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Cytokinetics of secondary neurulation in chick embryos: Hamburger and Hamilton stages 16–45

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Abstract *Objectives:* In an attempt to understand the events in the secondary neurulation in embryonic stage, we investigated cytokinetic changes in the tail bud of normal developing chick embryos. *Materials and methods:* We investigated chick embryos of Hamburger and Hamilton stages 16–45 by staining for proliferating cell nuclear antigen (PCNA) and applying the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) method. *Results and conclusions:* The cytokinetics of secondary neurulation have several distinctive properties: a general chronological delay compared to primary neurulation; absence of ventrodorsal differences; larger rostrocaudal differences; and degeneration of the distal end of the spinal cord in the later stages of development. These differences in morphogenetic and cytokinetic behavior between primary and secondary neurulation may help to improve our understanding of anomalies originating during secondary neurulation.

Keywords Secondary neurulation · Chick embryo · Cytokinetics · Cell proliferation · Apoptosis · Proliferating nuclear cell antigen (PCNA) · TUNEL

Introduction

In most vertebrates, the cranial portion of the neural tube is derived from the neural plate that forms after gastrulation by a process called primary neurulation. Subsequent to primary neurulation, the caudal or secondary neural tube develops by the cavitation of a solid mass of cells, the tail bud, to form a neurocele.

The morphological changes that take place during the development of the tail bud of chick embryos were extensively studied [9, 11, 12, 15]. However, there are only a few studies of the cytokinetic changes occurring during secondary neurulation [3, 7]. We carried out the present study to examine cellular kinetics throughout the period of chick embryo tail development.

Materials and methods

Fertile leghorn chicken eggs, purchased from a commercial supplier (Pulmuone, Eumseong, Korea), were incubated at 38–39°C with humidity in excess of 80%. At least four chick embryos at each of Hamburger and Hamilton (HH) stages 16, 18, 20, 22, 24, 26, 30, 35, 40, and 45 were harvested, fixed in 10% neutral-buffered formalin for 1 day, and were embedded in paraffin. Subsequently, the

embryos were sectioned into 4 μm thickness and were stained with hematoxylin–eosin. The peroxidase–antiperoxidase immunohistochemical staining method was used to analyze proliferating cell nuclear antigen (PCNA) using primary antigen PC10 (DAKO, Carpinteria, USA). To observe the pattern of apoptosis, we carried out terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) by using ApopTag In Situ Apoptosis Detection Peroxidase Kits (Oncor, Gaithersburg, USA). All the slides were processed in the same conditions for each staining procedure to make reliable comparisons between the groups of embryos of different ages. The density and distribution of the stained cells were evaluated under light microscope (BX-51, Olympus, Japan). The slides were evaluated using ×40, ×100, and ×400 magnifications.

Observations and results

There were many mitoses in the caudal cell mass at HH stage 16 but no detectable mitotic activity was observed in the notochord. This paucity of proliferative activity in the notochord persisted throughout the period of secondary neurulation. At HH stages 18 and 20, PCNA-positive cells were observed in the caudal cell mass and tail gut. At HH stage 20, the frequency of PCNA-positive cells observed in the caudal cell mass and in the tail bud mesenchyme was higher when compared to the distal neural tube and the somites. At HH stage 20, the proliferative activity in the distal neural tube was observed to be slightly greater when compared to HH stage 16 (Fig. 1).

PCNA-positive cells were mainly located at the periphery of the caudal cell mass at HH stage 22 and there were no PCNA-positive cells in the tail gut. At HH stage 24, less proliferative activity was found in the undifferentiated caudal cell mass compared to the distal neural tube and somites (Fig. 1).

At HH stage 30, the rate of cell division in the neural tube was observed to be higher in the proximal regions of the tail where it had the shape of a spinal cord close to regions formed by primary neurulation (Fig. 2). However, the rate of cell division was observed to be significantly lower around the tip of the tail. The number of PCNA-positive cells had further increased in the neural tube in the proximal part of the tail at HH stage 35. There were no PCNA-positive cells in the more distal part of the neural tube where it bulged dorsally (Fig. 2).

