Proliferation and Differentiation of Ependymal Cells after Transection of the Carp Spinal Cord

Authors: Yamada, Hajime, Miyake, Toshihiko, and Kitamura, Tadahisa

Source: Zoological Science, 14(2) : 331-338

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.14.331

ZOOLOGICAL SCIENCE **14**: 331–338 (1997)

Proliferation and Differentiation of Ependymal Cells after Transection of the Carp Spinal Cord

Hajime Yamada*, Toshihiko Miyake and Tadahisa Kitamura

Shionogi CNS Research Laboratories, 5-12-4 Sagisu, Fukushima-ku Osaka 553, Japan

ABSTRACT—Proliferation and differentiation of ependymal cells in the injured carp spinal cord were studied by immunohistochemistry using proliferating cell nuclear antigen (PCNA) and ^{3}H-thymidine (^{3}H-TdR) autoradiography. A surge of proliferation of ependymal cells occurred in the lesion with a peak around 6th day after surgical operation (complete transection) of the spinal cord. The proportion of PCNA-positive ependymal cells at 6th post-operative day (6 POD) was over 14 times that in the normal state. Electron microscopic autoradiography revealed that most of the ependymal cells incorporating ^{3}H-TdR at 5 POD, especially those located in the caudal side of the transection site, contained numerous free ribosomes in their apical portion, but the other organellae, such as rough endoplasmic reticulum and Golgi apparatus, were poorly developed in this portion. In three weeks thereafter, the ependymal cell layer was reconstructed through the lesion, and the apical part of the ^{3}H-TdR-labeled ependymal cells became elongated and morphologically differentiated: that is, it had free ribosomes decreased and glial filaments increased in number. Many bundles of regenerating axons were observed to course within the reconstructed ependymal cell layer. These results may suggest that proliferation, differentiation and reconstruction of the ependymal cell layer following injury of the carp spinal cord are requisite to make the permissive milieu for elongation of the regenerating axons.

INTRODUCTION

In the central nervous system (CNS) of the lower vertebrates such as amphibians and fishes, axonal regeneration occurs after injury (Coggeshall *et al.*, 1982; Coggeshall and Youngblood, 1983; Stensaas, 1983; Reier *et al.*, 1983; Davis *et al.*, 1989). In the case of the spinal cord, the restoration of the central canal and the proliferation and migration of ependymal cells have been reported to take place at first after traumatic injury, i.e., transection (Nordlander and Singer, 1978; Michel and Reier, 1979; Singer *et al.*, 1979; Simpson, 1983; Yamada *et al.*, 1995). We have previously reported that there were numerous regenerated axons widely within the reconstructed ependymal cell layer at the repair stage of the carp spinal cord lesion caused by transection, and thus suggested that ependymal cells may offer the circumstances beneficial to elongation of the regenerating axons in the lower vertebrates (Yamada *et al.*, 1995).

In the present study, we investigated in greater detail the proliferative response and morphological change of ependymal cells after transection of the carp spinal cord, and the possible roles of these cells in the axonal regeneration is discussed.

* Corresponding author: Tel. +81-6-458-5861; FAX. +81-6-458-0987.

MATERIALS AND METHODS

Surgical procedure

Carps (*Cyprinus carpio*) of 18-21 cm in body length were used. They were anesthetized with 0.02% tricaine methanesulfonate (Nacalai Tesque), and laminectomy was performed at the caudal end of the dorsal fin. Then, the spinal cord was transected completely with a micro-dissecting scissors at the site between the vertebral segments 7 and 8 counted from the caudal end. The fish were kept in an ordinary water aquarium after the surgery.

PCNA (proliferating cell nuclear antigen) immunohistochemistry

At 3th, 6th and 10th post-operative day (POD), three fish per group were anesthetized, and the transection lesion (seg. 7-8) of the spinal cord was removed and was fixed by immersion in either Carnoy's fluid or methacarn for 4 hr at room temperature. Tissue was embedded in paraffin. Semi-serial 4 μm-thick transverse sections were cut from the caudal or rostral sides of the transection. Sections were incubated with anti-PCNA antibody (1:200 dilution by PBS, Novocastra Lab. Ltd., UK) for 2 hr at room temperature, and the immunoreaction product was visualized using immunostaining reagents (Vectastain ABC Kit: Vector Lab., Inc., USA) by the avidin-biotin-peroxidase complex (ABC) method. After counterstained with methyl green, sections were observed under a light microscope.

The total numbers of PCNA positive and negative ependymal cells were counted on every section (approximately 80 sections for each animal).

Light and electron microscopic ^{3}H-TdR autoradiography

Flash labeling (2 hr exposure to ^{3}H-TdR): A total of eight fish were used, including two intact fish as controls. Two fish each were

sacrificed at 1.5, 3 and 5 POD. All fish received an intraperitoneal injection of ^{3}H-TdR (10-20 μCi/g b.w.; specific activity 3.3 TBq/mmol, DuPont/NEN) 2 hr before sacrifice. They were fixed by perfusion of a fixative containing 2.4% paraformaldehyde, 2.2% glutaraldehyde and 10 mM $CaCl_2$ in 0.1 M cacodylate buffer (pH 7.4).

