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Morphological diversity of dying cells during regression of the human tail

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Summary. During normal human development a number of transient structures form and subsequently regress completely. One of the most prominent structures that regress during development is the human tail. We report here a histological and ultrastructural study of cell death in the cranial and caudal (tail) parts of the neural tube in 4 to 6-week-old human embryos. Initially, the human tail is composed of tail bud mesenchyme which differentiates into caudal somites, secondary neural tube, notochord and tail gut. Later on, these structures gradually regress by cell death. During the investigated period, we observed two morphologically distinct types of dying cells. The well-described apoptotic type of cell death was observed only in the cranial neural tube that forms during primary neurulation. The other type of cell death characterized by necrotic morphology was observed in the tail mesenchyme and in the caudal neural tube that forms during secondary neurulation. This morphological diversity suggests that besides differences in origin and fate there are different mechanisms of developmental cell death between two parts of the human neural tube. We can speculate that the apoptotic type of cell death is associated with the precise control of cell numbers and that the other morphologically distinct type of cell death is responsible for the massive removal of transitory structures.

Key words: Apotosis – Developmental cell death – Human embryo – Tail

Introduction

The human tail is one of the most prominent structures that regress during development. It is located distal to the posterior neuropore or so called transitional or overlap zone. The pluripotent nature of this tissue is related to its origin from the primitive streak and Hensen's node (Griffith et al. 1992). As such, it may afford insight into the mechanisms of malformation and provide a valuable model for the study of cell differentation.

Recent investigations confirm that neural tube formation in humans is similar to that of some animals, in that there are two types of neurulation mechanisms – primary and secondary. The human brain and spinal cord down to the lumbosacral region appear to differentiate from the neuroectoderm of the neural tube during the process of primary neurulation. The caudal (tail) part of the human spinal cord derives from mesenchymal cells in the tail bud during secondary neurulation (Nievelstein et al. 1993; Saraga-Babić et al. 1995, 1996). Similar findings have been shown for the neural tube in different animals (Schoenwolf and Delongo 1980; Schoenwolf 1984).

The human tail develops during the third week, acquires its maximal length in the fifth week, and disappears during the seventh week (Nievelstein et al. 1993). Three subsequent developmental processes can be distinguished in the caudal neural tube during development; closure of the caudal neuropore, secondary neurulation and degeneration of the secondary neural tube (Nievelstein et al. 1993). Initially, the human tail is composed of undifferentiated mesenchyme which gradually transforms into somites, secondary neural tube, notochord and tail gut. These structures regress by cell death thus leading to the disappearance of the human tail (Fallon and Simandl 1978; Saraga-Babić et al. 1995, 1996).

According to prevailing current opinion, there are only two types of cell death (see Waux 1999). The proponents of this classification postulate a radical dichotomy between necrotic cell death, which is supposed to occur only in pathological situations, and apoptotic (programmed or physiological) cell death (Hetts 1998). This systematiza-

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tion (beside its nomenclatural inconsistency) neglects the fact that apoptosis is primarily a morphological characteristic of dying cells, and that this system cannot cover the morphological diversity and multiple mechanisms of cell death encountered in various experimental models and animals.

In many cases during normal development programmed cell death does not follow the apoptotic pattern described by Kerr et al. (1972). The morphological diversity of dying cells during development is not a new concept. Schweichel and Merker (1973) proposed three main types of cell death in developing tissue; a) isolated cell death, b) autophagic disintegration and c) non-lysosomal cell disintegration. Later many authors accepted and tested this concept but exclusively for experimental animals (for review see Clarke 1990). Investigations and reports on human conceptuses are rare. Here we report on a light microscope and an ultrastructural study of the human tail structures in the 4 to 6-week-old human embryo with a special emphasis on the morphological distinction and diversity of the dying cells.

Material and methods

Human embryonic material: Human embryonic material was collected after artificial abortions in compliance with the regulations of the Ethical Committee of the Clinical Hospital in Split. The material was collected immediately after abortion. The time between the end of the procedure and the fixation was no more than five minutes. All included material was collected following so called "social indications", which reduces chance of inclusion of the abnormal embryos. The postovulatory age was estimated from menstrual data and correlated with the crown-rump length and with the Carnegie stages. In the cases of embryos that were damaged during the abortion procedure, the developmental stage was determined by matching the structures with corresponding descriptions from O'Rahilly and Gardner (1971). In this study, we used embryos between 4–6 weeks of development (Table 1). Tissues were prepared according to the specific requirements for each of the employed methods.

