Apoptosis in human embryo development:

1. Cerebral cortex

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Abstract

We investigated the apoptosis at the beginning of human cerebral cortex development, in the 6th week of embryogenesis, Carnegie stages 16 and 17. Attention was focused on the dorsal wall of the telencephalon to the ventricular zone of proliferation and to the postmitotic zone with beginning of neuronal migration. We identified apoptotic cells in tissue sections by propidium iodide staining, TUNEL and immunohistochemistry for Fas(APO-1/CD95). We determined the distribution and the percentage (reported to the propidium iodide stained nuclei) of apoptotic TUNEL-positive and Fas(APO-1/CD95)-positive cells. TUNEL-positive apoptotic cells in the proliferative zone were 20% in stage 16 and 60% in stage 17. TUNEL-positive apoptotic cells in the postmitotic zone were 8% in stage 16 and 30% in stage 17. CD95-positive apoptotic cells in the proliferative zone were 5% in stage 16 and 2% in stage 17. There were no CD95-positive cells in the postmitotic zone. We evidenced the presence of the suicide receptor Fas(APO-1/CD95) only on a small population of apoptotic neuroblasts in the proliferative zone. The differences between apoptotic distribution and receptors in early corticogenesis suggest that different apoptotic pathways drive the selection of neuronal populations.

Keywords: apoptosis • human • embryo • cerebral cortex • TUNEL • Fas(APO-1/CD95)

Introduction

A key event in the development of mammalian cerebral cortex is the generation and differentiation of neuronal population during embryonic life. Previous studies have revealed many details of cortical neurons development including cell birthdates, migration patterns and lineage relationships [1, 2].

The requisite steps are the 'birth' of neurons within proliferative zones adjacent to the ventricles (e.g. the ventricular zone or vz) and migration of these neurons to a superficial location consisting of postmitotic cells (e.g. the cortical plate or cp; for terminology see Boulder Committee, 1970) [3].

During central nervous system (CNS) development, apoptosis has been viewed traditionally as a fate reserved for differentiating neurons that are in the process of making synaptic connections [4-6]. Recent studies in the embryonic cerebral cortex have shown that many neuroblasts in the proliferative ventricular zone undergo apoptosis [7-9].

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The existence of alternative pathways of neuronal death, the involvement of multiple triggers of apoptosis suggest that the apoptotic pathways in neuronal development are far from being fully understood. Recent studies provide evidence that Fas could function as a death receptor in neurons *in vitro* and *in vivo* [10, 11].

It is important to note that there is significant variation between different types of cells in the nervous system, regarding the level of apoptosis that can be induced *via* the Fas receptor [12-14].

We examined the apoptosis in the development of cerebral cortex, in the embryonic period. Attention was focused on the wall of the telencephalon during a relatively early stage of corticogenesis (week 6, Carnegie stages 16 and 17) corresponding to the time of major proliferation in the ventricular zone and to the beginning of neuronal migration; both these mechanisms are crucial for corticogenesis [15].

We identified apoptotic cells in tissue sections by propidium iodide staining, TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling) [16-18] and immunohistochemistry for Fas(APO-1/CD95).

We proved the presence of the suicide receptor Fas(APO-1/CD95) in human cerebral cortex during embryonic period in a population of apoptotic neuroblasts of the proliferative zones. This finding suggests that different apoptotic pathways drive the selection of cells which begin to differentiate.

Materials and methods

Tissue sampling and preparation

Two human embryos (6 weeks old, Carnegie stages 16 and 17) provided by ectopic pregnancy were fixed in pH 7 buffered formalin with phosphate buffer, embedded in paraffin and sectioned. The tissues sections (4 μ m) were deparaffinized, hydrated and treated for apoptosis detection.

Propidium iodide staining

Tissue sections were treated with trypsin in citrate stabilizing buffer, followed by trypsin inhibition, enzymatic digestion the cellular RNA, stabilization of the nuclear chromatin with spermine and then propidium iodide staining, using the Cycle TEST™ PLUS DNA Reagent Kit (Becton Dickinson, USA) and following the manufacturer instructions. Propidium iodide-stained nuclei

with excitation at 536 nm and emission at 617 nm were examined with a Nikon -Eclipse TE-200 microscope.

TUNEL method

In situ detection of apoptotic cells in tissue sections by TUNEL has been performed using DIG Nucleic Acid Detection Kit (Boehringer Mannheim, Germany) following the manufacturer instructions. DIG-11-UTP oligonucleotides were incorporated enzymatically by terminal deoxynucleotidyl transferase (TdT) at the 3'-end apoptotic DNA. DIG labeled DNA was detected by enzyme-linked immunoassay using an antibody conjugate (anti-digoxigenine alkaline phosphatase conjugate, anti-DIG-AP). Subsequently, alkaline phosphatase produced a color reaction with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue-tetrazolium salt (NBT) which developed an insoluble blue-purple precipitate. The NBT stained nuclei were analyzed by optical microscopy.