Pulse labeling (long survival period after labeling): Four fish received three successive injections of ^{3}H-TdR injections (10 μCi/g b.w.) at 6-hr intervals at 5 POD. They were sacrificed and fixed at 21 and 56 days thereafter in the same manner as above.

Tissue processing: After fixation, the transection lesion of the spinal cord, the film terminale and the wall of the third ventricle were removed, cut into small pieces, and osmificated. They were dehydrated in ethanol and embedded in Epon 812. Semi-thin sections, about 1-μm-thick, were cut out of the Epon block of each material and processed for light microscopic autoradiography. For electron microscopic autoradiography, ultrathin sections were cut and stained with uranyl acetate, and then they were coated with Ilford L4 emulsion (1:2.5 dilution, Ilford Ltd., UK) using a small wire-loop. After exposure for 1-2 months, sections were developed with Microdol-X (Eastman Kodak, USA), fixed and stained with lead citrate. Ultrathin sections were observed under a JOEL JEM-1200 EX electron microscope.

RESULTS

PCNA immunohistochemistry

PCNA is expressed in cell nuclei of the G1-S phases of proliferation cycle, and PCNA immunostaining has been proved to be useful to analyze the mode of cell proliferation (Hall *et al.*, 1990). In the intact spinal cord, PCNA-positive ependymal cells were scarcely found: they constituted approximately 0.3% to 0.4% of all ependymal cells. At 3 POD, the ratio of PCNA-positive to all ependymal cells (PCNA/all ratio) became slightly elevated up to 1.0% in the sides both rostral and caudal to the transection. At 6 POD, a number of PCNA-positive ependymal cells were easily found in the caudal side of the transection (Fig. 1), and the PCNA/all ratio became markedly increased: the ratio in the caudal side was 6.4%, higher than that in the rostral side, 2.2%. At 10 POD, the number of PCNA-positive ependymal cells was decreased and the ratios in the caudal and rostral sides were 2.3% and 1.5%, respectively. Throughout the experimental period, the numbers of PCNA-positive ependymal cells counted in the regions 1.6 cm caudal and 2.0 cm rostral to the transection were almost the same as that of the normal spinal cord (Fig. 2).

Light and electron microscopic ^{3}H-TdR autoradiography

Flash labeling (2 hr exposure to ^{3}H-TdR)

In all the unoperated control spinal cords, no ^{3}H-TdR-labeled ependymal cells were found by light microscopic autoradiography using semi-thin sections, except that a few labeled ependymal cells were observed in the frontal wall of the third ventricle and in the film terminale, i.e., the anterior and posterior ends of the central canal.

At 1.5 and 3 POD, the apical part of most ependymal cells, which faces the central canal was elongated in the side caudal to the transection. A very few of these ependymal cells were found to be labeled with ^{3}H-TdR on light microscopic autoradiography.

At 5 POD, in the side caudal to the transection, some ^{3}H-TdR-labeled ependymal cells were observed by electron microscopic autoradiography. At this day, the ependymal cell layer of the caudal side was composed of tightly aligned ependymal cells, and the central canal was extremely narrow or obliterated (Fig. 3a). The apical part of the labeled ependymal cells contained numerous free ribosomes and a few mitochondria, but the other organelles such as rough endoplasmic reticulum and Golgi apparatus were poorly developed (Fig. 3b). Only in the processes of these cells, glial filaments, which are usually seen in the perikaryal cytoplasm of normal ependymal cells, were observed. In the rostral side of the transection, the central canal was dilated and ependymal cells were flattened in shape. Although the profiles of intracellular organelles of these cells were almost the same as those of the ependymal cells observed in the caudal side, bundles of glial filaments were also seen in the perikaryal cytoplasm (Fig. 3c).

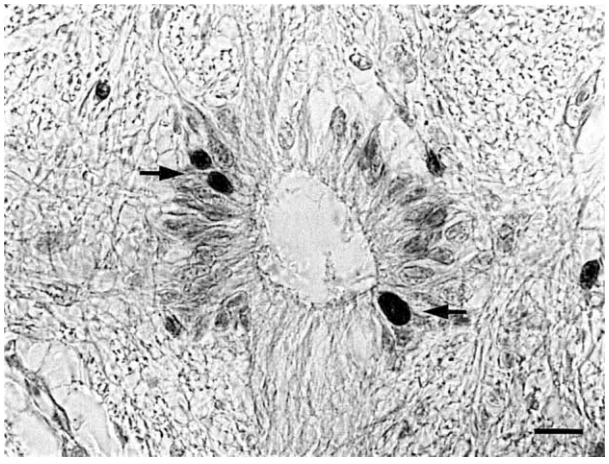

Fig. 1. PCNA immunohistochemistry of the carp spinal cord at 6th post operative day (POD). Three PCNA-positive ependymal cells (arrows) are seen in the ependymal layer. Bar=10 μm.

