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Cytokinetic Pattern in the Thoracic Spinal Cord of Chick Embryos (Incubation Day 5-13) Using PCNA Staining and TUNEL Method

For the cytokinetic studies using spinal cords of chick embryos, chronological patterns of cell proliferation and programmed cell death (apoptosis) should be known. Information in the early stages of chick embryos is available while data on later stages are seldom available. To investigate the chronological patterns of cell proliferation and apoptosis in the thoracic spinal cord of normal chick embryos on incubation day 5, 6, 8, 10 and 13 (Hamburger and Hamilton stage 26-40), proliferating cell nuclear antigen (PCNA) staining and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method were used. Cell proliferation was active at the germinal layer on days 5 and 6. It markedly declined on day 8 and became negligible on day 13. TUNEL-positive cells were mainly found in the germinal layer, the ventrolateral part of the mantle layer and the dorsal root ganglion. Compared to PCNA-positive cells, TUNEL-positive cells were sparse, especially after day 10, when only a few positive cells were scattered. These results will be used as a control data for the studies such as an experimental research for neural tube defects.

Key Words : *Spinal cord; Chick embryo; Cell cycle, cytokinetics; Proliferating cell nuclear antigen; DNA nucleotidylexotransferase; Apotosis*

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INTRODUCTION

For experimental studies in the fields of neuroscience such as development, tumor biology, degenerative disorders and ischemia, data on cytokinetics is essential not only for the understanding of normal and disease processes but also for the development of preventive and treatment strategies.

Spinal cords of chick embryos are widely used for the neural development research. The proliferative activities in the spinal cord of chick embryos observed by classical methods using hematoxylin-eosin (HE) staining have been described (1). The patterns of programmed cell death, apoptosis, in the spinal cord of chick embryos, however, have seldom been reported. In 1994, Homma et al. (2) described the patterns of apoptosis during the period of Hamburger and Hamilton (HH) stages 16-20 (3).

The sugical experimental model for neural tube defect research has several advantages. For the studies where the exact time of neural tube defect formation should be known, the surgical model is necessary. Investigators can easily modify the time of neural tube incision as well as the location and the size of the lesion. We planned to compare the cytokinetic features in the normal chick embryos and those with neural tube defects using the surgical model. For the incision of already closed neural tubes, embryos incubated for at least 3 days were used. Thereafter cell dynamics were observed for several days.

To obtain the control data for cytokinetic studies in surgically induced neural tube defect, we observed the chronological patterns of cell proliferation and apoptosis in the thoracic spinal cord of normal chick embryos of incubation days 5, 6, 8, 10, and 13 (HH stages 26-40) using proliferating cell nuclear antigen (PCNA) staining (4, 5) and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method (6).

MATERIALS AND METHODS

Eggs of commercial source (Pulmuone, Korea) were used. Eggs were incubated in 38-39℃ with humidity of more than 70%. For each of incubation days 5, 6, 8, 10 and 13, two embryos were sampled and staged (HH stage 26 on day 5; HH 28 on day 6; HH 30 or over on day 8; HH 35 on day 10; HH 40 or less on day 13; Stages

between 30 and 35, and between 35-40 were not determined in detail). The embryos were fixed with 10% neutral-buffered formalin solution for one day and embedded in paraffin. The embryos were sectioned by 4 μm thickness at the level of wing buds and stained. Adjacent sections were stained with HE, PCNA and TUNEL. The peroxidase-antiperoxidase immunohistochemical staining method was used for anaysis of PCNA using primary antigen PC10 (DAKO, Carpinteria, U.S.A.). To observe the pattern of apoptosis, we carried out TUNEL using ApopTag® In Situ Apoptosis Detection Peroxidase Kit (Oncor, Gaithersburg, U.S.A.). For each staining, all the slides were processed under the same condition for better comparison between groups of each incubation days. For TUNEL, lung tissue in the same section was used as the positive control. Using a light microscope, density and distribution of positively stained cells were observed.

RESULTS

Representative sections were shown in Figs. 1-10.

Cell proliferation

On incubation day 5 (Figs. 1, 2), PCNA-positive cells were frequently found in the germinal layer and the dorsal root ganglia. Though the mitotic figures were seen only along the luminal surface area, PCNA-positive cells were scattered throughout the whole thickness of the germinal layer. The ventral portion of the germinal layer which is less prominent than the dorsal portion became narrower on incubation day 6 (Fig. 3). Compared to day 5, the PCNA-positive cells decreased in the ventral part of the germinal layer. However, the dorsal part still showed active proliferation. The medial part of the dorsal root ganglia showed more positive cells. On incubation day 8 (Fig. 4), the germinal layer became thin at the posterior end. Its middle portion was the thickest. Generally PCNA-positive cells markedly decreased in number and were found mainly in the thick part of the germinal layer. No active proliferation was found in the ventral part of the germinal layer and the dorsal root ganglia. On incubation day 10 (Fig. 5), the germinal layer became less dense than on day 8. Only a few positive cells were seen on the middle portion of the germinal layer. On incubation day 13 (Fig. 6), posterior horns were formed. No PCNA-positive cells were visible in the whole spinal cord section.