At HH stage 40, proliferative activity had increased in the neural tube apart from the tip of the tail and most of the PCNA-positive cells were in the germinal layer. The rate of cell division was observed to be significantly lower at the caudal levels of the proximal tail. At HH stage 45, proliferative activity was found to be decreased (Fig. 2).

At HH stages 16 and 18, TUNEL-positive cells were found in the caudal cell mass adjacent to the surface epithelium and there were also a few positive cells in the notochord. Whether it was composed of multiple small cavities or not, no TUNEL-positive cell was observed in the neural tube. At HH stage 18, TUNEL-positive cells were also noticed at the tail bud mesenchyme where the

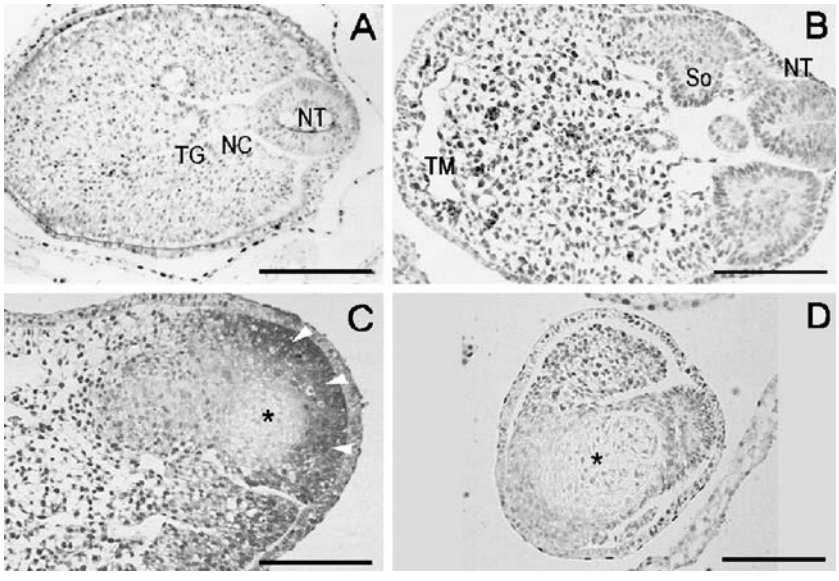

Fig. 1 PCNA staining of Hamburger and Hamilton stages 18 (**a**), 20 (**b**), 22 (**c**), and 24 (**d**). The photographs are arranged so that the left side of each corresponds to the ventral side of the embryo. (**a**) There are many proliferating cells in the neural tube (*NT*), paraxial mesoderm, tail gut (*TG*), and tail bud mesenchyme, but few in the notochord (*NC*). (**b**) The proliferating activity of the tail bud mesenchyme (*TM*) surpasses that of the neural tube (*NT*) and somites (*So*). (**c**) Labeled cells are mainly located at the periphery of the caudal cell mass (*arrowheads*). There are few PCNA-positive cells in the central portion (*asterisk*). (**d**) The PCNA-positive reactivity of caudal cell mass is lower than any other structures in the tail (*asterisk*). *Bar*=100 μm