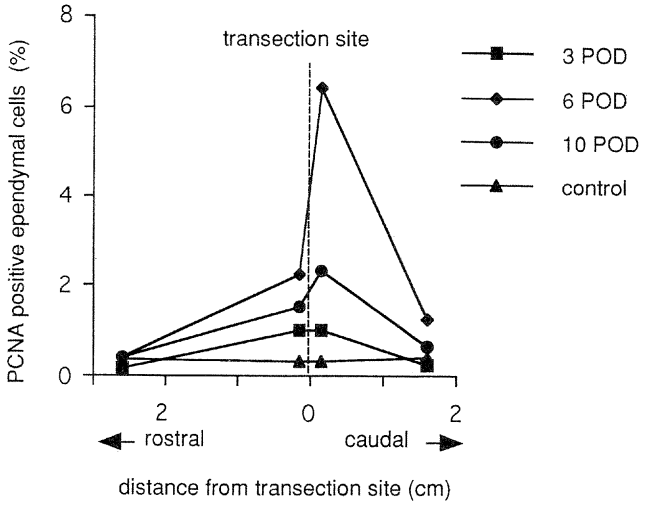

Fig. 2. Temporal and spatial change in proportion of PCNA-positive ependymal cells after spinal cord transection. POD: post-operative day.