Electron microscopy: For electron microscopy small pieces of tissue ($2 \times 2 \times 2$ mm) were preserved using a double fixation technique. The primary fixative was Karnovsky's fixative (Karnovsky 1965) (2.5% glutaraldehyde, 2% paraformaldehyde) solution for 2–5 hours. Secondly, the samples were postfixed with 1% osmium-tetroxide for 1 hour, dehydrated in an ascending series of ethanol/water until 100% ethanol. The specimens were then transferred to propylene oxide/epon and embedded in epon (Robinson 1982). Semithin sections, approximately 1 µm thick were cut and stained with Toluidine Blue. The ultrathin sections were made and stained with uranyl acetate and lead citrate for transmission electron microscopy (TEM).

Table 1. Human embryos analyzed in this study

Number of embryos	Age (weeks)	CRL (mm)	Carnegie stage
2	4	5	12
3	5	7–9	13–15
3	6	10–15	16–18

Light microscopy: For light microscopy, the tissue was fixed in 4% paraformaldehyde in phosphate buffer and dehydrated until 100% ethanol. Samples were finally embedded in paraffin wax and serially sectioned transversely and/or longitudinally at 8 µm and mounted on glass slides. Sections were stained with toluidine blue or hematoxylin and eosin.

Results

In the 4-week human embryo, two types of partly overlapping neurulation processes (primary and secondary) can be observed along the developing neural tube. Primary neurulation is found in the areas cranial to the closing caudal neuropore and secondary neurulation in the more caudal areas. The region caudal to the still open caudal neuropore forms a temporary human tail.

In the region close to the caudal neuropore, the cranial neural tube is still in the stage of neural groove. The wall of the neural groove consists of neuroepithelial cells and a thin layer of neuroblasts. The spinal ganglia, ventral and dorsal roots are not yet developed. The notochord is separated from the neural groove by the loose mesenchyme (Fig. 1 A).

At the tip of the tail the caudal neural tube is formed of a single lumen surrounded by the neuroepithelium, with the nuclei aligned in several rows. The neural tube is situated in the mesenchyme of the tail bud. The surface of the tail is covered by a thin epithelium.

The process of degeneration in the tail region is already present. Numerous dying cells are observed in the neuroepithelium of the caudal neural tube, as well as in the surrounding mesenchyme (Fig. 1 B).

During the 5th week the tail reaches its maximum length. This developmental period is characterised by further differentation and thickening of the primary neural tube. The lumen of the secondary neural tube is irregular. Sclerotomal cells surround the notochord, thus forming caudal vertebrae.

In the 6-week human embryos, signs of the regression of the tail structures are obvious; the secondary (caudal) neural tube consists of an irregular lumen surrounded by a layer of neuroepithelial cells. Connection between the cranial and caudal neural tube forms a transitional zone. The mesenchyme of the tail bud has differentiated into the sclerotomal cells of caudal vertebrae. The tail is short and covered with epithelium (Fig. 1 C).

More cranial parts of the neural tube derived during primary neurulation consist of three distinct layers: the neuroepithelial, mantle and marginal layers, respectively. Dying cells characterized by heterochromatic nuclei are found in the neuroepithelial and mantle layers of this region (Fig. 1 D).

In the tail part of the neural tube dying cells are more numerous than in more cranial regions and are distributed throughout the wall of neural tube, as well as in the surrounding mesenchyme.

On the ultrastructural level, initial signs of cell death in the cranial (primary) neural tube are characterized by numerous condensations of chromatin near the nuclear membrane. The cytoplasm of such cells is pale with fewer organelles than in the neighboring cells and with well preserved cell membrane (Fig. 2 A).

In the more advanced phase of cell death the nucleus acquires a characteristic half-moon shape, while the cytoplasm fragments into apoptotic bodies (Fig. 2 B). The final outcome of this process is cell fragmentation (i. e. the formation of the apoptotic bodies).