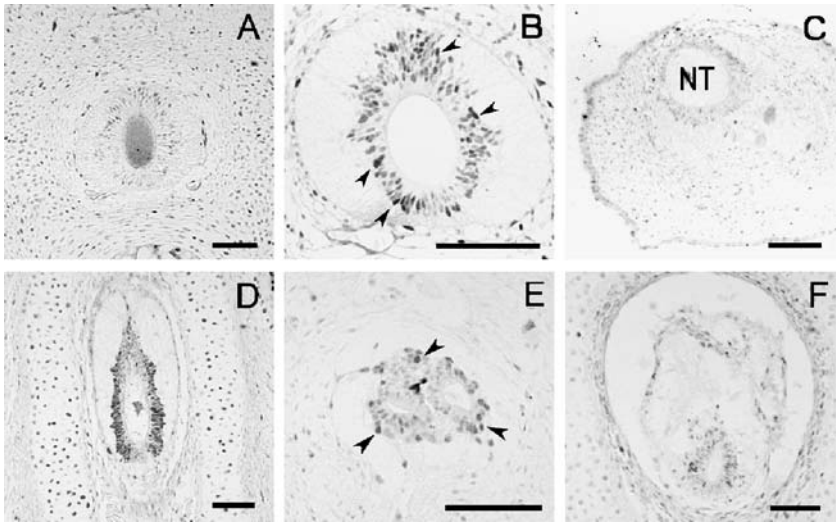

Fig. 2 PCNA staining of Hamburger and Hamilton stages 30 (**a**), 35 (**b**, **c**), 40 (**d**, **e**), and 45 (**f**). The photographs are arranged so that the lower side of each corresponds to the ventral side of the embryo. (**a**) There are several labeled cells in the neural tube at proximal part of the tail. (**b**) There are many labeled cells in the proximal region of the tail (*arrowheads*). (**c**) There are no labeled cells in the distal region of the tail where the lumen of neural tube (*NT*) is enlarged to dorsal side. (**d**) Proliferative activity in the neural tube of the proximal tail is further increased at this stage. (**e**) There are a few weakly labeled cells at the tip of the tail (*arrowheads*). (**f**) The number of labeled cells has decreased by this stage in the proximal tail. *Bar*=50 μm

incorporation into somites has not yet occurred. The number of TUNEL-positive cells was found to be further increased at HH stage 20. However, no TUNEL-positive cell was found in the tail gut (Fig. 3).

At HH stage 22, most TUNEL-positive cells were found in the caudal cell mass but there were also a few positive cells in the distal neural tube and tail gut. At HH stage 24, TUNEL-positive cells were mainly located in the center of the caudal cell mass and in the degenerating tail gut. The

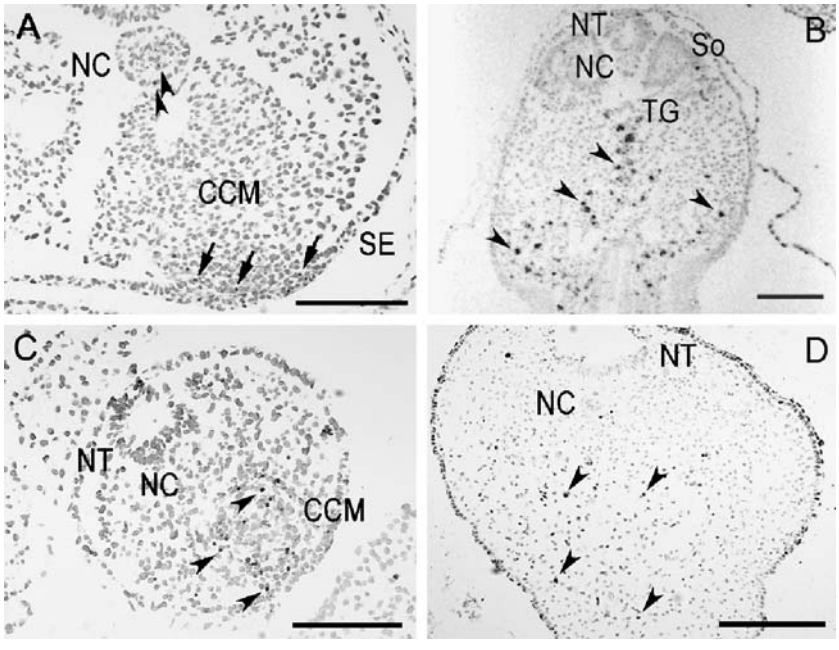

Fig. 3 TUNEL assays of Hamburger and Hamilton stages 16 (**a**), 20 (**b**), 26 (**c**), and 30 (**d**). The photographs are arranged so that the lower side of each corresponds to the ventral side of the embryo. (**a**) TUNEL-positive cells are found in the caudal cell mass (*CCM*) adjacent to the surface epithelium (*SE*) (*arrows*). There are also a few labeled cells in the notochord (*NC*) (*arrowheads*). (**b**) There are many labeled cells in the ventral and ventrolateral regions of the tail (*arrowheads*). There are no labeled cells in neural tube (*NT*), notochord (*NC*), and somites (*So*). Although labeled cells are in close proximity to the tail gut (*TG*), there are no positively labeled cells in itself. (**c**) The number of labeled cells has increased further in the caudal cell mass (*CCM*) (*arrowheads*). (**d**) There are several labeled cells in the tail bud mesenchyme (*arrowheads*). *Bar*=100 μm