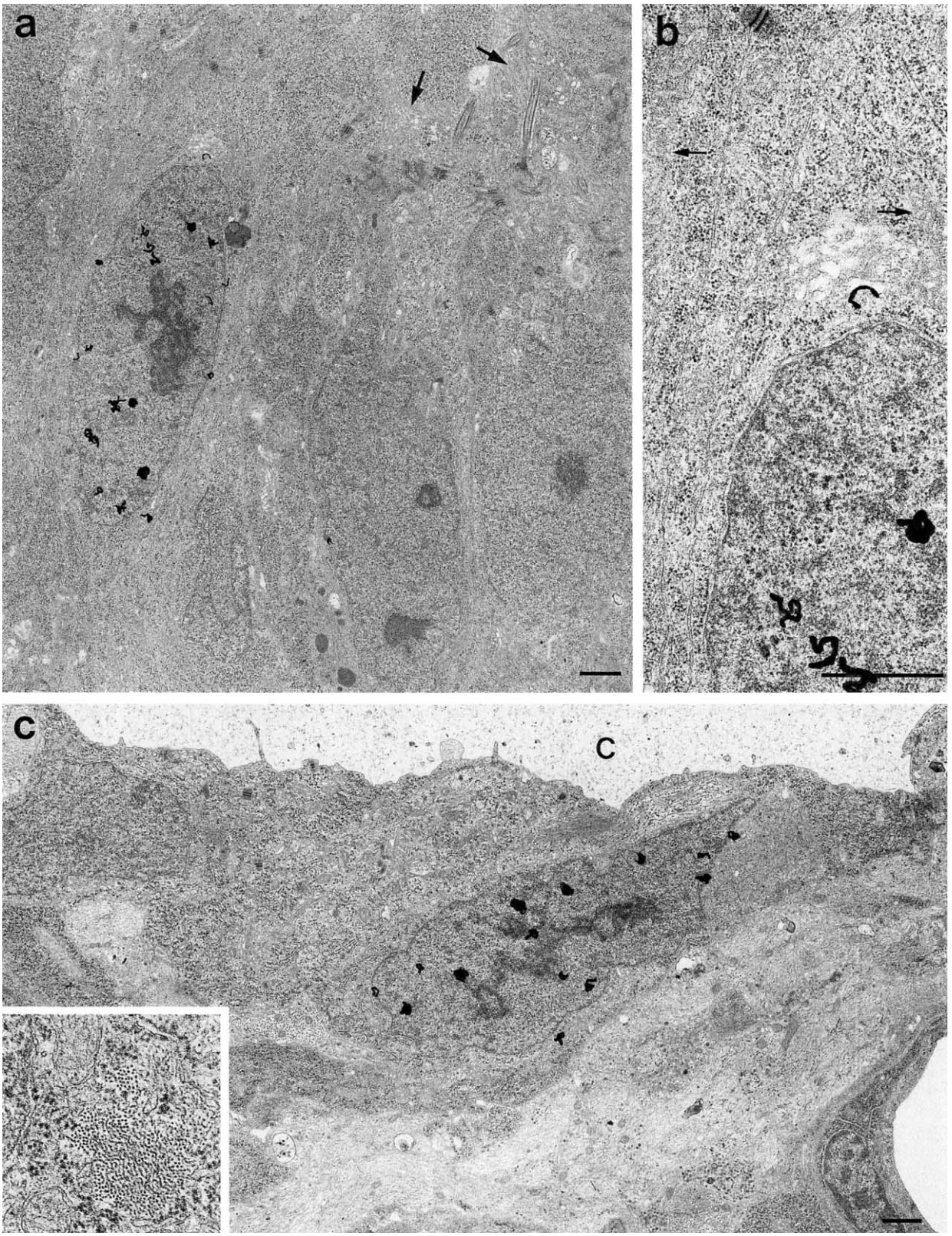

Fig. 3. Electron microscopic autoradiograms of ependymal cells in the carp spinal cord at 5 POD and at 2 hr exposure to ^{3}H-TdR. (**a**) A heavily labeled ependymal cell observed in the caudal side of the transection site, whose nucleus is localized near the obliterated central canal (arrows). Bar=1 μm. (**b**) Higher magnification of part of a labeled ependymal cell (**a**). Numerous free ribosomes and a few mitochondria (arrows) are seen. Bar=1 μm. (**c**) A labeled ependymal cell at the rostral side of the transection site. Inset: higher magnification of the cytoplasm showing glial filaments. C: central canal. Bar=1 μm.

Pulse labeling (labeling with ^{3}H-TdR at 5 POD and long term survival up to 26 and 61 POD)

26 POD: In the caudal side of the transection, the nuclei of most ependymal cells, usually spheroid in shape, were located far from the luminal surface of the central canal, which was still extremely narrow in diameter (Fig. 4a). Electron microscopic features of the ^{3}H-TdR-labeled ependymal cells were of better differentiated cells: free ribosomes in the apical part of these cells were decreased in number, while glycogen particles were increased and bundles of glial filaments appeared (Fig. 4b). These cells had small Golgi apparatus, a few mitochondria and relatively well developed rough endoplasmic reticulum. In the rostral side, the central canal was widely dilated.

In both rostral and caudal sides, large bundles of regenerating axons, most of which were unmyelinated, were seen within the ependymal cell layer consisting of both ^{3}H-TdR-labeled and non-labeled ependymal cells. Many small unmyelinated regenerating axons were also seen at the abluminal region of the ependymal layer (Fig. 5).

