects of apoptosis antagonists (BCL-2, etc.).

High numbers of apoptotic myoblasts were detected in the endomyocardial (spongious) layer of the primitive myocardium. These apoptotic myoblasts were shown by the TUNEL method after 4 weeks of intrauterine development, i.e., at the time when the spongious layer was still fully functional. These apoptotic myoblasts were not observed in electron micrographs before the 5th week.

In the epithelial layer of embryonal gut mucosa, a higher number of cells undergoing apoptosis were found in the apical zone of differentiating villi. A much lower number of apoptotic cells were observed in the basal parts of differentiating villi where Lieberkühn's crypts were to develop. This gradient was maintained in all the samples studied *(Fig. 2).*

In the other layers of the primitive intestine wall, the level of apoptosis was conspicuously higher in the mesothelium of primitive serosis.

Only single apoptotic cells were observed in the cortex of primitive hemispheres. In the spinal cord, the level of apoptotic cells was also low, as demonstrated by single positive cells only.

The expression of BCL-2 and BAX corresponded to the level of apoptosis in the structures mentioned above.

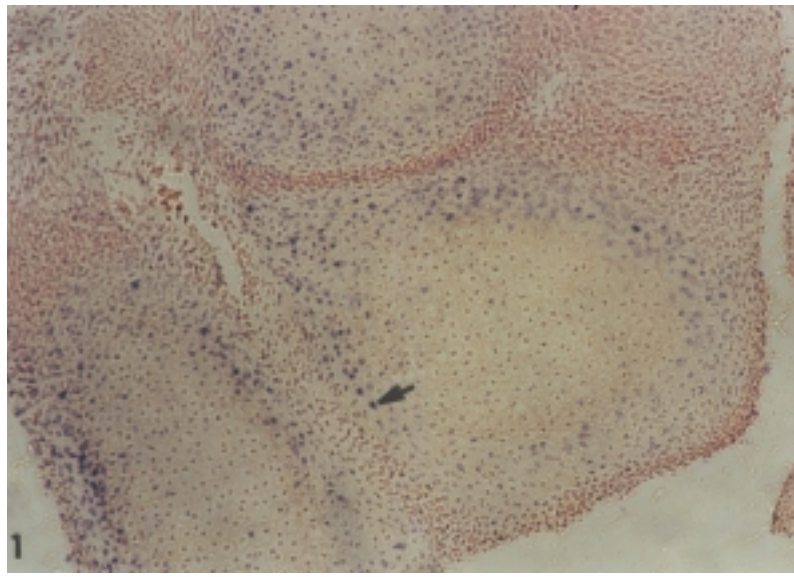

Fig. 1
High level of apoptosis in the interdigital part of the hand plate (arrow).
7-week-old embryo. Magnification: x100.

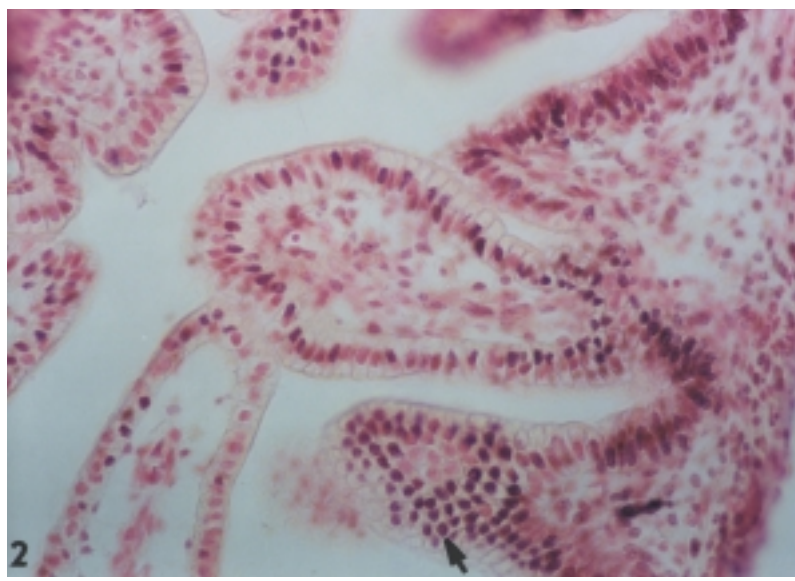

Fig. 2
Apoptotic cells (arrow) in the apical part of an intestinal villus.
11-week-old fetus. Magnification: x480.