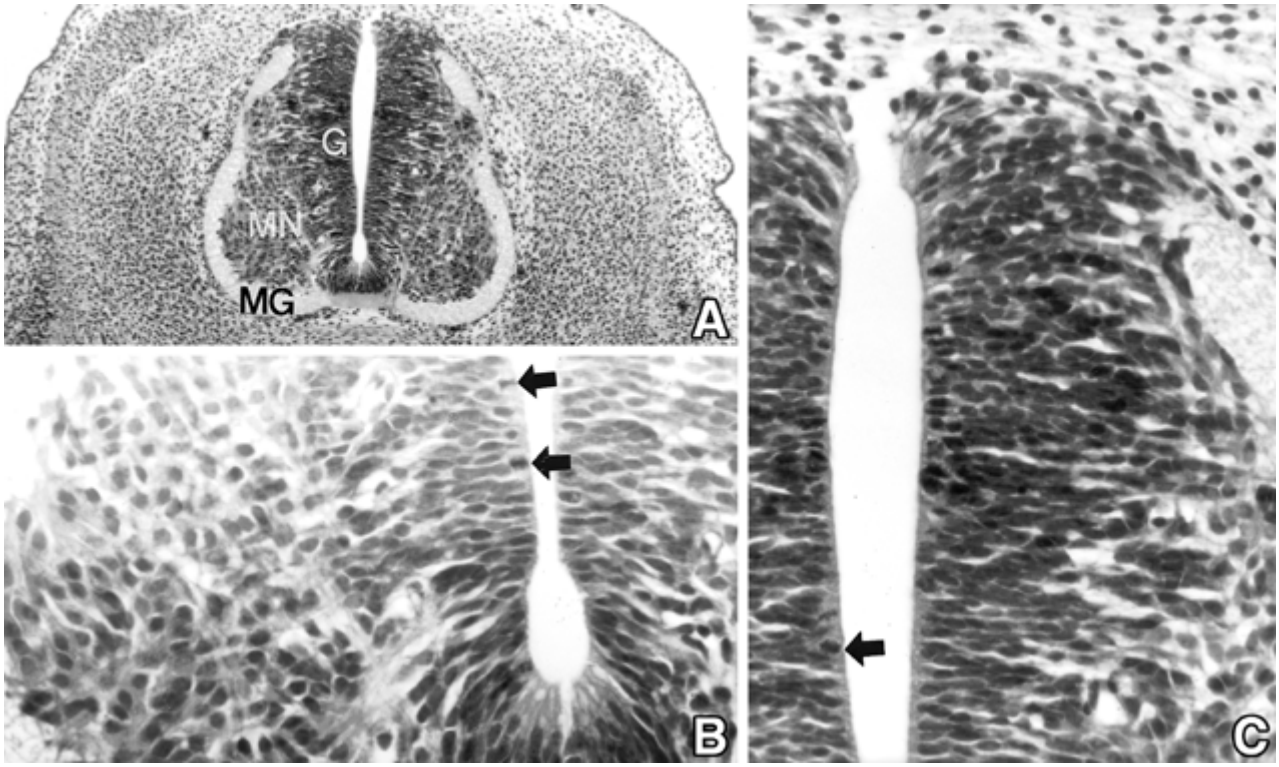

Fig. 1. Hematoxylin-eosin staining on incubation day 5. The germinal layer is prominent and mitoses are frequently seen along the inner surface of the germinal layer (arrows). Mantle layer was formed at the ventrolateral part. Marginal layer is thin (original magnification: A, ×100; B and C, ×400; letters in A: G=germinal layer, MN=mantle layer, MG=marginal layer).