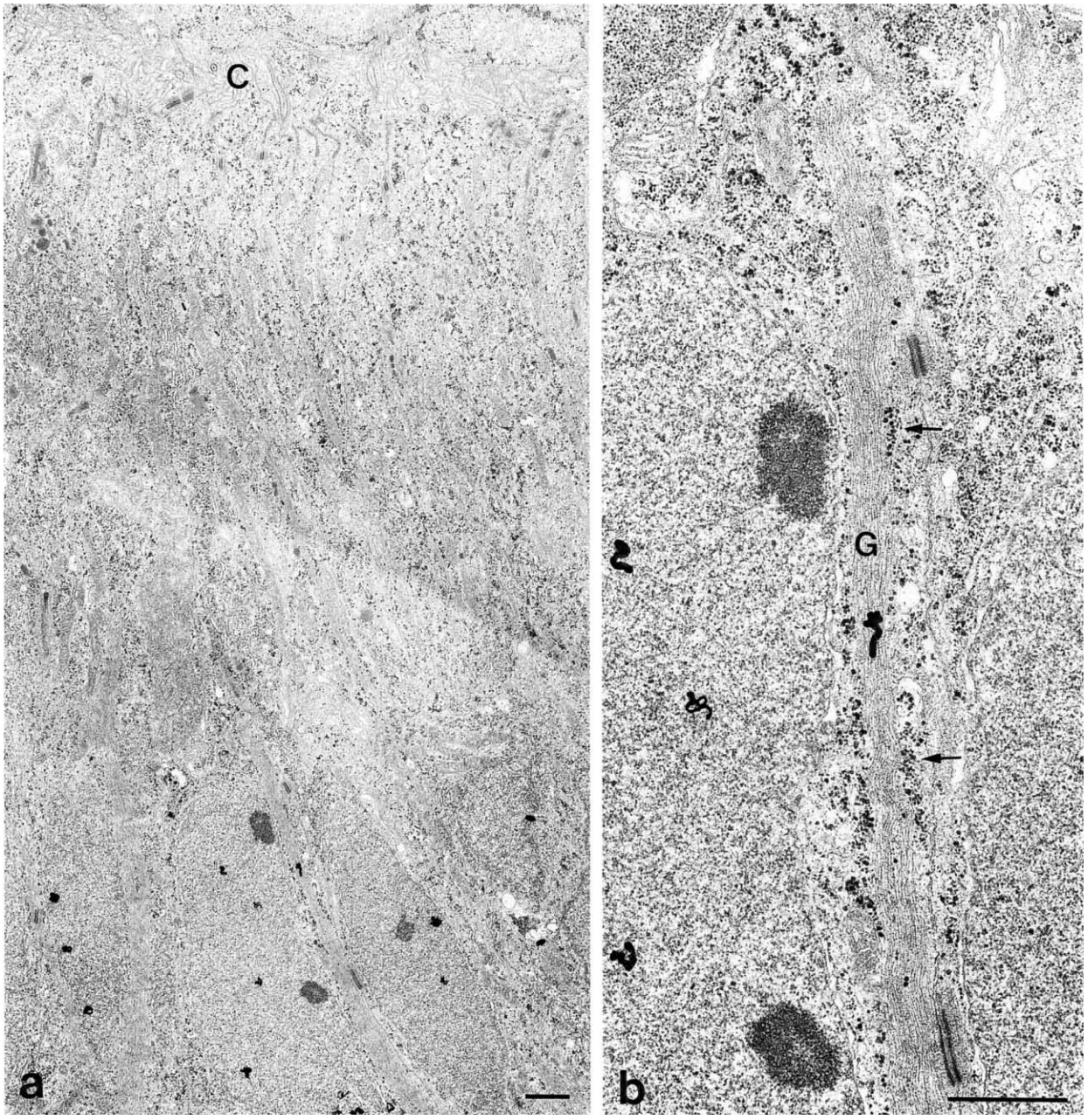

Fig. 4. Electron microscopic autoradiograms of ependymal cells in the carp spinal cord at 26 POD and at 21 days after labeling with ^{3}H-TdR. (**a**) Labeled ependymal cells with elongated apical cytoplasm observed in the caudal side of the transection site. C: central canal. Bar=1 μm. (**b**) Higher magnification of a labeled ependymal cell (**a**). Notice the numerous glycogen particles (arrows) and a bundle of glial filaments (G) in the perikaryal cytoplasm. Bar=1 μm.

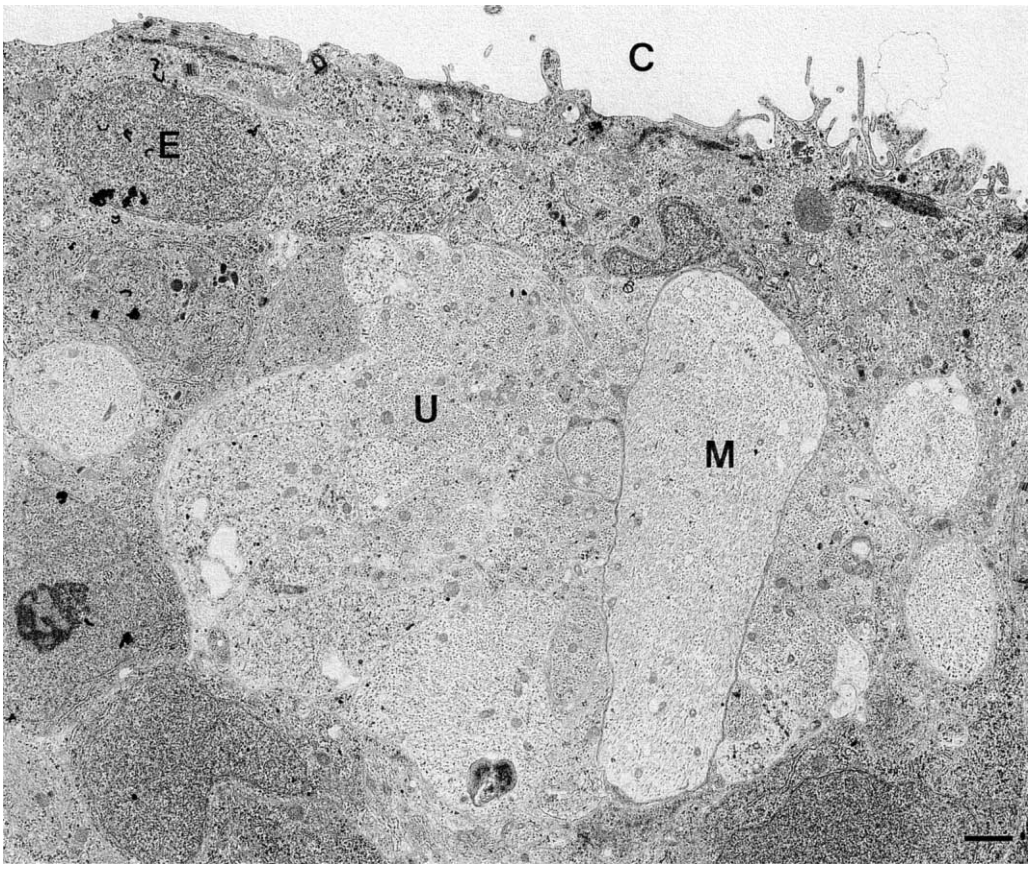

Fig. 5. Electron microscopic autoradiogram of the carp spinal cord at 26 POD and at 21 days after labeling with ^{3}H-TdR in the rostral side of the transection site. Many bundles of regenerating axons, unmyelinated (U) and poorly myelinated (M), are seen in the ependymal cell layer. These axons are in contact with a labeled ependymal cell (E). C: central canal. Bar=1 μm.