The process of cell death observed in the caudal region of secondary neurulation differs from that seen in the cranial regions: it begins with the chromatin condensation in the nucleus, appearance of large cytoplasmic vacuoles and swelling of the intracellular organelles (Fig. 2 C). Empty spaces in the cytoplasm are joined with each other and with the extracellular spaces. In the advanced stages of this process, the nucleus becomes pyknotic, while the cell membrane disrupts into fragments and organelles disintegrate (Fig. 2 D).

Observed morphological presentations of dying cells in the caudal and cranial neural tube are schematically presented in Fig. 3.

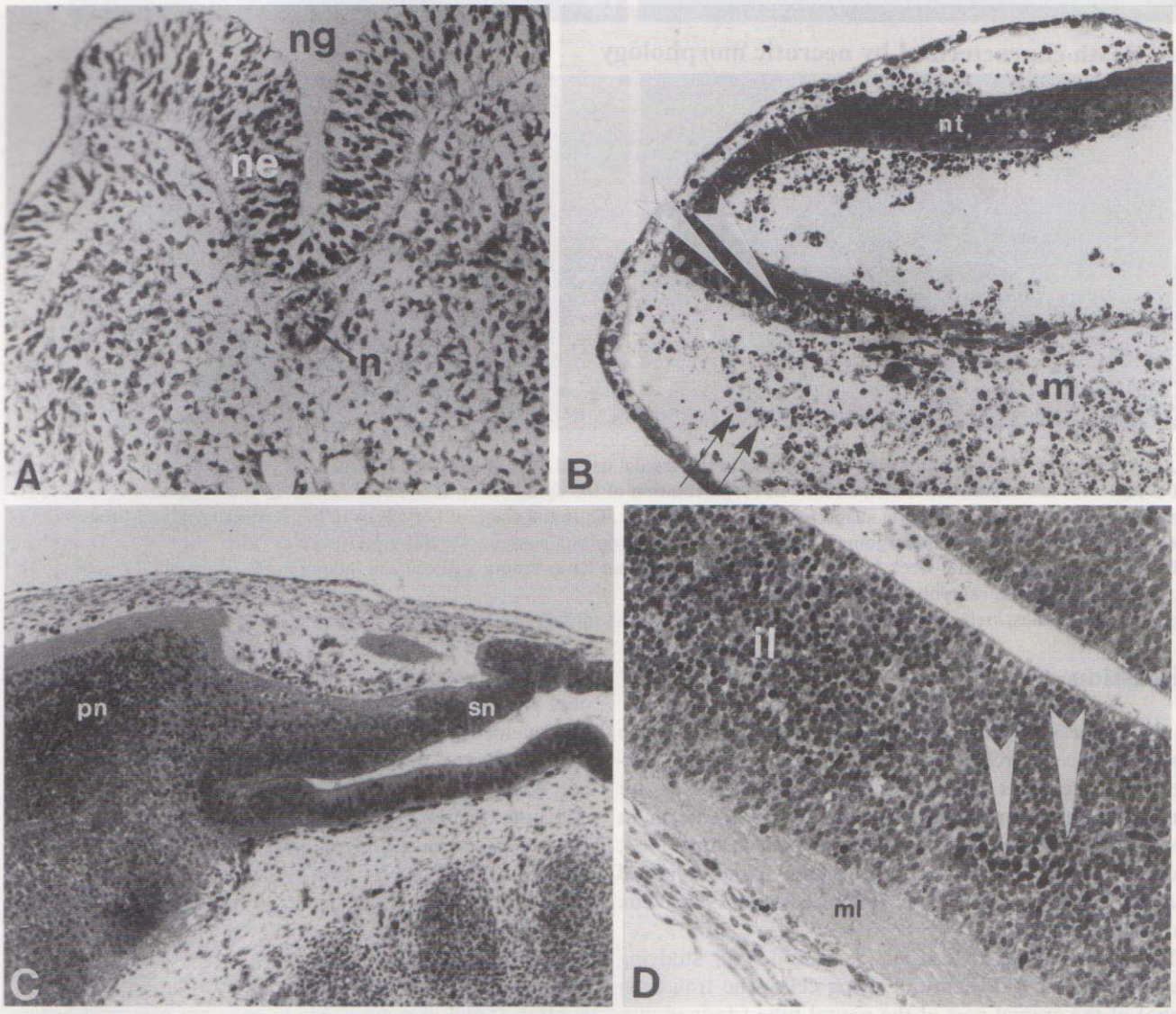
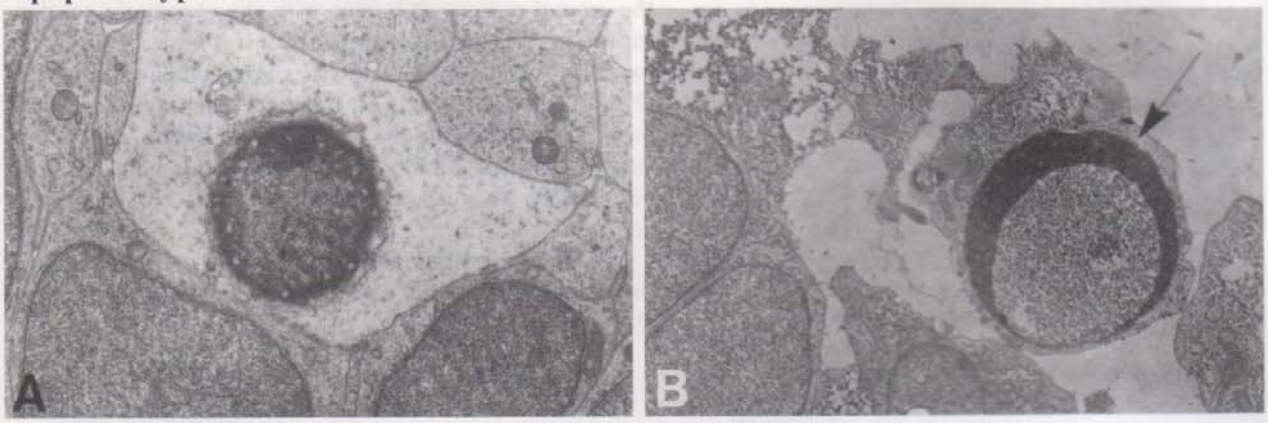