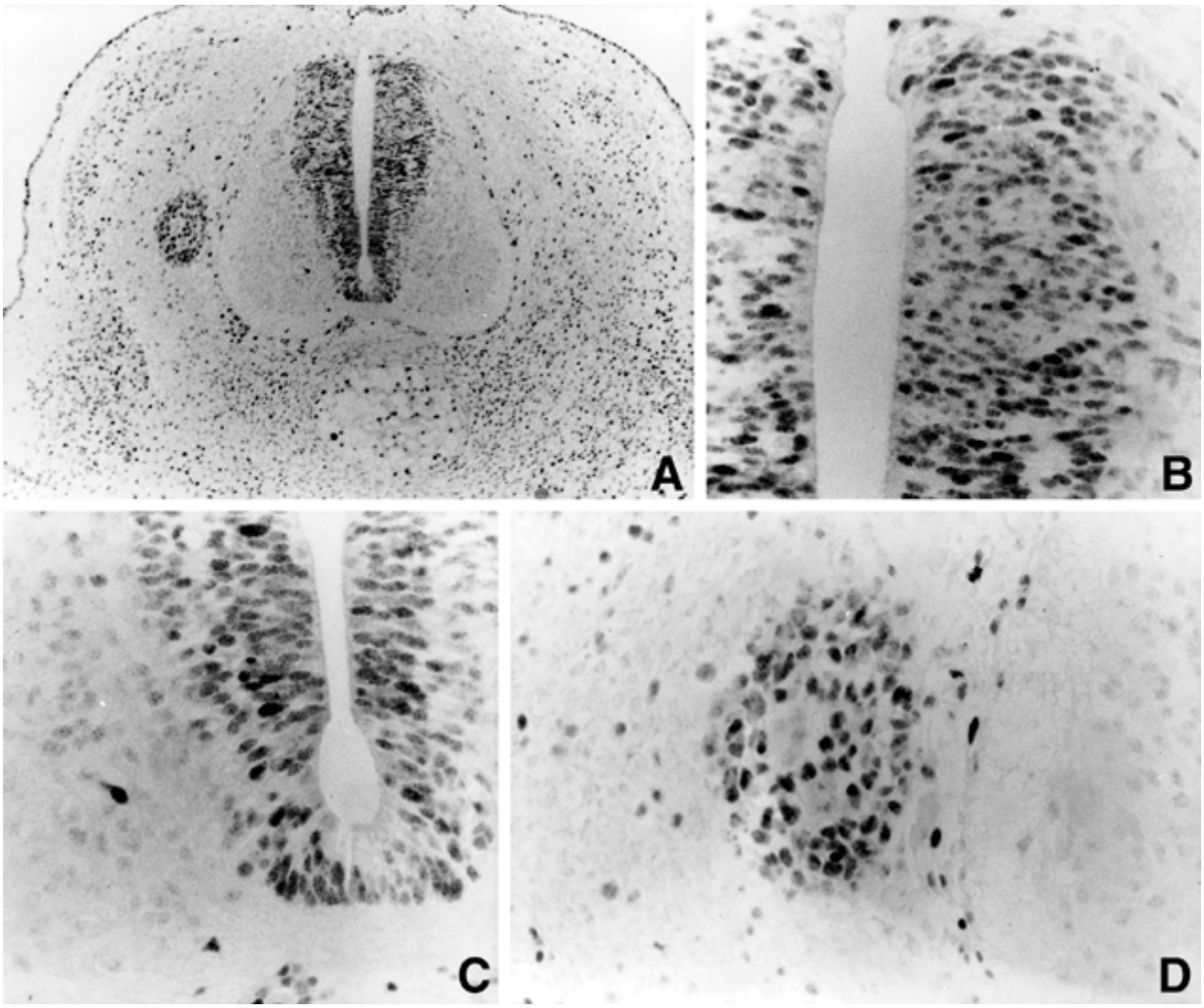

Fig. 2. PCNA staining on incubation day 5. The PCNA-positive cells are seen mainly in the dorsal root ganglion and germinal layer, which is more prominent at the dorsal half of the spinal cord (original magnification: A, ×100; B, C and D, ×400).

Apoptosis

On incubation day 5 (Fig. 7), TUNEL-positive cells were found in the narrow ventral part of the germinal layer, ventrolateral areas of the mantle layer and dorsal root ganglia. On incubation day 6 (Fig. 8), positive cells decreased markedly and prominence in the ventral part of the germinal layer disappeared. On incubation day 8 (Fig. 9), TUNEL-positive cells further decreased in number and were only occasionally found in the ventral horn motor neuron area. At the dorsal root ganglion, still a number of positive cells were noted. On incubation day 10 (Fig. 10), still several TUNEL-positive cells were seen in the whole cross section of the spinal cord and the dorsal root ganglia. The number of positive cells decreased further in both areas. On incubation day 13 (Fig. 6), only a few positive cells were found in the gray matter of the spinal cord.

DISCUSSION

Although PCNA staining is not strictly specific for the proliferating cells (7-9), it is generally accepted as one of the methods to investigate cell proliferative activity. The protein molecule is highly conserved in evolution. According to the classical study done by Hamburger (1), the developing spinal cord of the chick embryo shows active cell proliferation at the germinal layer. As in the brain, mitoses in the developing spinal cord were found along the luminal side of the germinal matrix. PCNA-positive cells were, however, seen throughout the whole