61 POD: The gap caused by transection was already connected with the regenerated nervous tissue, through which the central canal was reconstructed. Elongated ependymal cells made up the ependymal layer of this reconstructed central canal. Numerous microvilli and a few cilia were seen on the surface of each ependymal cell (Fig. 6a). The overall morphological appearance of the ^{3}H-TdR-labeled ependymal cells was similar to that observed in the fish at 26 POD (Fig. 6b), and organellae such as Golgi apparatus, mitochondria and rough endoplasmic reticulum were well developed (Fig. 6c). Many regenerating axons, both myelinated and unmyelinated, were seen within the ependymal cell layer.

DISCUSSION

In the present study, using PCNA immunohistochemistry and light and electron microscopic ^{3}H-TdR autoradiography, it was clearly demonstrated that ependymal cells of the carp spinal cord proliferate actively in response to transection injury.

The proliferative reaction of the carp ependymal cells was weak up to 3 POD and became very intense at 6 POD. This is in contrast to the general belief that mature ependymal cells of the human spinal cord have little, if any, capacity for reactive proliferation (Duchen, 1984). Ependymal cells of the rodent spinal cord have also been reported to show strong proliferation in response to injury (Adrian and Walker, 1962; Kraus-Ruppert *et al.*, 1975; Gilmore and Leiting, 1980), our preliminary study with BrdU immunohistochemistry of the rat spinal cord showed that the number of BrdU-labeled ependymal cells was increased up to about 7 times with a peak at 10th day after hemisection of the spinal cord (our unpublished data). On the other hand, the peak of proliferation of GFAP (glial fibrillary acidic protein)-positive astrocytes in the cortex of rat cerebrum was observed 3 to 4 days after injury (Miyake *et al.*, 1988). Proliferative response of ependymal cells in the carp and rat spinal cords seem to occur more slowly compared with that of astrocytes in rat brains.

The PCNA/all ratio of ependymal cells in the caudal side of the transection was about 3 times higher than that in the rostral side at 6 POD, and the ependymal cells were usually cylindrical in shape in the caudal side while they were flattened in the rostral side. These differences might depend on some chemical trophic factors such as NGF and would be worth another study. It seems to safe that no fine structural observation can answer this question.

At 5 POD, the central canal in the caudal side of the transection was seen to be obliterated, and elongated ependymal cells were arranged tightly with each other, forming