A massive occurence of apoptotic mesenchymal cells was observed in the interdigital parts of limb anlages (*Fig. 1*). Apoptotic hepatocytes were also found in substantial numbers in the left lobe of the liver anlage.

A high frequency of apoptosis was apparent in mesenchymal cells and primitive chondroblasts of the marginal zone of the cartilaginous anlage of limb bone and did not show any pronounced changes between the 7th and 14th weeks of intrauterine development; this may be related to growth and development of the bone collar. Apoptotic chondroblasts were observed in the region of primary ossification centers of the anlages of long limb bones. Our observations also confirmed a one-week acceleration of upper limb development.

A lot of apoptotic cells were found in the anlages of vertebral bodies. However, apoptotic cells were almost absent in the area of the developing intervertebral discs.

In the anlage of the axial skeleton and limbs, similar low expression of both BCL-2 and BAX proteins was observed in all the samples examined.

DISCUSSION

Our findings obtained by means of the TUNEL technique prove an important role of apoptosis in the early stage of human embryogenesis. This method enabled us to detect the cells undergoing apoptosis during early and middle phases of this process. The avialability of fresh embryonic materials is generally limited and the TUNEL method offers us an advantage of using archival materials fixed by Baker's fluid. This method allows us to observe apoptotic cells at earlier stages of IUD than is possible with electron microscopy technique *(5, 6, 7)*.

The highest level of apoptosis was found in the tissues and organs undergoing regression or becoming extinct during intrauterine development, e.g., in a spongious layer of the primitive myocardium, interdigital parts of primitive autopodia and the left liver lobe.

In our previous paper (5) we have investigated the importance of p53 protein, a product of the tumour supressor gene, for embryonic development and also its relation to apoptosis *(8)*. Our findings of high levels of apoptosis in the left lobe of liver anlage or in the spongious layer of the myocardium give support to the views of Donehower et al. *(8)* who suggest that, after detection of DNA damage, p53 induces a cell cycle block which provides time for the cell to repair its DNA. If this fails, apoptosis is triggered by p53. Until now, mechanisms which account for the occurrence of apoptosis in the absence of p53 remain unclear. We have failed to prove p53 expression in selected organs of older fetuses *(5)*. Obviously, other mechanisms involved in apoptotic regulation are worth considering.

We demonstrated an inverse relationship between apoptosis and BCL-2 in some organs (spongious layer of myocardium, CNS), thus confirming the antiapoptotic effect of BCL-2. In these organs, there was also an inverse

relationship between BCL-2 and BAX. These findings are in agreement with the opinions of *Merino et al. (9)* who assume that products of BCL-2 and BAX genes heterodimerise or homodimerise and that the relative levels of dimerisation partners available shift the balance of cell fate in favour of either viability or cell death.

In the other organs (skeletal muscles, lungs and axial skeleton) the inverse relationship was not convincing. Therefore we can speculate about some other mechanisms involved in the multilevel regulation of apoptosis.

ACKNOWLEDGMENTS

This work was supported by grant no. 717/99 from the Promotion of Research in Universities project and by grant no. 151100001 from the Ministry of Education, Youth and Physical Training of the Czech Republic.

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APOPTÓZA A EXPRESE BCL-2 A BAX V PRŮBĚHU ČASNÉ EMBRYOGENEZE ČLOVĚKA

S o u h r n

Apoptóza je proces, který hraje velmi důležitou roli v embryonálním vývoji. Cílem práce bylo studium úlohy apoptózy a exprese jejích regulačních genů v embryogenezi člověka. Detekce apoptózy metodou TUNEL byla provedena u 42 lidských zárodků ve stáří od 4. do 30. týdne intrauterinního vývoje.

K imunohistochemickému průkazu proteinů BCL-2 a BAX byla použita standartní nepřímá třístupňová imunohistochemická reakce s použitím komerčních protilátek (Biogenex, Santa Cruz Biotechnol.).

Vysoká úroveň apoptózy byla nalezena v části základů obratlů, v interdigitálních zónách základů končetin, v levém laloku základu jater a apexu diferencujících se střevních klků. V těchto orgánech se vysoká úroveň apoptózy shodovala s nízkou expresí BCL-2 a vysokou expresí BAX. V dalších orgánech (skeletní svaly, plíce, osifikační centra dlouhých kostí) nebyly popsané relace tak výrazné. Ze získaných údajů je možno spekulovat o výskytu dalších regulačních mechanismů apoptózy.

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