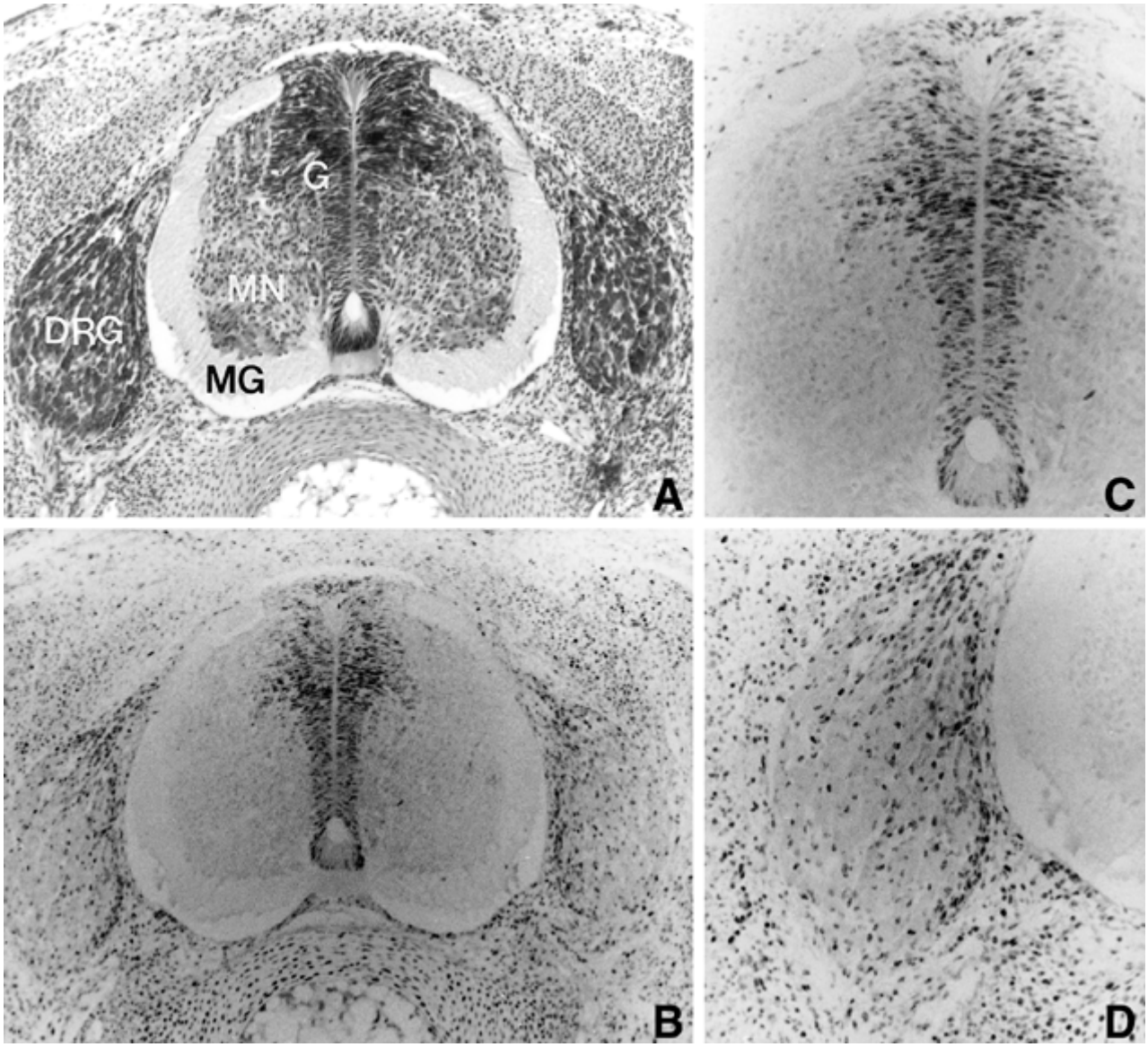

Fig. 3. Hematoxylin-eosin and PCNA staining on incubation day 6. Compared with incubation day 5, relative proportions of the mantle and marginal layers are larger. Motor neurons are seen at the ventrolateral corners of the mantle layer. Though the cell proliferative activity is still active in the germinal layer, it is decreased in the ventral portion. PCNA positive cells are more frequent in the medial part than in the lateral part of the dorsal root ganglion (original magnification: A, HE, ×100; B, PCNA, ×100; C and D, PCNA, ×200; letters in A: G=germinal layer, MN=mantle layer, MG=marginal layer, DRG=dorsal root ganglion).

thickness of the germinal layer at the early embryonic period up to incubation day 6 because the PCNA staining shows mainly S-phase of cell division and the molecule has a long half life. According to the classical study (1), cell proliferation is more active in the dorsal part of the germinal layer. It reaches its peak by incubation day 5 or 6 and declines thereafter. In contrast, the ventral part of the germinal layer demonstrates less active cell proliferation and from incubation day 3 it gradually decreases. The mitosis count is consistent with the morphological findings that the dorsal half of the germinal layer stayed active longer than the ventral half, and the proliferative activity fades out as the germinal layer becomes less prominent and almost negligible after HH stage 35. The chronological changes of cell proliferative activity in the present study corresponds to Hamburger's (1) description. Until incubation day 6, the PCNA reactivity parallels the prominence of the germinal layer. From incubation day 8, PCNA positivity markedly decreased though the germinal layer covered a considerable area on the HE-stained slides. Around incubation day 13, the normal spinal cord almost loses its proliferative activ-

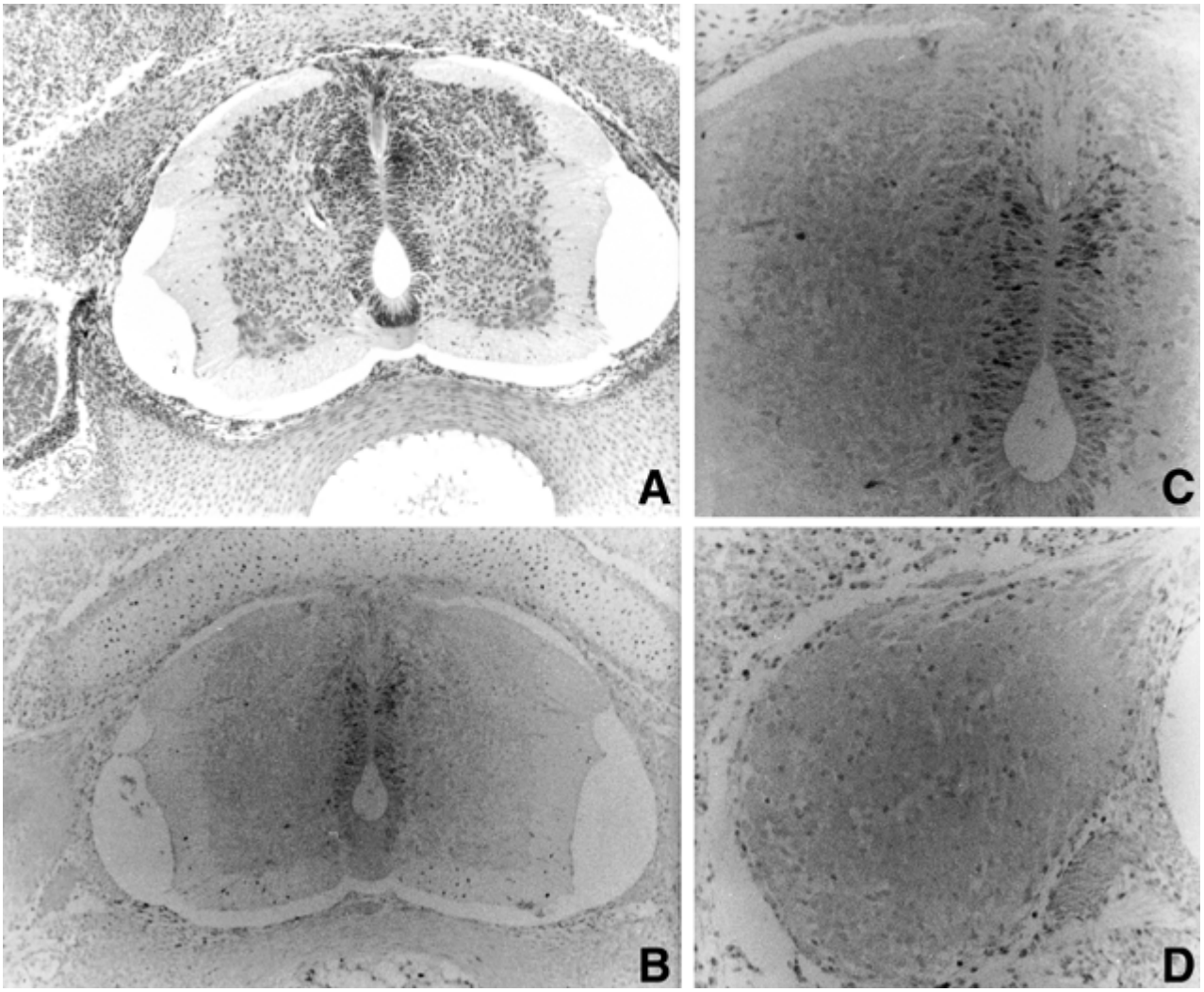

Fig. 4. Hematoxylin-eosin and PCNA staining on incubation day 8. Compared with incubation day 6, the germinal layer became less prominent while the mantle and marginal layers became wider. The density of PCNA cells decreased compared with that on incubation day 6 (original magnification: A, HE, ×100; B, PCNA, ×100; C and D, PCNA, ×200).

ities.