Fig. 1. **A:** A cross-section through the neural groove (ng) of a 4-week human embryo in the region close to the caudal neuropore. The neural groove consists of neuroepithelial cells (ne). The notochord (n) is surrounded by mesenchymal cells. Hematoxylin-eosin, ×40. **B:** Semi-thin cross section through the tip of the caudal neural tube (nt) of a 4-week human embryo. Numerous dying cells with dark nuclei can be observed in the neural tube (white arrows) and in the surrounding mesenchyme (black arrows). Toluidine blue, 40×. **C:** Longitudinal section through the human tail in a 6-week human embryo. A junction is seen between cranial (primary) (pn) and caudal (secondary) (sn) neural tube in the 6-week human embryo. Hematoxylin-eosin, 20×. **D:** Apoptotic cells with condensed chromatin (arrows) in the neural tube epithelium. Toluidine blue, 40×.

Legend: ng = neural groove; ne = neuroepithelial cells; n = notochord; nt = neural tube; m = mesenchyme; pn = primary neural tube; sn = secondary neural tube; il = intermediate layer; ml = marginal layer.

Apoptotic type of cell death



Cell death characterized by necrotic morphology

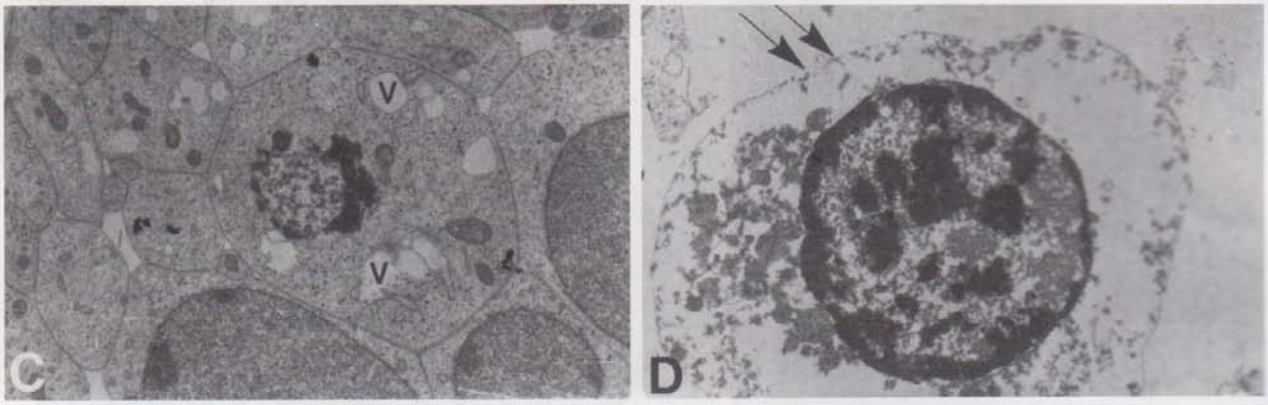


Fig. 2. **A:** Initial pyknosis as a first sign of apoptosis in the cranial neural tube of a 6-week human embryo. The cytoplasm of the dying cell is pale with few organelles. TEM, 1 600×. **B:** Fragmentation of the dying cell in the cranial cranial (primary) neural tube. The nucleus has a characteristic half-moon shape (arrow). TEM, 6 600×. **C:** Initial stage of karyolysis in the secondary neural tube of the 6-week embryo. The nucleus is undergoing karyolysis, while the cytoplasm contains several large vacuoles (v). TEM 8 300×. **D:** A dying cell in the caudal part of a 3-week human embryo. The cytoplasm looks empty and cell the membrane is fragmented (arrows). The nucleus is disintegrated (karyolysis). TEM 16 000×
Legend: v = vacuoles; arrow on Fig. 2 B = half moon shape; arrows on Fig. 2 D = fragmented cell membrane

Discussion

In the process of development and in adult life, large numbers of cells are known to die in many different tissues. In some cases, whole regions or entire organs are eliminated. In other cases, scattered dying cells occur in developing tissues destined to survive (Glucksman 1951; Saunders 1966). These facts are well known and reviewed elsewhere (Clarke and Clarke 1996; Lockshin 1997; Vaux and Korsmeyer 1999).

The human tail provides an ideal model for studying the morphological diversity of dying cells. The transition (zone) of the cranial part of the neural tube (derived during primary neurulation) into the caudal part (derived during secondary neurulation) of the neural tube seems to reflect the change of one pattern of cell death into another. While in the cranial tube a typical apoptotic type of cell death prevails, the caudal part of the neural tube displays a morphologically different type of cell death.

The observed morphological diversity of developmental cell death in this study corresponds to the cell death types described previously on different animal models. Schweichel and Merkel (1973) conducted a morphological study of developmental cell death in rat and mouse embryos and identified three morphologically distinct groups of cell death. Type one (I) is characterized by the early condensation of the nucleus and cytoplasm followed by cell fragmentation and phagocytosis by the neighboring cells. This cell death type is always found in isolated cells. The second type (II) (later named autophagic degeneration, see Clarke 1990) is characterized by the early appearance of large inclusions in the cytoplasm derived from autophagic vacuoles or autolysosomes. This type of cell death is often found in regions where cells are removed *in toto*. The third type (III) of cell death (later named non-lysosomal disintegration, see Clarke 1990) is characterized by the swelling of cavities within a membrane border, such as mitochondria, followed by extensive fragmentation of the cells into fragments so small that cell debris can no longer be observed. This type of cell death does not include a lysosomal system and is observed in regions of vacuolated cartilage during mineralization.