number of TUNEL-positive cells in the caudal cell mass was found to be progressively increased at HH stage 26. A similar increment was also observed in the neural tube. There were several TUNEL-positive cells in the caudal cell mass and in the tail bud mesenchyme at HH stage 30 (Fig. 3) and a few number of TUNEL-positive cells were noticed in the notochord near the tip of the tail. At HH stage 35, there was no TUNEL-positive cell in the neural tube where it bulged dorsally (Fig. 4).

At HH stage 40, the TUNEL-positive cells in the neural tube were located mainly at the ventral pole and in the dorsolateral regions. At HH stages 40 and 45, there were also many TUNEL-positive cells at the tip of the tail in the neural tube where numerous small cavities existed (Fig. 4). At HH stage 45, there were TUNEL-positive cells scattered in the neural tube, which were mainly located in the mantle layer of the dorsal half of the spinal cord (Fig. 4).

Discussion and conclusions

The cytokinetics of primary neurulation were studied by several investigators [3, 5, 14] and although there were several investigations of the cytokinetics of secondary neurulation, they did not cover the entire period [6–8]. We therefore extended the period of our observations to detect changes in cell dynamics covering the period of secondary neurulation up to hatching.

There was considerable cell division in the caudal cell mass in the early stages of secondary neurulation, although there is controversy about whether it is a true proliferation center [7, 8, 13]. By HH stage 22, the rate of cell division declined, especially in the central region, and there were many TUNEL-positive cells. Thereafter, cell division was overtaken by apoptosis and this situation was kept until the time when the caudal cell mass had disappeared.

The notochord itself did not show any proliferative activity. There were a few TUNEL-positive cells, especially in the distal portion near the tip of the tail at HH stages 16, 18, and 30. These foci are thought to be involved in the separation of the notochord from its precursors and its remodeling. The cytokinetic "inertness" of the notochord means that it is formed from terminally differentiated cells that have already passed the stage of proliferation and that the cells are not doomed to degenerate.

The TUNEL-positive cells that we observed in this study in the caudal cell mass adjacent to the surface epithelium at HH stages 16 and 18 were also noted by other investigators [7, 9, 11]. This supported the idea that the separation of the ectoderm from the tail bud, which is spatially correlated with the process of cavitation, is the result of cell death. This same observation was made at the equivalent stages of mouse and human development, suggesting that separation of the caudal cell mass from the surface ectoderm is a highly conserved event in secondary neurulation [1, 2].

The fan-shaped distribution of TUNEL-positive cells in the tail gives the impression that cells that are not destined to differentiate into structures such as neural tube, notochord, and somites degenerate as early as HH stage 20 while the caudal cell mass is actively proliferating. The cytokinetic changes in the tail gut observed in this study are in accord with morphological observations of its development and degeneration [6, 11, 15].

We observed maximal proliferative activity in the neural tube at HH stage 40, whereas during primary neurulation, the peak of proliferation occurs at HH stages 26 and 28 [3, 14]. This difference is probably due to the general delay of secondary neurulation relative to primary neurulation. The