Apoptosis is a well-known mechanism of histogenesis in the field of developmental biology. Introduction of in situ labeling method of apoptotic cells by Gavrieli et al. (6) allowed investigation of spatial distribution of the apoptotic process. In 1994, Homma et al. (2) described the patterns of programmed cell death in the spinal cord of the early stage chick embryos by observation of pyknotic cells in HE-stained slides. According to them, the density of pyknotic cells rapidly increased from HH stage 16 reaching its sharp peak at stage 18, followed by a rapid decline until stage 20. At stage 16, a significant number of pyknotic cells were scattered throughout the germinal layer. At stage 17 and 18, the apoptotic cells were found mainly at the ventral and dorsal poles of the germinal layer. At stage 19, as the number of pyknotic cells decreased, the polar distribution of pyknotic cells became less prominent. Only a few pyknotics cells were noted at stage 20, dominantly in the ventral part.

In the present study, for the specific and clear demonstration of apoptotic cells, TUNEL method was applied. Because Homma et al. (2) already described the pattern of apoptosis in early stages of development, we investigated the apoptotic pattern of the spinal cord in chick embryos of stage 26-40 (incubation days 5-13). As we expected from Homma et al. (2), the apoptotic process in the stages investigated in the present study was not active. Apoptosis decreased as the embryo got older. Throughout the stages examined, the apoptosis occurred mainly in the germinal layer, ventrolateral motor neuron area and dorsal root ganglia.

The TUNEL reactivity of the germinal matrix mark-

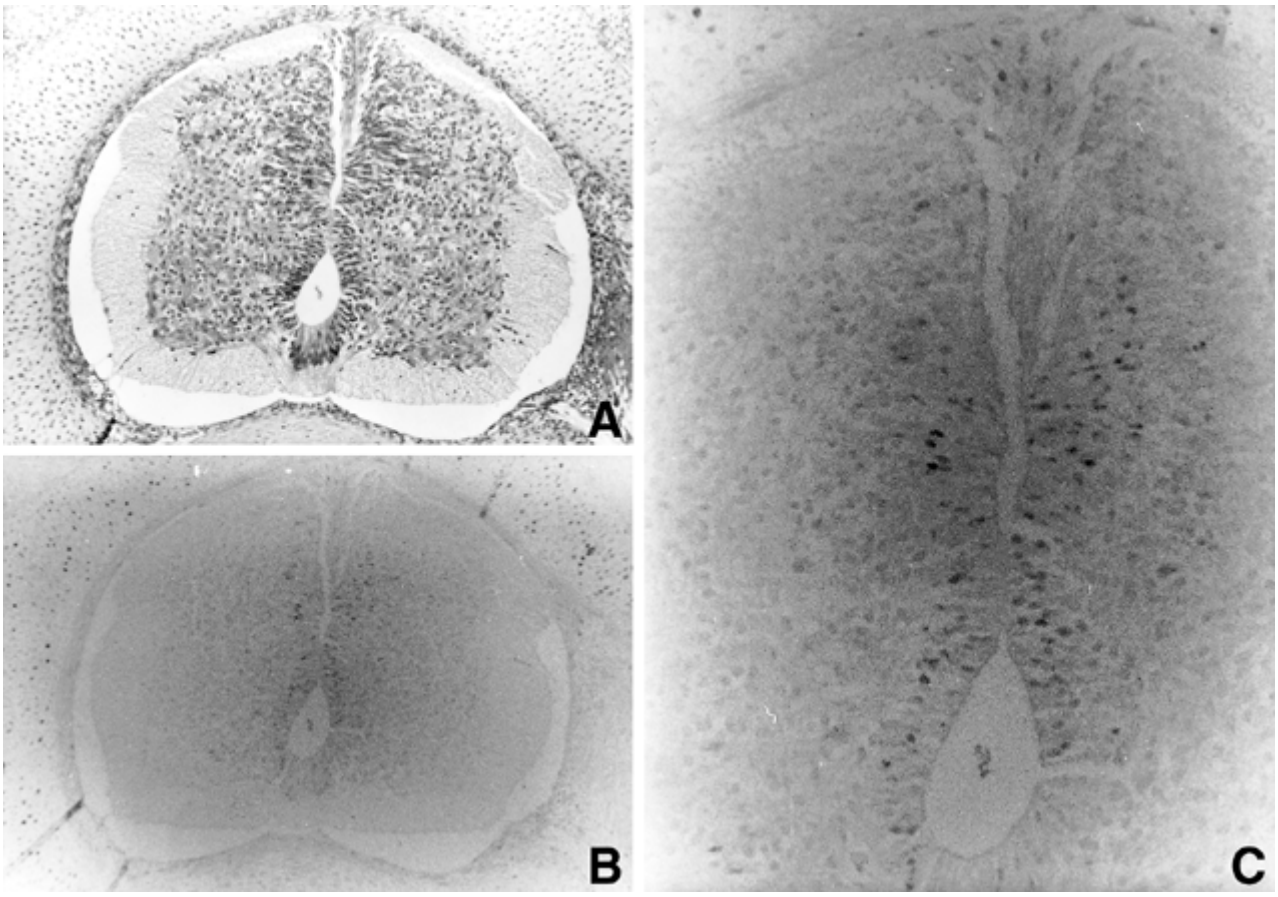

Fig. 5. Hematoxylin-eosin and PCNA staining on incubation day 10. Compared with incubation day 8, cell density of the germinal layer decreased and cell proliferation became less active (original magnification: A, HE, ×100; B, PCNA, ×100; C, PCNA, ×200).