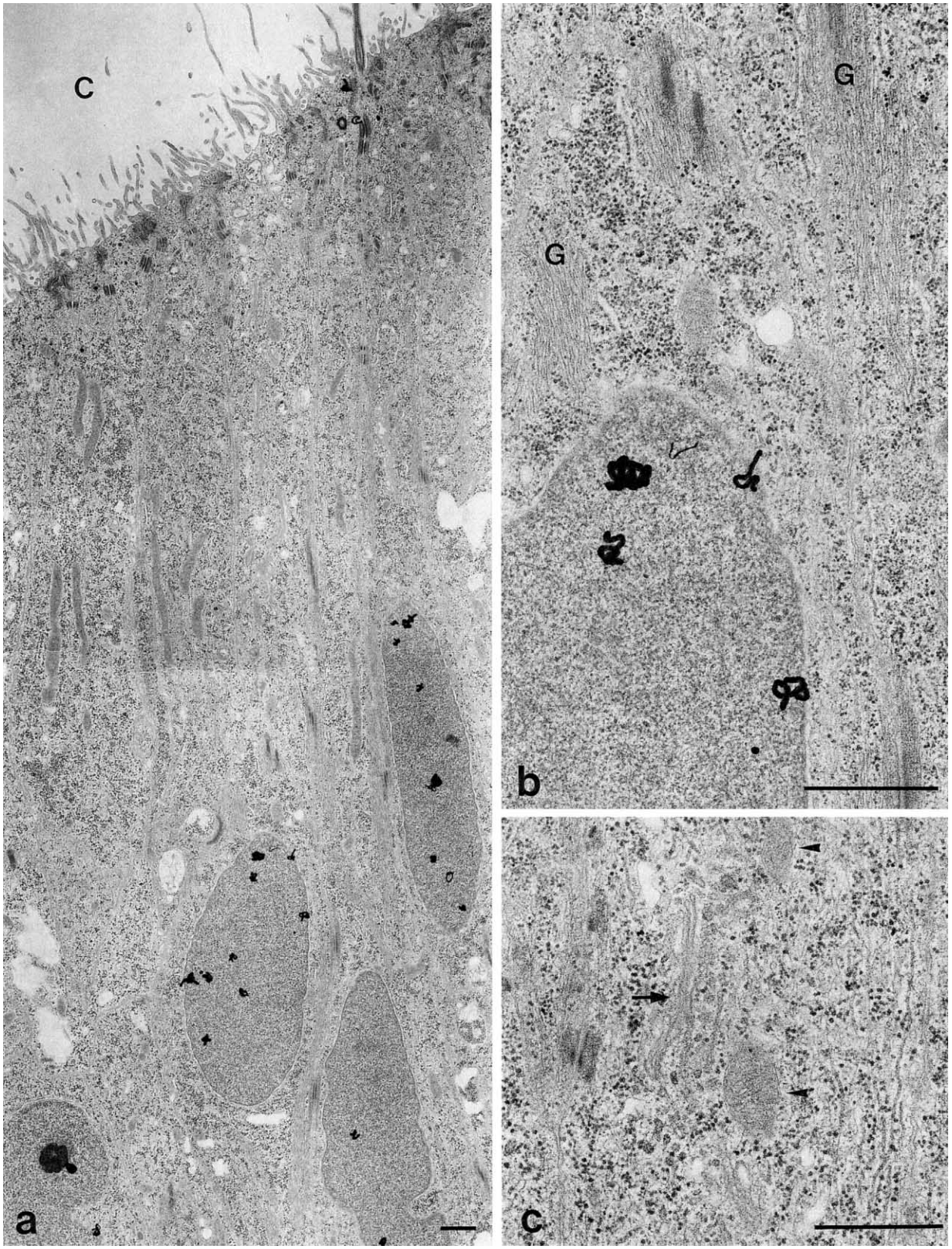

Fig. 6. Electron microscopic autoradiograms of ependymal cells in the carp spinal cord at 61 POD and at 56 days after labeling with ^{3}H-TdR. (**a**) Elongated ependymal cells make up an ependymal layer. A lot of microvilli projecting from the cell surface and many desmosomes are seen at the upper region of the ependymal cells. C: central canal. Bar=1 μm. (**b**) Higher magnification of part of a labeled cell in (**a**). Many bundles of glial filaments (G) and numerous glycogen particles are seen in the cytoplasm. Bar=1 μm. (**c**) Higher magnification of the apical cytoplasm of an ependymal cell. Well developed Golgi apparatus (arrow) and mitochondria (arrowheads) are seen. Bar=1 μm.

a thick ependymal cell layer. The apical part of these ependymal cells contained numerous free ribosomes but few other intracellular organellae. Although this electron microscopic feature was similar to that of immature cells (matrix cells) of the early embryonic brain (Fujita, 1963), the ependymal cells observed in this study characteristically contained glial filaments in their cytoplasmic processes. This means that these cells were not immature matrix cells but were more mature cells differentiated into ependymal cells. Some of these ependymal cells have proliferative activity, as revealed by the flash labeling with ^{3}H-TdR.

Neurogenesis of the lower vertebrates continues even in adulthood, as demonstrated, for example, in the optic tectum of goldfish (Raymond and Easter, 1983), the telencephalon of lizards (Perez-Sanchez *et al.*, 1989) and the hypothalamus of frogs (Chetverukhin and Polenov, 1993; Polenov and Chetverukhin, 1993). Anderson and Waxman (1985) reported that some ependymal cells of the spinal cord of teleosts, *Sternarchus albifrons* retained their capability to divide, and these cells proliferate to generate nerve and glial cells following amputation of the spinal cord. Furthermore, Anderson and Waxman (1985) detected signal of ^{3}H-TdR on nerve cells after long term exposure to ^{3}H-TdR of the cultured spinal cord explant. In the present study, the cells in the ependymal cell layer which had proliferated in response to injury maintain the characteristic figure of glial nature such as glial intermediate filaments, and we could not find any ^{3}H-TdR-labeled nerve cells. This fact suggests that the production of nerve cells does not occur in the adult carp spinal cord.

Numerous regenerated axons were observed in the ependymal cell layer which was reconstructed after transection of the carp spinal cord. In rats as well as in fish, proliferation of the ependymal cells occurs after spinal cord injury as mentioned above. However, the gap of the rat spinal cord caused by transection is not connected with regenerated axons (our unpublished data). Thus, proliferation of ependymal cells by itself is likely to be insufficient for axonal regeneration. Astroglial processes are known to be the major impediment to axonal regeneration in the CNS of higher mammals (Reier *et al.*, 1983; Liuzzi and Lasek, 1987; Stensaas *et al.*, 1987). However, we have previously shown that the processes of ependymal and astroglial cells, which have common fine structural characteristics, do not prevent elongation of regenerating axons in the spinal cord of carp (Yamada *et al.*, 1995). The key to axonal elongation might be found in the physicochemical differences of the cell membrane of ependymal and glial cells between higher and lower vertebrates.

ACKNOWLEDGMENTS

We thank Dr. Hisao Fujita for his helpful comments and discussion.