Fas(APO-1/CD95) immunohistochemistry

Embryo sections were hydrated with phosphate buffer saline (PBS, pH7.2) and incubated with anti-Fas(APO-1/CD95) [mouse Ig G 3] (Boehringer Mannheim, Germany). The Fas-anti Fas complexes were detected by a double-sandwich immunohistochemical reaction using a FITC-coupled second antibody to mouse IgG 3 subclass (anti-mouse-Ig G - FITC, Sigma). After both antibodies incubations (1 h, 37°C), the tissue sections were washed with PBS-Tween20 0,05 %, 3x10 min.

The FITC labeled positive cells were visualized by excitation at 495 nm and emission at 519 nm with a Nikon -Eclipse TE-200 microscope.

Quantitation of labeled cells

Cells were counted in images taken with a videocamera mounted on a Nikon -Eclipse TE-200 microscope, using the Lucia software for image analysis (Laboratory Imaging, Czech Republic).

Results and discussions

We investigated the spatial distribution of apoptotic cells in the human embryonic cerebral cortex in the 6th week, Carnegie stages 16 and 17.

The architecture of telencephalic wall and the distribution of TUNEL-positive nuclei are shown in Fig 1.

The apoptotic cells were observed throughout the cerebral cortical wall but the majority were found within zones of cell proliferation rather than in regions of postmitotic neurons [15].

Fig. 1 Distribution of apoptotic cells in human cerebral cortex in the 6th week of embryogenesis, Carnegie stages 16 (A, B) and 17 (C, D)

(A), (C) Fluorescent images of propidium iodide stained nuclei (red). Note the yellow staining of picnotic nuclei. (B), (D) TUNEL positive nuclei of apoptotic cells appear dark-purple. The cerebral wall of telencephalon consists of two distinct zones: a proliferative one and a postmitotic one. The proliferation of neuroepithelial layer is revealed by the increasing in thickness from the 16th to the 17th Carnegie stages. VZ - ventricular zone (proliferative zone adjacent to the ventricles), IZ - intermediare zone, MZ - marginal zone. The ventricle (V) is at the lower edge of each photograph. Nikon-Eclipse TE-200 microscope. A: $\times 600$; B, D: $\times 1000$; C: $\times 400$.

Fig. 2 Distribution of apoptotic cells with Fas(APO-1/CD95) receptors in human cerebral cortex during embryonic period in the 6th week of embryogenesis, Carnegie stages 16 (A) and 17 (B) Fas(APO-1/CD95) positive cells appear green. VZ - ventricular zone, IZ - intermediate zone, MZ - marginal zone, V - ventricle. Fluorescent microscopy, Nikon-Eclipse TE-200 microscope, $\times 1000$.

We determined the distribution and the percentage (reported to the propidium iodide stained nuclei) of apoptotic TUNEL-positive cells. TUNEL-positive apoptotic cells in the proliferative zone were 20% in stage 16 and 60% in stage 17. TUNEL-positive apoptotic cells in the postmitotic zone were 8% in stage 16 and 30% in stage 17.

The presence of Fas(APO-1/CD95)-positive apoptotic cells in the ventricular zone of the cerebral cortex in the same stages is shown in Fig. 2. The percentage of Fas(APO-1/CD95)-positive apoptotic cells in proliferative zone was 5% in stage 16 and 2% in stage 17. We did not detect Fas(APO-1/CD95)-positive cells in the postmitotic zone.

Therefore, the suicide receptor in human cerebral cortex was present only on a population of apoptotic neuroblasts in the proliferative zone.

The different sensitivities and targets of the two used methods for apoptosis detection did not permit the calculation of ratio Fas(APO-1/CD95)-positive cells versus TUNEL-positive cells, but the difference in proportion is obvious.

Fas expression was more frequent in embryonic cortical neuroblasts in dissociated culture compared to *in situ* within the developing brain, suggesting that the environmental factors partly suppress Fas expression at this developmental stage [10].

The differences between apoptotic distribution and receptors in early corticogenesis suggest that the selection of neuronal populations is driven by diverse apoptotic pathways.

Concomitantly, significant cell production must occur to replace the lost cells and to produce the observed increases in cortical cell number [8]. Recent studies [9] on the rate of cortical neurogenesis have provided evidence for exponential increases in cell production that could compensate for the extent of cell death observed here.

Interestingly a single blast cell is estimated to give rise to over than 250 neurons and, for some blasts, theoretically as many as 2^{11} or 2048 neurons may be produced.

This appears to be inconsistent with the modest increase observed in cortical size during the period of cortical neurogenesis, and the extent of apoptosis that we observe may in part explain this discrepancy.

Extensive apoptosis in proliferative and postmitotic zones might well be a general feature of embryonic CNS development, requiring cells to survive several phases of selection before reaching maturity.

Conclusions

We examined the developmental distribution of apoptotic cells in the embryonic cerebral cortex for the period spanning neurogenesis, week 6, Carnegie stages 16 and 17. Apoptotic cells were observed throughout the cerebral wall, but the majority were located within proliferative zones. We found that, during this period, an average of 30% of cortical cells are undergoing apoptosis.

The presence of Fas(APO-1/CD95) receptor only on a small population of apoptotic cells in the proliferative zone suggests that apoptotic pathways in cerebral cortex development are multiple.

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