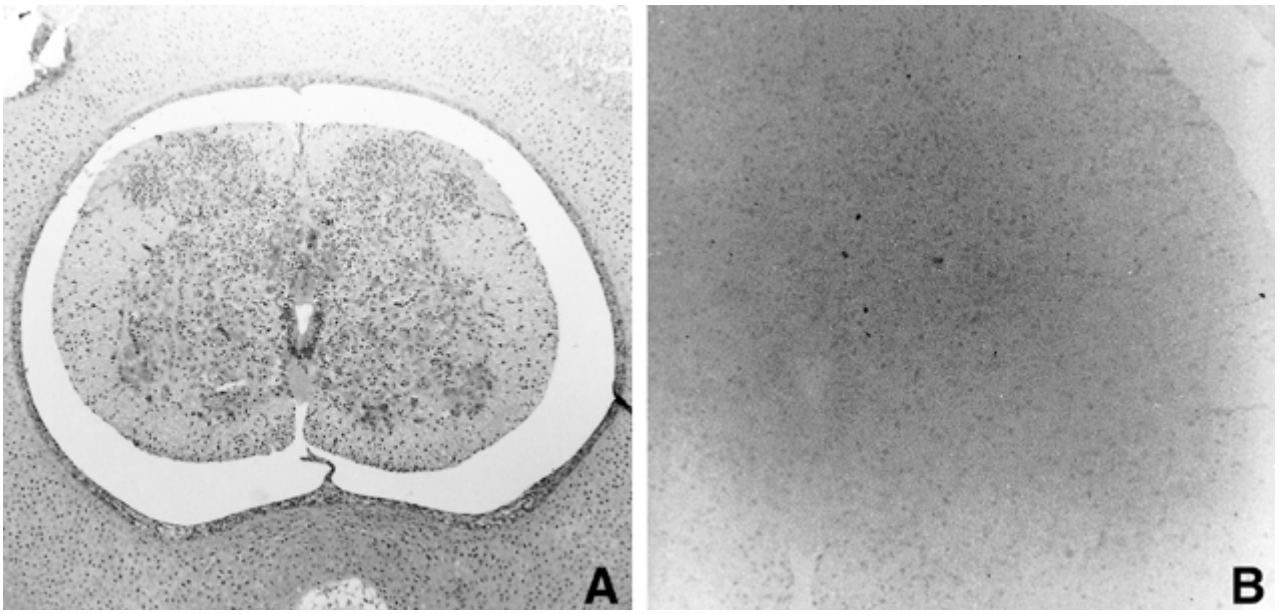

Fig. 6. Hematoxylin-eosin staining (A) and TUNEL (B) on incubation day 13. The contour of the dorsal half of the gray matter was formed well. PCNA-positive cells are invisible (not shown). Only a few TUNEL-positive cells are seen (original magnification: A, ×40; B, ×100).

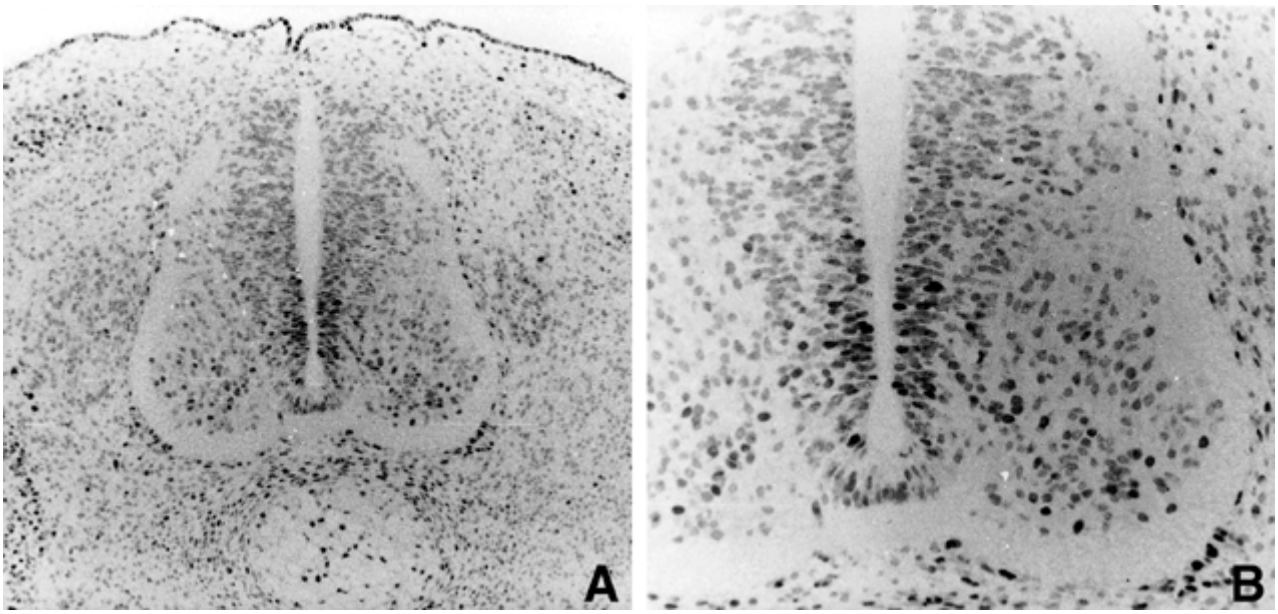

Fig. 7. TUNEL on incubation day 5. The TUNEL-positive cells are seen in the germinal layer, ventrolateral part of the mantle layer, and there are some in the dorsal root ganglion (not shown in this figure) (original magnification: A, ×100; B, ×200).