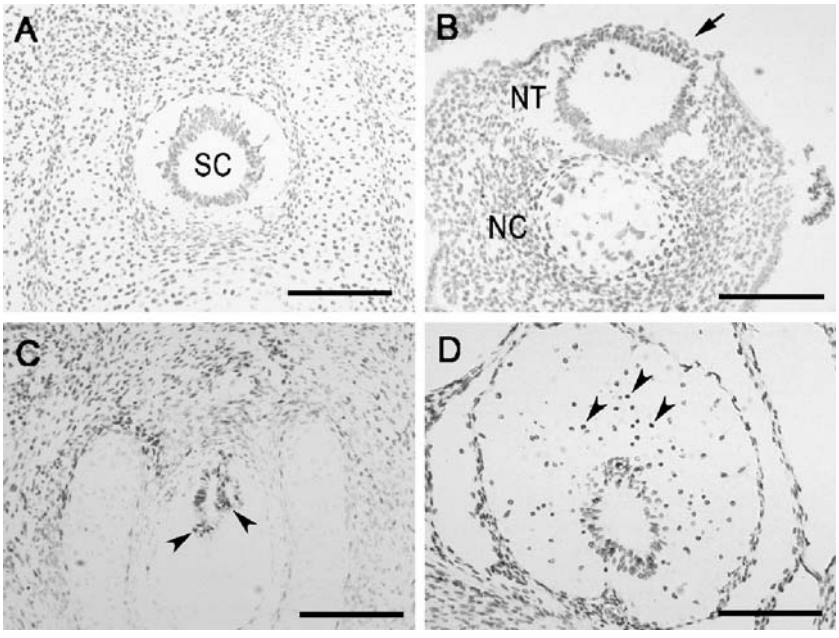

Fig. 4 TUNEL assays on Hamburger and Hamilton stages 35 (**a**, **b**), 40 (**c**), and 45 (**d**). Photos are arranged as before. (**a**) There are no labeled cells in spinal cord (*SC*) at the proximal part of the tail. (**b**) No labeled cells are visible at the distal part of the tail in notochord (*NC*) or neural tube (*NT*) where it bulges dorsally (*arrow*). (**c**) There are several labeled cells at the tip of tail where the neural tube is composed of multiple small cavities (*arrowheads*). (**d**) Labeled cells are mainly located in the mantle layer (*arrowheads*) *Bar*=100 μm

fact that we found no dorsoventral differences in proliferative activity in the spinal cord formed by secondary neurulation is probably due to the different mode of neural tube formation. Rostrocaudal differences are more marked in secondary neurulation than in primary neurulation. In primary neurulation, fusion of the neural folds begins at HH stage 8 (26–29 h of incubation) and is completed by HH stage 13 (48–52 h of incubation) [4, 10]. Secondary neurulation begins at HH stage 16 (51–56 h of incubation) and is completed by HH stage 35 (8–9 days of incubation) [15]. Because of this delay, at HH stage 35 the neural tube at the tip of the tail has just finished coalescing when the neural tube in the proximal tail already has the appearance of the developing spinal cord. This rostrocaudal time gradient is probably the cause of the morphological and cytokinetic differences at different levels of the tail.

The fact that there were no TUNEL-positive cells in the neural tube where it bulged dorsally at HH stage 35 suggests that the dorsal bulging of neural tube is not an active degenerative process involving apoptosis, but a passive dilatation caused by mechanical weakness [15].

In primary neurulation, there are three pycnotic zones in the spinal cord between HH stage 12 and 22 [5] and after that, TUNEL-positive cells are mainly found in the germinal layer, ventrolateral parts of the mantle layer, and the dorsal root ganglion [14]. The neural tube formed by secondary neurulation is delayed in development compared to that formed by primary neurulation. Even immediately before hatching, the distal neural tube only has a scanty mantle layer in the proximal tail. Therefore, the cell death observed in the present study is comparable to the early stages of primary neurulation described by Homma et al. [5]. Morphological studies of secondary neurulation of chick embryos led to the idea that the small multiple cavities detected at HH stages 40 and 45 at the tip of the tail are in the process of degeneration [15]. This notion is supported by the results of our cytokinetic study.

The cytokinetics of secondary neurulation have several distinctive properties: a general chronological delay compared to primary neurulation; absence of ventrodorsal differences; larger rostrocaudal differences; and degeneration of the distal end of the spinal cord in the later stages of development. These differences in morphogenetic and cytokinetic behavior between primary and secondary neurulation may help to improve our understanding of anomalies originating during secondary neurulation.

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