REFERENCES

Adrian EK, Walker BE(1962) Incorporation of thymidine ^{3}H by cells in normal and injured mouse spinal cord. J Neuropathol Exp Neurol 21: 597–609

Anderson MJ, Waxman SG(1985) Neurogenesis in tissue cultures of adult teleost spinal cord. Dev Brain Res 20: 203–212

Anderson MJ, Waxman SG(1983) Caudal spinal cord of the teleost *Sternarchus albifrons* resembles regenerating cord. Anat Rec 205: 85–92

Chetverukhin VK, Polenov AL(1993) Ultrastructural radioautographic analysis of neurogenesis in the hypothalamus of the adult frog, *Rana temporaria*, with special reference to physiological regeneration of the preoptic nucleus. I. Ventricular zone cell proliferation. Cell Tissue Res 271: 341–350

Coggeshall RE, Birse SG, Youngblood CS(1982) Recovery from spinal transection in fish. Neurosci Lett 32: 259–264

Coggeshall RE, Youngblood CS(1983) Recovery from transection in fish: regrowth of axons past the transection. Neurosci Lett 38: 227–231

Davis BM, Duffy MT, Simpson Jr SB(1989) Bulbospinal and intraspinal connections in normal and regenerated salamander spinal cord. Exp Neurol 103: 41–51

Duchen LW(1984) General pathology of neurons and neuroglia. In "Greenfield's Neuropathology, 4th ed" Ed by JH Adams, JAN Corsellis, LW duchen, Edward Arnold, London, pp 1–52

Fujita H(1963) Electron microscopic studies on the thyroid gland of domestic fowl, with special reference to the mode of section and the occurrence of a central flagellum in the follicular cell. Zeitshrift für Zellforschung 60: 615–632

Gilmore SA, Leiting JE(1980) Changes in the central canal area of immature rats following spinal cord injury. Brain Res 201: 185–189

Hall PA, Levison DA, Woods AL, Yu CC-W, Kellock DB, Watkins JA, Barnes DM, Gillett CE, Camplejohn R, Dover R, Waseem NH, Lane DP(1990) Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections: An index of cell proliferation with evidence of deregulated expression in some neoplasms. J Pathol 162: 285–294

Kraus-Ruppert R, Laissue J, Bürki H, Odartchenko N(1975) Kinetic studies on glia, schwann and capsular cells labeled with [^{3}H] thymidine in cerebrospinal tissue of young mice. J Neurol Sci 26: 555–563

Liuzzi FJ, Lasek RJ(1987) Astrocytes block axonal regeneration in mammals by activating the physiological stop pathway. Science 237: 642–645

Michel ME, Reier PJ(1979) Axonal-ependymal associations during early regeneration of the transected spinal cord in *Xenopus laevis* tadpoles. J Neurocytol 8: 529–548

Miyake T, Hattori T, Fukuda M, Kitamura T, Fujita S(1988) Quantitative studies on proliferative changes of reactive astrocytes in mouse cerebral cortex. Brain Res 451: 133–138

Nordlander RH, Singer M(1978) The role of ependyma in regeneration of the spinal cord in the urodel amphibian tail. J Comp Neurol 180: 349–374

Perez-Sanchez F, Molowny A, Garcia-Verdugo JM, Lopez-Garcia C(1989) Postnatal neurogenesis in the nucleus sphericus of the lizard, *Podarcis hispanica*. Neurosci Lett 106: 71–75

Polenov AL, Chetverukhin VK(1993) Ultrastructural radioautographic analysis of neurogenesis in the hypothalamus of the adult frog, *Rana temporaria*, with special reference to physiological regeneration of the preoptic nucleus. II. Types of neuronal cells produced. Cell Tissue Res 271: 351–362

Raymond PA, Easter SS Jr(1983) Postembryonic growth of the optic tectum in goldfish. J Neurosci 3: 1077–1091

Reier PJ, Stensaas LJ, Guth L(1983) The astrocytic scar as an

impediment to regeneration in the central nervous system. In "Spinal Cord Reconstruction" Ed by CC Kao, PB Bunge, PJ Reier, Raven Press, New York, pp 163–195

Simpson SB Jr(1983) Fasciculation and guidance of regenerating central axons by ependyma. In "Spinal Cord Reconstruction" Ed by CC Kao, RP Bunge, PJ Reier, Raven Press, New York, pp 151–162

Singer M, Nordlander RH, Eger M(1979) Axonal guidance during embryogenesis and regeneration in the spinal cord of the newt: The blueprint hypothesis of neuronal pathway patterning. J Comp Neurol 185: 1–22

Stensaas LJ(1983) Regeneration in the spinal cord of the newt *Notopthalmus (Triturus) pyrrhogaster*. In "Spinal Cord Reconstruction" Ed by CC Kao, PB Bunge, PJ Reier, Raven Press, New York, pp 121–149

Stensaas LJ, Partlow LM, Burgess PR, Horch KW(1987) Inhibition of regeneration: the ultrastructure of reactive astrocytes and abortive axon terminals in the transection zone of the dorsal root. Prog Brain Res 71: 457–466

Yamada H, Miyake T, Kitamura T(1995) Regeneration of axons in transection of the carp spinal cord. Zool Sci 12: 325–332

(Received November 18, 1996 / Accepted January 30, 1997)