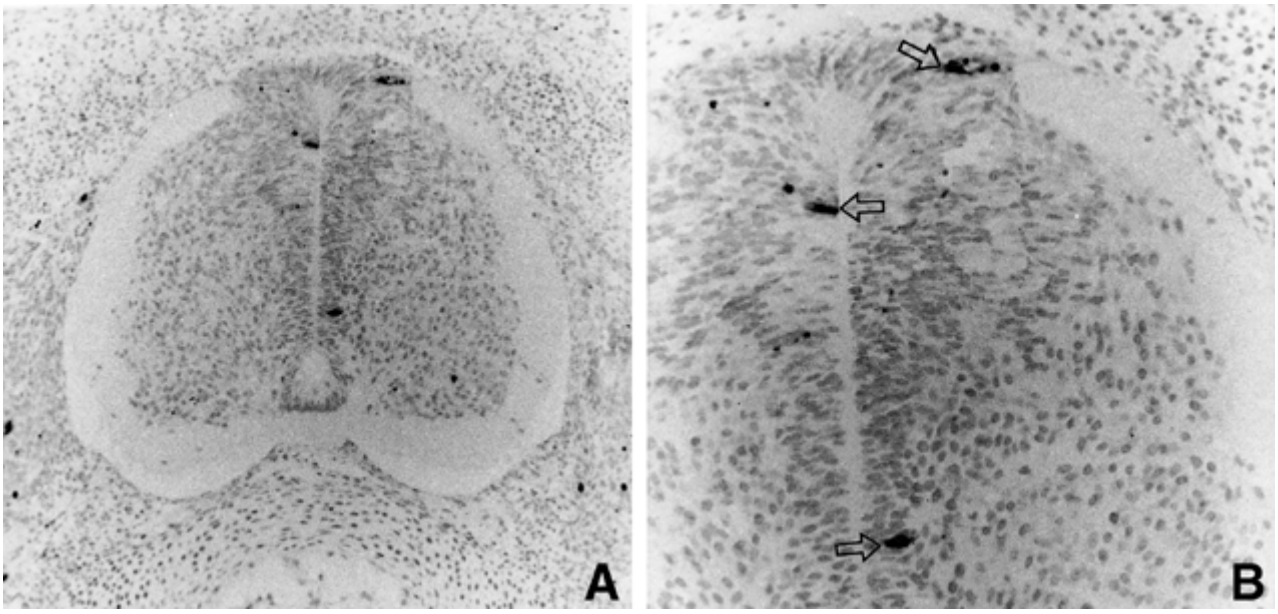

Fig. 8. TUNEL on incubation day 6. The TUNEL-positive cells are decreased in number and seen in the germinal layer, ventrolateral part of the mantle layer, and the dorsal root ganglion. Black dots indicated by open arrows are artifacts (original magnification: A, ×100; B, ×200).

edly decreased on day 6, which preceded a marked decline of proliferative activity by a few days. It is conceivable that as the germinal layer loses its role of cytogenesis, the general cell dynamics becomes rather static. Because the involution of the germinal layer did not accompany a massive cell death, it is our speculation that the cells of the germinal layer mature or change to other cells at least in some parts.

The ventrolateral motor neuron area is believed to be related to the growth factor dependent selection (10-12). As the neuromuscular connection is established, unconnected excessive motor neurons are designated to die.

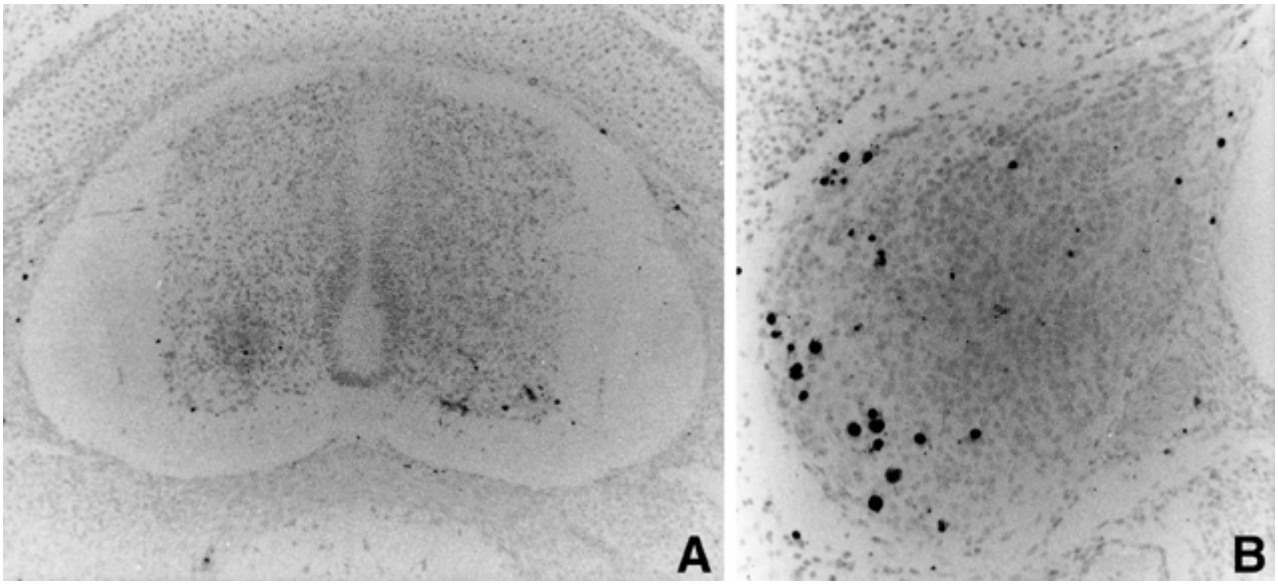

Fig. 9. TUNEL on incubation day 8. The apoptotic activity declined compared with that on incubation day 6. The TUNEL-positive cells are seen in the ventrolateral part of the mantle layer. Still a number of TUNEL-positive cells are seen at the dorsal root ganglion (original magnification: A, ×100; B, ×200).