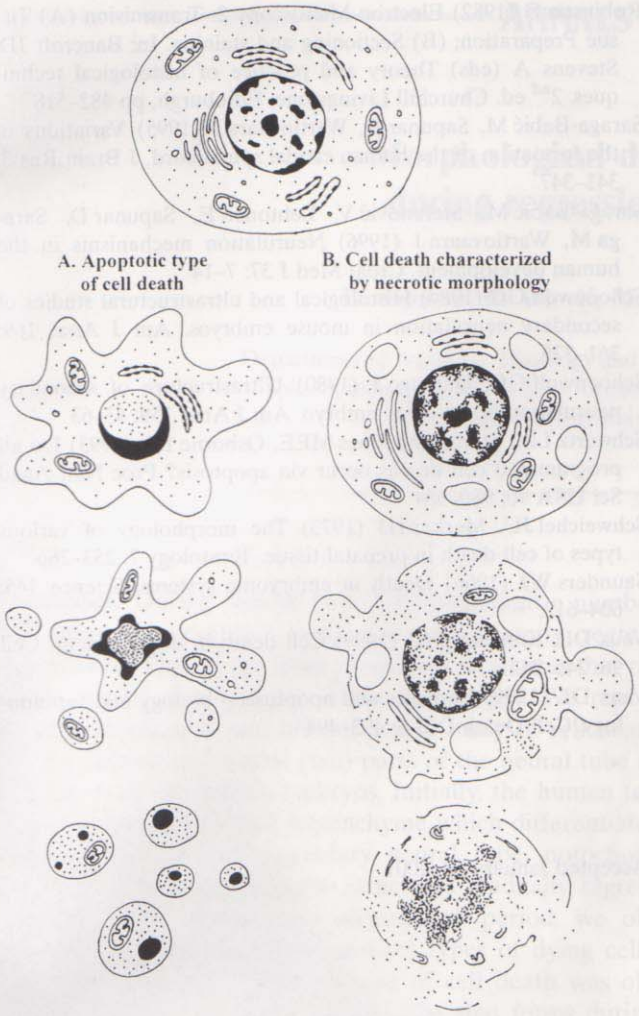


Fig. 3. Schematic representation of the observed morphologically distinct types of cell death.

The morphological characteristics of type I cell death are identical to apoptosis described by Kerr et al. (1972) and include cell shrinkage, dense chromatin condensation, nuclear fragmentation, cytoplasmic blebbing and cellular fragmentation into small apoptotic bodies. The cell death that we observed in the present study in primary neural tube of the human tail is comparable to type I (apoptotic) cell death.

The other two types of cell death according to Schweichel and Merkel (1973) can be vaguely morphologically classified as necrotic. These types of cell death have been previously described only in numerous experimental animal models (Decker RS 1974; Clarke PG 1984; Lamborghini JE 1987; Schwartz LM et al. 1993) and not in the human embryo. In their publications, the necrotic type of cell death is not connected with the *in toto* removal of vestigial structures. Morphologically, our description of cell death in the secondary neural tube corresponds to their classification of necrotic. However, we would not draw the same conclusion as to the importance of lysosomal activity as they did in their studies. In our study lysosomes appear to be relatively stable organelles; following the initiation of the self destruction process they burst only after cell death. This was also observed by Hawkins et al. (1972).

We can speculate that the apoptotic type of cell death is associated with the precise control of cell numbers and morphogenesis of the cranial tube. The other morphologically distinct type of cell death is associated with the massive removal of transitory structures in the caudal neural tube and also includes a vast involvement of macrophages. The relatively small numbers of observed apoptotic cells are probably the result of the rapid phagocytosis of those dying cells by their neighbouring cells. This observed morphological diversity of cell death also coincides with differences in the origin and fate of the cranial and caudal neural tubes, respectively.

Recent advances in molecular biology can help us in the molecular characterization of these types of cell death. Borner et al. 1999 summarized current data on signaling pathways on at least three intertwined pathways for cell death: a) the receptor-activated path, b) the mitochondrial path, and c) the caspase-independent path (Borner et al. 1999).

The first two pathways are characterised by an apoptotic morphology and have the same effectors; i. e. caspases. Their proteolytic activity provides a molecular basis for an apoptotic morphology. These two pathways could explain the appearance of the apoptotic cell death in the present study in the cranial neural tube.

In contrast, caspase independent programmed cell death is characterized by a necrotic morphology in dying cells. This caspase independent programmed cell death might be responsible for the destruction of the human tail and the secondary neural tube in the present report.

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