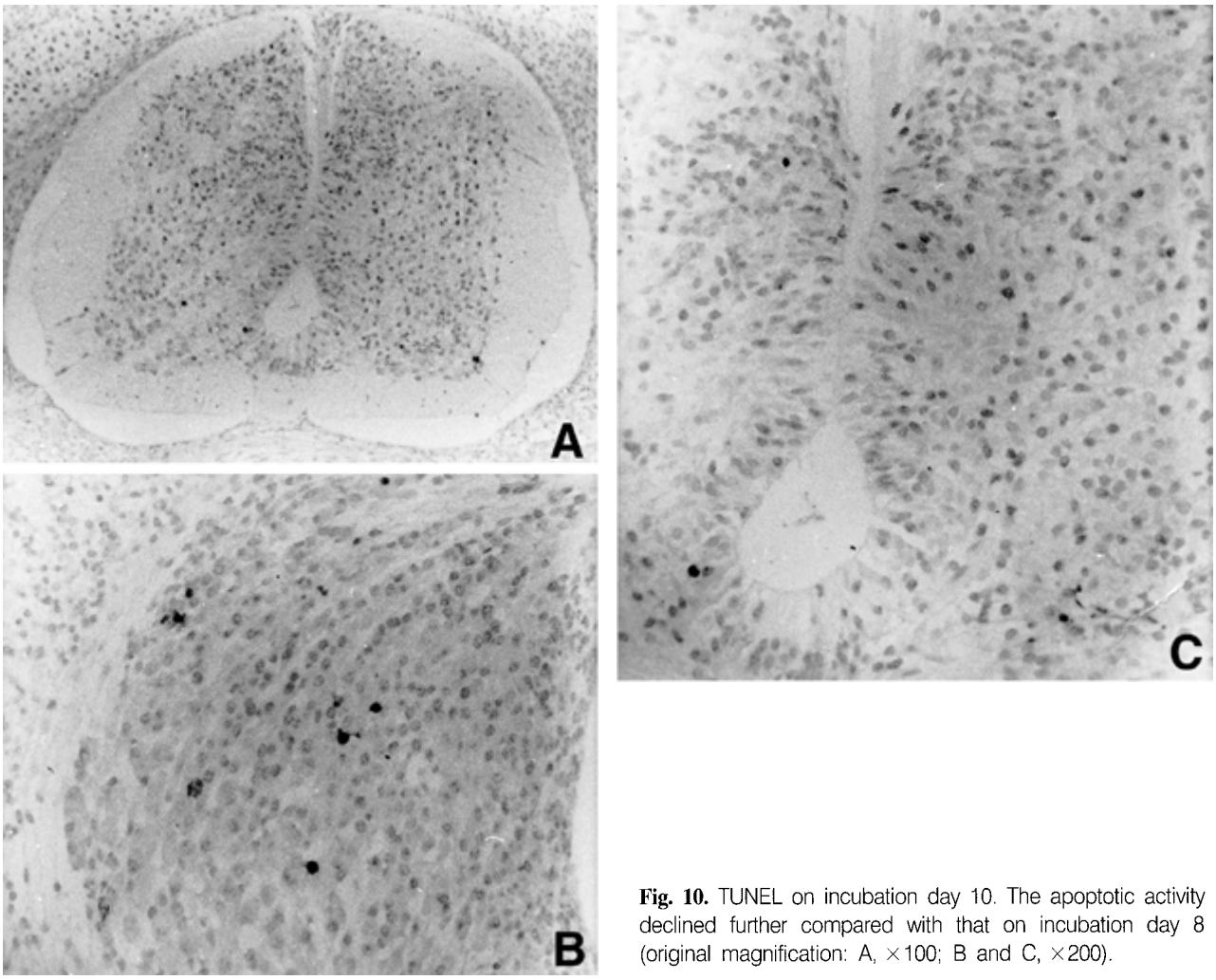

Fig. 10. TUNEL on incubation day 10. The apoptotic activity declined further compared with that on incubation day 8 (original magnification: A, ×100; B and C, ×200).

This process seems quite active on day 5 and much less active thereafter.

Dorsal root ganglion is one of the active site of apoptosis during the stage examined. A significant number of apoptotic cells were still visible on incubation day 10 though they were markedly reduced in number. On day 13, the TUNEL-positive cells were invisible at the dorsal root ganglia. The apoptotic process in the dorsal root ganglia was speculated to have a relationship with the completion of the neural network. If the speculation is right, one could surmise that the majority of neural network formation and related selection processes are completed by day 13.

To compare the PCNA staining and TUNEL results between the various groups, conditions of staining procedures should be identical. For this reason, each of the staining processes was performed simultaneously as much as possible. However, the difference of fixation time between embryos of different stages and difference of duration of proteinase K exposure according to the age of the embryos are possible sources of bias.

In the present study, embryos older than 13 days were not expected to show positive reactions to PCNA. TUNEL studies did not seem to reveal significant activities, either. So observation of older embryos were not carried out.

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